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Contents

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Contents

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EDITORIAL

Obstructive sleep apnea syndrome and fatty liver: Association or causal link?

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Abstract

Obstructive sleep apnea (OSA) is a complex disorder that consists of upper airway obstruction, chronic intermittent hypoxia and sleep fragmentation. OSA is well known to be associated with hypoxia, insulin resistance and glucose intolerance, and these factors can occur in the presence or absence of obesity and metabolic syndrome. Although it is well established that insulin resistance, glucose intolerance and obesity occur frequently with non-alcoholic fatty liver disease (NAFLD), it is now becoming apparent that hypoxia might also be important in the development of NAFLD, and it is recognized that there is increased risk of NAFLD with OSA. This review discusses the association between OSA, NAFLD and cardiovascular disease, and describes the potential role of hypoxia in the development of NAFLD with OSA.

Key words: Sleep apnea syndrome; Hyperlipidemia; Non-alcoholic fatty liver disease; Insulin resistance

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INTRODUCTION

Obstructive sleep apnea (OSA) is a condition that affects 1%-4% of the general population and 25%-35% of obese individuals. OSA is more common in men than women and is characterized by loud and frequent snoring, periods of apnea during sleep and excessive day somnolence[1]. Initially, OSA was thought to be due to failure to maintain small upper airway tone, which causes airway collapse and apnea, but recently, unstable ventilatory control and changes in lung volume have been implicated. An OSA disorder is generally defined as five or more apnea-hypopnea episodes per hour of sleep [i.e. the apnea-hypopnea index (AHI)][1,2]. OSA is associated with insulin resistance and hyperlipidemia and both conditions are associated with non-alcoholic fatty liver disease (NAFLD)[3,4]. Importantly, and potentially relevant to OSA, hypoxia is now considered as one of the aggravating factors for development of NAFLD[4], and interestingly, OSA is also regarded as one of the factors that accelerate the progression of NAFLD to non-alcoholic steatohepatitis (NASH)[5].

NAFLD is emerging as an important public health problem across the globe[6]. NAFLD refers to a wide spectrum of liver damage, which ranges from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis. NAFLD is strongly associated with insulin resistance and is defined by accumulation of liver fat > 5% per liver

weight, in the presence of < 10 g daily alcohol consumption[7]. The diagnosis of NAFLD can be established by ultrasound and can be confirmed by liver biopsy. The characteristic histology of NAFLD resembles that of alcohol-induced liver injury, but occurs in people who consume minimal or no alcohol. NAFLD is regarded as the most common cause of increased liver enzyme concentrations and is associated with type 2 diabetes, obesity and hyperlipidemia[8]. The reported prevalence of obesity with NAFLD varies between 30% and 100%, whereas the prevalence of NAFLD with type 2 diabetes varies between 10% and 75%[7]. In routine clinical practice, most cases of fatty liver disease are attributable to alcohol excess; however, fatty liver disease can also occur in association with a wide range of toxins, drugs, and diseases, such as morbid obesity, cachexia, type 2 diabetes, hyperlipidemia, and after jejuno-ileal bypass surgery. As important risk factors for NAFLD such as obesity and type 2 diabetes are increasing in prevalence this could explain the marked increase in numbers of individuals with NAFLD[9].

NAFLD can progress silently to cirrhosis, portal hypertension, and liver-related death in early adulthood. Importantly, NAFLD is also associated with an increased risk of all-cause death and predicts future cardiovascular disease (CVD) events, independently of age, sex, low-density lipoprotein (LDL)-cholesterol, smoking and the cluster of features of the metabolic syndrome[9]. Currently, there are no sensitive and specific biochemical markers for NAFLD. An increase (or decrease) in alanine aminotransferase (ALT) is often used as a biochemical marker to monitor progression (or amelioration) of NAFLD, despite the fact that ALT concentrations can be misleading and do not reflect the severity or outcome. Mass screening for significant liver injury in patients with NAFLD will be an important medical challenge in the years to come because of the epidemics of obesity and diabetes[10].

We have previously summarized the studies that have shown that NAFLD is associated with an increase in incidence of CVD[7]. Importantly, considerable numbers of studies have shown an increase in incidence of CVD with OSA[11-13]. The subsequent discussion focuses on the association of OSA with hypoxia, insulin resistance and hyperlipidemia, and how ultimately this can lead to NAFLD.

OSA AND FATTY LIVER DISEASE

Experimental studies have shown that OSA can lead to an increase in insulin resistance and an alteration in lipid metabolism and can precipitate NAFLD[14-16]. Savransky *et al*[14] have exposed lean C57BL/6J mice (n = 15) on a regular chow diet to chronic intermittent hypoxia (CIH) for 12 wk and compared these mice with pair-fed mice, exposed to intermittent air (IA, n = 15). CIH caused liver injury with an increase in serum ALT (224 ± 39 U/ L *vs* 118 ± 22 U/L in the IA group, $P < 0.05$). CIH also induced hyperglycemia, lipid peroxidation of liver tissue, and increased activity of nuclear factor (NF)-κB but not an inflammatory response [as tumor necrosis factor (TNF)-α was not detectable], which suggests that CIH induces oxidative stress in the liver. Liver histology shows swelling of hepatocytes, with marked accumulation of glycogen in hepatocytes, but no evidence of hepatic steatosis. CIH greatly exacerbates acetaminophen-induced liver toxicity, which causes fulminant hepatocellular injury[14]. It is therefore likely that in the absence of factors that induce obesity as a primary stressor on the liver, IH *per se* leads to mild liver injury. The same authors have repeated the same experiment in C57BL/6J mice on a high-fat, high-cholesterol diet, exposed to CIH for 6 mo. CIH caused liver injury with an increase in serum ALT (461 ± 58 U/L *vs* 103 ± 16 U/L in the control group, $P < 0.01$) and aspartate aminotransferase (AST) (637 ± 37 U/L *vs* 175 ± 13 U/L in the control group, $P < 0.001$). Histology revealed evidence of inflammation and fibrosis in the liver, which was not evident in the control mice. CIH caused marked increases in lipid peroxidation in serum and liver tissue; marked increases in hepatic levels of myeloperoxidase, pro-inflammatory cytokines interleukin (IL)-1β, IL-6, the chemokine macrophage inflammatory protein-2; a trend towards an increase in TNF-α; and an increase in α1 (I)-collagen mRNA[15]. Thus, it is plausible that a high-fat diet that occurs in the presence of hypoxia with OSA promotes NAFLD. Furthermore, in a rat model of NAFLD (a choline-deficient high-fat diet) IH has been shown to induce NASH[16]. The metabolic disorders that predispose patients to NASH include insulin resistance and obesity but the mechanism by which repeated hypoxic events, such as occur in OSA, can lead to the progression of liver disease is unclear. It has been shown that hypoxia decreases insulin sensitivity in mice and might ultimately increase expression of the lipogenic genes sterol-regulatory-element-binding protein-1c (SREBP-1c), peroxisome-proliferator-activated receptor-γ (PPAR-γ), acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2). Furthermore, hypoxia also decreases expression of genes that regulate mitochondrial β oxidation [e.g. PPAR-α and carnitine palmitoyltransferase-1 (CPT-1)][17], which suggests that fat oxidation is also inhibited. Therefore, hypoxia can increase lipogenesis and inhibit fat oxidation; both factors that promote fat accumulation and development of NAFLD.

Human studies have shown that OSA is associated with an increase in liver enzymes, and treatment of OSA has been shown to decrease liver enzymes. For example, Chin *et al*[18] have shown that OSA is associated with an increase in liver enzyme concentrations in 14 of 44 (35%) obese individuals. Furthermore, continuous positive airway pressure (CPAP) therapy decreases concentrations of liver enzymes (ALT and AST). In contrast, in a randomized controlled trial, administration of CPAP for 4 wk had no effect on liver enzymes[19]. In a cohort of morbidly obese patients who required bariatric surgery, OSA was found to be a risk factor for increased liver enzyme concentrations but not for NASH[20]. However, Kallwitz *et al*[21] have shown that, in obese patients with NAFLD, OSA is associated with elevated ALT levels and a trend toward histological evidence of progressive

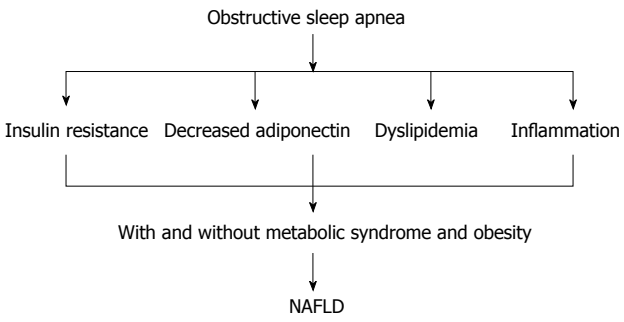

Figure 1 Obstructive sleep apnea can induce non-alcoholic fatty liver disease through increasing insulin resistance, dyslipidemia and inflammation. The presence of metabolic syndrome and obesity with obstructive sleep apnea (OSA) can aggravate non-alcoholic fatty liver disease (NAFLD). OSA might aggravate NAFLD in the absence of obesity and metabolic syndrome.

liver disease. This finding has been endorsed by Mishara *et al*[22], who have shown that, in 101 patients awaiting bariatric surgery, OSA was a risk factor for progression of NAFLD to NASH. Histopathological evidence from 20 obese individuals has shown that OSA is associated with NASH and insulin resistance[23]. Importantly, in 109 patients with OSA, serum aminotransferase levels were better predicted by markers of oxygen desaturation than by factors traditionally associated with the metabolic syndrome[24]. Markers of hypoxia were correlated significantly with AST and ALT levels, whereas the AHI, body mass index (BMI), blood pressure, fasting glucose, triglyceride, and cholesterol levels did not[24]. Importantly, in obese children, OSA has also been shown to be associated with hepatic steatosis and insulin resistance[25], which suggests that early exposure to relative hypoxia also has a deleterious impact on the liver (Figure 1).

OSA AND INSULIN RESISTANCE

There is a strong link between insulin resistance and excessive deposition of triglyceride in hepatocytes, which is the hallmark of NAFLD[26]. Although clinical and experimental studies have shown an association between OSA and insulin resistance, whether CPAP therapy improves insulin resistance remains a controversial issue[27]. In 14 obese individuals with OSA, there were marked increases in leptin, insulin resistance, TNF-α and IL-6, compared with non-apneic obese men. The sleep apnea patients had a significantly greater amount of visceral fat compared to obese controls ($P < 0.05$) and indexes of sleep disordered breathing were positively correlated with visceral fat, but not with BMI, or total or subcutaneous fat. Furthermore, a greater degree of insulin resistance was observed in the group of apnea patients than in BMI-matched non-apneic controls[28]. This finding suggests that OSA is not only associated with insulin resistance but also with inflammation.

Punjabi *et al*[29] have shown that the prevalence of sleep-disordered breathing in 150 healthy mildly obese men, without diabetes, ranged from 40% to 60%, and impairment in glucose tolerance was related to severity of oxygen desaturation. For a 4% decrease in oxygen saturation, the associated OR for worsening glucose tolerance was 1.99 (95% CI: 1.11-3.56) after adjusting for percent body fat, BMI, and AHI. Multivariate linear regression analyses revealed that increasing OSA was associated with worsening insulin resistance independent of obesity[29]. Ip *et al*[30] also have shown that OSA is independently associated with insulin resistance. Furthermore, Meslier *et al*[31] have carried out a cross-sectional study in 494 patients with OSA and have found that the prevalence of type 2 diabetes was 30% and impaired glucose tolerance was 20%, and importantly, diabetes and BMI were independent predictors of OSA[32]. Mallon *et al*[33] have shown in a 12-year follow-up study that difficulty maintaining sleep, or short sleep duration ($\leqslant$ 5 h), was associated with an increased incidence of diabetes in men; whereas in contrast, the Finnish type 2 diabetes survey (FIN-D2D) (a large population study in Finland) has shown that sleep duration of $\leqslant$ 6 h, or $\geqslant$ 8 h, was independently associated with type 2 diabetes in middle-aged women, but not in men[34]. Taken together, these data suggest that an alteration in the normal sleep pattern increases risk of diabetes in men and women.

Data from experimental animal models have shown that IH is also associated with insulin resistance. In obese mice, short-term IH led to a decrease in blood glucose levels accompanied by a marked increase in serum insulin levels, and intriguingly, this effect was completely abolished by prior leptin infusion. Obese mice exposed to IH for 12 wk developed a time-dependent increase in fasting serum insulin levels (from 3.6 ± 1.1 ng/mL at baseline to 9.8 ± 1.8 ng/mL at wk 12, $P < 0.001$) and worsening glucose tolerance, consistent with an increase in insulin resistance[35]. However, in lean C57BL/6J mice, exposure to IH for 5 d did not induce the same metabolic changes seen in obese mice[35]. Furthermore, in lean C57BL/6J mice IH induced insulin resistance. This effect was seen during the time of exposure to IH[36]. These data suggest that the presence of obesity or metabolic syndrome (as a first insult) in association with OSA (a second insult) might lead to NAFLD and ultimately NASH. Therefore, it is plausible to suggest that OSA in association with insulin resistance increases risk of type 2 diabetes. Several mechanisms are thought to contribute to the development of insulin resistance with OSA (Figure 2).

Death of adipose tissue and associated excess release of free fatty acid

Adipose tissue hypoxia (ATH) is a new concept in understanding the pathogenesis of insulin resistance and inflammation in OSA. The concept suggests that inhibition of adipogenesis and triglyceride synthesis by hypoxia might be a mechanism for the increased free fatty acid (FFA) concentrations in obesity that occurs with insulin resistance. Gross obesity might be associated with ATH and adipocyte death. However, the exact cause of adipocyte death in obesity is not known. It has been suggested that gross obesity *per se* is associated with a reduction in

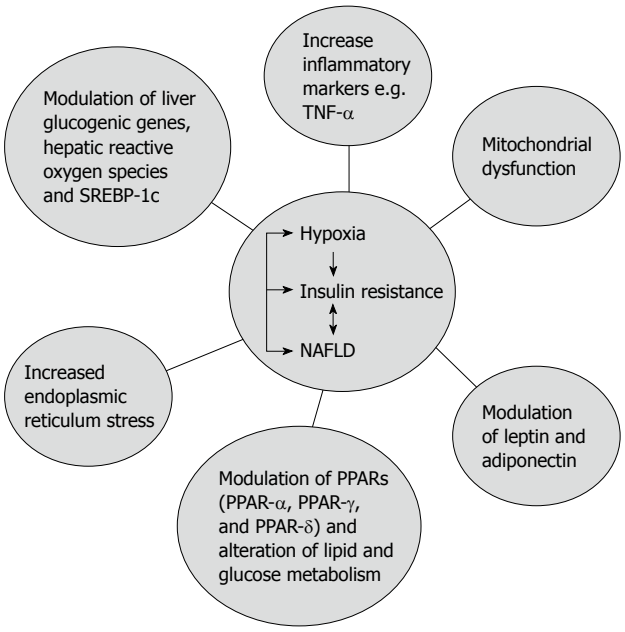

Figure 2 The complex relationship between non-alcoholic fatty liver disease, obstructive sleep apnea and insulin resistance. TNF: Tumor necrosis factor; NAFLD: Non-alcoholic fatty liver disease; PPAR: Peroxisome-proliferator-activated receptor; SREBP-1c: Sterol-regulatory-element-binding protein-1c.

blood flow to adipocytes due to diminished angiogenesis or vasoconstriction[37]. Yin *et al*[38] have shown that hypoxia might inhibit insulin-induced glucose uptake by reducing concentrations of the insulin-signaling molecules insulin receptor β and insulin receptor substrate-1 in mice. Hypoxia might also stimulate lipolysis and inhibit uptake of FFA in adipocytes, which leads to FFA elevation in the plasma of obese subjects, because the increase in FFA might occur as a result of the inhibitory effect of hypoxia on the fatty acid transporters (FATP1, CD36) and the transcription factor (PPAR-γ)[38]. It is tempting to speculate that OSA might act in synergism with gross obesity to accelerate the process of adipocyte death that could ultimately aggravate the course of insulin resistance.

Inflammation

Numerous studies have shown that OSA is a trigger for inflammation. This might explain the associated increase in insulin resistance, dyslipidemia and hypertension in OSA[36]. NF-κB is the transcription factor that is involved in inflammatory pathways and might be involved in modulating insulin sensitivity[39]. Importantly, NF-κB is also increased not only with OSA but also with obesity and metabolic syndrome. NF-κB is the master regulator of inflammatory process and its activation with hypoxia also leads to activation of TNF-α, IL-1, IL-6, monocyte chemoattractant protein-1, macrophage migration-inhibition factor, inducible nitric oxide synthase and matrix metalloproteinase 9. Some of these mediators are also activated by the hypoxia inducible factor (HIF)[40-43]. TNF-α and IL-1 are known to be increased not only with OSA, but also with obesity and metabolic syndrome[40], and the increase in TNF-α and IL-1 is known to be associated with an increase in insulin resistance[44].

Modulation of transcription factors

The modulation of transcription factors has a crucial role in the development of insulin resistance. Evidence is now emerging that hypoxia stimulates SREBP-1c, which is a positive transcription factor for activity of ACC and fatty acid synthase genes, both genes whose activity can promote development of fatty liver[45]. Hypoxia-induced fatty liver has been shown to be associated with an increase in the expression of SREBP-1c[45]. Insulin-resistant ob/ob mice have increased concentrations of SREBP-1c and also develop spontaneous fatty liver[45]. We have presented evidence that suggests strongly that abnormalities of SREBP-1c function play an important pathogenetic role in contributing to the NAFLD phenotype[46]. PPAR-γ is required for maintenance of insulin sensitivity and lipid metabolism[47,48]. Importantly, hypoxia and the associated increase of NF-κB, TNF-α, IL-1 and IL-6 are all known to inhibit PPAR-γ[49]. Overexpression of hepatic PPAR-γ leads to lipid accumulation and it is suggested as a mechanism for hypoxia-induced fatty liver. Furthermore, PPAR-α is also reduced by hypoxia. PPAR-α is highly expressed in the liver, and animal models deficient in PPAR-α develop NAFLD and insulin resistance[49]. In addition, hypoxia also decreases the expression of mitochondrial fatty acid transporter CPT-1[17], which might decrease fat oxidation and promote lipid accumulation. Hypoxia might also modulate AMP-activated protein kinase through mitochondrial respiration or oxidative stress, and ultimately, this might enhance insulin resistance[50].

Adiponectin

Adiponectin is a cytokine that is produced by adipocytes. Serum levels of adiponectin correlate with systemic insulin sensitivity[51]. A reduction in adiponectin contributes to insulin resistance in obesity. However, it is still not clear why adiponectin concentrations are decreased in obesity[52]. Decreased adiponectin is known to be associated with NAFLD[53] and studies have now shown that hypoxia reduces adiponectin expression in adipocytes[54,55]. In adipose tissue, the inhibitory effect of hypoxia on adiponectin might result in increased expression of inflammatory cytokines[56]. Furthermore, TNF-α has been shown to inhibit adiponectin in adipocytes[56]. Thus, these data suggest that hypoxia might directly inhibit adiponectin expression, directly or indirectly, through TNF-α, although whether the decrease in adiponectin causes NAFLD is still uncertain.

Leptin

Leptin is a hormone that is secreted by adipose tissue and increases with obesity. The main role of leptin is to reduce appetite[57]. OSA is known to be associated with an increase in leptin plasma levels, and the increase in leptin occurs in proportion to the severity of OSA[57]. Therefore, it is likely that adipose tissue hypoxia might in part

contribute to the increase in plasma leptin level. HIF-1α is associated with increased leptin level[58]. Despite the increase in plasma leptin in the majority of obese individuals with OSA, there is no improvement in appetite due to leptin resistance associated with excess fats[59-61]. CPAP has been shown to be associated with a decrease in leptin[59-61], which suggests that hypoxia might modulate insulin sensitivity at least in part *via* changes in leptin concentrations. In contrast, other studies have suggested that hypoxia is associated with a decrease in leptin level[62-64]. Yasumasu *et al*[62] have shown that hypoxia is associated with a decrease in leptin secretion in cultured rat adipocytes[62]. Furthermore, short-term hypoxia does not affect leptin in humans. Hypoxia for 8 wk in a neonatal animal model was not associated with marked changes in plasma leptin levels[64]. Therefore, further research is needed to establish the impact of hypoxia on leptin.

Mitochondrial dysfunction and endoplasmic reticulum stress

Hypoxia is known to inhibit biogenesis and respiration of the mitochondria[65]. Furthermore, hypoxia might also gradually decrease the number and function of the mitochondria and this could lead to insulin resistance[65]. We have shown that alteration in mitochondrial function is associated with NAFLD[66]. Hypoxia is known to induce endoplasmic reticulum stress and inhibition of this has been found to protect mice against insulin resistance. OSA is thought to induce endoplasmic reticulum stress in obesity[67-69]. An increase in endoplasmic reticulum stress is also associated with NAFLD[70].

OSA AND METABOLIC SYNDROME

NAFLD is regarded as the hepatic component of the metabolic syndrome[71]. In October 2009, a joint interim statement from the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and the International Association for the Study of Obesity was published that defined diagnostic criteria for identifying the presence of the metabolic syndrome, without having to resort to measurements that require sophisticated equipment[72]. Metabolic syndrome was defined by the presence of three of five criteria, including: increased waist circumference, elevated triglycerides, reduced high-density lipoprotein (HDL)-cholesterol levels, elevated blood pressure, and elevated fasting-glucose levels. In this new definition, waist circumference is not an obligate requirement for defining the syndrome and is one of five criteria that physicians can use when diagnosing the metabolic syndrome[72]. Furthermore, Vgontzas *et al*[73] have shown that sleep apnea patients have a significantly greater amount of visceral fat and insulin resistance compared to obese controls ($P < 0.05$), and indexes of sleep disordered breathing are positively correlated with visceral fat, but not with BMI or total or subcutaneous fat. This finding has led to the suggestion that OSA should be considered as part of the metabolic syndrome[73]. Coughlin *et al*[74] have shown that subjects with OSA are more obese, have higher blood pressure and fasting insulin concentrations, are more insulin resistant, and have lower HDL-cholesterol concentrations, which provides evidence that there is increased prevalence of metabolic syndrome (87% *vs* 35%, $P < 0.0001$). A regression analysis adjusted for age, BMI, smoking and alcohol consumption, has demonstrated that OSA is independently associated with increased systolic and diastolic blood pressure, higher fasting insulin and triglyceride concentrations, decreased HDL-cholesterol, increased cholesterol:HDL ratios, and a trend towards higher homeostasis model assessment values. Importantly, the authors have concluded that, in individuals with OSA, the prevalence of metabolic syndrome was 9.1 times higher (95% CI: 2.6-31.2, $P < 0.0001$) than in the general population. These data suggest that OSA is independently associated with an increase in the cardiovascular risk factors, and are supported by the work of Tkacova *et al*[75] who have shown that severe OSA is associated with an increased incidence of CVD independent of insulin resistance and obesity.

There could be a differing contribution of risk factors for OSA between ethnic groups. In 819 Japanese patients with OSA (719 men and 100 women) and 89 control subjects without OSA, metabolic syndrome was significantly more common in patients with OSA than in the controls (49.5% *vs* 22.0% for men, $P < 0.01$; 32.0% *vs* 6.7% for women, $P < 0.01$)[70]. Men and women with moderate and severe OSA have a higher risk of metabolic syndrome compared with controls. In men, age, BMI and OSA are significantly associated with metabolic syndrome, whereas, in women, BMI is the only risk factor for metabolic syndrome[76].

In a small study in China, the independent determinants of OSA in men and women were age, sex, BMI and the metabolic syndrome[77]. In another study in the Japanese population with OSA, the concurrent presence of metabolic syndrome constituted an additional cardiovascular risk factor[78]. From the evidence mentioned above, it is possible to conclude that OSA is associated with an increase in risk of CVD in the presence, (or absence), of metabolic syndrome. In addition, when the metabolic syndrome (including NAFLD) occurs in association with OSA, there might be a further increase in risk of CVD. Therefore, the complex relationship between OSA, metabolic syndrome and NAFLD to increase risk of CVD suggests the importance of identifying and treating NAFLD in individuals with metabolic syndrome and OSA.

OSA, NAFLD AND HYPERLIPIDEMIA

NAFLD is not only associated with insulin resistance but also with dyslipidemia[79]. Importantly, in numerous studies, NAFLD has been shown to be associated with an increase in risk of CVD. NAFLD is associated with increased incidence of CVD in type 2 diabetes[80-87]. Furthermore, OSA is associated with significant cardio-

vascular morbidity and mortality[88]. There is increasing evidence that OSA is associated with dyslipidemia in animal models as well as human studies. Acute exposure to hypoxia increases LDL-cholesterol concentrations but does not influence the concentration of cholesterol and fatty acids in rats[89]. Repeated exposure to hypobaric hypoxia causes a significant increase in the concentration of cholesterol, fatty acids, chylomicron, LDL-cholesterol and very-low-density beta-lipoproteins (VLDL) in rats, whereas the level of HDL-cholesterol decreases[89]. Furthermore, Li *et al*[90] have shown that, in leptin-deficient obese C57BL/6J-Lep(ob) mice, exposure to IH increases fasting serum levels of total cholesterol, HDL-cholesterol, and triglycerides, as well as liver triglyceride content. These changes are not observed in obese mice, which have hyperlipidemia and fatty liver at baseline. In lean mice, IH increases SREBP-1c levels in the liver, increases mRNA and protein levels of stearoyl-coenzyme A desaturase 1 (SCD-1), an important enzyme that is involved in desaturation of fatty acids, controlled by SREBP-1, and increases monounsaturated fatty acid content in serum, which indicates augmented SCD-1 activity. In addition, in lean mice, IH decreases protein levels of scavenger receptor B1, which regulates uptake of cholesterol esters and HDL by the liver[91]. In mice with a conditional knockout of SREBP cleavage-activating protein (SCAP) in the liver, which exhibits low levels of an active nuclear isoform of SREBP-1c (nSREBP-1c), IH does not have any effect on serum and liver lipids, and expression of lipid metabolic genes is not altered[89]. In wild-type mice, IH increases fasting levels of serum total and HDL-cholesterol, serum triglycerides, serum and liver phospholipids, mRNA levels of SREBP-1c and mitochondrial glycerol-3-phosphate acyltransferase (mtGPAT), and protein levels of SCAP, nSREBP-1, and mtGPAT in the liver. These data suggest that hyperlipidemia in response to IH is mediated in part *via* the SREBP-1c pathway[91], and we have previously suggested that modulation of SREBP provides a potential treatment of NAFLD[46].

In contrast, Savransky *et al*[92] have shown that C57BL/6J mice exposed to CIH and a high-cholesterol diet develop dyslipidemia, aortic atherosclerosis, and upregulation of SCD-1. Therefore, inhibition of SCD-1 might have the potential to prevent dyslipidemia and atherosclerosis during OSA. In another study by Savransky *et al*[93] in mice and in obese humans, C57BL/6J mice were exposed to CIH or normoxia for 10 wk while being treated with SCD-1 or control antisense oligonucleotides. In mice, hypoxia increased hepatic SCD-1 and plasma VLDL levels and induced atherosclerotic lesions in the ascending aorta (the cross-section area of 156 514 ± 57 408 μm^2, and descending aorta (7.0% ± 1.2% of the total aortic surface). In mice exposed to CIH and treated with SCD-1 antisense oligonucleotides, dyslipidemia and atherosclerosis in the ascending aorta were abolished, whereas there was a 56% decrease in lesions in the descending aorta. None of the mice exposed to normoxia developed atherosclerosis. Furthermore, Savransky *et al*[93] have studied obese human subjects who have undergone an intraoperative liver biopsy at the time of bariatric surgery for treatment of sleep apnea and obesity. In these patients, hepatic SCD mRNA levels correlated with the degree of nocturnal hypoxemia ($r = 0.68$, $P = 0.001$) and patients who showed oxyhemoglobin desaturation at night showed higher plasma triglyceride and LDL-cholesterol levels, compared to subjects without hypoxemia[93].

Modulation of HIF-1 activity could also be a precipitating factor for dyslipidemia with OSA. HIF-1 is a master transcriptional regulator of genes that are involved in physiological responses to hypoxia, including erythropoiesis, angiogenesis, and glucose metabolism. Li *et al*[94] have hypothesized that HIF-1 might be involved in dyslipidemia associated with OSA. They have performed a 5-d IH experiment using C57BL/6J (wild-type) or heterozygous *Hif1*$\alpha^{+/-}$ mice (with partial HIF-1α deficiency). During IH, *Hif1*$\alpha^{+/-}$ mice experienced blunted rises in serum triglycerides, liver triglycerides, light-phase fasting insulin, and glucose level, and attenuated transcription or translation of several liver lipid biosynthesis enzymes. HIF-1α deficiency diminished the rise of SREBP-1 and SCD-1 protein levels during IH without affecting serum cholesterol[94]. This suggests that, besides obesity, insulin resistance and a high intake of dietary cholesterol, modulation of HIF-1α could represent another factor that mediates hypoxia-induced dyslipidemia. In summary, hypoxia might lead to an increase in plasma and hepatic lipid profile through different factors and this could precipitate fatty liver.

Clinical studies have shown that CPAP is associated with a reduction in cholesterol and C-reactive protein. In two studies, CPAP was associated with a reduction in cholesterol, LDL-cholesterol, C-reactive protein and homocysteine[95,96]. In children with OSA, tonsillectomy improves parameters of the lipid profile such as LDL-cholesterol, apolipoprotein B and HDL-cholesterol[97].

OSA AND NAFLD AND MODULATION OF CVD RISK

Recently, Floras and Bradley have reviewed the association between OSA and CVD[98]. Their conclusion was that OSA is associated with an increased risk of CVD and this has been demonstrated in epidemiological, clinical and physiological studies. Epidemiological studies have shown a significant independent association between OSA and hypertension, coronary artery disease, arrhythmias, heart failure and stroke[99-105]. Although the association between NAFLD and OSA and CVD is not yet fully elucidated, from the evidence presented, it is tempting to postulate that the association between OSA and NAFLD accelerates atherosclerosis development. The complex interaction between OSA and NAFLD, and the fact that they share similar metabolic pathways that are well known to be associated with an increase in the incidence of CVD, suggest the need for clinical trials in this field (Figure 3).

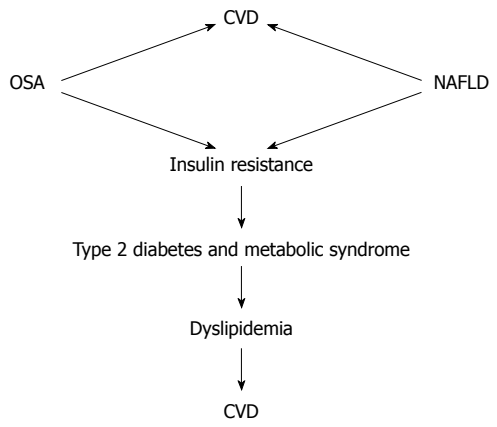

Figure 3 Association of non-alcoholic fatty liver disease and obstructive sleep apnea with cardiovascular disease. Whether the combination of non-alcoholic fatty liver disease (NAFLD) and obstructive sleep apnea (OSA) has a synergistic effect in the incidence of cardiovascular disease (CVD) needs to be demonstrated.

CONCLUSION

OSA is associated with NAFLD in experimental animals and in humans. Importantly, OSA can aggravate the development of NAFLD to NASH in obese individuals or those with metabolic syndrome. OSA might induce NAFLD in the absence of obesity and metabolic syndrome, and the link with hypoxia might be instrumental in precipitating fatty liver development. We suggest that the relationship between CVD, OSA and NAFLD requires further study to elucidate the precise nature of these relationships. Importantly, individuals with OSA require a full evaluation of their CVD risk, and clinicians should be aware that these individuals are also at increased risk of NAFLD.

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EDITORIAL

Endoscopic ultrasound in chronic pancreatitis: Where are we now?

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Abstract

Endoscopic ultrasonography (EUS) is well suited for assessment of the pancreas due to its high resolution and the proximity of the transducer to the pancreas, avoiding air in the gut. Evaluation of chronic pancreatitis (CP) was an early target for EUS, initially just for diagnosis but later for therapeutic purposes. The diagnosis of CP is still accomplished using the standard scoring based on nine criteria, all considered to be of equal value. For diagnosis of any CP, at least three or four criteria must be fulfilled, but for diagnosis of severe CP at least six criteria are necessary. The Rosemont classification, more restrictive, aims to standardize the criteria and assigns different values to different features, but requires further validation. EUS-fine needle aspiration (EUS-FNA) is less advisable for diagnosis of diffuse CP due to its potential side effects. Elastography and contrast-enhanced EUS are orientation in differentiating a focal pancreatic mass in a parenchyma with features of CP, but they cannot replace EUS-FNA. The usefulness of EUS-guided celiac block for painful CP is still being debated with regard to the best technique and the indications. EUS-guided drainage of pseudocysts is preferred in non-bulging pseudocysts or in the presence of portal hypertension. EUS-guided drainage of the main pancreatic duct should be reserved for cases in which endoscopic retrograde cholangiopancreatography has failed owing to difficult cannulation of the papilla or difficult endotherapy. It should be performed only by highly skilled endoscopists, due to the high rate of complications.

Key words: Endoscopic ultrasonography; Pancreatic neoplasms; Chronic pancreatitis; Contrast agents; Nerve block; Pancreatic pseudocyst; Drainage; Elastography; Main pancreatic duct

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INTRODUCTION

Chronic pancreatitis (CP) is an irreversible and progressive inflammatory process featuring pathological modifications of fibrosis, inflammatory infiltration, and destruction of exocrine and endocrine tissue, resulting in characteristic morphological changes in the parenchyma and pancreatic ducts. These modifications vary in intensity and distribution (diffuse or patchy). This has several consequences: (1) Biopsy specimens are difficult to obtain and not always

relevant, because they do not fully display the signs of CP; moreover, duct biopsy is usually avoided due to the risk of acute pancreatitis; (2) Most imaging methods reflects only partially the CP modifications, especially those typical for late stages of the disease; some methods, such as endoscopic retrograde cholangiopancreatography (ERCP) and magnetic retrograde cholangiopancreatography (MRCP) detect only the ductal features of CP; and (3) The findings of pancreatic function tests are not modified until a late stage in the natural history of the disease. Endoscopic ultrasonography (EUS) accomplishes the quality of being an imaging method able to detect both early and late changes in the parenchyma and pancreatic ducts.

The pancreas is well assessed by EUS due to the method's high resolution and the proximity of the transducer to the pancreas with the possibility of avoiding air in the gut. In patients with CP, EUS was performed initially for diagnosis, then for differential diagnosis, and later for therapeutic purposes (Figure 1).

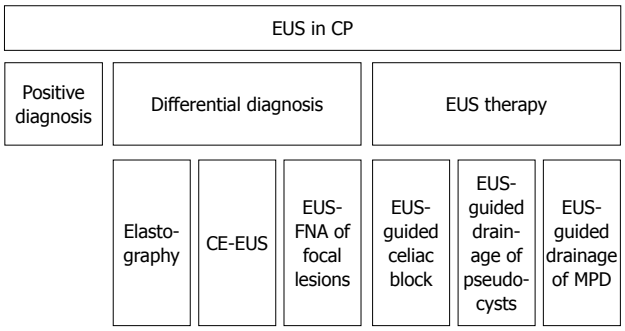

Figure 1 Flowchart of the endoscopic ultrasonography utility in chronic pancreatitis. EUS: Endoscopic ultrasonography; CP: Chronic pancreatitis; CE-EUS: Contrast enhanced endoscopic ultrasonography; MPD: Main pancreatic duct.

POSITIVE DIAGNOSIS

Despite its advantage of assessing the pancreas at very close range, EUS, being operator dependent, is still imperfect in establishing the diagnosis of chronic pancreatitis. The various pathological aspects of the disease are shown as different EUS features, and the same importance for diagnosis has been attributed to all of them. There have been several attempts to define the disease on ductal and parenchymal criteria, initially embracing 11 criteria[1,2], then focusing on nine factors corresponding to histopathological changes[3]: five parenchymal criteria (hyperechoic foci, hyperechoic strands, parenchymal lobularity, cysts, calcifications) and four ductal criteria (pancreatic duct dilatation, pancreatic duct irregularity, hyperechoic pancreatic duct walls, visible pancreatic side branches) (Figure 2). Very rarely are all these manifestations present simultaneously. Some of these features have been found also in elderly people[4], males (OR = 1.8, 95% CI: 1.3-2.55), persons with a history of alcohol consumption abuse (OR = 5.1, 95% CI: 3.1-8.5), smokers (OR = 1.7, 95% CI: 1.2-2.4), and those with history of acute pancreatitis[5-9]. Some features, like gland atrophy or lobularity aspect, can impede the complete assessment of all features (e.g. visualization of side branches of pancreatic ducts).

The interobserver agreement in one study using these criteria was moderate (k = 0.45), with good agreement only for duct dilatation and lobularity; the main drawback of the study was the limited experience of some examiners with pancreatic EUS. The most important criterion for the diagnosis was considered by all experts to be pancreatic stones, followed by visible side branches and lobularity, and the least significant was main pancreatic duct (MPD) dilatation[9]. In an EUS study in which both digital linear and radial echo endoscopes were employed, the interobserver variability also moderate (k = 0.50 and 0.61 respectively); the best concordance between the two methods was found for detection of cysts, calcifications, and visible side branches[10].

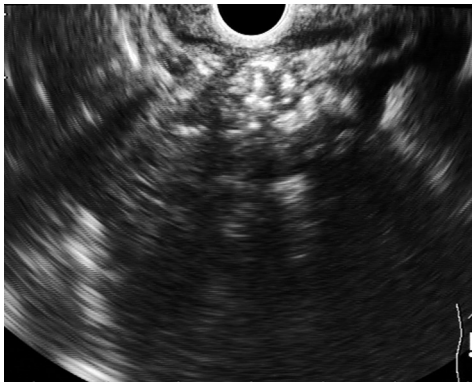

Figure 2 Chronic pancreatitis. Parenchymal and ductal pancreatic stones as hyperechoic structures with shadowing and stenosis of the main pancreatic duct.

Because histological evaluation of the pancreas is usually difficult, different gold standards have been used to establish the optimum number of EUS criteria for diagnosis of CP. The secretin direct pancreatic test has 85% sensitivity and 85% specificity for CP diagnosis, and the false-negative results are due to preserved pancreatic exocrine function[11]. Using one or two criteria for mild pancreatitis, three to five for moderate pancreatitis, and more than five for severe forms, the agreement with the secretin test as gold standard was 100% for normal parenchyma and severe disease, 50% for moderate forms, and 13% for mild disease[2]. On comparison of both EUS radial and linear assessment with the endoscopic secretin test during the same procedure, the best EUS accuracy was obtained for a cut-off point of more than four criteria (accuracy of 84% and 74%, respectively)[10]. The same group obtained lower sensitivity and specificity for diagnosis using four EUS criteria when cholecystokinin was used instead of secretin to test pancreatic function[12]. Comparison of assessment by non-blinded EUS (three to five criteria for diagnosis) and endoscopic retrograde cholangiopancreatography (ERCP; Cambridge classification) showed quite similar sensitivity (72% *vs* 68%) and specificity (76% *vs* 79%) for either mild or severe chronic pancreatitis, with the secretin endoscopic direct pancreatic test as the reference. However, the odds ratio for exocrine insufficiency was higher for EUS assessment than for ERCP[13]. To obtain the best specificity and

Table 1 Diagnostic value of endoscopic ultrasonography in chronic pancreatitis

Author	No. of pts	No. of EUS criteria	Threshold for CP diagnosis	Comparison	Sn	Sp	PPV	NPV	Acc
Wiersema *et al*[4]	69	11	> 3 = dg	EUS *vs* ERCP	100	79			
				EUS *vs* ERCP + secretin test	70	33			
				EUS *vs* ERCP + history	90	66			
Catalano *et al*[2]	80	11	1-2 mild	EUS *vs* secretin test	84	78			
			3-5 moderate	EUS *vs* ERCP	86.1	95.4			
			> 5 severe	EUS *vs* ERCP + secretin test	84.2	97.6			
Sahai *et al*[8]	126	9	> 2 for any CP	EUS *vs* ERCP			> 85	< 85	
			< 3 = fibrosis						
			> 6 = severe				> 85		
Conwell *et al*[14]	56	9	4-5 = equivocal	EUS *vs* ePFT	36	94	93	41	
			> 6 = definite		26	100	100	39	
Stevens *et al*[13]	83	9	3-5 = dg	EUS *vs* ERCP	68	79	83	62	
			6-9 = severe						
Stevens *et al*[10]	100	9	> 4	Radial EUS *vs* ePFT	68	95			84
				Linear EUS *vs* ePFT	44	95			74
Stevens *et al*[12]	50	9	> 4	EUS *vs* secretin ePFT	71	92			
				EUS *vs* CCK ePFT	63	85			
Zimmermann *et al*[23]	21	9	> 4	EUS *vs* histology (surgery)	78	73			
Varadarajulu *et al*[24]	21	9	> 4	EUS *vs* histology[1] (surgery)	90	85.7			88.1
Chong *et al*[25]	71	9	> 3 = dg	EUS *vs* histology[1] (surgery)	83.3	80			
			> 4 = severe fibrosis						
Bhutani *et al*[22]	11	9	> 3	EUS *vs* histology (autopsy)					

[1]Non-calcific chronic pancreatitis. ePFT: Endoscopic pancreatic function test; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; Sn: Sensitivity; Sp: Specificity; Acc: Accuracy; CCK: Cholecystokinin; PPV: Positive predictive value; NPV: Negative predictive value.

the best negative predictive value for diagnosis, six criteria were needed, however, the sensitivity was only 26%[8,14]. Secretin-stimulated EUS detected the features of CP better than EUS without secretin (12/13 patients) and the sensitized EUS seemed to be able to predict a favorable outcome or success of endoscopic treatment[15] (Table 1).

Using ERCP as gold standard, more than two criteria or three criteria, respectively, were found to be optimal for diagnosis[4,8]. The EUS sensitivity for diagnosis varied between 68% and 100% and the specificity was 78%-97% when ERCP was considered the gold standard (Table 1). The overall agreement with ERCP was $k = 0.51$, but the concordance for mild forms on EUS was only 83%. The factors most predictive for abnormal ERCP were ductal stones and parenchymal calcifications[4]. Among patients with a normal pancreatogram, 84.2% were found to have parenchymal changes of CP (accentuation of lobular pattern, focal areas of reduced echogenicity, hyperechoic foci) or increased ductal wall echogenicity. During follow-up (median 18 mo), 68% of patients with initially normal findings on ERCP progressed to an abnormal pancreatogram, supporting the importance of EUS description for early CP. However, this evolution was not confirmed in a second study of alcoholic chronic cirrhosis and CP[16,17]. Evaluation of images can be improved by computer-assisted image analysis[18].

The patient's history may be suggestive of CP. More than five features of CP were seen in 49.9% of 156 patients with persistent or non-specific dyspepsia[19]. Another study showed that there were more criteria for CP in the group with pain and steatorrhea than in the group with pain but no steatorrhea, so they concluded that history can be helpful in diagnosing CP[20].

Table 2 Correspondence between standard endoscopic ultrasonography criteria and pathologic features in chronic pancreatitis (adapted from Sahai AV 2002[21])

Standard EUS criteria	Pathologic features
Parenchymal criteria	
Hyperechoic foci	Small calcifications
Hyperechoic strands	Fibrosis
Lobularity	Edema or fibrosis
Cysts	Pseudocysts
Calcifications	Calcifications
Ductal criteria	
MPD dilatation	MPD dilatation
MPD irregularity	MPD irregular
Hyperechoic MPD walls	Ductal fibrosis or edema
Visible side branches	Dilated secondary branches

EUS: Endoscopic ultrasonography; MPD: Main pancreatic duct.

Pathologic diagnosis, the ideal gold standard, is rarely obtained from surgical specimens, EUS fine needle aspiration (EUS-FNA) or Tru-Cut core biopsies. The correspondence of EUS criteria to pathologic changes is shown in Table 2[21,22]. One recent paper showed that in postmortem pancreatic specimens the presence of more than three EUS standard criteria of CP correlated with the histologic diagnosis, but these features were also present in elderly persons dying of diseases other than CP[22] and in 59% of asymptomatic alcohol abusers[5].

Comparing the EUS standard criteria with the histologic findings from specimens obtained during surgery, fulfillment of five or more criteria was associated with sensitivity of 60% and specificity of 83%, compared with 87% and 64% respectively when three criteria were

Table 3 Rosemont consensus definitions

Rank	Features		Definition	Location
	Parenchymal features			
1	Major A	Hyperechoic foci with shadowing	Echogenic structures ≥ 2 mm in length and width that shadow	Body and tail only
2	Major B	Lobularity with honeycombing	Well-circumscribed, ≥ 5 mm structures with enhancing rims and relatively echo-poor centers, with ≥ 3 lobules	Body and tail only
	Minor	Lobularity with honeycombing	Well-circumscribed, ≥ 5 mm structures with enhancing rims and relatively echo-poor centers, with non-contiguous lobules	Body and tail only
3	Minor	Hyperechoic foci without shadowing	Echogenic structures ≥ 2 mm in length and width with no shadowing	Body and tail only
4	Minor	Cysts	Anechoic, rounded/elliptical structures with or without septations	Head, body and tail only
5	Minor	Stranding	Hyperechoic lines ≥ 3 mm in length in at least two different directions with respect to the imaged plane	Body and tail only
	Ductal features			
1	Major A	MPD calculi	Echogenic structures within the MPD with acoustic shadowing	Head, body and tail only
2	Minor	Irregularity of MPD contour	Uneven or irregular outline and ectatic course	Body and tail only
3	Minor	Dilated side branches	3 or more tubular anechoic structures each measuring ≥ 1 mm in width, budding from MPD	Body and tail only
4	Minor	MPD dilation	≥ 3.5 mm in body or > 1.5 mm in tail	Body and tail only
5	Minor	Hyperechoic duct margin	Echogenic, distinct structure greater than 50%of the entire MPD	Body and tail only

MPD: Main pancreatic duct.

used[23]. Good correlation with histology obtained during surgery of non-calcific CP was also found for the presence of four pancreatic features and for EUS findings of foci, stranding, lobulation, or ductal modifications. A limitation of this study was its use of surgical specimens secondary to neoplastic pancreatic disease[24]. Using surgical specimens obtained after preoperative EUS, three criteria were shown to differentiate abnormal from normal pancreatic tissue, but four criteria represented the limit for identification of severe fibrosis[25]. Again, the use of four EUS criteria compared with the association of ERCP, surgical pathology, and/or long-term clinical follow-up showed that EUS was more sensitive than MRCP but equally specific, and when both tests were abnormal the specificity was 100%[26]. Therefore, three or four criteria seems to suffice to rule out CP, but to establish the diagnosis at least six criteria are necessary[27].

The diagnosis of autoimmune pancreatitis is based on the same criteria, but for early stages (corresponding to Cambridge grade 0 to 2) the characteristic criteria are lobularity and hyperechoic pancreatic duct walls[28]. One study found diffuse hypoechoic areas, diffuse enlargement of the parenchyma, focal hypoechoic areas, and bile duct wall thickening as supplementary features characterizing autoimmune pancreatitis; these manifestations resolved after steroid treatment and were helpful in differentiation from ductal adenocarcinomas[29]. EUS-FNA is able to show a stromal structure with high lymphoid cellularity[30]. Lymphoplasmocytic sclerosing pancreatitis can be more accurately detected in tissue samples obtained by Tru-Cut biopsy[31]. With regard to the assessment of severity, preliminary data have pointed to significant diagnostic EUS features: hyperechoic foci for mild CP; hyperechoic foci, visible side branches, and duct dilatation for moderate CP; and visible side branches, duct dilatation, duct irregularity, and calcifications for severe CP[32].

Table 4 Rosemont diagnostic stratification

Stratum	Criteria
Consistent with CP	1 major feature A + ≥ 3 minor features 1 major feature A + major feature B 2 major feature
Suggestive of CP	1 major feature A + < 3 minor features 1 major feature B + ≥ 3 minor features ≥ 5 minor features (any)
Indeterminate for CP	3 or 4 minor features major feature B alone or with < 3 minor features
Normal	≤ 2 minor features[1]

[1]Excludes cysts, dilated main pancreatic duct, hyperechoic non-shadowing foci, dilated side branch. CP: Chronic pancreatitis.

Because the different pathological characteristics of CP vary in importance, the nine-criteria scheme assigning each criterion the same importance is insufficiently reliable and its diagnostic accuracy doubtful. The Rosemont classification, elaborated by international consensus, uses parenchymal and ductal criteria divided into major and minor features (Table 3). On this basis the findings are classified as "consistent with CP", "suggestive of CP", "indeterminate for CP", or "normal" (Table 4)[33]. This system, quite complicated and more restrictive in diagnosing CP, proved to agree with the diagnostic classification of the nine-criteria scheme in 74% of cases, increasing to 84% when "suggestive of CP" was included[34,35]. Using this system, the findings were similar for radial and linear EUS, with good results for parenchymal criteria (cysts 100%, hyperechoic foci 98%, lobularity/dilated ducts 94%) and modest results for dilated side branch, irregular pancreatic duct and hyperechoic wall of MPD[36]. In a recent multicenter study, 14 experts evaluated 50 recorded videos using the standard nine EUS criteria (diagnostic: > 4 criteria) and the Rosemont criteria (diagnostic: suggestive of CP or consistent

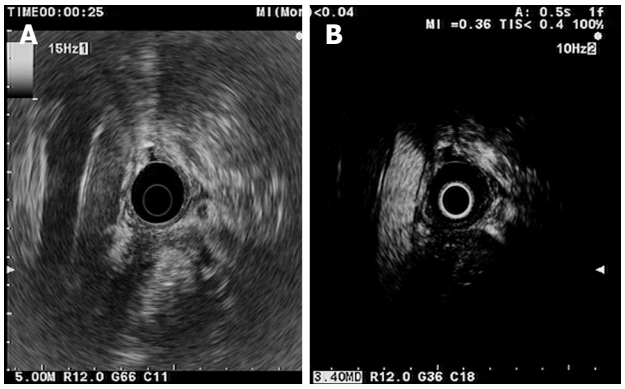

Figure 3 Mass resembling chronic pancreatitis. A: Conventional endoscopic ultrasonography (EUS). Hypoechoic inhomogeneous mass in the pancreatic head. Aorta and inferior caval vein are also seen; B: Contrast-enhanced harmonic-EUS. During the arterial phase (25 s after contrast injection) the abdominal aorta becomes hyperechoic and the mass is hypovascular compared with surrounding parenchyma.

with CP). They obtained substantial interobserver agreement for the Rosemont classification (k = 0.65) and moderate agreement for the standard classification (k = 0.54); the difference was not significant. The best agreement was noted for calcifications (standard scoring), pancreatic duct calcifications (Rosemont classification) and pancreatic duct dilation (both systems). The least agreement was seen for lobularity without honeycomb (Rosemont classification). This study used computed tomography (CT) and endoscopic pancreatic function test (ePFT) as gold standard, without histology. The patients were correctly classified as "definite CP" in 91.2% of cases (standard scoring) and 83.5% (Rosemont scoring); as "mild CP" in 50% (standard scoring) and 42.9% (Rosemont scoring); and "no CP" in 83.3% and 95.2% of cases respectively[37]. Further validation of the Rosemont classifications is needed.

Using EUS-FNA for diffuse CP, the negative predictive value increased to 100% against 75% for EUS, the specificity increased to 67% *vs* 60%, with higher concordance for severe disease than for mild CP[38]. Tru-Cut biopsy should not be recommended for non-focal CP because of complications[39], but its utility has been proved in autoimmune pancreatitis[31,40].

DIFFERENTIAL DIAGNOSIS

If focal hypoechoic lesion are found in the pancreatic parenchyma, the differential diagnosis includes primary or secondary pancreatic tumor, focal CP, and autoimmune pancreatitis. Several methods have been developed for this purpose.

Elastography

Elastography evaluates tissue strain resulting from compression and that strain is smaller in harder tissue than in softer tissue. Different tissue elasticity patterns are marked supplementary on the grey-color scale with different colors (blue for hard tissue and red for soft tissue). EUS elastography in CP shows a honeycomb aspect with predominantly hard strands, corresponding to fulfillment of four standard diagnostic criteria. The sensitivity, specificity, and accuracy were found to be 66%, 57% and 60%, respectively, and the method was considered useful in cases of equivocal EUS (three criteria or fewer)[41,42]. Further studies overcame the limitations of qualitative image analysis by means of digital image quantification, which helps to differentiate benign (normal pancreas and chronic pseudotumoral pancreatitis) from malignant lesions (pancreatic cancer and neuroendocrine tumors) with higher sensitivity, specificity, and accuracy (91.4%, 87.9% and 89.7%, respectively)[43]. Using a scoring system based on different color patterns in the images, the differentiation between benign and malignant pancreatic masses had sensitivity of 92.3% and specificity of 80%[44]. However, another study concluded that elastography did not allow complete delineation of the border of lesions greater than 35 mm in diameter or of lesions situated at some distance from the transducer, yielding poor sensitivity (41%), specificity (53%), and accuracy (45%) for predicting the nature of pancreatic focal lesions[45]. Because elastographic images are still difficult to obtain and interpret, although interobserver agreement is good (k = 0.725)[44], further improvement of the equipment with the possibility of quantification is expected. EUS elastography could have a special role in autoimmune pancreatitis, where the whole pancreas shows a typical, unique homogeneous stiffness, distinct from the circumscribed mass lesion in ductal adenocarcinoma[46].

Contrast-enhanced EUS

Ultrasound contrast agents increases the signal from the blood and improves the detectability of small vessels flow during ultrasound examinations. Before and after injection of Sonovue® (Bracco), the focal pancreatitis shows no detectable vascularization or the vessels appear regular over a distance of at least 20 mm, with detection of both arterial and venous vessels in the contrast-enhanced phase[47] (Figure 3). Based on the perfusion characteristics of microvessels, contrast-enhanced US facilitates differential diagnosis between inflammatory lesions and ductal adenocarcinoma. The specificity of the discrimination between benign and malignant focal pancreatic lesions was found to be 93.3% using power Doppler contrast-enhanced EUS (CE-EUS) compared with 83.3% for conventional EUS[47]. The hypovascular aspect of lesions under power Doppler CE-EUS seemed highly sensitive and specific (91.1% and 93.3%, respectively) for adenocarcinoma[48]. During power Doppler CE-EUS examinations the ultrasound frequency returned to the transducer is the same with that transmitted, but the method is associated with artifacts resulting from turbulent flow (blooming and overpainting). The use of contrast agents is preferred using harmonic frequencies which result from non-linear and non-symmetrical oscillation of the microbubbles. This yields an image with complete "subtraction" of the tissue-derived signal, optimized by using a low mechanical index, which allows continuous real-time assessment of the microvascularization during contrast medium uptake.

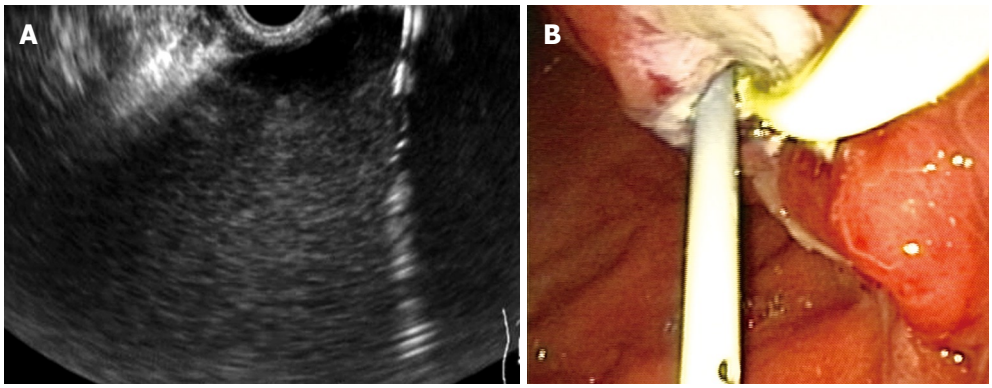

Figure 4 Endoscopic ultrasonography-guided pseudocyst drainage. A: The cystostomy is seen as a hyperechoic parallel structure inside the hypoechoic well-delineated pseudocyst; B: Endoscopic view of a stent and a nasocystic drainage placed transgastric into a pseudocyst.

Harmonic CE-EUS shows an iso vascular homogeneous pattern of CP[49] or, in severe forms, a hypovascular pattern, due to extensive fibrosis[50] (Figure 4A). Our results confirmed that severe CP may be hypovascular on harmonic CE-EUS, and quantitative assessment of images can improve differentiation between adenocarcinomas and chronic pancreatitis (accuracy of 86%) (unpublished data), but, similar to elastography, cannot replace the use of EUS-FNA.

EUS-FNA of focal lesions

The EUS sensitivity for detection of suspected pancreatic mass in a parenchyma with CP modifications was 100%, but the positive predictive value of pancreatic malignancy in these situations was only 60%, because some malignant masses present internal or peripheral calcifications, similar to focal CP[51]. The sensitivity of EUS-FNA for malignancy in parenchymal masses with features of CP is only 54%-74%, compared with 89% when the surrounding parenchyma is normal[51-55]. However, in the event of high suspicion of malignancy with negative EUS-FNA, repeated FNA yields a positive diagnosis in 84% of cases, whereas half of the failures of first biopsies are attributed to the presence of CP[56]. Kras mutation and allele deletion of the microsatellite or of the tumor suppressors can be reliably detected in EUS-FNA samples from pancreatic masses, improving the diagnostic accuracy[57,58]. The search for codon-12 Kras mutation revealed no cases in patients with pseudotumor CP, in contrast to the adenocarcinoma group, although 6%-12% of patients with diffuse CP and PanIN lesions had presented Kras mutations in a previous meta-analysis[59,60].

EUS THERAPY

EUS-guided celiac block

One of the therapeutic uses of EUS in CP is celiac plexus blockade, i.e. temporary inhibition of the celiac plexus using a combination of local anesthesia and steroids, with the aim of reducing pain and improving the quality of life[61]. This guidance is preferred to CT-guided blockade because the details of the region are better appreciated and the side effects are fewer and less severe[62]. Frequently the celiac ganglia can be seen as a unique or concatenate hypoechoic structure, less well delineated, with some whitish strands inside[63].

Some issues regarding EUS-guided celiac block remain to be resolved. The indication is pain in CP, but some studies included pain accompanying moderate pancreatitis[64] or patients with pain that had not responded to other forms of treatment[65]. Another unclarified issue is the technique of injection (central or bilateral) and the quantity of steroid needed. The majority of studies used the bilateral injection technique, considered equal in safety to central injection, but the results of the two techniques concerning the alleviation of pain were close and contradictory[64,66], showing the need for a placebo-controlled trial[67]. Direct injection of triamcinolone within the celiac ganglia (13 patients) compared with alcohol injection (5 patients) yielded disappointing results in respect of pain alleviation for steroid use (38% *vs* 80%)[68]. A comparative study of results between the celiac region injection and celiac ganglia injection for EUS-guided celiac block is still lacking.

The question of cost-effectiveness remains unresolved. Some studies followed up the patients for only 1-4 wk[66,68]. The only study with an extended follow-up period showed duration of pain relief of up to 673 d. This raises the question of whether the natural course of the disease may have been responsible, because there were no data indicative of the level of severity of CP: duration of disease from onset of pain, presence of diabetes, or calcifications[64].

In many studies, the alleviation of pain varied from 55% to 70% with a short duration of follow-up[64-66,69]. Persistence of pain alleviation for as long as 24 wk was seen in no patients[65] or in only 10% of patients[69]. Two meta-analyses showed efficacy in managing chronic abdominal pain in 51.46%[70] and 59.45%[71] of patients respectively. The rate of major complications seemed very low (0.6%), being represented by retroperitoneal abscess[72].

EUS-guided drainage of pseudocysts

Therapeutic intervention in patients with chronic pancreatic pseudocysts is indicated when at least one complica-

tion is present (compression of large vessels, obstruction of duodenum, stomach, or common bile duct, infection, hemorrhage into pancreatic pseudocyst, pancreatico-pleural fistula) or when symptoms occur (satiety, pain, nausea or vomiting, upper gastrointestinal bleeding)[73,74]. Since 1996, several series of EUS-guided drainage have been reported, especially for collections without bulging onto the gut wall or with parietal vessels due to portal hypertension[75-77]. The main limitation is location of the fluid collection further than 1 to 1.5 cm from the gut wall[78-80] (Figure 4).

This method is preferred to surgical drainage, which is associated with a high rate of mortality and morbidity[81]. However, a non-randomized case-control study showed the same rates of treatment success, complications, and reinterventions for surgical and EUS-guided drainage, but with lower costs and shorter hospital stay for the EUS-guided procedure[82].

Conventional endoscopic drainage and EUS-guided drainage were compared in four papers. In a prospective non-randomized study the two approaches seemed equally safe and effective[83], but this was not confirmed in a second non-randomized study, where EUS represented a salvage method in the case of failure of conventional endoscopic drainage owing to non-bulging pseudocysts or location in the tail of the organ, but was a more time-consuming procedure[84]. The conclusion of this second study was that EUS should be reserved for pseudocysts located in the tail of the pancreas, because these are unlikely to cause luminal compression or are technically difficult to access. Also, EUS assessment would identify a tumor in 5% of pseudocysts[84]. A third randomized clinical trial showed a significantly better success rate for EUS- than for conventional endoscopic-guided drainage (100% *vs* 33%), despite the small number of patients, even after statistical adjustment for luminal compression[85]. A fourth randomized study confirmed also a significant advantage for EUS over conventional endoscopic drainage (94% *vs* 72%); both were considered first-line methods for treatment of bulging pseudocysts, but the authors recommended that EUS-guided drainage should be preferred for non-bulging pseudocysts[86].

Several aspects of EUS-guided drainage remain to be elucidated. First among these is the issue of the means used to create the communication between gut and pseudocyst. There are two major techniques for obtaining this communication: (1) balloon dilatation of a previous puncture site, with a 93%-100% success rate[83,84,87-89]; and (2) coagulation of the communication site by means of a cystostomy (success rate of 95% when two procedures per patient were performed[90] and 71%-82% with one procedure per patient[91,92]), a Giovannini needle (success rate of 94%[93,94], but only 84% after the first attempt[86]), or a needle-knife, with the same success rate as balloon dilatation but a higher perforation rate[88,89,95,96]. Larger comparative studies will be necessary to assess the best device with the highest success rate and the lowest complication rate. The prototype "transluminal balloon accessotome", which combines a needle-knife and a dilating balloon, will probably allow easier drainage in one single step, reducing the exchange of accessories and simplifying the procedure[97]. Moreover, the use of the prototype three-layer puncture kit, which allows the simultaneous insertion of two guidewires at the initial puncture in one step, or the use of a larger working channel in the echo-endoscope, would allow safer and faster drainage[98]. Furthermore, the use of a forward-viewing echoendoscope seems promising for drainage of pseudocysts, even those inaccessible with a conventional therapeutic side-viewing EUS endoscope[99].

A further issue to be resolved is that of the morphological or biological factors that predict therapeutic success. Knowledge of such factors would facilitate selection of patients suitable for direct surgery. Moreover, to avoid pseudocyst relapse, described in 4%-17% of cases after 6-9 mo follow-up[94,96,100], communication with a secondary pancreatic duct, should be assessed very carefully.

EUS-guided drainage of main pancreatic duct

EUS-guided drainage of the MPD is a second-line procedure indicated when ERCP is unsuccessful owing to inability to cannulate the MPD (severe inflammation, previous surgery, postsurgical stricture) or difficult endotherapy (tight stenosis, large stone, MPD rupture, pancreas divisum). In practice, there are only few cases in which ERCP cannot be successfully performed by an experienced endoscopist, and recent studies suggests the superiority of surgery in managing pain. Thus, only a very small number of patients, namely those in whom ERCP fails and surgery cannot be performed safely, are good candidates for this procedure[101]. Using the transluminal approach or the transpapillary rendezvous approach, EUS-guided drainage of the MPD remains technically challenging because of difficulty in orienting the endoscope along the axis of the duct, difficult dilatation of the transmural tract due to pancreatic fibrosis, or the acute angle of the needle in relation to the MPD. Despite success rates of 68%-71%, the complication rates were important in all four series published (5%-43%); the complications included perforations, bleeding, pancreatitis, fever, and postprocedural pain[102-105]. EUS-guided drainage of the MPD should continue to be confined to tertiary care centers and very experienced endoscopists.

CONCLUSION

The diagnosis of CP is still accomplished using the standard scoring based on nine criteria each considered as having the same value. For diagnosis of any CP, at least three or four of these criteria must be present, but for diagnosis of severe CP more than six criteria must be fulfilled. The more restrictive Rosemont classification aims to standardize the criteria and assigns different values to different features, but requires further validation. EUS-FNA is less advisable for diagnosis of diffuse CP due to the possible side effects. Elastography and contrast-enhanced EUS are orientation in differentiating focal pancreatic mass, but they cannot replace EUS-FNA. The utility of EUS-guided celiac block for painful CP is still a matter of debate with

regard to best technique and the indications. EUS-guided drainage of pseudocysts is preferred especially in non-bulging pseudocysts or presence of portal hypertension. EUS-guided drainage of the MPD should be reserved for cases of unsuccessful ERCP caused by difficult cannulation of the papilla or difficult endotherapy. It should be performed only by highly skilled endoscopists, due to the high risk of complications.

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REVIEW

Intestinal epithelial cells in inflammatory bowel diseases

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Abstract

The pathogenesis of inflammatory bowel diseases (IBDs) seems to involve a primary defect in one or more of the elements responsible for the maintenance of intestinal homeostasis and oral tolerance. The most important element is represented by the intestinal barrier, a complex system formed mostly by intestinal epithelial cells (IECs). IECs have an active role in producing mucus and regulating its composition; they provide a physical barrier capable of controlling antigen traffic through the intestinal mucosa. At the same time, they are able to play the role of non-professional antigen presenting cells, by processing and presenting antigens directly to the cells of the intestinal immune system. On the other hand, immune cells regulate epithelial growth and differentiation, producing a continuous bi-directional cross-talk within the barrier. Several alterations of the barrier function have been identified in IBD, starting from mucus features up to its components, from epithelial junctions up to the Toll-like receptors, and altered immune responses. It remains to be understood whether these defects are primary causes of epithelial damage or secondary effects. We review the possible role of the epithelial barrier and particularly describe the role of IECs in the pathogenesis of IBD.

Key words: Intestinal epithelial cells; Epithelial barrier; Tight junctions; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel diseases (IBDs) - mostly represented by Crohn's disease (CD) and ulcerative colitis (UC) - are a group of inflammatory disorders of the gastrointestinal tract characterized by an abnormal immune response to antigens of the intestinal content that leads to a persistent inflammatory state[1].

Intestinal homeostasis in healthy subjects is ensured by a complex system called "intestinal barrier", a dynamic structure that separates intestinal contents from the host tissues, regulates nutrient absorption and allows interactions between the resident bacterial flora and the mucosal immune system.

The intestinal barrier is composed of a thick mucus layer containing antimicrobial products, a monolayer of intestinal epithelial cells (IECs) and an underlying set of cells (mesenchymal cells, dendritic cells, lymphocytes and macrophages)[2].

IECs are exactly at the centre of this system because of their anatomical and functional position: on the lumi-

nal side they secrete and regulate the composition of the mucus layer, while on the basolateral side they interact and cross-talk with the underlying cells.

By putting the IECs at the centre of the barrier system we can divide it into an "upper barrier" and a "lower barrier". The former constitutes a physical barrier, which prevents bacterial adhesion and paracellular diffusion to the underlying host tissues, and a functional barrier, which is able to discriminate commensal bacteria from pathogens; the latter operates by regulating antigen traffic through an intensive cross-talking with immune cells of the lamina propria (Figure 1A).

Given the importance of the epithelium in intestinal immune regulation mechanisms, it is clear how defects at one of these levels could be the primary pathogenetic mechanism that causes the loss of oral tolerance and therefore the establishment of an inflammatory response against luminal antigens, as happens in IBDs (Figure 1B).

Our purpose is to review the state of the art in understanding the key role played by IECs within the epithelial barrier system, in both healthy subjects and IBD patients.

THE UPPER BARRIER

The "upper barrier" is the intestinal epithelial single layer of columnar cells consisting of four IEC types: the absorbent enterocytes, the goblet cells, the Paneth cells and the enteroendocrine cells[3]. Upper barrier features are similar in small and large bowel. The main difference is constituted by the presence of elevations and projections (circular folds, villi and microvilli) in duodenum, jejunum and ileum that allows the increase of the absorption surface. This is not observed in the colon, which instead shows a flat surface.

Amongst the mucous membrane protrusions termed villi, there are inflections called crypts of Lieberkühn, which are distinct glandular invaginations.

Enterocytes are the most representative type of cells, which present finger-like projections, known as microvilli. These arise on the luminal side of the cells, constituting the so-called buffy coat, and are completely coated by glycocalyx and joined by apical junctional complexes, which prevent the entry of pathogens by keeping the cells tight.

In addition, the enterocyte monolayer is interrupted by the presence of goblet cells, secreting mucus, and by the presence of enteroendocrine cells that produce peptide hormones. These hormones are involved in cellular trophism, tissue repair, angiogenesis, enterocyte differentiation and polarization along the crypt-villus axis.

The epithelial cells derive from multipotent stem cells located at the base of the crypts. When these cells reach their maturity, they migrate toward the top of the villus, where aged cells are expelled in the intestinal lumen.

In the depth of the crypts there are also the Paneth cells that have regulatory functions since they produce antimicrobial peptides - called defensins - which constitute an inducible system against pathogens.

Hence, it is clear that the epithelial layer represents an anatomical and functional barrier, an upper interface,

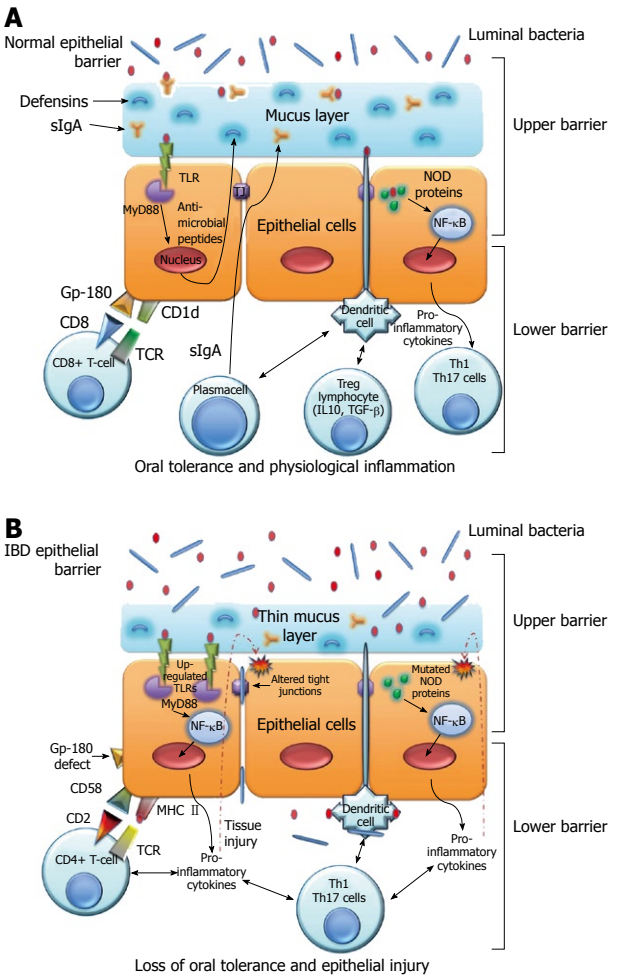

Figure 1 The epithelial barrier system. A: Normal epithelial barrier; B: Inflammatory bowel disease (IBD) epithelial barrier. TLR: Toll-like receptors; MyD88: myeloid differentiation factor 88; TJ: Tight junctions.

which is needed to maintain the whole intestinal homeostasis.

Evidence demonstrates that impairment of the upper barrier plays a key role in the pathogenesis of IBD. In fact, alterations of the mucus layer, disabled epithelial junctions, increased permeability and a defective production of antimicrobial peptides have been observed in CD as well in UC[2].

Mucus layer

The upper barrier presents a mechanical external protection, which consists of a thick mucus layer (about 150 μm) synthesized by goblet cells and full of several molecular peptides. The latter allow mucus to act as a chemical boundary, preventing pathogen invasion.

The most important role of mucus in protecting the mucosa is carried out by its viscosity, due to the concomitant presence of glycosylated mucins and trefoil factors (TFFs) which modulate the defensive properties of the mucus layer, most likely through the check of mucin polymerization and by trapping microbes, which would otherwise be conveyed by the peristaltic process[4]. TFFs are a family of small, yet abundant, secreted proteins

which maintain the epithelial continuity and restitution[5].

Furthermore, Mashimo *et al*[6] demonstrated that mice lacking intestinal trefoil factors (ITF) had impaired mucosal healing and died after oral administration of dextran sulfate sodium (DSS).

Taken together, these findings highlight the central role for TFFs in the maintenance and repair of the intestinal mucosa.

As mentioned previously, mucus is composed of a large and complex variety of molecules. The most representative element is mucin, a glycoprotein encoded by the *MUC2* gene and synthesized by goblet cells. Another important component is constituted by trefoil factors (mainly TFF3), which are small protease-resistant peptides, secreted together with the mucin by the same kind of cells[7,8]. A recent finding demonstrates that TLR2 activation induces synthesis of TFF3 which protects the inflamed mucosa during acute intestinal injury, such as IBD[9].

The other constitutive elements of the mucus are secretory immunoglobulins, especially sIgA, produced by B lymphocytes; antimicrobial peptides such as defensins and lectins, secreted by Paneth cells; antimicrobial protease inhibitors, synthesized by epithelial cells; and enterocyte hydrophobic phospholipids[2,10].

In IBD, a substantial reduction of trefoil factors, which leads to the production of less viscous mucus, has been found. This fact proves that mucus viscosity and not its thickness, as thought previously, is the most important factor in the protection of the epithelium[11]. For instance, in CD we observe goblet cell hypertrophy with an increase of mucus production and a weakness of antimicrobial activity of defensins and peptides[12].

UC is instead characterised by a reduction of MUC2 expression, by a thinning of the mucus layer and by a decreased goblet cell number. Despite this, UC is often clinically represented by a mucus diarrhea, because of a worsening of mucus quality, probably due to an accumulation of non-glycosylated mucin[13].

These alterations could lead to a lessened capability of the mucus layer to limit antigenic traffic and bacterial translocation in the lamina propria, but it is still to be clarified if they are primary defects or secondary effects of the inflammatory state[14].

Epithelial junctions

The absorbent IECs regulate intestinal permeability through the epithelial junctions, by limiting access of microbes to host tissues and mediating the antigenic traffic from the lumen to the lamina propria, where antigens are processed, presented and eliminated. The junctions consist of desmosomes, adherent junctions (AJs) and tight junctions (TJs), necessary for maintenance of intercellular adhesion and to regulate paracellular transport[3].

The adhesive junctional complexes are characterized by transmembrane proteins that interact with adjacent cells and with intracellular adaptor proteins, which are linked to the cytoskeleton. All together they form a connecting network[15].

The most representative structure of AJs is formed by cadherin-catenin interactions, which not only connect the junctional complex to the cellular cytoskeletal network but also help to maintain cell polarity by regulating epithelial migration and proliferation[16,17].

A dysfunction of AJ proteins has been described and consists of a down-regulation of E-cadherin, which weakens intercellular adhesion. This could be responsible for promoting intestinal inflammation, such as in IBD[18]. However, the most important impact on IBD pathogenesis seems to be the impairment of the tight junctions[19].

The tight junctions, located at the apical end of the intercellular space, consist of a complex structure composed of different proteins, such as hyperphosphorylated occludins, proteins of the zonula occludens and proteins of the claudin family[20]. Far from being static structures, tight junctions are highly regulated by cytokines, which play a central role in modulating intestinal barrier function.

Recent studies have showed that proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, induce a downregulation of the constituent proteins of the tight junctions, mainly zonula occludens-1. This phenomenon can produce an actin-cytoskeletal disarrangement. Probably these proinflammatory cytokines induce internalization of the apical-junctional complex due to an increase in macropinocytosis of the tight junction proteins[21-23].

Other recent studies have demonstrated that IFN-γ and TNF-α can cause a reorganization of numerous tight junction proteins such as zonulin-1, JAM-A, occludin, claudin-1 and claudin-4, increasing intestinal permeability[24].

A downregulation of occludin, claudin-5 and claudin-8 has been found in CD; an upregulation of claudin-2 has been observed in UC. The expression of this pore-forming protein is due to the stimulation of IECs by interleukin (IL)-13. This cytokine induces an increase of barrier permeability and promotes the epithelial apoptotic events in UC[25-27].

However, the extent of defects in the barrier is more significant in UC than in CD, because of an early presence of apoptotic foci that degenerate into erosion and ulcer-type lesions, which has already occurred in the mild stage of disease[28,29].

Overall, the increase of proinflammatory cytokines leads to an impairment of tight junctions and consequently to a loss of barrier function.

Transforming growth factor (TGF)-β, prototype of anti-inflammatory cytokines and produced by Th3 regulatory cells, can preserve the integrity of the tight junctions by acting directly on IECs, probably through a cAMP-dependent mechanism[30]. Hence, regulatory T cells are involved not only in suppressing inflammatory responses but also in preserving the integrity of the tight junctions.

Many studies have shown that TGF-β also plays a role in epithelial restitution, which occurs after an injury, and its secretion is promoted by the wounded epithelium itself[31].

Defensins

The intestinal mucosa produces antimicrobial peptides, called defensins, which contribute to maintaining host im-

munity and protect from pathological flora. The antimicrobial activity of defensins is expressed by the formation of micropores in the bacterial membranes that cause the lost of pathogen integrity.

In IBD there is a deficiency in defensin expression; however it is not clear whether this alteration contributes to the pathogenesis or is a secondary phenomenon[32].

The gastrointestinal mucosa produces ten types of defensins that help protect the epithelium from microbes. They play an important role, especially in protecting epithelial stem cells, thanks to their location in small bowel at the base of the crypts of Lieberkühn[33].

Defensins can be differentiated into two groups: α-defensins and β-defensins. Their type-expression is modulated along the different intestinal sections. For instance, α-defensins (HD) - especially HD5 and HD6 - are synthesized by Paneth cells positioned in the crypts, while β-defensins (HBD) are produced by colonic epithelial cells[34,35].

These peptides are produced as pro-peptides, a precursor form that needs an enzymatic digestion by trypsin to turn them into their active form. This process is necessary to allow a conformational folding of these proteins, helpful to accomplish their function.

Evidence demonstrates that in CD there is a defective expression of HD5 and HD6 with a release of non-functional peptide that forms an unfolded structure, probably due to a defective enzymatic digestion[36]. A decreased concentration of defensins is responsible for the presence of a less efficient mucus membrane as a biochemical barrier for pathogenic bacteria.

The cause of deficient defensin production has not been clearly determined but the NOD2 signalling pathway could be involved in this process. In fact, NOD2 receptors are highly expressed in Paneth cells and an association between *NOD2* gene mutation and a reduced expression of HD5 mRNA in Paneth cells of CD patients has been found by Wehkamp *et al*[37]. However, it should be pointed out that the primary cause of the α-defensin deficiency is due to an epithelial cell loss[38].

As stated above, HBD are produced by colonic epithelial cells. The type HBD1 is constitutively expressed in all subjects and its concentration does not change, in spite of the presence of inflammatory cytokines or bacteria. In contrast, HBD3 and HBD4 are minimally represented in normal intestinal mucosa and their expression is maintained in CD, and increased in UC. The most important difference in IBD patients with respect to controls is provided by HBD2 production, which is significantly increased in these patients, especially in active UC. This fact is probably due to an increase of pro-inflammatory cytokines[39].

Conversely, in CD, it seems that genetic factors induce a lower expression of inducible β-defensins by means of a suppression of nuclear factor (NF)-κB, which is caused by a direct mechanism or a *NOD2* mutation[40].

IECs as sentinels of innate immunity: Toll-like receptors and NOD signaling

The innate immune system is able to recognize a limited set of conserved bacteria and viral motifs known as pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs), including above all the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) families.

TLRs are a family of receptors which recognize specific PAMPs and activate signal transduction through the NF-κB pathway. As a consequence, a pro-inflammatory cascade initiates to induce cytokine and chemokine genes[41]. Activation of the TLR pathway occurs through an adapter molecule, myeloid differentiation factor 88 (MyD88)[42,43].

IECs express several members of the TLR family: TLR2, which recognizes peptidoglycan (PGN), a component of the bacterial cell walls of Gram+ bacteria; TLR3, a receptor for viral double-stranded RNA; TLR4, which recognizes lipopolysaccharide (LPS), the major component of the outer membrane of Gram- bacteria; and finally, TLR5, which binds bacterial flagellin. TLR1, TLR3 and TLR4 are all located on the apical surface of IECs; alternatively, TLR5 is restricted to the basolateral surface of IECs and it is only activated when bacteria invade the epithelium[42].

These findings suggest that PRRs are positioned in order to trigger a response in the event of bacterial penetration of the epithelium. Therefore, pathogens, which unlike commensals are able to penetrate the barrier, are recognized by basolateral TLRs[44]. Moreover, TLRs bind saturated fatty acids in acetylated form, which are essential for the agonistic activity.

Wolowczuk *et al*[45] report that saturated fatty acids are able to induce the activation of TLR2 and TLR4, whereas unsaturated fatty acids - such as PUFAs - inhibit the TLR-mediated signaling pathway and gene expression by suppressing NF-κB activation and inflammation. These data suggest the protective role of unsaturated fatty acids such as omega-3 and the regulation of immune responses by fatty acid types.

Recognition by TLRs protects against pathogens and is carefully regulated to shut down a proinflammatory response to commensal organisms[41].

An interesting observation is that various TLRs are also expressed in cells of the adaptive immune system, such as B and T cells and dendritic cells (DCs), inducing differentiation and cytokine production, connecting innate to adaptive immunity. We can consequently consider the TLRs as a link from upper to lower barrier function.

For all these reasons it is clear that TLRs may have a dual role. Under normal conditions they maintain oral tolerance and eliminate pathogens, while in IBD they can also amplify inappropriate immune responses.

Several polymorphisms of TLRs have been associated with IBD, such as those of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9. However, the functional effects of these variants are not well defined[43].

Szebeni *et al*[46] have demonstrated that in IECs of IBD patients, there is an abnormal production of certain TLR subtypes with a significant upregulation of TLR2 and TLR4 expression in the inflamed mucosa. These alterations could compromise the capability to distinguish

commensals from pathogens, or amplify inappropriate immune responses. It is still to be clarified whether this upregulation is one of the causes of IBD or just one of the consequences or even a concomitant factor.

NOD2 is a gene encoding for a cytoplasmatic protein (also known as CARD15), which recognizes bacterial muramyl dipeptide (MDP). This is the major component of peptidoglycan (PGN) and it is present in both Gram+ and Gram- bacteria. The binding of MDP to NOD2 results in the activation of the NF-κB pathway and IL-12 production[47].

In recent years researchers have identified a large number of *NOD2* polymorphisms (SNPs) and the most common are associated with susceptibility to CD[48].

It seems that NOD2 variations lead to an impaired intracellular microorganism recognition and a consequent perpetual nuclear translocation of NF-κB, which results in an inadequate phlogosis state.

Using transgenic mice, Watanabe *et al*[49] have demonstrated that mice overexpressing *NOD2* exhibit greatly decreased IL-12 responses to systemic administration of PGN but not to LPS, indicating that defects in NOD2 signaling lead to excessive TLR2-dependent inflammatory responses. Indeed, under normal conditions, PGN from commensal bacteria leads to innate immune responses, which are subsequently made weak by NOD2 modulation and other regulatory responses[49]. In the case of a NOD2 signaling defect, a TLR2-dependent inflammatory response cannot be controlled, leading to mucosal injuries[50].

Strober's group demonstrated that mice with cells with increased NOD2 function have decreased responses to TLR stimulation, resulting in protection against DSS-induced colitis. They showed that prestimulation of cells with NOD2 ligand renders them unresponsive to TLR stimulation, because of an inhibitor of TLR-induced inflammatory pathways (IRF4)[51,52].

Greater understanding of the relationship between NOD2 variations and the pathomechanisms of IBD is required, but recent studies indicate that these mutations could participate, together with other barrier dysfunctions described previously, in the progression to CD[48].

Bacterial-epithelial interactions

TLRs and NOD proteins are also key sensors of bacterial-epithelial interactions. The intestinal microbiota contribute to protecting the host against invasions by pathogenic bacteria, competing for nutrients and stimulating immune responses, and play a crucial role in correct epithelial cell development. In return, commensal bacteria derive benefits from this association with the host since they can inhabit a protected environment from which they receive nutrients[53].

Within the bacterial-epithelial interface there is a continuous cross-talk which is enabled by PRRs; components of the mammalian innate immune system continuously sample the composition of commensal communities. However, only pathogens can activate the innate immune system since they can survive within host tissues.

A recent study showed that the immune status of the host can influence the composition of the commensal community. For instance, in the intestinal epithelium of *Drosophila melanogaster*, the dysregulation of NF-κB-dependent expression of antimicrobial peptides results in the outgrowth of a pathogenic commensal community[54].

In turn, scientific evidence also shows that commensal bacteria modulate IEC function by inhibiting the NF-κB pathway, through blocking the ubiquitylation and degradation of IκB or by hijacking the peroxisome-proliferation-activated receptor-γ (PPARγ) pathway[44].

Several pathologic features of IBD suggest that they derive in part from dysregulated control of bacterial interactions with the mucosal surface. For example, as reported by Duerkop *et al*[55] demonstrated that IBD patients exhibit increased numbers of mucosal surface-associated bacteria. This evidence suggests a failure of the mechanisms to prevent the microbiota from direct contact with the surface epithelium.

In 2006, Mizoguchi found that chitinase 3-like1 (CHI3L1), a molecule characterized by a strong binding affinity to chitin (a polymer of N-acetylglucosamine richly found in microorganisms), is specifically up-regulated under intestinal inflammatory conditions; it plays a pathogenic role in acute colitis by enhancing bacterial adhesion and invasion into colonic epithelial cells[56].

Many studies have investigated the presence of specific potentially pathogenic microorganisms in the mucosa of IBD patients; Darfeuille-Michaud *et al*[57] have demonstrated the association between ileal CD and adherent-invasive *Escherichia coli* (AIEC), not only as secondary invaders but also as possibly responsible for the initiation of the inflammatory process.

Moreover, it was demonstrated that CD-associated AIEC strains adhere to the brush border of primary ileal enterocytes isolated from CD patients but not from controls; AIEC adhesion is dependent on type 1 pili expression on the bacterial surface and on carcinoembryonic antigen (CEA)-related cell adhesion molecule 6 (CEACAM6) expression on the apical surface of ileal epithelial cells in CD patients. CEACAM6 is up-regulated in intestinal epithelial cells of CD patients and acts as a receptor for AIEC adhesion. Finally, this study suggests that AIEC can promote its own colonization in CD patients, since it is able to increase CEACAM6 expression in infected epithelial cells[58].

THE LOWER BARRIER

A continuous epithelium: lymphocytes cross-talking

Recent advances regarding an active role of intestinal epithelial cells within the mucosal immune system have revealed that they act as non-professional antigen presenting cells (APCs), activating subsets of T-cells with regulatory function. On the other hand, lamina propria lymphocytes are able to influence epithelial cell growth and differentiation along the crypt/villus axis by mediating intercellular interactions and by secreting cytokines and other fac-

tors[41]. In IBD patients, the cross-talking between IECs and mucosal lymphocytes is changed by altered production of these factors. This complicated dialogue contributes to promoting mucosal inflammation.

IECs are able to directly process and present antigens to lymphocytes by a highly polarized system with apical antigen sorting, processing and exclusively basolateral presentation.

IECs do not express conventional costimulatory molecules such as CD80 and CD86, but express new B7 family members, such as B7h [inducible costimulatory ligand (ICOS-L)] and B7-H1 [programmed death ligand (PD-1 L)], as well as several non-classical MHC class Ib molecules such as MICA/B, HLA-E, and CD1d[42,54].

By using *in vitro* IEC: peripheral blood T-cells (PBT) co-cultures, Allez *et al*[59,60] have demonstrated that intestinal epithelial cells preferentially activate CD8+ regulatory T-cells, in particular a CD28- and CD101/CD103+ subset, characterized by the biased usage of the T cell receptor (TCR) Vβ5.1 chain. These data have been confirmed by the same group *in vivo* by using CD8+ T-cells isolated from the lamina propria (LP) of healthy subjects.

Hence, under normal conditions, luminal antigens presented by IECs cause a suppression rather than an increase of the immune response.

For the activation of the restricted regulatory T-cell subset, the role is crucial of a unique complex formed by gp-180 (a CEA family member glycoprotein) and by CD1d, which bind CD8 and the TCR on the T-cell surface, respectively. Indeed, blocking gp-180 with a monoclonal antibody (B9) suppresses the proliferation of regulatory T-cells[61-63]. This helps to explain the oral tolerance and controlled inflammation phenomena.

Results from both *in vitro* IEC:PBT co-cultures and from *in vivo* within the lamina propria of IBD patients demonstrated a reduced amount of CD8+ regulatory T cells, that might be linked to glycoprotein gp-180. Indeed, the frequency of Vβ5.1 cells among the LP CD8+ T cells is significantly decreased in IBD patients with respect to healthy subjects[64].

In IBD IECs, especially in CD, the same group observed a defective expression of gp-180, and moreover, IECs from IBD patients preferentially stimulate CD4+ T-cell proliferation and secretion of IFN-γ, through MHC class Ⅱ[65,66].

In a subsequent study, by using freshly isolated IECs and lamina propria lymphocytes (LPLs), as well as T84 cell lines, we have correlated SOX9 to *CEACAM5* gene expression: SOX9 is able to downregulate CEACAM5. The former is a transcription factor involved in the differentiation of several tissues such as chondrocytes, male gonads, neural crest and spinal cord glial cells, while the latter is a member of the CEA family. We speculate that LPLs, the main source of cytokines within the gastrointestinal mucosa-associated lymphoid tissue (GALT), influence the nuclear translocation of SOX9 in IECs and consequently the downregulation of the CEA family member gp-180, together with a lack of activation and/or expansion of regulatory cells[67,68].

As mentioned previously, the described cross-talk between epithelium and LPLs has also a role from the standpoint of IECs, in particular affecting their proliferation and differentiation along the crypt/villus axis in the colon. Indeed, starting from the concept that IECs can promote regulatory T-cell responses in the mucosa, Dahan *et al*[41] have demonstrated that lympho-epithelial interactions occur and play a role in IBD. By using freshly isolated LPLs derived from healthy subjects and CD patients, they suggest that cross-talk leads to an enhanced IEC differentiation, a pattern restricted to CD, which seems to involve the transcription factor CDX2 and PI3K/p38 MAPK pathways.

Moreover, T84 cells co-cultured with CD LPLs display a greater increase of differentiation and CDX2 mRNA levels with respect to normal LPLs.

These data were confirmed *in vivo*, through immunostaining both in human colonic mucosa and in RAG1-/- mice lacking lymphocytes; studies in which an altered IEC differentiation was observed[41,69,70].

CONCLUSION

Our purpose was to review the state of the art in understanding the key role of intestinal epithelial cells in maintaining gut homeostasis and the possible role in the pathogenesis of IBD.

Intestinal epithelial cells, because they act as a functional barrier and as non-professional antigen presenting cells, represent important elements in the development and maintenance of immune oral tolerance.

In IBD, we observe a global defect in the mucosal immune system: barrier function, innate and adaptive responses.

Two main strands of research on these defects exist: one is focused on the impairment of the epithelial barrier, the other on defects of epithelial-lymphocyte cross-talk. In both these lines of investigation, IECs occupy a prominent place within the complex and dynamic system of the intestinal barrier. Despite much progress in this area of research, it remains to be clarified whether defects involving IECs are fundamental or a consequence of abnormal signals coming from the lamina propria.

A better understanding of these regulatory mechanisms, which allow us to see intestinal epithelial cells at the interface between an "upper" and "lower" barrier, could help to identify new therapeutic targets.

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ORIGINAL ARTICLE

α,β-amyrin, a natural triterpenoid ameliorates L-arginine-induced acute pancreatitis in rats

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Abstract

AIM: To study the beneficial effects of triterpene α,β-amyrin and the underlying mechanisms in an experimental pancreatitis model.

METHODS: Acute pancreatitis was induced in five groups of rats (n = 8) by L-arginine (2 × 2.5 g/kg, intraperitoneal, 1 h apart) and 1 h later, they received a single oral dose of α,β-amyrin (10, 30 and 100 mg/kg), methylprednisolone (30 mg/kg) and vehicle (3% Tween 80). A saline (0.9% NaCl) treated group served as a normal control. Efficacy was assessed at 24 h by determination of serum levels of amylase, lipase and pro-inflammatory cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-6], pancreatic myeloperoxidase (MPO) activity, lipid peroxidation [thiobarbituric acid reactive substances (TBARS)], nitrate/nitrite levels, and the wet weight/body weight ratio. Tissue histology and the immunoreactivity for TNF-α and inducible nitric oxide synthetase (iNOS) were performed.

RESULTS: α,β-amyrin and methylprednisolone treatments significantly (P < 0.05) attenuated the L-arginine-induced increases in pancreatic wet weight/body weight ratio, and decreased the serum levels of amylase and lipase, and TNF-α and IL-6, as compared to the vehicle control. Also, pancreatic levels of MPO activity, TBARS, and nitrate/nitrite were significantly lower. Histological findings and TNF-α and iNOS immunostaining further confirmed the amelioration of pancreatic injury by α,β-amyrin.

CONCLUSION: α,β-amyrin has the potential to combat acute pancreatitis by acting as an anti-inflammatory and antioxidant agent.

Key words: Acute pancreatitis; L-arginine; Cytokines; Lipid peroxidation; α,β-amyrin

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INTRODUCTION

Acute pancreatitis (AP) is a life-threatening inflammatory disorder with a significant impact on patient health. Although its pathogenesis is not fully understood, microcirculatory disturbances, leukocyte activation, and oxidative stress are the main events in AP that is characterized by activation of digestive proteases, a widespread inflammatory cell infiltration, leukocyte activation and the release of various kinds of inflammatory mediators and reactive oxygen and nitrogen species[1-4]. Repeated attacks of acute pancreatitis have the potential to evolve into chronic disease that is characterized by fibrosis and loss of pancreatic function[5]. There are no specific therapies for acute pancreatitis. Medical management is aimed at the control of symptoms with anti-inflammatory agents, steroids, and analgesics. As a result of the limitations of conventional therapy, many ethnobotanical agents are being pursued as alternative sources to develop novel and safe therapeutic agents to treat pancreatitis[6-11].

The resin (oily amorphous exudate) obtained from the trunk wood of *Protium heptaphyllum* (*P. heptaphyllum*) (Aubl.) March (Burseraceae) that grows abundantly in Brazil and South America is a reputed folk medicinal agent because of its analgesic and anti-inflammatory properties[12,13]. Chemical investigations have revealed the presence of α,β-amyrin, a pentacyclic triterpene as the major component of resin, and pharmacological studies have revealed its anti-inflammatory, antipruritic, gastroprotective and hepatoprotective effects[14-17]. Also, a few other studies have shown its efficacy in suppressing the acute visceral and orofacial nociception and bladder inflammation[17,18]. Recently, Vitor *et al*[19] have demonstrated that α,β-amyrin exerts a marked and rapid suppression of inflammatory cytokines and cyclooxygenase-2 levels in a murine model of trinitro-benzene-sulfonic acid (TNBS)-induced colitis. Since these studies have established the anti-inflammatory, antinociceptive, and antioxidant properties of α,β-amyrin at non-toxic doses, which range from 10 to 100 mg/kg, the present study evaluated its potential to ameliorate pancreatic injury in a rat model of acute pancreatitis induced by L-arginine, wherein inflammation and oxidative stress play a pathogenic role.

MATERIALS AND METHODS

Plant material and isolation of α,β-amyrin

The resinous exudate from the trunk wood of *P. heptaphyllum* (March.) was collected from the municipal areas of Timon, Maranhão state of Brazil, after its identification by botanist Professor Roseli Farias de Melo Barros. A voucher sample (#18247) has been deposited at the Herbarium Graziela Barroso of the Federal University of Piauí, Teresina, Brazil. The extraction and isolation of α,β-amyrin from the crude resin of *P. heptaphyllum* (March.) was carried out as described earlier[20] and its structural identity was confirmed by ^{1}H- and ^{13}C-NMR spectral analysis, based on the method developed by Gallegos *et al*[21] and in comparison to literature data[22]. The ratio of α- and β-amyrin in this mixture was 63:37.

Animals and animal procedures

Forty-eight male Wistar rats obtained from the Central Animal House of Federal University of Ceara, Fortaleza were maintained at a constant room temperature (23 ± 2℃) with light-dark cycles of 12/12 h and free access to water and standard laboratory chow. The rats were randomly divided into six groups of eight in each and experiments were performed after 12 h of fasting. Their body weights ranged between 180 and 200 g at the time of experimentation.

Experimental protocols were approved by the Institutional Committee on Care and Use of Animals for experimentation (No. 84/08) in accordance with the guidelines of the National Institutes of Health, Bethesda, MD, USA.

Chemicals

L-arginine, hexadecyltrimethylammonium bromide (HETAB), *o*-dianisidine dihydrochloride, thiobarbituric acid and methylprednisolone were from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and reagents were of the highest commercial grade available.

L-arginine-induced pancreatitis model

Acute pancreatitis was induced in five groups of rats (n = 8/group) by two intraperitoneal (ip) injections of L-arginine (2.5 g/kg, 1 h apart)[23]. One hour following the last injection of L-arginine, the rats were treated orally as follows: group 1 received the vehicle (3% Tween 80) of α,β-amyrin (vehicle control); groups 2, 3 and 4 were treated with α,β-amyrin (10, 30 and 100 mg/kg, respectively); and group 5 acted as positive control and received methylprednisolone (30 mg/kg), all in a volume of 10 mL/kg.

A sixth group (n = 8) of rats that received saline (0.9%, NaCl, ip) in place of L-arginine served as a normal control. Twenty-four hours after the last injection of L-arginine or saline, a midline laparotomy was performed in rats under ketamine anesthesia and blood samples were collected from the inferior vena cava, the rats were then exsanguinated, the whole pancreas was quickly removed and stored at -70℃ until use. The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema (mg/g)[24].

Serum analysis

For serum assays, blood samples were centrifuged at 3000 × g at 4℃ for 10 min. The serum amylase and lipase were determined by routine colorimetric methods using the commercial kits for amylase (Labtest Diagnostica SA, Lagoa Santa, Brazil) and lipase (Bioclin-Quibasa, Belo Hor-

izonte, Minas Gerais, Brazil) and expressed as U/dL and U/L, respectively. Serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured using an ELISA kit according to the manufacturer's instructions (Quantikine®; R&D Systems, Minneapolis, MN, USA). The cytokine levels were calculated from the standard curve and expressed as pg/mL.

Determination of myeloperoxidase activity and thiobarbituric acid-reactive substances

The degree of neutrophil infiltration was quantified by the measurement of pancreatic myeloperoxidase (MPO) activity[25]. Pancreatic tissue (50 mg) was minced and homogenized in 0.5 mL of 50 mmol/L phosphate buffer solution (PBS) (pH 6) that contained 0.5% HETAB. The homogenate was subjected to three cycles of freezing (-30℃) and thawing (37℃) and brief periods (15 s) of sonication, after which, they were centrifuged at 12 000 × *g* for 15 min at 4℃. The supernatant (0.1 mL) was mixed with 2.9 mL of 50 mmol/L PBS, pH 6, which contained 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 470 nm was then measured for 5 min using a Beckman spectrophotometer (Beckman DU 640B; CA, USA).

Thiobarbituric acid-reactive substances (TBARS) level in the pancreatic tissue was determined as an indicator of lipid peroxidation according to a previously described method[26]. Briefly, 500 μL of 10% tissue homogenate in 0.15 mol/L KCl was mixed with 200 μL 8.1% SDS, and then incubated at room temperature for 5 min. The reaction mixture was heated at 95℃ for 1 h after the addition of 1.5 mL 20% acetic acid (pH 3.5) and 1.5 mL 0.8% thiobarbituric acid. After the mixture had cooled, 1.0 mL distilled water and 5.0 mL butanol/pyridine (15:1) solution were added under agitation using a vortex. This solution was centrifuged at 1000 × *g* for 15 min, and the resultant colored layer was measured at 532 nm using a Beckman DU 640B spectrophotometer.

Determination of nitrate/nitrite levels

Total nitrate/nitrite levels were determined as a measure of nitric oxide with the use of Griess reagent[27]. The pancreatic tissue was homogenized in 50 mmol/L potassium phosphate buffer (pH 7.8) and centrifuged at 11 000 × *g* for 15 min at 4℃. One hundred microliters of the supernatant was mixed with 100 μL Griess reagent [0.1% N-(1-naphthyl) ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid], and after 10 min, the absorbance was measured at 540 nm using a Beckman DU 640B spectrophotometer. The standard curve was obtained by using sodium nitrite. The results were calculated from a standard curve by using sodium nitrite and expressed as micromoles of nitrate/nitrite.

Pancreatic histology and immunohistochemistry

Samples of pancreatic tissue were fixed in 10% buffered formalin solution, embedded in paraffin by standard methods, cut into 5-μm sections, stained with hematoxylin-eosin, and then assessed under light microscopy and examined blind by a morphologist for grading the histological alterations. Pancreatic edema, leukocyte infiltration, hemorrhage, acinar vacuolization and necrosis were described with scores ranging from 0 to 3 as described previously[28].

Immunohistochemical analysis of the expression of TNF-α and inducible nitric oxide synthase (iNOS) was performed. Sections of pancreas (4 μm) were transferred to a gelatin-coated slide. The tissue sections were deparaffinized, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide (30 min). Nonspecific protein binding was blocked by incubating the tissue sections with goat serum (0.5% in PBS for 45 min). The slides were then incubated overnight with primary rabbit anti-TNF-α or rabbit anti-iNOS (Sigma), diluted 1:400 in PBS plus bovine serum albumin. For TNF-α, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate (Vectastain® ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min, and TNF-α was visualized with the chromogen 3,3' diaminobenzidine and counterstained with Harry's hematoxylin. For iNOS, the slides were incubated with alkaline-phosphatase-conjugated secondary antibody (EnVisionTM/AP, K1396; Dako, Carpinteria, CA, USA). The reaction was developed by applying on the slides a solution containing levamisole and Fast Red Substrate (EnVisionTM/AP, K1396; Dako).

Statistical analysis

Statistical analysis was performed by analysis of variance followed by Kruskal-Wallis or Student Newman Keul's as post-hoc tests using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). The non-parametric data are expressed as median (with low and high ranges), and parametric data, expressed as mean ± SE. Differences were considered to be statistically significant when P was < 0.05.

RESULTS

Serum biochemical parameters and pancreatic edema

Figure 1 shows the levels of serum amylase and lipase activities and the pancreatic edema in rats under different treatments. When compared to the saline-treated group, the levels of serum amylase and lipase were significantly (P < 0.05) higher in the L-arginine-induced acute pancreatitis group. However, L-arginine caused a much higher increase in lipase as compared to amylase. Besides, L-arginine markedly increased the pancreatic wet weight/body weight ratio, an index of pancreatic edema. While treatment with α,β-amyrin (10, 30 and 100 mg/kg) significantly (P < 0.05) lowered the L-arginine-induced elevation of serum amylase and lipase (Figure 1A and B) at all doses, a significant decrease in pancreatic edema was observed only at 30 and 100 mg/kg (Figure 1C). Methylprednisolone (30 mg/kg), the reference anti-inflammatory drug included in the study, manifested similar reductions in serum amylase and lipase activities, as well as in pancreatic edema (Figure 1).

Figure 2 depicts the serum levels of pro-inflammatory cytokines TNF-α and IL-6 in rats under different treatments. Both TNF-α and IL-6 were significantly elevated

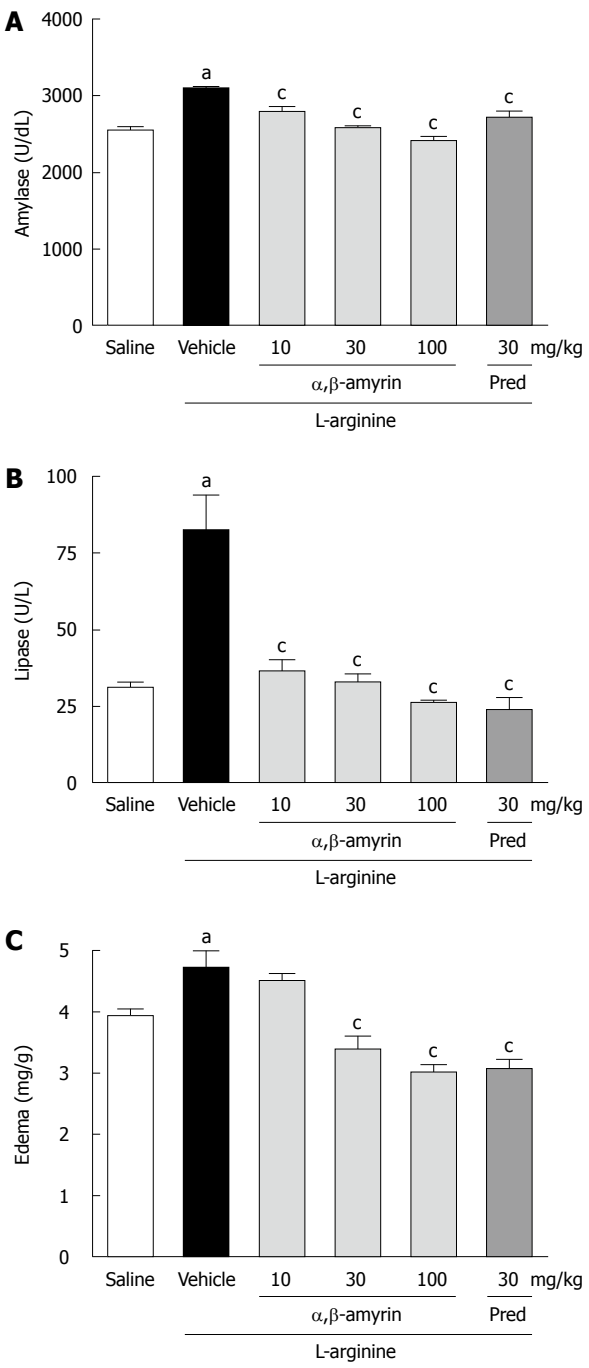

Figure 1 Effects of α,β-amyrin treatment on serum amylase (A), lipase (B) and on pancreatic edema (C) in rats on L-arginine-induced acute pancreatitis. Each column represents mean ± SE (n = 8). $^aP < 0.05$ *vs* saline control group; $^cP < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone.

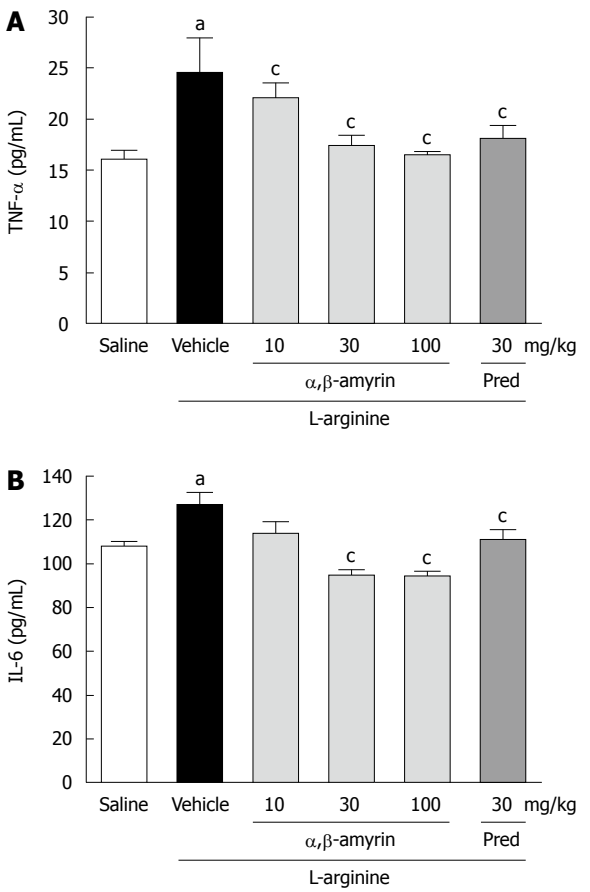

Figure 2 Effect of α,β-amyrin treatment on the serum tumor necrosis factor α (A) and interleukin-6 (B) in L-arginine induced acute pancreatitis. Each column represents mean ± SE (n = 8). $^aP < 0.05$ *vs* saline control group; $^cP < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone; TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6.

in the L-arginine-induced pancreatitis control group when compared to the saline group. Treatment with α,β-amyrin (10, 30 and 100 mg/kg) effectively decreased the enhanced levels of TNF-α at all doses, however, IL-6 inhibition was statistically significant only at 30 and 100 mg/kg of α,β-amyrin.

Pancreatic MPO activity, and levels of TBARS and nitrate/nitrite

In the L-arginine-induced acute pancreatitis group, pancreatic MPO activity, TBARS, and nitrate/nitrite levels were significantly elevated when compared to the saline-treated group (Figure 3A-C). Treatment with α,β-amyrin (10, 30 and 100 mg/kg) and methylprednisolone significantly ($P < 0.05$) reduced the L-arginine-evoked increase in pancreatic MPO activity, TBARS and nitrate/nitrite levels.

Pancreatic histology and immunostaining

Representative TNF-α and iNOS immunostaining of the pancreas for different treatments are shown in Figures 4 and 5. In saline-treated control rats, the pattern of TNF-α and iNOS staining was very mild (Figures 4A and 5A). On the other hand, there was a high intensity staining for TNF-α and iNOS in the acinar cells, inflammatory cells and blood vessels of the pancreas in the L-arginine-induced acute pancreatitis group that received only the vehicle (Figures 4B and 5B), however, in rats treated with α,β-amyrin (100 mg/kg) or methylprednisolone (30 mg/kg), immunostaining intensity for TNF-α and iNOS was much less (Figures 4C, D and 5C, D).

Histological examination of the saline-treated controls showed normal architecture and absence of edema, leukocyte infiltration, acinar vacuolization, hemorrhage and necrosis (Figure 6A and Table 1). In contrast, pancreatic sec-

Table 1 Effects of α,β-amyrin treatment on morphological signs of pancreatic damage

Group	Edema (0-3)	Inflammatory infiltration (0-3)	Acinar vacuolization (0-3)	Hemorrhage (0-3)	Necrosis (0-3)
Saline control	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Vehicle + L-arginine	3 (2-3)[a]	2 (2-3)[a]	3 (3-3)[a]	2 (2-3)[a]	3 (3-3)[a]
α,β-amyrin (100 mg/kg) + L-arginine	0 (0-0)[c]	0 (0-0)[c]	0 (0-1)[c]	0 (0-0)[c]	0 (0-0)[c]
Pred + L-arginine	0 (0-1)[c]	0 (0-0)[c]	1 (0-1)[c]	0 (0-1)[c]	0 (0-0)[c]

Median scores with ranges (min-max) of the results in six animals in each group are shown. [a]$P < 0.05$ *vs* saline control group; [c]$P < 0.05$ *vs* vehicle + L-arginine group. Pred: Methylprednisolone.

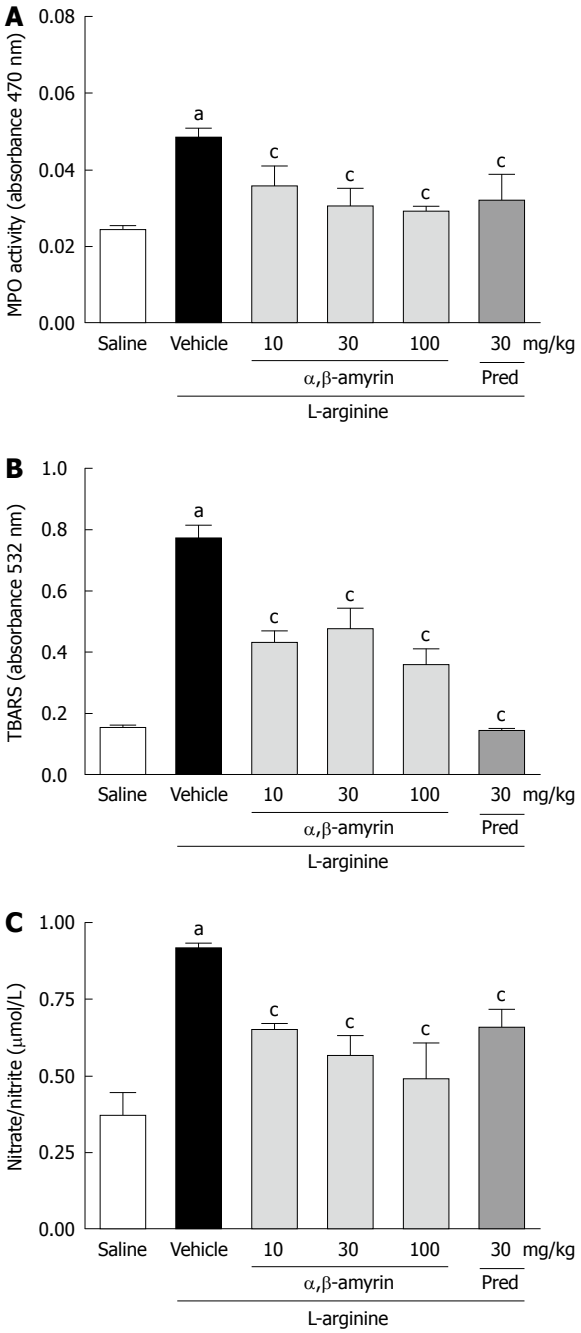

Figure 3 Effects of α,β-amyrin treatment on the pancreatic myeloperoxidase activity (A), thiobarbituric acid-reactant substances (B) and nitrate/nitrite levels (C) in L-arginine induced acute pancreatitis. Each column represents mean ± SE ($n = 8$). [a]$P < 0.05$ *vs* saline control group; [c]$P < 0.05$ *vs* vehicle control group. Pred: Methylprednisolone; MPO: Myeloperoxidase; TBARS: Thiobarbituric acid-reactant substances.

tions from the L-arginine-induced acute pancreatitis group of rats revealed extensive tissue damage that was characterized by significant disruption of normal architecture, with massive edema, acinar cell vacuolization, necrosis, hemorrhage and inflammatory cell infiltration, and thus received significantly higher scores (Figure 6B and Table 1). Treatment with α,β-amyrin (100 mg/kg) and methylprednisolone (30 mg/kg) resolved the inflammation, and most strikingly the edema, and protected the pancreas from histological damage induced by L-arginine. In addition, the total pathological scores were significantly decreased by α,β-amyrin treatment (Figure 6C and D, Table 1).

DISCUSSION

Our study demonstrated that the natural triterpene α,β-amyrin has the potential to attenuate the severity of L-arginine-induced pancreatitis in rats. In agreement with previous studies[29,30], we found significant increases in serum amylase and lipase levels, neutrophil infiltration, massive edema, necrosis and hemorrhage in this experimental model of pancreatitis. Besides, we noticed an increase in the serum levels of pro-inflammatory cytokines TNF-α and IL-6, and a higher expression of TNF-α and iNOS in pancreatic tissue, consistent with earlier reports that have described similar increases in experimental pancreatitis and in clinical patients as well[23,31,32]. TNF-α plays a pivotal role in severe acute pancreatitis, acting early in the disease course[33], and IL-6 constitutes the principal mediator in the synthesis of acute-phase proteins, in addition to transitioning the acute inflammatory response to a chronic response[34]. These findings suggest that inflammatory cytokines and neutrophil-mediated oxidative stress have a central role in the pathogenesis of acute pancreatitis induced by L-arginine, and it also implies that compounds that combat inflammation and oxidative stress ameliorate acute pancreatitis. Treatment with triterpene α,β-amyrin and glucocorticoid methylprednisolone resulted in a significant decrease of serum amylase and lipase, pancreatic edema, and serum TNF-α and IL-6 levels, as well as the TNF-α and iNOS expression induced by L-arginine. Both drugs effectively improved the pancreatic morphology, and these results clearly point out their anti-inflammatory potential in obliterating the pancreatic inflammation and the associated tissue injury.

The glucocorticoids are useful for the treatment of a wide range of inflammatory and autoimmune condi-

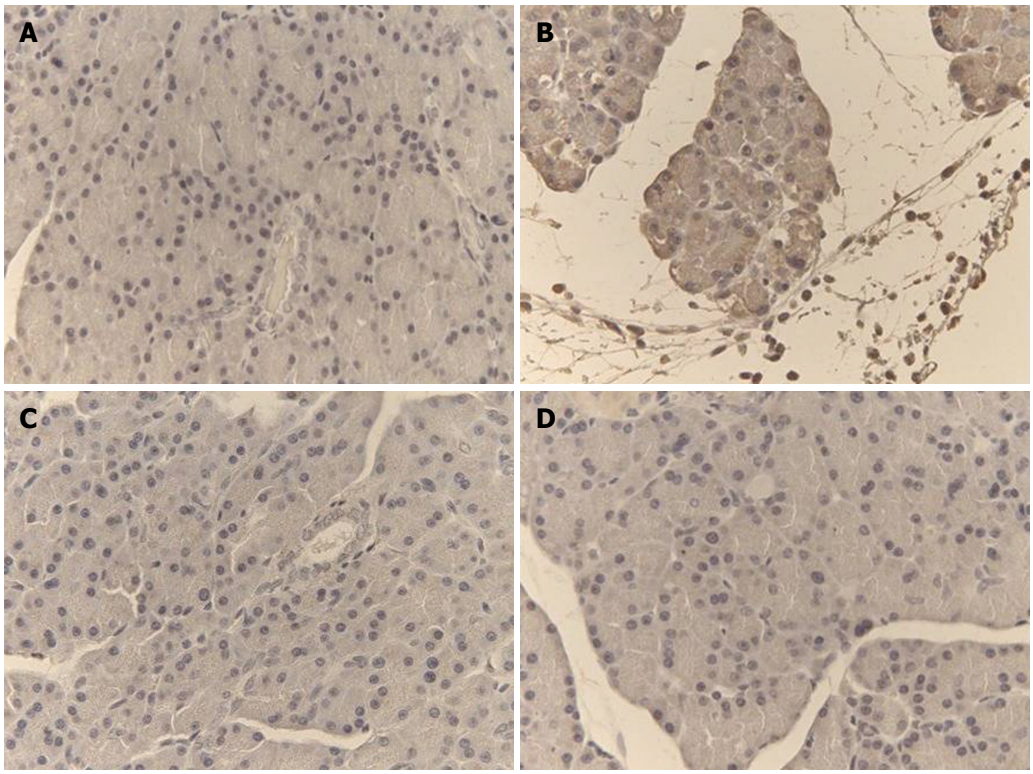

Figure 4 Effect of α,β-amyrin on tumor necrosis factor α immunoreactivity in L-arginine-induced acute pancreatitis (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

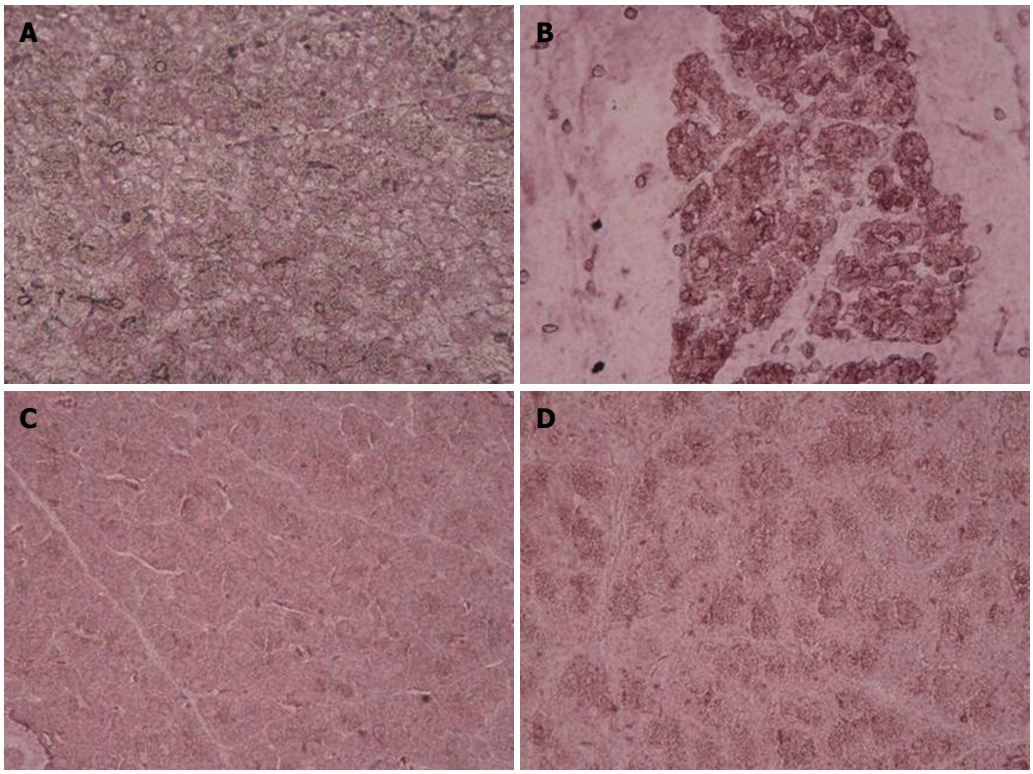

Figure 5 Effect of α,β-amirin on inducible nitric oxide synthetase immunoreactivity in L-arginine-induced acute pancreatitis (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

tions[35]. The effect of α,β-amyrin treatment is the same as that of glucocorticoid methylprednisolone that has been previously shown to be effective in the L-arginine model of experimental pancreatitis[36]. Since α,β-amyrin

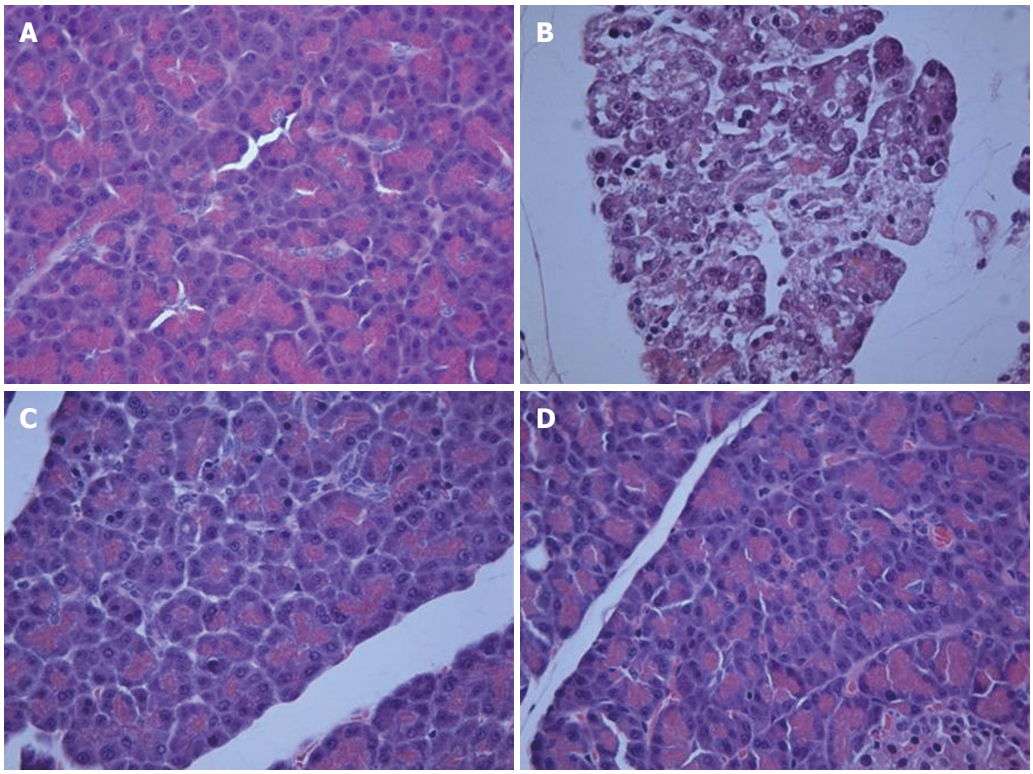

Figure 6 Representatives microphotographs of pancreatic sections (× 400). A: Normal control group; B: Vehicle + L-arginine; C: α,β-amyrin (100 mg/kg) + L-arginine; D: Methylprednisolone (30 mg/kg) + L-arginine.

belongs to the group of ursane and oleanane pentacyclic triterpenes that have a chemical structure that resembles glucocorticoids, it might exercise similar anti-inflammatory effects by affecting the transcription of inflammatory mediators[37].

α,β-amyrin therapy significantly reduces the extent of edema and the total pathological score, possibly due to its anti-inflammatory action[17-19]. NO produced *via* activity of NOS is one of the factors that is involved in the regulation of the rate of perfusion of the pancreatic microvessels[38]. These authors have suggested that excess arginine can induce iNOS activity, which results in high tissue levels of NO that might cause a direct toxic effect on pancreatic acinar cells. The ability of NO to increase the vascular/microcapillary permeability might contribute to the occurrence of pancreatic edema. Total nitrate/nitrite, a marker of endogenous NO, was markedly increased by L-arginine treatment, and was found to be significantly reduced in rats pretreated with α,β-amyrin (100 mg/kg). Furthermore, immunohistochemical staining for iNOS showed that α,β-amyrin could inhibit their expression. It implies that inhibition of NO also participates in the protective effect of triterpenoid.

In the present study, possibly, α,β-amyrin inhibited neutrophil infiltration, TNF-α and IL-6 production, and iNOS expression in pancreatic tissue, probably *via* inhibition of nuclear factor (NF)-κB activity. In this context, recent studies have shown that α,β-amyrin can inhibit NF-κB activation and thereby the production of inflammatory mediators[19]. Oxidative stress plays an important role in the pathophysiology of acute pancreatitis and the beneficial effects of α,β-amyrin might also be associated with the inhibition of NF-κB activity. Excessive reactive oxygen and nitrogen species produced by NOS and isoforms of NADPH oxidase, or as by-products of the mitochondrial electron-transport chain, have been implicated in the pathogenesis of acute pancreatitis[4]. α,β-amyrin potently suppressed neutrophil-mediated MPO and lipoperoxidation, as demonstrated by reduced TBARS formation; events that reflect its antioxidant action. Thus, the present study reveals that α,β-amyrin ameliorates acute pancreatitis by suppressing pro-inflammatory cytokines TNF-α and IL-6, and iNOS expression.

In conclusion, the study provides the first evidence to show that α,β-amyrin attenuates the development of L-arginine-induced acute pancreatitis by reducing the infiltration of neutrophils, generation of inflammatory cytokines and iNOS. This study might provide a basis for future investigations of the therapeutic role of α,β-amyrin in severe necrotizing pancreatitis.

ACKNOWLEDGMENTS

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COMMENTS

Background

The growing incidence of acute pancreatitis in developing and developed countries

has a significant impact on the healthcare system. As a result of the limitations of conventional therapy, many ethnobotanical agents are being pursued as alternative sources to develop novel and safe therapeutic agents for acute pancreatitis. The present experimental study investigated α,β-amyrin, a natural triterpenoid from *Protium heptaphyllum* as a treatment option for acute pancreatitis.

Research frontiers

α,β-amyrin exhibits anti-inflammatory and antioxidant effects. Both inflammation and oxidant stress play a pathogenic role in a rat model of acute pancreatitis induced by L-arginine. α,β-amyrin (100 mg/kg) effectively ameliorates L-arginine-associated pancreatic injury through inhibition of neutrophil infiltration, tumor necrosis factor α and interleukin (IL)-6 production, and inducible nitric oxide synthase expression in pancreatic tissue, probably *via* inhibition of nuclear factor-κB activity.

Innovations and breakthroughs

This is believed to be the first study that has demonstrated the beneficial effect of α,β-amyrin treatment in a rat model of L-arginine-induced acute pancreatitis. Inhibition of pro-inflammatory cytokine IL-6 by α,β-amyrin might arrest the transition of acute pancreatitis to a chronic state, and thus, progression to the more severe form of pancreatitis that is characterized by fibrosis and loss of pancreatic function.

Applications

In this study, acute pancreatic injury caused by L-arginine was ameliorated by α,β-amyrin treatment. This could represent a basis for future investigations on the therapeutic role of α,β-amyrin in severe necrotizing pancreatitis. Clinical studies have suggested that prophylactic administration of anti-inflammatory drugs is useful in preventing pancreatitis in patients undergoing therapeutic endoscopic retrograde cholangiopancreatography (ERCP). α,β-amyrin is an anti-inflammatory and antioxidant agent, therefore, it could also serve as a prophylactic agent in the prevention of ERCP. However, more in-depth experimental studies are warranted to support our observations of its beneficial effects.

Terminology

α,β-amyrin, is a pentacyclic triterpene that is isolated from the resin of the traditional medicinal plant, *Protium heptaphyllum*. α,β-amyrin has anti-inflammatory, antinociceptive, gastroprotective and hepatoprotective properties and is non-toxic at the doses employed in this study.

Peer review

The article describes the beneficial effects of two natural triterpenoids, α and β-amyrin on experimental acute pancreatitis induced by L-arginine in rats. The results presented are very clear, and the authors have been able to demonstrate that these triterpenoids produce anti-inflammatory, antinociceptive, and antioxidant properties at doses that range from 10 to 100 mg/kg, which are non-toxic.

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ORIGINAL ARTICLE

Antitumor effect of matrine in human hepatoma G2 cells by inducing apoptosis and autophagy

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Abstract

AIM: To study the antitumor effect of matrine in human hepatoma G2 (HepG2) cells and its molecular mechanism involved in antineoplastic activities.

METHODS: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect viability of HepG2 cells. The effect of matrine on cell cycle was detected by flow cytometry. Annexin-V-FITC/PI double staining assay was used to detect cellular apoptosis. Cellular morphological changes were observed under an inverted phase contrast microscope. Transmission electron microscopy was performed to further examine ultrastructural structure of the cells treated with matrine. Monodansylcadaverine (MDC) staining was used to detect autophagy. Whether autophagy is blocked by 3-methyladenine (3-MA), an autophagy inhibitor, was evaluated. Expression levels of Bax and Beclin 1 in HepG2 cells were measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Matrine significantly inhibited the proliferation of HepG2 cells in a dose- and time-dependent manner, and induced G1-phase cell cycle arrest and apoptosis of HepG2 cells in a dose-dependent manner. The total apoptosis rate was 0.14% for HepG2 cells not treated with matrine. In contrast, the apoptosis rate was 28.91%, 34.36% and 38.80%, respectively, for HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL. The remarkable morphological changes were observed under an inverted phase contrast microscope. Abundant cytoplasmic vacuoles with varying sizes were observed in HepG2 cells treated with matrine. Furthermore, vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. Electron microscopy demonstrated formation of abundant autophagic vacuoles in HepG2 cells after matrine treatment. When the specific autophagic inhibitor, 3-MA, was applied, the number of autophagic vacuoles greatly decreased. MDC staining showed that the fluorescent density was higher and the number of MDC-labeled particles in HepG2 cells was greater in matrine treatment group than in control group. Fewer autophagic vacuoles were observed in the combined 3-MA and matrine treatment group when 3-MA was added before matrine treatment, indicating that both autophagy and apoptosis are activated when matrine-induced death of hepatoma G2 cells occurs. Real-time quantitative RT-PCR revealed that the expression levels of Bax gene, an apoptosis-related molecule, and Beclin 1 gene which plays a key role in autophagy were higher in matrine treatment group than in control group, indicating that Beclin 1 is involved in matrine-induced autophagy and the pro-apoptotic mechanism

of matrine may be related to its upregulation of Bax expression.

CONCLUSION: Matrine has potent antitumor activities in HepG2 cells and may be used as a novel effective reagent in treatment of hepatocellular carcinoma.

Key words: Matrine; Autophagy; Apoptosis; Bax; Beclin 1; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an important global health issue. Owing to the dissemination of hepatitis B and C virus infection, the overall incidence of HCC remains alarmingly high in developing countries and increases steadily in most developed countries[1,2]. HCC is the sixth most common cancer and the third leading cause of cancer-related death worldwide[3]. Its incidence rate is different in various countries, accounting for 55% of all cases (and deaths) in China[3]. Its age-adjusted annual incidence is 5.5-14.9 people per 100 000 population in the world[4,5]. The prognosis of HCC patients is generally very poor and their 5-year relative survival rate is 3%-5% in most countries[3]. Current major treatment modalities for HCC include surgical resection, liver transplantation and local ablation therapies[6]. Many patients who are diagnosed with HCC at its advanced stage, are only candidates for palliative care[2,7]. Furthermore, since no effective palliative chemotherapy is available, the prognosis of advanced HCC patients is dismal. Therefore, it is necessary to find new effective medications for HCC. Development of pharmacologically effective agents with little toxicity or few side effects from natural products has become a new trend.

Matrine is one of the main alkaloid components extracted from the Sophora root, with a molecular formula of $C_{15}H_{24}N_2O$ (Figure 1)[8], which was first isolated and identified in 1958 from Sophora flavescens Ait (also known as kushen), subprostrata (shandougen) and alopecuroides (kudouzi)[9-12]. Matrine has been widely used in treatment of viral hepatitis, hepatic fibrosis, cardiac arrhythmia and skin diseases, such as atopic dermatitis and eczema in China, because it has a wide range of pharmacological effects, such as anti-inflammatory[13,14], antiviral[15,16], immunoinhibitory[17], antifibrotic[18,19], analgesic[20], antiarrhythmic[21-23] and anti-diarrhea effects[24]. Recently, interest has been generated in its antitumor activity. It has been reported that matrine exerts its antitumor effects by inhibiting proliferation and inducing apoptosis of gastric and cervical cancer cells, leukemia and glioma cells[10,12,25-29]. Matrine can also inhibit invasiveness and metastasis of human malignant melanoma cell line A375 and cervical cancer HeLa cells, and induce differentiation of leukemia K-562 cells[12,30]. In addition, matrine-induced autophagy in rat C6 glioma cells has been observed by electronic microscopy[29]. However, the precise mechanism underlying the anticancer activity of matrine remains unclear. Therefore, we designed this study to investigate the antitumor effect of matrine in human hepatoma G2 cells, and further elucidate its molecular mechanism involved in antineoplastic activities.

MATERIALS AND METHODS

Reagents

Fetal bovine serum was purchased from Sijiqing Biological Engineering Company Limited (Hangzhou, China). RPMI medium 1640 was bought from Gibco (USA). Sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-glutamine, Annexin V-FITC/PI (propidium iodide) apoptosis detection kit, and monodansylcadaverine (MDC) were purchased from Sigma (USA). TRIzol reagent was bought from Invitrogen (USA). Primescript™ reverse transcription (RT) reagent kit and SYBR® Premix Ex Taq™ Ⅱ were purchased from TaKaRa (Dalian, China).

Matrine was purchased from Xi'an Tianyuan Biologics Plant (China), with a purity of over 99% as proved by high-performance liquid chromatography. Matrine was dissolved in sterile double distilled water at a stock concentration of 40 mg/mL, stored at -20℃ in the dark, and then diluted in RPMI-1640 medium to obtain the desired concentration. 3-methyladenine (3-MA) (Sigma, USA) was dissolved in heated sterile double distilled water to make a 100 mmol/L stock solution and then added to the medium after heated for a final concentration of 2 mmol/L. Three hours later, matrine was added for treatment.

Cell line and cell culture

Human hepatoma G2 (HepG2) cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37℃ in a 5% CO_2 incubator. The cells in mid-log phase were used in experiments.

Cell viability assay

Viability of HepG2 cells was assessed by MTT assay as previously described[31]. The cells were seeded in 96-well flat bottom microtiter plates (Costar 3599, Corning Inc., Corning, NY) at a density of 5×10^3 cells per well, allowed to adhere overnight, and then treated with matrine at the con-

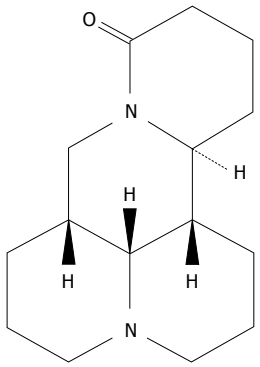

Figure 1 Chemical structure of matrine with a molecular formula of $C_{15}H_{24}N_2O$ and a molecular weight of 248.37.

centration of 0.25, 0.5, 1.0, 2.0 mg/mL for 24, 48 and 72 h, respectively. Control group and zero adjustment well were set as well. A MTT solution (5 mg/mL) was added 4 h before the end of incubation and the reaction was terminated by adding 10% acidified SDS. The absorbance value per well at 570 nm was read using an automatic multiwell spectrophotometer (PowerWave x, Bio-Tek Instruments Inc, USA). All MTT assays were performed in triplicate. The inhibitory rate for the proliferation of HepG2 cells was calculated according to the formula: (1-experimental absorbance value/control absorbance value) × 100%.

Cell cycle analysis

To determine cell cycle distribution, HepG2 cells were treated with matrine at the concentration of 0, 0.5 and 1.0 mg/mL, respectively. After 24 h of treatment, both floating and attached cells were collected and centrifuged before washed with cold phosphate-buffered saline (PBS), and then fixed in 70% cold ethanol overnight at 4℃. A fluorochrome solution containing 50 μg/mL PI, 3.4 mmol/L sodium citration, 20 μg/mL RNase A and 1% Triton X-100 was added and then incubated in the dark at room temperature for 30 min. Cell cycle analysis was performed using an EPICS XL flow cytometer (Beckman Coulter, California, USA). All experiments were performed in triplicate.

Detection of apoptosis

Annexin-V-FITC/PI double staining assay was performed to detect apoptosis of HepG2 cells. The cells were exposed to matrine at the concentration of 0, 0.5, 1.0 and 2.0 mg/mL, respectively, for 24 h, then harvested and resuspended in Annexin-V binding buffer. The suspension was incubated with 5 μL of Annexin V-FITC and 10 μL of PI for 10 min at room temperature in the dark, followed by cytometric analysis (EPICS XL, Beckman Coulter, USA) within 30 min of staining (as soon as possible). All experiments were performed in triplicate.

Morphologic observation under inverted phase contrast microscope

HepG2 cells were equally seeded in 24-well flat bottom microtiter plates (Costar 3524, Corning Inc., Corning, NY), and then treated with matrine at the concentration of 0, 0.25, 0.5, 1.0 and 2.0 mg/mL, respectively. After 24 h of treatment, the morphology of HepG2 cells was observed under an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Observation of cell ultrastructure under transmission electron microscope

HepG2 cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) for 90 min at room temperature, and post-fixed in 1% osmium tetraoxide for 30 min. After washed with PBS, the cells were progressively dehydrated in a 10% graded series of 50%-100% ethanol and propylene oxide, and embedded in Epon 812 resin. The blocks were cut into ultrathin sections with a microtome, which were then stained with saturated uranyl acetate and lead citrate. The ultrastructure of the cells was then observed under a transmission electron microscope (JEM-1230, JEOL, Japan).

MDC staining of autophagic vacuoles

MDC staining of autophagic vacuoles was performed for autophagy analysis as previously described[32]. HepG2 cells were divided into control group, 3-MA treatment group, matrine treatment group, and combined 3-MA and matrine treatment group. The cells were incubated for 48 h on coverslips. Autophagic vacuoles were labeled with 0.05 mmol/L MDC in PBS at 37℃ for 10 min. After incubation, the cells were washed three times with PBS and immediately analyzed under a fluorescence microscope (IX-81; Olympus, Japan). Fluorescence of MDC was measured at the excitation wavelength 380 nm with an emission filter at 530 nm.

Real-time quantitative RT-polymerase chain reaction

HepG2 cells were cultured in 35 mm dishes and then collected after treatment with matrine for 24 and 72 h, respectively. Total RNA was isolated from cells using Trizol reagent (Invitrogen, USA) according to its manufacturer's protocol. RNA concentration and purity were measured with a spectrophotometer at A260 and A260/280, respectively. RNA was reverse-transcribed into cDNA using a Primescript™ RT reagent kit (TaKaRa, Dalian, China) according to its manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) was carried out with the SYBR Green I fluorescent dye method (SYBR® Premix Ex Taq™ II, TaKaRa, Dalian, China) and a Rotor Gene 3000 real-time PCR apparatus (Corbett Research Company, Australia). The sequences of primers used are as follows: forward: 5'-TGCTTCAGGGTTTCATCCAG-3' and reverse: 5'-GGCGGCAAT CATCCTCTG-3' for Bax; forward: 5'-GAGGGATGGAAGGGTCTAAG-3' and reverse: 5'-GCCTGGGCTGTGGTAAGT-3' for Beclin 1; forward: 5'-TGGCACCCAGCACAATGAA-3' and reverse: 5'-CTAAGTCATAGT CCGCCTAGAAGCA- 3' for β-actin. β-actin was used as an internal control to evaluate the relative expressions of Bax and Beclin 1. The PCR conditions were as follows:

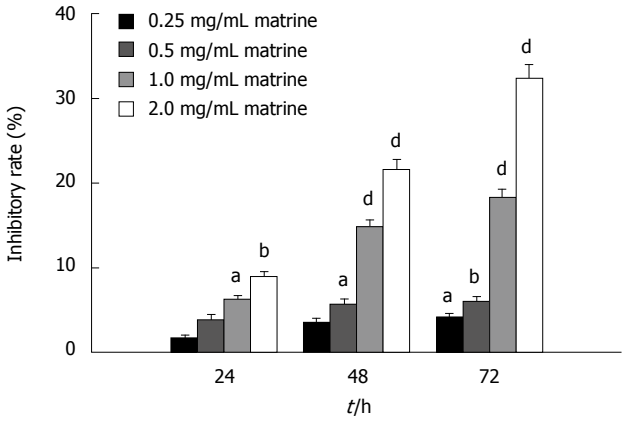

Figure 2 MTT assay showing the inhibitory effect of matrine on growth of HepG2 cells. HepG2 cells were treated with matrine at the concentration of 0.25, 0.5, 1.0 and 2.0 mg/mL, respectively for 24, 48 and 72 h. Matrine inhibited the growth of HepG2 cells in a dose- and time-dependent manner. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control group.

a pre-denaturing at 95℃ for 2 min, followed by 45 cycles of denaturation at 95℃ for 10 s, annealing/extension at 60℃ for 20 s. The amplification specificity was checked by melting curve analysis. The PCR products were visualized by gel electrophoresis to confirm the presence of a single product with a correct size. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative abundance of target gene expression generated by Rotor-Gene Real-Time Analysis Software 6.1.81. For each cDNA, the target gene mRNA level was normalized to β-actin mRNA level. Results were expressed as the ratio of normalized target gene mRNA level in cells treated with matrine to that in cells not treated with matrine. The experiments were performed in triplicate.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using the SPSS 16.0 for Window. One-way analysis of variance (ANOVA) was used to analyze statistical differences between groups under different conditions. $P < 0.05$ was considered statistically significant.

RESULTS

Matrine inhibited proliferation of HepG2 cells in a dose- and time-dependent manner

The antiproliferative effect of matrine on HepG2 cells was detected by MTT assay. The results showed that matrine inhibited the proliferation of HepG2 cells in a dose-dependent and time-dependent manner. The inhibitory rate of matrine on growth of HepG2 cells was 6.28% ± 0.42%, 14.81% ± 0.81%, and 18.25% ± 0.99%, respectively, after the cells were treated with matrine at the concentration of 1.0 mg/mL for 24, 48 and 72 h (Figure 2).

Matrine induced G1-phase cell cycle arrest in HepG2 cells

To better understand the inhibitory effect of matrine on growth of HepG2 cells, cell cycle distribution was analyzed by flow cytometry. Matrine significantly increased the number of cells in G0/G1 phase and decreased the number of cells in the S phase in a dose-dependent manner (Figure 3), indicating that matrine can induce the G0/G1 phase cell cycle arrest in HepG2 cells.

Matrine induced apoptosis of HepG2 cells

Annexin-V-FITC/PI double staining assay showed that matrine induced apoptosis of HepG2 cells in a dose-dependent manner (Figure 4A). Flow cytometry showed that the total apoptosis rate was 0.14% in HepG2 cells not treated with matrine, and was 28.91%, 34.36%, and 38.80%, respectively, in HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL (Figure 4B-E). Early apoptosis was observed in HepG2 cells treated with matrine at the concentration of 0.5 and 1.0 mg/mL, while late apoptosis was observed in HepG2 cells treated with matrine at the concentration of 2.0 mg/mL. The late apoptosis rate of HepG2 cells treated with matrine at the concentration of 2.0 mg/mL increased from 1.06% to 16%, which may be due to the direct cytotoxic effect of matrine on HepG2 cells.

Observation of vacuolization in cytoplasm by inverted phase contrast microscopy

Inverted phase contrast microscopy showed the morphological characteristics of HepG2 cells. Control cells not treated with matrine were well adhered, showing the normal morphology of HepG2 cells, while the tumor cells treated with matrine for 24 h demonstrated remarkable morphological changes (Figure 5). Abundant cytoplasmic vacuoles with varying sizes were observed. Vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. Moreover, the majority of cells treated with matrine at the concentration of 2.0 mg/mL became round and shrunken, and could not be affixed to the wall and suspended in culture medium.

Transmission electron microscopy revealed formation of autophagosomes in matrine-treated HepG2 cells

To further clarify whether the cell vacuolization induced by matrine is involved in autophagy, transmission electron microscopy (TEM) was performed to detect the cells treated with matrine at the concentration of 1.0 mg/mL. The HepG2 cells not treated with matrine exhibited the normal ultrastructural morphology of cytoplasm, organelles and nuclei (Figure 6). The most prominent morphological change in matrine-treated cells was the formation of abundant autophagic vacuoles sequestrating cytoplasm and organelles, such as mitochondria and endoplasmic reticulum. Double-membranes, giant autophagosomes filled with degraded organelles and autolysosomes were frequently observed. TEM, the standard method to detect autophagy[33], was performed to detected the formation of autophagosomes, demonstrating that matrine could induce HepG2 cells to generate autophagy, which was consistent with the vacuolization obtained by inverted phase contrast microscopy. Moreover, 3-MA, a specific inhibitor

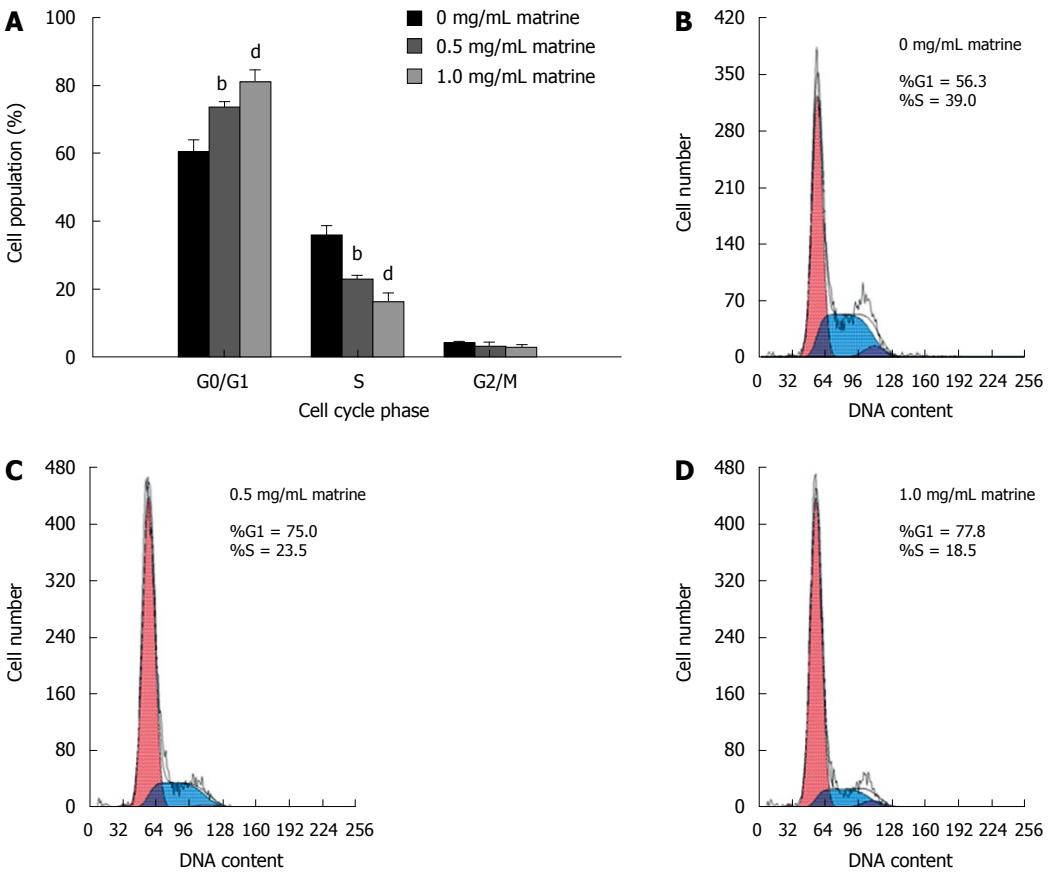

Figure 3 Effect of matrine on cell cycle distribution in HepG2 cells. A: Matrine treatment significantly increased the proportion of HepG2 cells in G0/G1 phase while decreased the number of HepG2 cells in the S phase. Results were expressed as mean ± SD (n = 3); B-D: Histograms of HepG2 cells incubated with matrine at the concentrations of 0, 0.5 and 1.0 mg/mL. ^{b}P < 0.01, ^{d}P <0.001 *vs* control group.

of autophagy, potently suppressed the matrine-induced autophagy (Figure 6E). The number of autophagic vacuoles was significantly lower while the formed vacuoles were smaller and appeared to be less developed in combined 3-MA and matrine treatment group than in single matrine treatment group.

MDC-labeled vacuoles in matrine-treated HepG2 cells

It has been reported that MDC is a specific marker for autophagic vacuoles[34]. When the cells were viewed under a fluorescence microscope, MDC-labeled autophagic vacuoles appeared as distinct dot like structures distributing in cytoplasm or in perinuclear. The fluorescent density and MDC-labeled particles of HepG2 cells were higher in matrine treatment group than in control group (Figure 7), indicating that matrine induces formation of MDC-labeled vacuoles. Fewer autophagic vacuoles were observed in combined 3-MA and matrine treatment group when 3-MA was added before matrine treatment, showing that 3-MA exerts its inhibitory effects on matrine-treated autophagy.

Matrine up-regulated mRNA expression of Bax and Beclin 1 in HepG2 cells

In order to understand the molecular mechanism underlying the apoptosis induced by matrine, the mRNA expression level of Bax gene, an apoptosis-related molecule, in HepG2 cells treated with matrine was measured by real-time quantitative RT-PCR, which showed that matrine up-regulated the Bax mRNA expression in HepG2 cells in a dose- and time-dependent manner (Figure 8A). Following 24 h of treatment, the Bax mRNA expression level increased nearly 3-fold and 30-fold, respectively, when the cells were treated with matrine at the concentration of 1.0 mg/mL for 24 and 72 h. At the same time, matrine gradually increased the Bax mRNA expression level in HepG2 cells when the concentration of matrine was increased.

To elucidate the mechanism underlying the autophagy induced by matrine, real-time quantitative RT-PCR was performed to evaluate the effect of matrine on mRNA expression of Beclin 1, which plays a key role in autophagy[35]. Real-time quantitative RT-PCR showed that matrine activated the Beclin 1 gene expression in a dose- and time-dependent manner (Figure 8B). In other words, the Beclin 1 mRNA expression level steadily increased with the increasing drug concentration and action time. It was interesting that the mRNA expression level of Bax and Beclin 1 was lower in HepG2 cells treated with matrine at the concentration of 2.0 mg/mL than in those treated with matrine at the concentration of 1.0 mg/mL, indicating that matrine at the concentration of 2.0 mg/mL exerts its direct cytotoxic effect on necrosis of HepG2 cells.

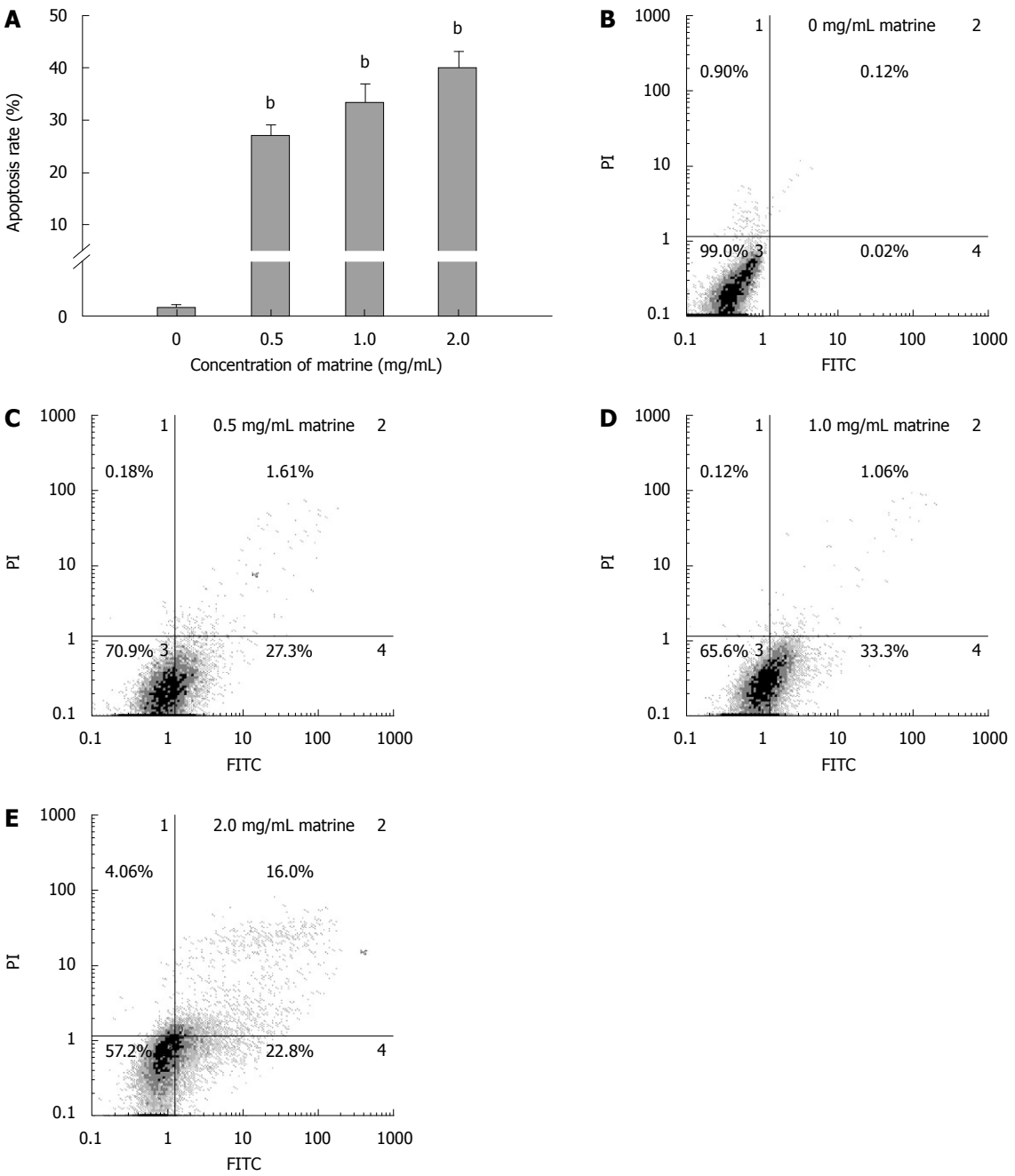

Figure 4 Matrine induces apoptosis of HepG2 cells. A: Apoptosis rate of HepG2 cells treated with matrine at the concentration of 0.5, 1.0 and 2.0 mg/mL, respectively, were significantly different from that in control group. Furthermore, the percentage of apoptotic cells increased when the concentration of matrine was increased. The data are expressed as mean ± SD (n = 3); B-E: Histograms of HepG2 cells incubated with matrine at the concentration of 0, 0.5, 1.0 and 2.0 mg/mL, respectively. Early apoptotic HepG2 cells were observed after incubated with matrine at the concentration of 0.5 and 1.0 mg/mL, the late apoptotic HepG2 cells were observed after incubated with matrine at the concentration of 2.0 mg/mL. $^{b}P < 0.001$ *vs* control group.

DISCUSSION

Both *in vivo* and *in vitro* studies showed that matrine inhibits the proliferation of tumor cells[10,27-29]. In this study, matrine obviously inhibited the growth of HepG2 cells in a dose- and time-dependent manner (Figure 2). Flow cytometry showed that matrine markedly arrested HepG2 cells in G0/G1 phase of cell cycle (Figure 3), which is consistent with the reported findings[8], suggesting that retardation of cell cycle progression may be one of the mechanisms underlying the antiproliferative effect of matrine.

It has been reported that matrine induces apoptosis in gastric cancer MKN45 and SGC-7901 cells, leukemia U937 and K562 cells, and C6 glioma cells[25,26,28,29,36]. In this study, matrine induced apoptosis of HepG2 cells in a dose-dependent manner (Figure 4). Consistent with the ability of matrine to kill cells *via* apoptotic processes, matrine up-regulated the expression of proapoptosis gene, Bax, in a dose- and time-dependent manner (Figure 8A), indicating that the increased Bax expression may trigger matrine-induced apoptosis of HepG2 cells, which is in agreement with the reported findings[28].

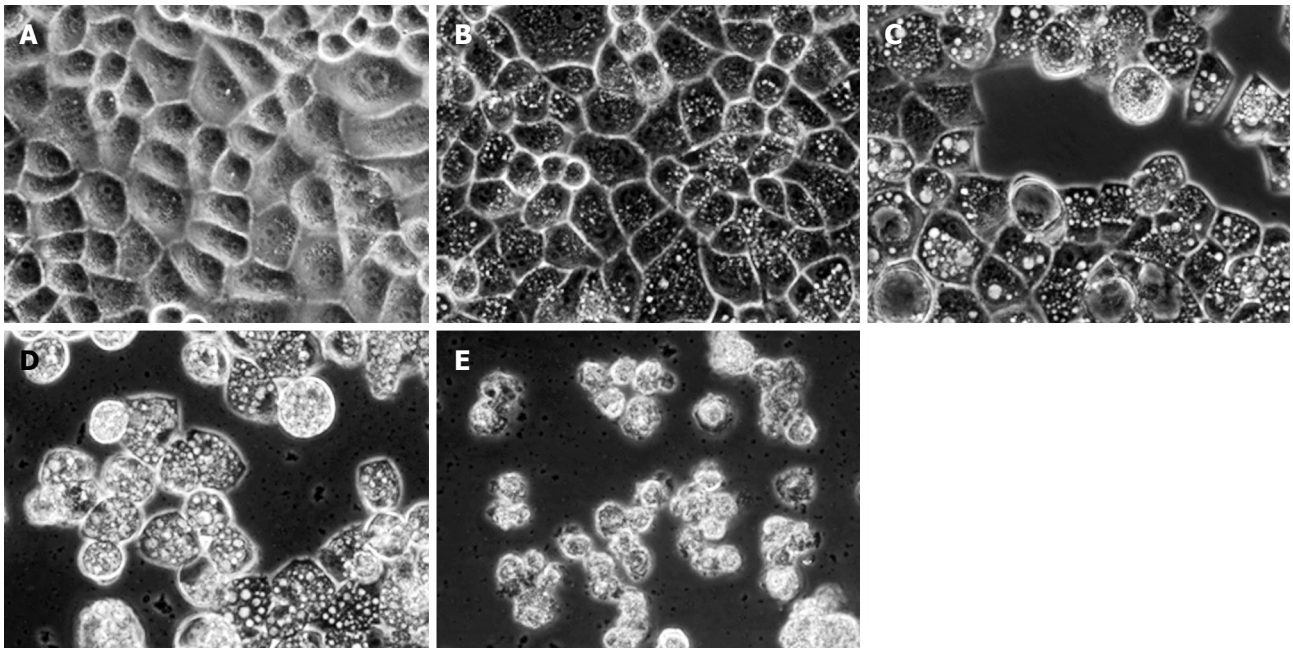

Figure 5 Inverted phase contrast microscopy showing matrine-induced morphologic changes of HepG2 cells. The control cells were well adhered, displaying the normal morphology of HepG2 cells. In contrast, abundant cytoplasmic vacuoles were observed in cells treated with matrine. Moreover, vacuolization in cytoplasm progressively became larger and denser when the concentration of matrine was increased. The majority of HepG2 cells treated with matrine at the concentration of 2.0 mg/mL became round and shrunken and could not be affixed to the wall and suspended in culture medium (× 400 magnification). A: 0 mg/mL matrine; B: 0.25 mg/mL matrine; C: 0.5 mg/mL matrine; D: 1.0 mg/mL matrine; E: 2.0 mg/mL matrine.

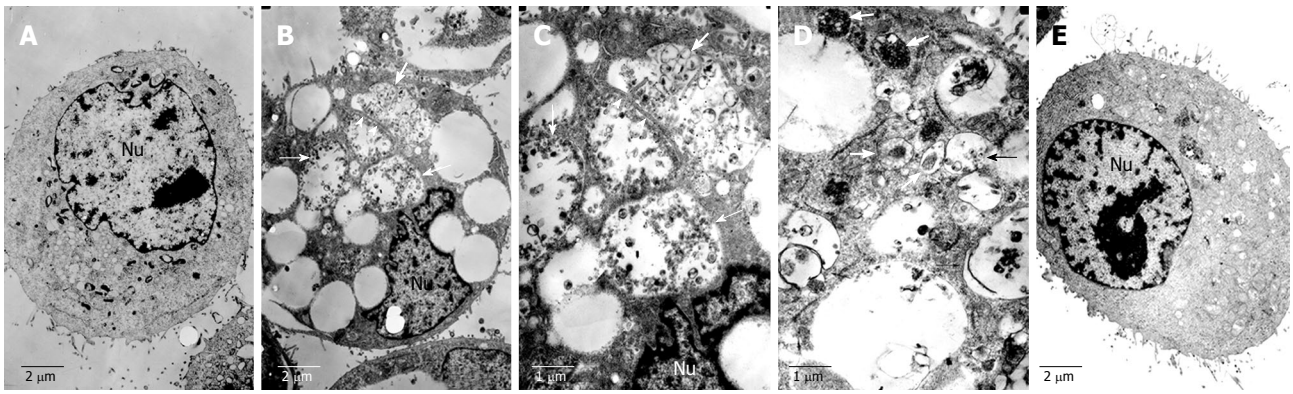

Figure 6 Transmission electron microscopy showing normal morphology of cytoplasm, cell organelles, and nuclei of HepG2 cells not treated with matrine (A), characteristic ultrastructural morphology of autophagy (B), double-membrane (C) and a large number of autophagic vacuoles (D) of HepG2 cells treated with matrine, and sharply decreased autophagic vacuoles (E) of HepG2 cells treated with combination of 3-MA and matrine (A, B, E, × 4000; C, D, × 8000). Arrowheads represent double-membrane, thick arrows represent autophagosomes, and thin arrows represent autolysosomes. Nu: Nucleus.

In this study, abundant cytoplasmic vacuoles were observed in matrine-treated HepG2 cells under an inverted phase contrast microscope (Figure 5). Electron microscopy showed autophagosomes in HepG2 cells treated with matrine at the concentration of 1.0 mg/mL (Figure 6). When the specific autophagic inhibitor, 3-MA, was applied, the number of autophagic vacuoles greatly decreased. MDC staining demonstrated independent evidence supporting the conclusion that matrine triggers autophagocytosis (Figure 7). The results of our study demonstrated that both autophagy and apoptosis were activated when death of HepG2 cells occurred after matrine treatment, revealing that the mechanism underlying the cytocidal action of matrine may be more complex than it has been reported. Autophagy, an evolutionarily conserved process, regulates cell death in both physiological and pathophysiological conditions[37-39]. In normal cells, autophagy contributes to the turnover of long-lived proteins and elimination of damaged or aged organelles to maintain cell homeostasis[40,41]. While under pathological conditions, autophagy is generally considered to play a prosurvival role. However, extensive autophagy or inappropriate activation of autophagy results in autophagic cell death (type Ⅱ programmed cell death), which is an important cell death process besides apoptosis. Recently, increasing evidence indicates that autophagy is closely associated with tumors and plays an important role in human tumor suppression[35,41,42]. Autophagy has been

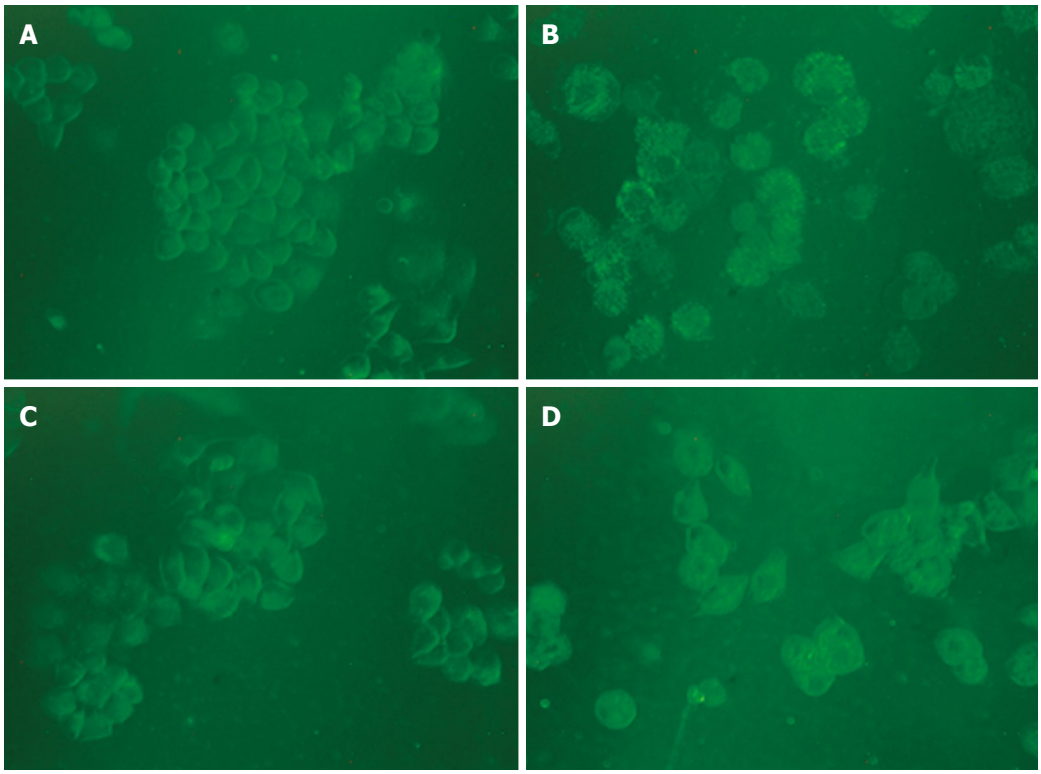

Figure 7 Monodansylcadaverine-labeled vacuoles in HepG2 cells. Autophagic vacuoles were labeled with 0.05 mmol/L monodansylcadaverine (MDC) in phosphate-buffered saline (PBS) at 37℃ for 10 min. The fluorescent density and the MDC-labeled particles in HepG2 cells were higher in matrine treatment group than in control group. The number of MDC-labeled particles in HepG2 cells was significantly lower in combined 3-MA and matrine treatment group than in single matrine treatment group (× 400 magnifications). A: Control; B: Matrine; C: 3-MA; D: 3-MA + matrine.

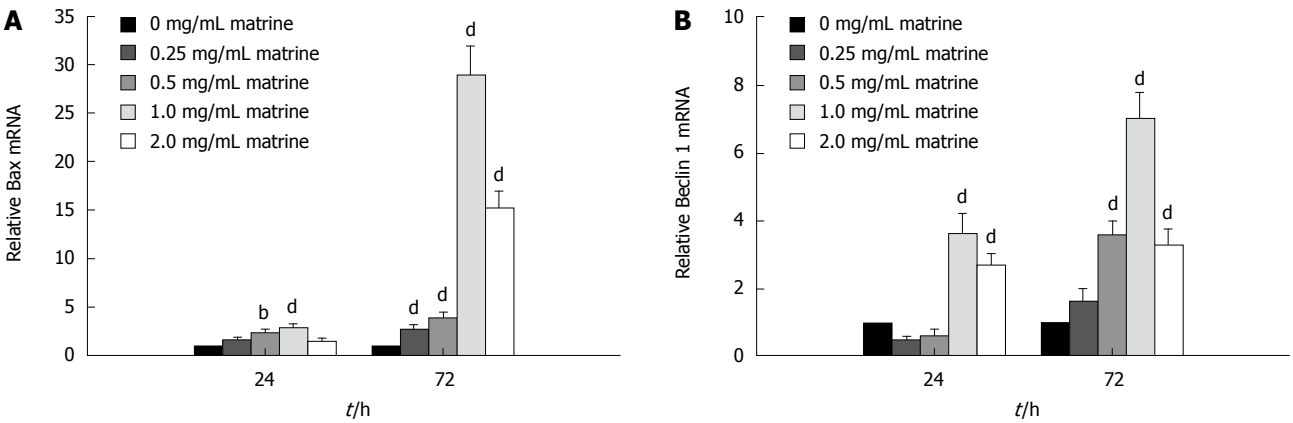

Figure 8 Matrine up-regulates the mRNA expression level of Bax (A) and Beclin 1 (B) in HepG2 cells. Data are shown as mean ± SD of three independent experiments. $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control group.

observed in response to anticancer agents, such as vitamin D analogues[43], resveratrol[44], arsenic trioxide[45], tamoxifen[46], temozolomide[47] and rapamycin[48], indicating that autophagy can be potentially used in treatment of cancer. Furthermore, it has been reported that agents can directly lead to autophagic cell death. For example, caspase inhibitor induces autophagic cell death of L929 and U937 cells [49], which is consistent with that of human leukemia HL60 cells treated with Eupalitin A[50]. In addition, 5-fluorouracil induces autophagic cell death of Bax or PUMA deficient human colon cancer cells[51], indicating that induction of autophagy may be a promising new approach to treatment of tumor cells. In this study, a novel activity of matrine was identified in HepG2 cells, namely the ability of matrine to induce autophagy. In line with our data, matrine-induced autophagy in rat C6 glioma cells has been reported[29], suggesting that autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality.

In this study, the mRNA expression level of Beclin 1 in HepG2 cells was measured. Beclin 1, a mammalian orthologue of the yeast Apg6/Vps30 gene, is the first identified mammalian gene to induce autophagy[35]. The Beclin 1 gene is mapped to the human chromosome 17q21[52],

which is monoallelically deleted in 40%-75% of human prostate, breast, and ovarian cancers[53]. Ectopic expression of Beclin 1 restores full autophagy potential in Beclin 1 deficient MCF-7 cells[35]. Moreover, the incidence of spontaneous tumors is high in Beclin $1^{+/-}$ heterozygous mice and Beclin $1^{-/-}$ homozygous embryonic stem cells exhibit a decreased number of autophagic vesicles, establishing that Beclin 1 is a critical component of mammalian autophagy and a haploinsufficient tumor suppressor gene[42,54]. Beclin 1 functions in autophagy as part of class Ⅲ phosphatidylinositol 3-kinase (PI3k) complex, which is necessary for the formation of autophagosome during the autophagic sequestration process[55,56]. In our study, matrine treatment increased the expression of Beclin 1 in HepG2 cells in a dose- and time-dependent manner (Figure 8B), indicating that Beclin 1 is involved in matrine-induced autophagy.

Additionally, although matrine simultaneously induced both apoptosis and autophagy in HepG2 cells in our study, the relation between apoptosis and autophagy remains unknown. Further study is needed to analyze the relation between related molecules at protein level.

In conclusion, matrine is a potent antitumor agent and exerts its antineoplastic action by inhibiting cell proliferation and inducing cell apoptosis and autophagy. Autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality. In particular, autophagy may provide leverage to treat HCC that is chemoresistant on the basis of ineffective apoptosis. Beclin 1 is involved in matrine-induced autophagy and the pro-apoptotic mechanism of matrine may be related to its upregulation of Bax expression.

COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) increases in the world while many patients are diagnosed with HCC at its advanced stage. Since no effective palliative chemotherapy is available for HCC, the prognosis of patients with advanced HCC is dismal. Recently, interest has been generated in the antitumor activity of matrine, which has been widely used in treatment of various diseases.

Research frontiers

Autophagy is closely associated with tumors and plays an important role in human tumor suppression. Furthermore, it has been reported that agents directly cause autophagic cell death. Matrine induces autophagy in human hepatoma G2 (HepG2) cells. This study investigated the effect of matrine on the proliferation, cell cycle, apoptosis and autophagy of HepG2 cells and its molecular mechanism involved in antineoplastic activities.

Innovations and breakthroughs

In this paper, the authors identified a novel activity of matrine in HepG2 cells, namely the ability of matrine to induce autophagy, revealing that the mechanism underlying the cytocidal action of matrine may be more complex than it has been previously reported. The authors further demonstrated that Beclin 1 was involved in matrine-induced autophagy and the pro-apoptotic mechanisms of matrine might be related to its upregulation of Bax expression. Autophagic cell death induced by matrine underlines its potential utility as a new cancer treatment modality.

Applications

Matrine may be used as a potentially promising reagent in treatment of hepatocellular carcinoma. In particular, autophagy may provide leverage to treat HCC that is chemoresistant on the basis of ineffective apoptosis.

Terminology

Autophagy, an evolutionarily conserved process, regulates cell death in both physiological and pathophysiological conditions. Autophagic cell death (type Ⅱ programmed cell death) is an important cell death process besides apoptosis. Beclin 1, a mammalian orthologue of the yeast Apg6/Vps30 gene, is the first identified mammalian gene to induce autophagy and a haploinsufficient tumor suppressor gene. Bax, a pro-apoptotic molecule, is closely related to apoptosis.

Peer review

This is an interesting study. The study investigated the effect of matrine, one of the main alkaloid components extracted from the Sophora root, on the proliferation, apoptosis and autophagy of HepG2 cells. Matrine has been widely used in treatment of various diseases. The authors showed that matrine inhibited the proliferation and stimulated the apoptosis and autophagy of HepG2 cells, indicating that matrine can be used as a potentially promising agent in treatment of cancer.

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BRIEF ARTICLE

Peripancreatic collections in acute pancreatitis: Correlation between computerized tomography and operative findings

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Abstract

AIM: To evaluate the ability of contrast-enhanced computerized tomography (CECT) to characterize the nature of peripancreatic collections.

METHODS: Twenty five patients with peripancreatic collections on CECT and who underwent operative intervention for severe acute pancreatitis were retrospectively studied. The collections were classified into (1) necrosis without frank pus; (2) necrosis with pus; and (3) fluid without necrosis. A blinded radiologist assessed the preoperative CTs of each patient for necrosis and peripancreatic fluid collections. Peripancreatic collections were described in terms of volume, location, number, heterogeneity, fluid attenuation, wall perceptibility, wall enhancement, presence of extraluminal gas, and vascular compromise.

RESULTS: Fifty-four collections were identified at operation, of which 45 (83%) were identified on CECT. Of these, 25/26 (96%) had necrosis without pus, 16/19 (84%) had necrosis with pus, and 4/9 (44%) had fluid without necrosis. Among the study characteristics, fluid heterogeneity was seen in a greater proportion of collections in the group with necrosis and pus, compared to the other two groups (94% *vs* 48% and 25%, P = 0.002 and 0.003, respectively). Among the wall characteristics, irregularity was seen in a greater proportion of collections in the groups with necrosis with and without pus, when compared to the group with fluid without necrosis (88% and 71% *vs* 25%, P = 0.06 and P < 0.01, respectively). The combination of heterogeneity and presence of extraluminal gas had a specificity and positive likelihood ratio of 92% and 5.9, respectively, in detecting pus.

CONCLUSION: Most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue. CECT has a somewhat limited role in differentiating the different types of collections.

Key words: Contrast-enhanced computerized tomography; Correlation; Pancreatic necrosis; Pancreatitis; Peripancreatic fluid collection; Surgery

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INTRODUCTION

Pancreatic necrosis and so called peripancreatic "fluid" collections are local complications of acute pancreatitis (AP) defined according to the Atlanta Criteria of AP, which categorizes AP as severe if these complications are present. It has been estimated that about 15% (4%-47%) of patients with AP will have necrotizing pancreatitis[1]; and 21%-46%[2-4] of patients will develop a peripancreatic "fluid" collection, including acute collections (during the first 4-6 wk), pseudocysts (after 4-6 wk of an acute attack with a defined wall with no or little necrotic tissue), necrotic collections and abscesses (collection after 4-6 wk with pus and a defined wall), according to the definitions of the Atlanta Classification. Earlier studies have demonstrated good correlation between contrast-enhanced computerized tomography (CECT) and operative findings with regard to pancreatic parenchymal necrosis in patients with AP[5-8]. Lack of vascular-based enhancement of the pancreatic parenchyma is the most characteristic finding on CECT that would suggest the presence of pancreatic parenchymal necrosis; and it has been suggested that the degree of pancreatic necrosis has important prognostic implications[9]. Problems, however, have been evident with much of the nomenclature of the Atlanta Classification with our more current understanding of the spectrum of necrotizing pancreatitis. The nomenclature of peripancreatic fluid collection is confusing due to various terms (as described above) and their clinical significance. It is not always possible in the first 4-6 wk to distinguish on CECT simple fluid collections (associated with acute edematous pancreatitis and better outcomes) from necrotic collections (associated with necrotizing pancreatitis and worse outcomes). No specific CT features that could detect necrosis of peripancreatic tissues (so called peripancreatic necrosis) have been reported so far. Moreover, studies evaluating CECT and operative correlation in the detection of peripancreatic necrosis and peripancreatic fluid collections are scant. Sarr *et al*[7] suggested that peripancreatic necrosis may not be seen on CECT and that a normal enhancement of the pancreas does not necessarily rule out the presence of peripancreatic necrosis. Therefore, assessment of the nature of these peripancreatic collections can be difficult even though these collections are fairly well imaged by CECT.

In this study, we evaluate the correlation between CT findings of peripancreatic collections and findings at operation and thereby assess the ability of CECT to characterize the nature of these peripancreatic collections. We have consciously and purposely elected to call the peripancreatic collections, not peripancreatic "fluid" collections, but peripancreatic "collections" because of the apparent difficulty of differentiating true fluid collections from necrotic areas and necrotic areas with some liquefaction necrosis. Thus, we will not refer to these collections as peripancreatic fluid collections as was done in the Atlanta Classification.

MATERIALS AND METHODS

In this retrospective study, we evaluated 25 patients who had one or more peripancreatic collections on CECT and who underwent operative intervention for severe acute pancreatitis (SAP) between 1995 and 2001 at the Mayo Clinic, Rochester. Severity of acute pancreatitis was determined according to the Atlanta Criteria. The CT slice thickness was 7 mm. Operative findings were used as an objective, gold standard for defining the nature of the peripancreatic collections. A single surgeon (MGS), blinded to the CT findings, interpreted the surgical notes and classified the peripancreatic collections into the following three groups: (1) necrosis without frank pus; (2) necrosis with pus; and (3) fluid without necrosis. The nomenclature of pancreatic collections is currently undergoing a revision (International Working Group); indeed the above three terms could mean the previous entities, peripancreatic necrosis, peripancreatic necrosis with pus, and pseudocysts. The presence of necrosis was confirmed from the operative findings and histopathologic review of the surgical specimens. Following this, a single radiologist (JGF), blinded to the operative findings, assessed the preoperative CTs of each patient for necrosis and peripancreatic collections. Pancreatic parenchymal necrosis was assessed in terms of extent (no necrosis, 30% necrosis, 30%-50% necrosis and > 50% necrosis), and location. Peripancreatic collections were described in terms of volume, location, number, heterogeneity, fluid attenuation, wall perceptibility, wall enhancement, presence of extraluminal gas, and vascular compromise. The volumes of the collections on CECT were measured using the formula $\pi/6 \times (d1 \times d2 \times d3)$, with d representing the maximum diameters in three different planes. Thereafter, the locations of the collections seen on CECT were matched to those identified at operation as per the operative notes. When two collections on CECT matched with only one collection seen at operation, then one of the collections on CT was randomly selected for further evaluation. If collections seen on CT did not match with collections seen at operation, they were excluded from further consideration.

Other parameters that were retrieved from the charts included demographic variables, presence of organ failure, presence of infection in the different groups of peripancreatic collections, and mortality. Approval by the Mayo Institutional Review Board approval was obtained prior to the study.

Statistical analysis

A database was generated in Microsoft Excel, and subsequently all statistical analyses were performed using the JMP software (version 7) from the SAS Institute, NC, USA. Continuous variables were expressed as median (IQR) and categorical variables as percentage. The different CT characteristics between the three groups of peripancreatic collections were compared by the chi square test (with Yates correction wherever applicable) and ordinal variables were analyzed with the Wilcoxon rank test. The '*t*' test was applied to compare continuous variables. A '*P*' value of less than 0.05 was considered statistically significant.

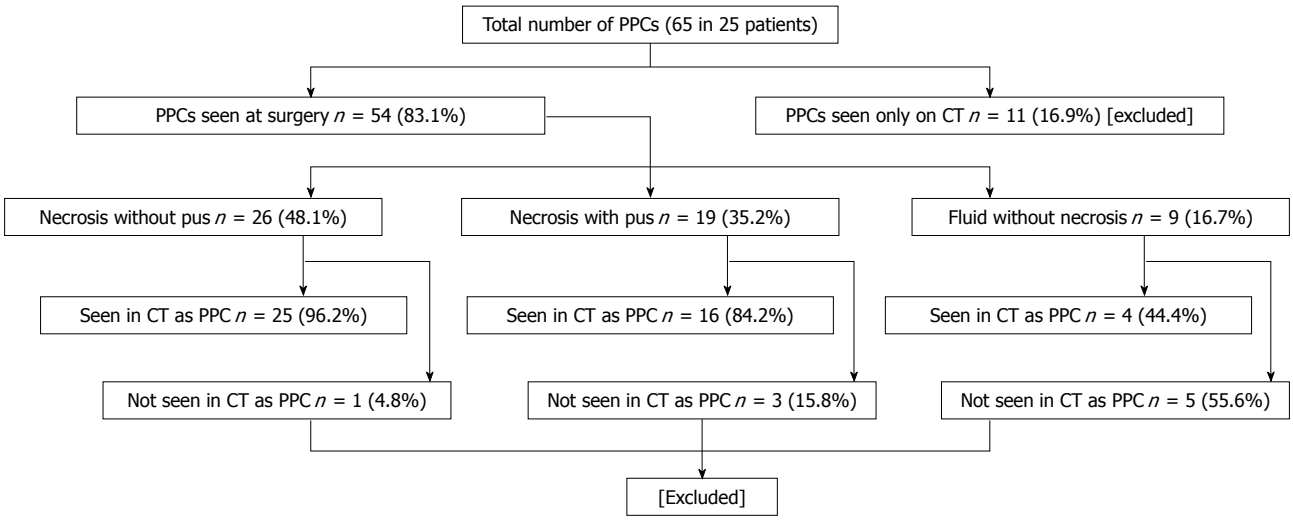

Figure 1 Distribution of different types of peripancreatic collections. PPCs: Peripancreatic collections; CT: Computerized tomography.

Table 1 Characteristics of the study population

Parameters	
Median (IQR) age (yr)	54 (50-67)
Male patients, *n* (%)	20 (80)
Contrast-enhanced CT, *n* (%)	23 (92)
Median (IQR) No. of PPCs	
Necrosis without pus	2 (1-2)
Necrosis with pus	1 (1-2)
Fluid without necrosis	1 (1-3)
Infected PPC, *n* (%)	12 (48)
OF in patients with PPC	
Total, *n* (%)	19 (76)
Persistent OF (> 48 h), *n* (%)	15 (60)
Multiple OF, *n* (%)	2 (8)
Mortality, *n* (%)	3 (12)

NB: Two patients in the group with necrosis and pus did not have a contrast injection prior to computed tomography scan due to renal failure. PPC: Peripancreatic collections; OF: Organ failure.

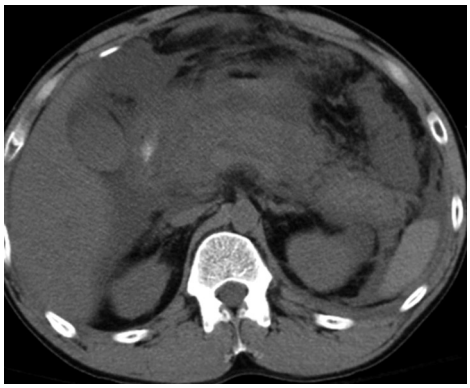

Figure 2 Representative computed tomography picture of a patient with peripancreatic necrotic collection observed at operation but which could not be identified as a discrete collection on preoperative computed tomography (i.e. false negative computed tomography).

RESULTS

We evaluated 25 individuals who had a total of 65 collections, of which 54 were identified specifically at operation. These patients underwent surgery because they had either deterioration in clinical and laboratory parameters or they had evidence of sepsis. Table 1 shows the patient characteristics and Figure 1 shows the distribution of the different types of peripancreatic collections.

Peripancreatic collections

We analyzed only the peripancreatic collections that were evaluated at operative exploration. Of the 54 seen at operation, 26 had necrosis without pus, 19 had necrosis with pus, and 9 had fluid without necrosis. Forty five (83%) of 54 collections were identified on CECT, among which 25/26 (96%) had necrosis without pus, 16/19 (84%) had necrosis with pus, and 4/9 (44%) had fluid without necrosis. Therefore, the majority (n = 41, 91%) of the collections seen on CT were actually peripancreatic necrosis when correlated with operative findings. Among the 54 collections seen at operative exploration, 9 (17%) could not be identified as discrete collections on CT (Figure 2). Five (55%) of these 9 collections had associated necrosis, and 4 (44%) had only fluid without necrosis and were read as ascites on CT.

CT characteristics of pancreatic necrosis

Eighteen (72%) out of 25 patients had pancreatic parenchymal necrosis. Among these patients, 9 (50%) had < 30% necrosis, 4 (22%) had 30%-50% necrosis, and 5 (28%) had > 50% necrosis. We did not observe a statistically significant relationship between the percentage and location of pancreatic necrosis and the number and size of peripancreatic collections.

CT characteristics of peripancreatic collections

All peripancreatic collections were seen around the pancreas, except for one collection in the group with necrosis without pus, which was present in the lower abdomen in the retroperitoneum. Among the characteristics studied,

Table 2 Computed tomography characteristics of peripancreatic collections

		Necrosis without pus (n = 25)	Necrosis with pus (n = 16)	Fluid without necrosis (n = 4)	'P' value
	Volume in cm^3, median (IQR)	193.4 (50-1129)	116.3 (88-389)	551.7 (324-937)	
Fluid characteristics	Heterogeneity, *n* (%)	12 (48)	15 (93.8)[c]	1 (25)[a]	0.004[1]
	Fluid attenuation, *n* (%)	21 (84)	13 (81.3)	4 (100)	0.69
Wall characteristics	Perceptible, *n* (%)	23 (92)	14 (88)	4 (100)	0.76
	Enhancing, *n* (%)	21 (84)	14 (88)	4 (100)	0.68
	Irregular, *n* (%)	18 (71)	14 (88)	1 (25)[a]	0.05
	Internal air, *n* (%)	4 (16)	9 (56)[c]	0 (0)[a]	0.009[1]
	Vessel involvement, *n* (%)	5 (20)	3 (19)	0 (0)	0.62

NB: [1]Indicates statistically significant value when all groups were compared. [a]$P < 0.05$ when compared individually with the group with necrosis and pus; [c]$P < 0.05$ when compared individually with the group with necrosis without pus. Difference between parameters in the group with necrosis without pus and the group with fluid without necrosis were not statistically significant ($P > 0.05$). Difference in volumes of peripancreatic collections in the three groups were not statistically significant ($P > 0.05$).

fluid heterogeneity was seen in a greater proportion of collections in the group with necrosis and pus, compared to the other two groups (94% *vs* 48% and 25%, $P = 0.002$ and 0.003, respectively); however, there was no difference in heterogeneity between the groups with necrosis without pus and fluid without necrosis (48% *vs* 25%, $P = 0.39$). Attenuation units were similar in all three groups (Table 2).

Among the characteristics of the "wall" of the collections studied, irregularity of the wall was seen in a greater proportion of collections in the groups with necrosis with and without pus, when compared to the group with fluid without necrosis (88% and 71% *vs* 25%, $P = 0.06$ and $P < 0.01$, respectively). This finding was not different between the groups with necrosis with and without pus (88% *vs* 71%, P = ns). The other wall characteristics, i.e. perceptibility and enhancement, were similar in all three groups. Two patients with necrosis and pus could not have intravenous contrast administered during CT due to renal failure; when these two patients were excluded from the group while comparing the wall characteristics, the results were unchanged.

The presence of internal, extraluminal gas was seen only in collections with necrosis and in a greater proportion of collections in the group with necrosis and pus when compared to the other two groups (56% *vs* 16% and 0%, $P < 0.01$ in each). In the group with necrosis and pus, the proportion of collections containing extraluminal gas was less than the proportion containing heterogeneity (56% *vs* 94%, $P = 0.04$). In the group with necrosis without pus, bacterial growth was present in cultures of the surgical specimens from 12/14 (86%) patients; while in the group with necrosis and pus, bacterial growth occurred in 10/12 (83%) patients. All these patients were on high-dose broad-spectrum antibiotics at the time of operation. Among the necrotic collections without pus, there were gram positive and gram negative infections in 50% and 14%, respectively, and mixed infections in 21%. On the other hand, among the necrotic collections with pus, there were gram positive and negative infections in 25% and 33%, respectively, and mixed infections in 25%. Table 3 shows the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios of fluid heterogeneity and the presence of extraluminal gas on CT in predicting the presence of pus in peripancreatic collections.

Table 3 Predictive value of heterogeneity and presence of air within necrotic collection on computed tomography scan in diagnosing the presence of pus

	Heterogeneity	Presence of air	Both
Sensitivity (%)	93.8	47.3	47.4
Specificity (%)	52.0	84.0	92.0
Positive predictive value (%)	55.5	69.2	81.8
Negative predictive value (%)	92.9	67.7	23.3
Positive likelihood ratio	1.9	2.9	5.9
Negative likelihood ratio	0.1	0.6	0.6

DISCUSSION

Earlier studies have demonstrated good correlation between CECT and operative exploration in diagnosing pancreatic necrosis in patients with AP[5-8]. The CECT feature that characterizes pancreatic necrosis most reliably is lack of enhancement of pancreatic parenchymal tissue[9]. In contrast, no specific CECT features that can differentiate peripancreatic necrosis from peripancreatic fluid without necrosis, especially early after the onset of pancreatitis, have been reported so far. MRI has been reported to be a more powerful tool for detecting necrotic debris within peripancreatic collections after acute pancreatitis[10], however, MRI is substantially more expensive than CECT and can be difficult to perform in the very ill patient, and requires radiologic experience and expertise in its interpretation. These considerations regarding MRI *vs* CECT increase the likelihood of the utility of CECT in the assessment of patients worldwide with severe acute pancreatitis for years to come. In this study, we evaluated the correlation between CECT findings of peripancreatic collections with objective findings at operative exploration in order to assess the ability of CECT to predict the nature of these collections. Such differentiation would be important from the standpoint

of better clinical decision-making, and thereby, possibly altering the treatment of these collections.

Importantly in this study, we specifically avoided the term "peripancreatic fluid collection" as was adapted by the Atlanta Classification. This term, in our opinion, has stifled progress in this field, led to controversy in the discussion of acute fluid collections *vs* peripancreatic necrosis, and has led to unnecessary controversy as well as confusion as to the appropriate use and misuse of purely drainage procedures (radiologic, endoscopic, or operative) *vs* procedures allowing a true necrosectomy. Therefore, we use the term peripancreatic "collection" rather than peripancreatic "fluid collection", because early in the course of necrotizing pancreatitis, some (perhaps most) pancreatic collections are composed primarily of necrosis without substantial fluid components, thereby differentiating them from acute fluid collections that lack necrosis. This study was designed, in part, to see if CT would be able to differentiate areas of necrosis (with or without a fluid component) from collections of fluid without necrosis.

We found that CT had a false positive rate (peripancreatic collections seen on CT but not found at operation) of 17% (11/65) and a false negative rate (collections found at operation but not seen on CT) of 17% (9/54). The false positive and false negative collections on CT were excluded from the comparative analysis. Operative findings and histologic evidence confirmed that over 90% of the peripancreatic collections detected on CECT in patients with necrotizing pancreatitis undergoing operative exploration/necrosectomy contained necrosis. In the current study, we found all the CT parameters (heterogeneity, attenuation, wall perceptibility, wall enhancement, wall irregularity, and presence of extraluminal gas) to be similar in the groups with necrosis without pus and fluid without necrosis. In contrast, heterogeneity and presence of extraluminal gas were significantly more common in the group with necrosis and pus, compared to the collections without necrosis. All other parameters were similar. Therefore, CT did not have any specific feature that would reliably detect the presence of necrosis within the peripancreatic collections. The presence of irregularity of the wall of the collection was more common in those with necrosis (88% and 71% with and without pus, respectively) but not enough to reliably differentiate necrotic collections from fluid collections, 25% of which had an irregular wall.

If we consider the groups with necrosis with and without pus, the presence of heterogeneity on CT was seen in a significantly greater proportion in the former group with a sensitivity and positive likelihood ratio of detecting pus in the collection of 94% and 1.9%, respectively. The presence of extraluminal gas within fluid collections is well-recognized as a marker of infection in the absence of a history of prior endoscopic, radiologic, or operative intubation. In the current study, as expected, internal air was seen in a significantly greater proportion of collections with pus (indeed no fluid collection without necrosis had gas within it), although significantly less than those with heterogeneity (P = 0.04). When both heterogeneity and the presence of extraluminal gas were combined, then the positive likelihood ratio increased substantially to 5.9, and the absence of both reliably excluded the presence of pus (specificity 92%). From these observations, it is clear that heterogeneity and presence of extraluminal gas in the peripancreatic collections in a patient with severe acute pancreatitis can suggest the presence of pus, i.e. purulent infection. The characteristics of the progression of infection in necrotic tissue is a dynamic process, and pus formation tends to occur in the later stages of the disease. In this study, bacterial growth occurred in 86% patients with a peripancreatic collection containing necrosis but no obvious pus. None of the CT features could, therefore, detect the presence of infection in this group of collections, i.e. infected necrosis without pus.

To our knowledge, this analysis is among the very few studies in the literature that have assessed the direct correlation between CECT and operative findings in the diagnosis and characterization of peripancreatic collections in the presence of severe acute pancreatitis. We used operative exploration as the gold standard to detect the presence of necrosis within these collections which was confirmed histologically. The operative data and the CECT findings were interpreted and analyzed by a single, blinded surgeon and radiologist, respectively. This approach helped to eliminate the chance of bias in the interpretations. This study, however, has a few limitations. First, this is a retrospective study, and the number of collections in the group with fluid without necrosis was only 4. This small number of fluid collections could have potentially skewed the CECT findings towards an inability to detect necrosis within the collections. Moreover, the CECT scans were performed between 1995 and 2001, when older generation machines with thick sections were used. The use of modern, multidetector CECTs with thinner slice sections might increase the ability of CECT to detect the presence of infection in these collections without pus. Second, the gold standard for the presence of a collection was operative exploration. We believe that this method was most appropriate, because at operative exploration, the operative approach at our institution is to expose the entire pancreas and open all areas of the peripancreatic retroperitoneum to ensure complete necrosectomy. In addition, CECT serves as a roadmap for surgery, and all collections noted on CECT were specifically sought in the operating room. Finally, the patients we studied were a skewed population with necrotizing pancreatitis, all of whom required operative intervention and thus represent only patients with necrotizing pancreatitis.

In conclusion, this study shows that most of the peripancreatic collections seen on CECT in patients with severe acute pancreatitis who require operative intervention contain necrotic tissue. It appears that CECT has a somewhat limited role in differentiating the different types of collections into necrosis with pus, necrosis without pus, and fluid collections without necrosis. While hetero-

geneity of the collection can reliably suggest the presence of necrosis, and extraluminal gas defines the presence of infection in a collection with necrosis, no CECT feature could suggest the presence of infection in a necrotic collection without pus.

COMMENTS

Background

Earlier studies have demonstrated good correlation between contrast-enhanced computerized tomography (CECT) and operative exploration in diagnosing pancreatic necrosis in patients with acute pancreatitis. Studies evaluating CECT and operative correlation in the detection of peripancreatic necrosis and peripancreatic fluid collections are scarce. Vege *et al* suggested that peripancreatic necrosis may not be seen on CECT and that a normal enhancement of the pancreas does not necessarily rule out the presence of peripancreatic necrosis.

Innovations and breakthroughs

There are very few similar studies in the literature so far. In this study, the authors objectively correlated peripancreatic collections seen on CT with those actually seen at surgery. The use of surgery as a gold standard adds maximum value to this study.

Applications

This study shows that while heterogeneity of the collection can reliably suggest the presence of necrosis, and extraluminal gas can define the presence of infection in a collection with necrosis, no CECT feature can suggest the presence of infection in a necrotic collection without pus. However, since the authors' sample size was not very large, the data could be validated in centers where a large volume of surgery is still performed for peripancreatic collections in patients with acute necrotizing pancreatitis.

Peer review

The Authors reported an interesting study which correlates CECT and intraoperative findings in patients who required surgical drainage for peripancreatic collections following severe acute pancreatitis. The paper is clear, well written, the aim of the study is covered, the methodology of the study despite being retrospective is correct, the statistical analysis is well done and explores the most important topics and the discussion is complete.

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BRIEF ARTICLE

Pre-illness changes in dietary habits and diet as a risk factor for inflammatory bowel disease: A case-control study

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Abstract

AIM: To evaluate whether symptoms of inflammatory bowel disease (IBD), before diagnosis modify dietary habits, and to investigate the pre-illness diet in patients with recent IBD in comparison with an age-matched healthy control group.

METHODS: Overall, 83 new cases of IBD (41 ulcerative colitis, 42 Crohn's disease) and 160 healthy controls were studied. Portions per week of 34 foods and beverages before onset of symptoms were recorded using a validated questionnaire. Duration of symptoms before IBD diagnosis, presence of specific symptoms and their impact on subjective changes in usual dietary habits were also recorded. The association between diet and IBD was investigated by multiple logistic regression and dietary patterns were assessed by factor analysis.

RESULTS: Changes in dietary habits, due to the presence of symptoms, were reported by 38.6% of patients and were not significantly related to specific symptoms, rather to long duration of symptoms, only in Crohn's disease patients. In IBD patients who did not change dietary habits, moderate and high consumption of margarine (OR = 11.8 and OR = 21.37) was associated with ulcerative colitis, whilst high consumption of red meat (OR = 7.8) and high intake of cheese were associated with Crohn's disease.

CONCLUSION: More than one third of IBD patients change dietary habits before diagnosis. Margarine, red meat and cheese increase the risk of ulcerative colitis and Crohn's disease.

Key words: Inflammatory bowel diseases; Diet; Symptoms; Factor analysis

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INTRODUCTION

The aetiology of inflammatory bowel disease (IBD) is

still unknown. Since genetically determined mechanisms have remained unchanged over historical time periods, the increase in incidence of ulcerative colitis (UC) and Crohn's disease (CD) during the 20th century suggests environmental influences. Epidemiological and clinical evidence support an association between IBD and many apparently unrelated environmental factors, including diet, smoking, geographical and social status, occupation, and microbial factors[1-4]. In particular, the role of dietary factors or components of diet in the pathophysiology have long since been taken into consideration, and immunological mechanisms linking food antigens to development of inflammation have also been postulated. However, this attractive explanation is far from proven, and studies investigating this potential link are few and unconvincing. In some reports, it has been suggested that increased consumption of sugar and refined carbohydrates might be a risk factor for CD[5-10], and has also been demonstrated in some UC patients[7,9,11-13], whereas protein and fat intake, as well as decreased consumption of fruit, vegetables and fibre, appear to increase the risk of IBD, but data reported have been more controversial[5,14-16]. Indeed, a causal relationship between diet and IBD is difficult to define, due to the possibility that early symptoms of the disease may lead to a modification in dietary habits and the inability of the patients to accurately remember their diet before the onset of symptoms. To date, only a few studies have examined the pre-illness diet, in incident cases, and have shown conflicting results[5,15-18].

The present study aimed to evaluate whether the signs and symptoms of IBD in patients before diagnosis led to a modification in their diet, and to investigate pre-illness dietary habits in patients with recent IBD in comparison to those in an age-matched control group.

MATERIALS AND METHODS

Between June and September 2003, and between September 2007 and June 2008, incident cases of UC and CD (diagnosis < 12 mo, median duration of symptoms: 5 mo, range: 0-84 mo) consecutively observed at the IBD Unit of the Gastroenterology Department of L. Sacco University Hospital were interviewed. Controls were recruited among healthy blood donors of the same Hospital and were frequency-matched (by quinquennial of age and sex) with the cases. None of the controls reported serious abdominal diseases that could have influenced their dietary habits. All patients were contacted by two L. Sacco University gastroenterologists (Cucino C and Bezzio C), who interviewed patients and controls, asking questions in an identical manner, with interviews lasting approximately 30 min each during which a validated questionnaire was employed which had been previously used for epidemiological studies concerning the relationship between cancer and diet[19].

This questionnaire included information on sociodemographic characteristics, anthropometric measures, lifestyle, including both tobacco smoking and alcohol habits, and personal and family medical history. Attention was focused, in particular, on long-term drinking, smoking, and dietary habits before diagnosis and onset of symptoms in IBD patients. Patients and healthy controls were asked about the number and size of portions of different food items consumed per week and whether 3 alcoholic and 4 non-alcoholic drinking items were consumed per day. IBD patients were also investigated to determine the duration of symptoms before diagnosis and the presence of specific symptoms such as diarrhoea, abdominal pain, rectal bleeding and weight loss > 3 kg. In IBD patients, an attempt was made to establish whether they had changed their usual dietary habits due to symptoms. The categorical answers to these questions were "yes", "no" or "don't know". The validated food frequency questionnaire investigated the usual dietary habits during the 5 years prior to diagnosis and onset of symptoms for IBD patients or during the last 5 years in healthy controls. The questionnaire included 34 food items and beverages commonly used in the Italian diet, grouped as follows: (1) bread and cereal dishes (first courses); (2) red meat and other main dishes such as fish, pork, poultry or rabbit (second courses); (3) vegetables (side dishes); (4) fruit; (5) sweets (including refined carbohydrates and sugar), desserts and soft drinks; (6) milk and hot beverages; and (7) alcoholic beverages. Furthermore, the consumption of coffee (non decaffeinated and decaffeinated), tea, olives and seed oils, margarine and butter were also investigated. Questions were also asked concerning the average weekly frequency of consumption of each dietary item; occasional intakes (< once a week but at least once a month) were coded as 0.5/wk.

Data analysis

Data were reported as mean and SD or as median and range, where appropriate. The association between symptoms and changes in dietary habit was evaluated by the Fisher exact test.

Consumption of foods and beverages, as well as other continuous variables, were subdivided into tertiles (low, moderate and high consumptions).

Odds ratios (OR) and corresponding 95% CI were defined using multiple logistic regression models. All the regression equations included terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ⩾ 25 cigarettes/d) and body mass index (BMI) (quintiles). Tests for trends were based on the likelihood ratio test between models with and without a linear term for the diet score.

Dietary patterns, based on nutrient intake, associated with IBD, were defined using factor analysis, a multivariate technique which analyses the underlying structure of a set of data in order to explain observed relationships between a large number of variables in terms of simpler relations.

Within a factor, negative loading indicates that foods

Table 1 Demographic and clinical features of inflammatory bowel disease cases and healthy controls

	Cases (n = 83)	Controls (n = 160)
Sex (M/F)	49/34	97/63
Age (mean ± SD)	37.5 ± 15.2	40.4 ± 14.6
Social status, n (%)		
Unmarried	50 (60.3)	83 (51.9)
Married	26 (31.3)	69 (43.1)
Divorced/widowed	7 (8.4)	8 (5.0)
Years of education (mean-SD)	12.4-3.5	11.5-4.2
Body mass index, kg^2/cm (mean-SD)	26.1-9.3	29.1-10.8
Smoking habit, n (%)		
Never	32 (38.6)	80 (50.0)
Smokers	29 (34.9)	41 (35.6)
Ex-smokers	22 (26.5)	39 (24.4)
No. cigarettes/d (smokers) mean (range)	13.4 (3-50)	14.7 (5-30)
Disease location (Crohn's disease) n = 42		
Ileum, n (%)	18 (42.9)	
Ileum and colon	13 (31.0)	/
Colon	11 (26.2)	
Disease location (ulcerative colitis) n = 41		
Proctitis	5 (12.2)	
Left sided colitis	22 (53.7)	/
Pancolitis	14 (34.1)	
Disease behaviour (Crohn's disease)		
Inflammatory (B1)	27 (64.3)	
Stricturing (B2)	8 (19.0)	/
Penetrating (B3)	7 (16.7)	

are inversely associated with the factor, while positive loading indicates a direct association with the factor. After varimax rotation, factor scores were saved from the principal components analysis for each individual. Factor scores were categorized into tertiles based on the distribution of the control.

RESULTS

The study population comprised 83 IBD patients (41 UC, 42 CD) and 160 sex- and age-matched healthy controls, comparable for social status, years of education, BMI and smoking habits. CD patients showed more frequent ileal localisation of disease and, as expected, an inflammatory behaviour, whilst UC patients showed more frequent left sided colitis (Table 1).

Symptoms and change of diet in IBD patients

The duration of symptoms before diagnosis was comparable in UC and CD patients [median (range): 5 (1-84) *vs* 6 (0-48) mo]. Weight loss, diarrhoea and abdominal pain at diagnosis were comparable, judging from the complaints referred to by the UC and CD patients. As expected, UC patients presented rectal bleeding more frequently than CD patients (Figure 1).

A conscious change of dietary habits due, before diagnosis, to the presence of symptoms, was reported by 32 patients (38.6%), 15 with UC and 17 with CD. The main dietary changes were reduction of fat and calorie intake (12 patients), while other patients reduced or stopped the consumption of fibre (18 patients) and milk or cheese (9 patients).

Table 2 Association between changes in pre-illness dietary habits and symptoms in inflammatory bowel disease patients

	Changes in diet			
	Ulcerative colitis		Crohn's disease	
	No (26)	Yes (15)	No (25)	Yes (17)
Duration of symptoms				
Short (≤ 3 mo)	14 (53.85)	4 (26.67)	8 (32.00)	4 (23.53)
Intermediate (4-10 mo)	7 (26.92)	4 (26.67)	13 (52.00)	4 (23.53)
Long (> 10 mo)	5 (19.23)	7 (46.66)	4 (16.00)	9 (52.94)[a]
Median, range (mo)	3 (1-15)	7 (1-84)	5 (1-30)	12 (1-48)[a]
Weight loss				
Absent/low (< 2 kg)	12 (46.15)	4 (26.67)	8 (32.00)	3 (16.65)
Moderate (2-6 kg)	8 (30.77)	3 (20.00)	11 (44.00)	8 (47.06)
Severe (> 6 kg)	6 (23.08)	8 (53.33)	6 (24.00)	6 (35.29)
Abdominal pain				
No	9 (34.61)	3 (20.00)	4 (16.00)	1 (5.88)
Yes	17 (65.39)	12 (80.00)	21 (84.00)	16 (94.12)
Diarrhoea				
No	5 (19.23)	1 (6.67)	4 (16.00)	1 (5.88)
Yes	21 (80.77)	14 (93.33)	21 (84.00)	16 (94.12)
Rectal bleeding				
No	9 (34.62)	5 (33.33)	13 (52.00)	12 (70.59)
Yes	17 (65.38)	10 (66.67)	12 (48.00)	5 (29.41)

[a]P < 0.05.

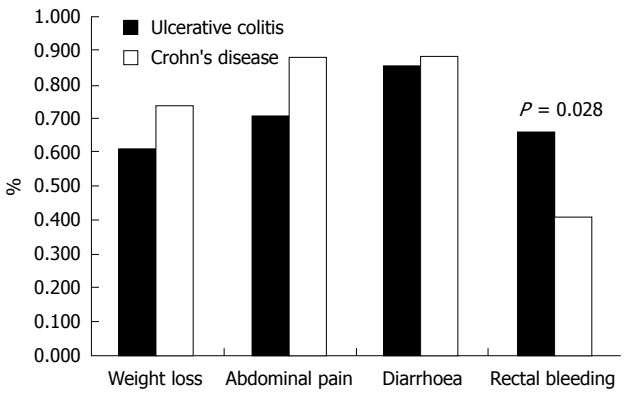

Figure 1 Prevalence of specific symptoms and changes in dietary habits in ulcerative colitis and Crohn's disease patients.

Change in dietary habits was not due to specific symptoms. However, in CD patients the long duration of symptoms was significantly correlated with changes in dietary habits (Table 2).

Food consumption and risk of IBD

To exclude any potential confounding influence of symptom-induced changes in diet (i.e. intake of milk in UC and fibre in CD) only patients who did not change dietary habits (26 UC and 25 CD patients) were taken into consideration (Tables 3 and 4). In these patients, an increased risk of UC (although not significant) was found for those reporting a high consumption of pasta and rice (OR = 3.38, 95% CI: 0.99-11.47) and with moderate or high consumption of margarine (OR = 11.8 and OR = 21.37, respectively). CD was significantly associated with moder-

Table 3 Association between pre-illness intake of foods and beverages and risk of ulcerative colitis in patients who did not modify the diet before diagnosis

	Controls	Ulcerative colitis	
	n (%)	*n* (%)	OR[1] (95% CI)
Pasta and/or rice			
Low	67 (41.88)	7 (26.92)	[2]
Moderate	68 (42.50)	12 (46.15)	1.89 (0.66-5.41)
High	25 (15.63)	7 (26.92)	3.38 (0.99-11.47)
Bread			
Low	60 (37.50)	6 (23.08)	[2]
Moderate	55 (34.38)	9 (34.62)	1.31 (0.42-4.13)
High	45 (28.13)	11 (42.31)	2.40 (0.80-7.26)
Sweets and cakes			
Low	52 (32.50)	10 (38.46)	[2]
Moderate	49 (30.63)	4 (15.38)	0.37 (0.10-1.33)
High	59 (36.88)	12 (46.15)	0.96 (0.36-2.51)
Red meat			
Low	73 (45.63)	12 (46.15)	[2]
Moderate	38 (23.75)	8 (30.77)	1.22 (0.45-3.32)
High	49 (30.63)	6 (23.08)	0.63 (0.20-1.94)
White meat			
Low	45 (28.13)	5 (19.23)	[2]
Moderate	70 (43.75)	17 (65.38)	2.04 (0.69-6.05)
High	45 (28.13)	4 (15.38)	0.75 (0.19-3.04)
Tuna fish			
Low	37 (23.13)	10 (38.46)	[2]
Moderate	48 (30.00)	6 (23.08)	0.43 (0.13-1.37)
High	75 (46.88)	10 (38.46)	0.49 (0.18-1.36)
Fish			
Low	61 (38.13)	17 (65.38)	[2]
Moderate	39 (24.38)	3 (11.54)	0.33 (0.09-1.26)
High	60 (37.50)	6 (23.08)	0.33 (0.11-0.92)
Processed meat			
Low	50 (31.25)	9 (34.62)	[2]
Moderate	57 (35.63)	10 (38.46)	0.82 (0.28-2.36)
High	53 (33.13)	7 (26.92)	0.63 (0.21-1.91)
Milk			
Low	45 (28.13)	13 (50.00)	[2]
Moderate	36 (22.50)	3 (11.54)	0.30 (0.08-1.19)
High	79 (49.38)	10 (38.46)	0.55 (0.21-1.40)
Cheese			
Low	43 (26.88)	8 (30.77)	[2]
Moderate	70 (43.75)	11 (42.31)	0.93 (0.33-2.63)
High	47 (29.38)	7 (26.92)	0.98 (0.31-3.12)
Eggs			
Low	44 (27.50)	11 (42.31)	[2]
Moderate	51 (31.88)	11 (42.31)	0.78 (0.30-2.06)
High	65 (40.63)	4 (15.38)	0.21 (0.06-0.73)
Potatoes			
Low	25 (15.63)	10 (38.46)	[2]
Moderate	78 (48.75)	11 (42.31)	0.39 (0.14-1.09)
High	57 (35.63)	5 (19.23)	0.24 (0.09-0.83)
Vegetables			
Low	44 (27.67)	11 (42.31)	[2]
Moderate	67 (42.14)	10 (38.46)	0.49 (0.18-1.35)
High	48 (30.19)	5 (19.23)	0.37 (0.11-1.21)
Fruits			
Low	48 (30.00)	9 (34.62)	[2]
Moderate	61 (38.13)	11 (42.31)	1.12 (0.42-3.01)
High	51 (31.88)	6 (23.08)	0.41 (0.13-1.35)
Refined sugar			
Low	57 (35.63)	9 (34.62)	[2]
Moderate	45 (28.13)	12 (46.15)	1.52 (0.57-4.09)
High	58 (36.25)	5 (19.23)	0.57 (0.17-1.87)
Butter			
Low	28 (17.50)	1 (3.85)	[2]
Moderate	88 (55.00)	18 (69.23)	6.02 (0.75-47.97)
High	44 (27.50)	7 (26.92)	5.18 (0.58-46.20)
Margarine			
Low	57 (35.63)	1 (3.85)	[2]
Moderate	84 (52.50)	19 (73.08)	11.80 (1.51-91.99)
High	19 (11.88)	6 (23.08)	21.37 (2.32-196.6)
Olive oil			
Low	47 (29.38)	6 (23.08)	[2]
Moderate	88 (55.00)	16 (61.54)	1.40 (0.50-3.93)
High	25 (15.63)	4 (15.38)	1.16 (0.29-4.63)
Seed oil			
Low	33 (20.63)	1 (3.85)	[2]
Moderate	97 (60.63)	22 (84.62)	7.26 (0.93-56.53)
High	30 (18.75)	3 (11.54)	3.82 (0.36-40.14)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ≥ 25 cigarettes/d) and body mass index (quintiles); [2]Reference category.

ate consumption of meat (OR = 7.8, 95% CI: 1.61-37.89) and high consumption of cheese (OR = 3.7, 95% CI: 1.14-12.01). High intake of fish and potatoes reduced the risk of IBD while the consumption of vegetables and tuna fish was negatively correlated with the risk of CD and high intake of eggs was negatively correlated with risk of UC.

In particular, as far as any possible correlation of diet with disease location in CD is concerned, tuna fish consumption was negatively correlated with both pure colonic location (OR = 0.33, 95% CI: 0.11-0.98) and ileal or ileocolonic CD (OR = 0.48, 95% CI: 0.26-0.91), while vegetable consumption showed protective effects in patients with ileal or ileocolonic location of CD (OR = 0.43, 95% CI: 0.21-0.88). Interestingly, we also found that an increased risk of ileal or ileocolonic (but not pure colonic) CD associated with high bread or cheese consumption (OR = 2.61, 95% CI: 1.26-5.41 and OR = 2.61, 95% CI: 1.26-5.41, respectively) (Table 5).

No correlation was found between beverages and risk of IBD.

Factor analysis identified three dietary-intake factors that, overall, explained 94% of total variability, accounting for 59%, 20% and 14%, respectively. A first dietary pattern, called "refined", was mainly correlated to pasta, sweets, red and processed meat, butter and margarine. The second pattern (prudent) loaded heavily on white meat, tuna fish, fish, eggs and potatoes. The last pattern, designated "healthy", loaded heavily bread, cheese and, in particular, fruit and vegetables, as well as olive oil (which was found to be negatively correlated with the two previously described patterns). Evaluation of the association between these specific dietary intake patterns and IBD showed that a "refined" diet was associated with an increased risk of UC and CD. In contrast, the "prudent" pattern was significantly associated with a decreased risk (Table 6). The "healthy" pattern was not consistently associated with UC, moreover, a non-significant increase in risk was present for CD.

DISCUSSION

In this case-control study analysing the relationship be-

Table 4 Association between pre-illness intake of foods and beverages and risk of Crohn's disease in patients who did not modify the diet before diagnosis

	Controls	Crohn's disease	
	n (%)	*n* (%)	OR[1] (95% CI)
Pasta and/or rice			
Low	67 (41.88)	8 (32.00)	[2]
Moderate	68 (42.50)	14 (56.00)	1.59 (0.61-4.17)
High	25 (15.63)	3 (12.00)	1.01 (0.24-4.34)
Bread			
Low	60 (37.50)	6 (24.00)	[2]
Moderate	55 (34.38)	8 (32.00)	1.25 (0.38-4.04)
High	45 (28.13)	11 (44.00)	2.47 (0.81-7.56)
Sweets and cakes			
Low	52 (32.50)	8 (32.00)	[2]
Moderate	49 (30.63)	4 (16.00)	0.43 (0.11-1.63)
High	59 (36.88)	13 (52.00)	1.21 (0.44-3.29)
Red meat			
Low	73 (45.63)	8 (32.00)	[2]
Moderate	38 (23.75)	6 (24.00)	1.25 (0.38-4.07)
High	49 (30.63)	11 (44.00)	2.42 (0.85-6.85)
White meat			
Low	45 (28.13)	8 (32.00)	[2]
Moderate	70 (43.75)	15 (60.00)	1.33 (0.50-3.52)
High	45 (28.13)	2 (8.00)	0.25 (0.05-1.27)
Tuna fish			
Low	37 (23.13)	13 (52.00)	[2]
Moderate	48 (30.00)	6 (24.00)	1.31 (0.35-4.87)
High	75 (46.88)	6 (24.00)	0.25 (0.08-0.77)
Fish			
Low	61 (38.13)	16 (64.00)	[2]
Moderate	39 (24.38)	6 (24.00)	0.57 (0.19-1.72)
High	60 (37.50)	3 (12.00)	0.18 (0.05-0.67)
Processed meat			
Low	50 (31.25)	2 (8.00)	[2]
Moderate	57 (35.63)	18 (72.00)	7.80 (1.61-37.89)
High	53 (33.13)	5 (20.00)	1.97 (0.35-11.03)
Milk			
Low	45 (28.13)	7 (28.00)	[2]
Moderate	36 (22.50)	2 (8.00)	0.41 (0.07-2.18)
High	79 (49.38)	16 (64.00)	1.55 (0.57-4.19)
Cheese			
Low	43 (26.88)	5 (20.00)	[2]
Moderate	70 (43.75)	5 (20.00)	0.54 (0.14-2.06)
High	47 (29.38)	15 (60.00)	3.70 (1.14-12.01)
Eggs			
Low	44 (27.50)	10 (40.00)	[2]
Moderate	51 (31.88)	9 (36.00)	0.85 (0.30-2.40)
High	65 (40.63)	6 (24.00)	0.42 (0.14-1.29)
Potatoes			
Low	25 (15.63)	7 (28.00)	[2]
Moderate	78 (48.75)	13 (52.00)	0.60 (0.19-1.83)
High	57 (35.63)	5 (20.00)	0.24 (0.06-0.91)
Vegetables			
Low	44 (27.67)	14 (56.00)	[2]
Moderate	67 (42.14)	7 (28.00)	0.26 (0.09-0.78)
High	48 (30.19)	4 (16.00)	0.21 (0.05-0.78)
Fruits			
Low	48 (30.00)	9 (36.00)	[2]
Moderate	61 (38.13)	8 (32.00)	0.78 (0.26-2.30)
High	51 (31.88)	8 (32.00)	0.76 (0.26-2.21)
Refined sugar			
Low	57 (35.63)	8 (32.00)	[2]
Moderate	45 (28.13)	12 (48.00)	1.61 (0.58-4.42)
High	58 (36.25)	5 (20.00)	0.58 (0.17-1.97)
Butter			
Low	28 (17.50)	1 (4.00)	[2]
Moderate	88 (55.00)	20 (80.00)	6.07 (0.75-49.20)
High	44 (27.50)	4 (16.00)	1.84 (0.18-18.19)
Margarine			
Low	57 (35.63)	8 (32.00)	[2]
Moderate	84 (52.50)	13 (52.00)	1.07 (0.40-2.84)
High	19 (11.88)	4 (16.00)	1.17 (0.29-4.74)
Olive oil			
Low	47 (29.38)	10 (40.00)	[2]
Moderate	88 (55.00)	10 (40.00)	0.60 (0.22-1.61)
High	25 (15.63)	5 (20.00)	0.98 (0.29-3.35)
Seed oil			
Low	33 (20.63)	6 (24.00)	[2]
Moderate	97 (60.63)	15 (60.00)	0.84 (0.29-2.43)
High	30 (18.75)	4 (16.00)	0.66 (0.16-2.76)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ≥ 25 cigarettes/d) and body mass index (quintiles). [2]Reference category.

Table 5 Odds ratios[1] and corresponding 95% CI for pre-illness intake of foods according to the disease location for Crohn's disease patients

	Small bowel	Colon
Pasta and/or rice	1.44 (0.71-2.92)	0.65 (0.20-2.13)
Bread	2.61 (1.26-5.41)[2]	0.27 (0.06-1.10)
Sweets and cakes	1.17 (0.64-2.14)	1.39 (0.53-3.63)
Red meat	1.58 (0.88-2.82)	1.23 (0.49-3.12)
White meat	0.74 (0.38-1.46)	0.40 (0.12-1.29)
Tuna fish	0.48 (0.26-0.91)[2]	0.33 (0.11-0.98)[2]
Fish	0.48 (0.24-0.93)[2]	0.41 (0.13-1.29)
Processed meat	1.31 (0.67-2.58)	0.74 (0.27-2.01)
Milk	1.30 (0.71-2.36)	1.13 (0.46-2.78)
Cheese	2.61 (1.26-5.41)[2]	1.94 (0.62-6.09)
Eggs	0.67 (0.36-1.23)	0.69 (0.27-1.80)
Potatoes	0.50 (0.23-1.07)	0.46 (0.15-1.41)
Vegetables	0.43 (0.21-0.88)[2]	0.60 (0.20-1.83)
Fruits	0.82 (0.42-1.58)	0.83 (0.29-2.36)
Refined sugar	0.80 (0.44-1.46)	0.77 (0.31-1.95)
Butter	0.83 (0.39-1.79)	1.44 (0.41-5.12)
Margarine	1.10 (0.51-2.36)	1.83 (0.58-5.76)
Olive oil	1.20 (0.58-2.51)	0.24 (0.06-1.00)[2]
Seed oil	0.81 (0.37-1.79)	1.05 (0.32-3.42)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ≥ 25 cigarettes/d) and body mass index (quintiles) and according to an increase of 1 tertile level; [2]Significant associations.

tween pre-illness diet and the risk of IBD, in incident cases of UC and CD, results showed a positive relationship between the intake of margarine and a non-significant trend for pasta and rice in UC, and meat and cheese in CD. IBD patients reported a significant reduction in intake of potatoes and fish, and CD patients also have reduced consumption of vegetables and tuna fish compared with healthy controls. An association between pasta, sweets, red and processed meat, butter and margarine and IBD was also observed by factor analysis.

Several studies have focused on the role of dietary factors in IBD, some of which were already implicated as influencing the development of IBD, starting from the study by Martini & Brandes which demonstrated a

Table 6 Food group loadings for 3 dietary patterns extracted by factor analyses

	Factor 1	Factor 2	Factor 3
Pasta and/or rice	0.30	-0.25	0.32
Bread	-0.01	-0.40	0.51
Soft, sweets and cakes	0.45	-0.28	0.09
Red meat	0.57	0.30	0.23
White meat	0.45	0.49	-0.02
Tuna fish	0.43	0.60	-0.11
Fish	0.33	0.58	-0.12
Processed meat	0.46	0.04	-0.02
Milk	0.03	-0.33	0.04
Cheese	0.02	0.19	0.56
Eggs	-0.05	0.69	0.08
Potatoes	0.09	0.58	0.01
Vegetables	-0.52	0.22	0.32
Fruit	-0.53	-0.01	0.25
Refined sugar	-0.01	-0.03	0.60
Butter	0.47	0.20	0.27
Margarine	0.46	0.29	-0.02
Olive oil	-0.17	-0.32	0.44
Seed oil	0.39	0.13	0.07
	Refined OR (95% CI)	Prudent OR[1] (95% CI)	Healthy OR (95% CI)
Ulcerative colitis			
Low[2]	1[3]	1[3]	1[3]
Moderate	2.29 (0.70-7.32)	0.32 (0.11-0.90)	0.80 (0.28-2.28)
High	2.46 (0.77-7.83)	0.15 (0.04-0.51)	0.95 (0.33-2.76)
Crohn's disease			
Low	1[3]	1[3]	1[3]
Moderate	1.51 (0.45-4.99)	0.47 (0.17-1.27)	0.57 (0.18-1.78)
High	2.15 (0.67-6.87)	0.13 (0.03-0.51)	1.39 (0.49-3.95)

[1]From unconditional logistic regression models including terms for age (5-year groups), sex, years of education, tobacco consumption (never, ex-smoker, current smoker of < 15, 15-24, ≥ 25 cigarettes/d) and body mass index (quintiles); [2]Tertiles of factor scores; [3]Reference categories.

higher intake of refined carbohydrates in CD patients compared to controls[20]. This report was then followed by several studies in which other dietary factors, such as fibre, proteins, and total calorie intake, were evaluated in patients with UC and CD[5,6,8,11-17]. Despite the large number of papers on this issue, it has been shown that studying the association between diet and chronic disease presents methodological problems, since dietary habits could have already been influenced by the pathological condition itself. This has been demonstrated also by the present data, which revealed that UC and CD patients had already modified their dietary habits by themselves before diagnosis. We found that most IBD patients remembered having avoided vegetables, milk and/or cheese, or having reduced calorie intake before diagnosis and the onset of symptoms. The influence of symptoms on dietary habits and the difficulties encountered in recalling foods eaten 5 years before diagnosis are the main problems emerging in studies investigating diet and IBD.

In previous studies IBD patients, when asked about their dietary habits, described an increased consumption of refined sugar and a decreased intake of fruit and vegetables. These findings have also been interpreted as a consequence of the disease, rather than a factor which could be implicated in its aetiology. Indeed, refined sugar is rapidly absorbed in the small intestine, avoiding bulking effects and giving the possibility to compensate loss of energy and weight, which are typical characteristics of UC and CD. On the other hand, a decreased consumption of fibres could easily be the effect of trying to avoid symptoms caused by the bulk-rich food. Due to this methodological bias, in some case-control studies efforts have been made to retrieve data on the patients' dietary habits many years before onset of the illness, unfortunately obtaining less reliable answers, due to the weakness of the memory of those replying as well as the influence that the symptoms themselves could have had on the pre-illness dietary habits, the so called "recall bias". One condition which should be met is, therefore, to interview the patients about pre-illness dietary habits as soon as the diagnosis of IBD is made, in order to consider those subjects as incident cases. Even new patients might have had symptoms for several months or years prior to the diagnosis. To date, only a few studies by-passed the recall bias interviewing incident cases of IBD, as soon as possible after diagnosis, collecting information on pre-illness dietary habits[5,15-18].

The consumption of refined sugar was found to be positively associated with IBD in two of these studies[5,17], while another reported a positive association with CD but not with UC[20]. The total intake of proteins and a high consumption of eggs were shown to be positively related with the risk of IBD in one study[17]. This study also showed that a high consumption of fruit, vegetables and fibres, in general, helps to protect against the risk of IBD, while two other studies did not show any difference between patients and controls[5,20]. The study by Thornton *et al*[16] did not find any striking relationship in the intake of sugar, fibre, carbohydrates and proteins between the new cases of UC and controls.

In our study, only patients in whom the diagnosis had been made < 1 year prior to the interview and who had not modified dietary habits were considered. We found a significant intake of margarine and a high, although not significant, intake of rice and pasta in UC and a significant consumption of red meat and cheese in CD patients. We also identified a dietary pattern at higher risk of IBD correlated to pasta, sweets, red and processed meat, butter and margarine intake.

The differences in collecting and grouping foods and nutrients make it difficult to compare our results with those of other studies. On the other hand, while some authors reported an increased intake of protein in CD patients[21,22] others did not[17], furthermore, the pre-illness consumption of red meat, as well as cheese, has not been previously considered in detail. It is worthwhile pointing out that we found a relationship between both these nutrients and CD and that these foods are also related to *Mycobacterium avium* subspecies paratuberculosis, a candidate as an infectious aetiological agent in CD[23,24]. We also found a correlation between UC and margarine, pasta and rice intake, but not with other carbohydrates such as bread and sugar. These findings confirm the results of another Italian study showing that UC patients had a high intake

of total carbohydrates but not with those of a Japanese study in which the intake of bread for breakfast tended to be positively associated with the risk of UC[25,26]. However, the latter study, together with a recent multicenter study, showed that margarine and polyunsatured fatty acid intake was positively and significantly associated with UC, an association also found in our study[26,27].

In conclusion, although based on only a small number of cases, our study is one of the few in the literature providing information on the changes in dietary habits before diagnosis in IBD patients, thus explaining the difficulties and uncertainties encountered in epidemiological studies on diet and IBD. Furthermore, the study also revealed the role played by some environmental dietary factors and identified, by factor analysis, a specific dietary pattern at risk of IBD.

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COMMENTS

Background

The aetiology of inflammatory bowel disease (IBD) is still unknown, but the role of dietary factors in the pathophysiology has long since been taken into consideration. However, studies investigating this potential link are few and unconvincing.

Research frontiers

To date, only a few studies have examined the pre-illness diet, in incident IBD cases, showing conflicting results. In the present study the authors showed that signs and symptoms of IBD in patients before diagnosis led to a modification in their diet, and confirmed that pre-illness dietary habits, namely margarine, red meat, and cheese significantly increased the risk of ulcerative colitis and Crohn's disease.

Innovations and breakthroughs

A causal relationship between diet and IBD is difficult to define, due to the possibility that early symptoms of the disease may lead to a modification in dietary habits and the inability of the patients to accurately remember their diet before the onset of symptoms. The present case-control study evaluated whether the signs and symptoms of IBD patients before diagnosis led to a modification in their diet and assessed the association between diet and IBD onset in patients who did not change dietary habits by using multiple logistic regression and factor analysis.

Applications

The results of this study could contribute to a better understanding of the impact and significance of diet in the pathogenesis of inflammatory bowel disease.

Terminology

In the present study, the association between diet and dietary pattern and IBD has been assessed using multiple logistic regression and factor analysis. Factor analysis is a multivariate technique which analyses the underlying structure of a set of data in order to explain observed relationships between a large number of variables in terms of simpler relations.

Peer review

Although the number of patients is rather small, it is a well written and interesting study with some weaknesses which need to be addressed in order to strengthen the manuscript.

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BRIEF ARTICLE

Exertional esophageal pH-metry and manometry in recurrent chest pain

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Abstract

AIM: To investigate the diagnostic efficacy of 24-h and exertional esophageal pH-metry and manometry in patients with recurrent chest pain.

METHODS: The study included 111 patients (54% male) with recurrent angina-like chest pain, non-responsive to therapy with proton pump inhibitors. Sixty-five (59%) had non-obstructive lesions in coronary artery angiography, and in 46 (41%) significant coronary artery narrowing was found. In all patients, 24-h esophageal pH-metry and manometry, and treadmill stress tests with simultaneous esophageal pH-metry and manometry monitoring were performed. During a 24-h examination the percentage of spontaneous chest pain (sCP) episodes associated with acid reflux or dysmotility (symptom index, SI) was calculated. Patients with SI > 50% for acid gastroesophageal reflux (GER) were classified as having GER-related sCP. The remaining symptomatic individuals were determined as having non-GER-related sCP. During the stress test, the occurrence of chest pain, episodes of esophageal acidification (pH < 4 for 10 s) and esophageal spasm with more than 55% of simultaneous contractions (exercise-provoked esophageal spasm or EPES) were noted.

RESULTS: Sixty-eight (61%) individuals reported sCP during 24-h esophageal function monitoring. Eleven of these (16%) were classified as having GER-related sCP and 53/68 (84%) as having non-GER-related sCP. The exercise-provoked chest pain during a stress test occurred in 13/111 (12%) subjects. In order to compare the clinical usefulness of 24-h esophageal function monitoring and its examination limited only to the treadmill stress test, the standard parameters of diagnostic test evaluation were determined. The occurrence of GER-related or non-GER-related sCP was assumed as a "gold standard". Afterwards, accuracy, sensitivity and specificity were calculated. These parameters expressed a prediction of GER-related or non-GER-related sCP occurrence by the presence of chest pain, esophageal acidification and EPES. Accuracy, sensitivity and specificity of chest pain during the stress test predicting any sCP occurrence were 28%, 35% and 80%, respectively, predicting GER-related sCP were 42%, 0% and 83%, respectively, and predicting non-GER-related sCP were 57%, 36% and 83%, respectively. Similar values were obtained for exercise-related acidification with pH < 4 longer than 10 s in the prediction of GER-related sCP (44%, 36% and 92%, respectively) and EPES in relation to non-GER-related sCP (48%, 23% and 84%, respectively).

CONCLUSION: The presence of chest pain, esophageal acidification and EPES had greater than 80% specificity to exclude the GER-related and non-GER-related causes of recurrent chest pain.

Key words: Chest pain; Diagnosis; Esophageal manom-

etry; Esophageal pH-metry; Treadmill test

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INTRODUCTION

Recurrent, effort-provoked chest pain is the most common among cardiac and esophageal symptoms. It is also one of the greatest problems in contemporary health care because of its prevalence, adverse effects on quality of life, morbidity, and the utilization of health care resources[1,2]. The other problem is the frequent overlapping of causes of chest pain[3]. The presence of gastroesophageal reflux (GER)-related chest pain was confirmed in about 60% of patients with normal coronary angiography[4] and in 35% of patients with coronary artery disease (CAD)[5]. On the other hand, patients with GER presented with many comorbidities, originating both from cardiac and noncardiac sources, which may cause chest pain[6]. For this reason, in the diagnostic procedures of chest pain, both in patients with and without significant coronary artery narrowing, it is very important to evaluate the temporal relationship between symptoms and electrocardiographic signs of myocardial ischemia and/or the occurrence of esophageal abnormalities. It has been proven that analysis of the symptom association probability (SAP), symptom index (SI) or symptom sensitivity index reproducibly increases the yield of 24-h esophageal pH-metry, manometry and impedance examination[7,8]. As a result, some provocative tests inducing symptoms, could probably make these diagnostic procedures more efficient. It was reported that esophageal testing during exercise[9-16], dynamic position changing[17] and bending[18] made the 24-h esophageal pH-metry more informative and more efficient in the detection of significant GER. Exercise can provoke symptoms and abnormalities originating both from the heart and the esophagus[11,16]. Therefore, it was made a hypothesis that the simultaneous monitoring of clinical, electrocardiographic and hemodynamic parameters, as well as esophageal pH and pressure during the treadmill stress test, might also provide a more accurate means to evaluate the temporal interrelation between chest pain occurrence and myocardial and esophageal disturbances than separate tests[19]. Such a procedure might be useful, especially in patients non-responsive to empirical therapy with proton pump inhibitors (PPI)[7], both with and without significant coronary artery narrowing in coronary artery angiography, because of the above mentioned overlap in the causes of chest pain[5,20]. In addition it may allow the possibility of diagnosing myocardial ischemia in patients with non-obstructive coronary artery lesions due to microvascular angina[2] or the ischemic effect of the cardio-esophageal reflex[21]. The cardio-esophageal reflex is a vagal, visceral neural reflex, which may be activated by changes in intra-esophageal pH, pressure or temperature. Its stimulation may lead to a decrease in myocardial perfusion, proven in invasive[21,22] and non-invasive examinations[23], as well as to the occurrence of electrocardiographic signs of myocardial ischemia[5,24] or arrhythmia[19,25-29]. The mentioned effects were confirmed in about 56% of subjects with a normal coronarography[21] and in some subjects with significant coronary artery narrowing[24]. On the other hand, products of anaerobic myocardial metabolism, especially bradykinin[30], or invasive procedures on coronary arteries[22] *via* neural pathways may lead to esophageal dysmotility and reflux. These relationships connect ischemic heart disease and esophageal disorders in a vicious circle.

It is known that the activation of vagal reflexes may change the autonomic nervous system balance. In this way, abnormalities in intraesophageal pH[31,32] and pressure may also lead to a decrease in pain threshold and hypersensitivity[33]. This may explain why, in many studies, time-dependence between GER, esophageal dysmotility and chest pain episodes was relatively small and amounted to 22%-65%, and why many of the patients with noncardiac chest pain remained symptomatic in spite of detailed diagnosis and appropriate treatment[4]. These complicated interrelations assumed the planning of further studies to evaluate the new diagnostic tools in patients with recurrent chest pain of suspected noncardiac origin, as well as to determine more easily, and in a shorter time, the causal associations between esophageal disorders and patients' symptoms.

The aim of this study was to estimate the diagnostic efficacy of esophageal pH-metry and manometry monitoring during a treadmill stress test in comparison to 24-h esophageal pH-metry and manometry in patients with recurrent angina-like chest pain. In other words, this study addresses whether it is possible to replace 24-h esophageal function monitoring by an examination limited only to a treadmill stress test.

MATERIALS AND METHODS

One hundred and twenty-nine consecutive patients diagnosed with recurrent angina-like chest pain of suspected noncardiac origin were investigated. The symptoms were suspected of being of noncardiac origin by the leading doctor, independently of the researcher, who referred his patients for gastroenterological diagnosis after a cardiac work-up because of recurrent symptoms resistant to standard treatment oriented to coronary reserve improvement and empirical therapy with PPI. The pre-referral cardiac diagnostics procedures covered history, physical examination, electrocardiogram (ECG), treadmill stress test, and coronary artery angiography (Table 1). An extracardiac source of chest pain was suspected because none of the referred patients presented with an association between chest pain and ischemic changes during a treadmill stress

Table 1 Demographic and clinical data of investigated patients with a comparison of subjects without and with significant (> 50%) coronary artery narrowing *n* (%)

Parameter	Significant coronary artery narrowing	
	No (*n* = 65)	Yes (*n* = 46)
Males/females	29/36 (45/55)	31/15 (67/33)
Age (yr)	55.0 ± 8.8	55.0 ± 8.8
BMI (kg/m^2)	28.7 ± 4.2	27.8 ± 3.9
WHR	0.94 ± 0.9	0.93 ± 0.07
Smoking	11 (17)	10 (21)
History of PCI	0	21 (46)[a]
History of CABG	0	9 (19)[a]
History of myocardial infarction	0	18 (40)
Hypertension	24 (39)	24 (52)
Systolic blood pressure (mmHg)	122.8 ± 17.7	123.4 ± 17.4
Diastolic blood pressure (mmHg)	82.0 ± 11.2	81.1 ± 9.3
Diabetes mellitus	4 (5)	7 (13)
Total cholesterol (mg/dL)	195.7 ± 28.1	218.9 ± 48.3[a]
LDL cholesterol (mg/dL)	122.7 ± 25.9	136.1 ± 39.6
HDL cholesterol (mg/dL)	52.1 ± 11.9	48.5 ± 12.9
Triglycerides (mg/dL)	100.1 ± 33.0	166.1 ± 84.5[a]
Blood glucose (mg/dL)	100.2 ± 15.8	95.0 ± 17.7
Stress test duration (s)	455 ± 172	549 ± 191[a]
ST interval depression > 1 mm without chest pain during treadmill (silent ischemia)	28 (43)	17 (37)
Chest pain without ST interval depression during stress test	9 (14)	4 (9)
GER-related chest pain	7 (11)	4 (9)
Non-GER-related chest pain	35 (54)	22 (48)
Erosive esophagitis in endoscopy	16 (25)	17 (37)
Pathological GER	19 (29)	16 (35)
DES	13 (20)	10 (22)
epGER	12 (18)	4 (9)
epDES	14 (22)	9 (20)

Data presented as *n* (%) or mean ± SD. [a]$P < 0.05$ in an unpaired Student *t*-test or Fisher exact test. BMI: Body mass index; WHR: Waist/hip ratio; LDL: Low density lipoprotein; HDL: High density lipoprotein; PCI: Percutaneous coronary intervention; CABG: Coronary artery bypass graft; GER: Gastroesophageal reflux (> 4.5% time of esophageal monitoring with pH < 4); DES: Diffuse esophageal spasm, defined as esophageal motility abnormality with more than 30% of simultaneous contractions; epGER; Exercise-provoked GER, defined as a decrease in esophageal pH during a stress test for more than 10 s.

test. However, in spite of the results of the pre-referral cardiological diagnostic procedures, angina-like chest pain connected with electrocardiographic signs of myocardial ischemia was observed during the treadmill stress test conducted in the clinic in 18 subjects with significant coronary artery narrowing in angiography. These patients were excluded from the analysis because it would be impossible to distinguish between cardiac and extracardiac sources of chest pain, especially in patients with significant coronary artery disease. Finally, 111 consecutive subjects were included in the analysis, and fulfilled the following inclusion criteria: (1) age between 40 and 70 years; (2) prior coronary angiography performance not earlier than 3 mo before gastroenterological work-up; (3) angina-like chest pain to a degree of class Ⅱ in accordance with the Canadian Cardiovascular Society; such a pattern of chest pain was defined as precordial symptoms induced by exercise of less than, for example, marching for a distance under 200 m, and receding after rest or taking nitroglycerine; the occurrence of such defined chest pain during a treadmill stress test cannot be accompanied by signs of myocardial ischemia in the ECG; and (4) persistent symptoms despite adequate anti-anginal treatment (in patients with significant coronary artery lesions) and at least 1 mo-long therapy with a double dose of omeprazole, both in patients with and without significant coronary artery narrowing. Such a course of symptoms justified a suspicion of an extracardiac cause of chest pain, resistant to empirical therapy with PPI, and provided reasons for gastroenterological diagnostics to be undertaken. The exclusion criteria were: the presence of changes in the resting ECG, which made it impossible to estimate signs of myocardial ischemia (e.g. left bundle branch block or pre-excitation syndrome). All patients were asked not to take histamine receptor type 2 antagonists (e.g. ranitidine and famotidine), PPI or prokinetics (metoclopramide, cisapride, trimebutine and mebeverine).

Finally, the study group consisted of 46 (40%) patients with significant coronary artery changes, more than 50% of them with narrowing of the arteries, although not suitable for revascularization, and 65 (60%) subjects showing a normal coronary arteriography or no obstructive coronary lesions. Clinical and demographic data of the studied patients were divided according to the presence of significant narrowing of the coronary vessels (Table 1). Neither group differed in relation to the majority of these (Table 1). During the investigation, patients continued taking the stable doses of previously prescribed drugs (i.e. for CAD, hypertension and diabetes).

In all subjects, the medical history, physical examination, panendoscopy with gastric and esophageal biopsy, 24-h esophageal pH-metry and manometry were performed "off-therapy". An investigation of ambulatory esophageal function was carried out using a multi-use antimony probe (Synetics Medical AB, Sweden), a manometry catheter (Synectics, Medtronic) with 3 pressure sensors separated by 5 cm, and a Synectics micro-Digitrapper. An esophageal pH-metric sensor, after calibration to pH 7 and 1, using nasal and esophageal intubation, was positioned 5 cm above a monometrically-determined lower esophageal sphincter (LES). Pressure transducers were located through the other nostril at 3, 8 and 13 cm above the LES. During esophageal pH and pressure monitoring, all patients recorded occurring symptoms. None of the patients reported disturbances in nasal breathing. Every chest pain appearing during 24-h esophageal function monitoring was recorded by the micro-Digitrapper and labelled spontaneous chest pain (sCP).

The following day, when patients had become accustomed to the presence of the pH-metric and manometric probes in their nostrils, a treadmill stress test on a running track was carried out at approximately 7 am during continuous esophageal pH-metry and manometry

monitoring. The exercise test was performed using a device manufactured by Schiller, Switzerland, according to the Bruce protocol (the speed and gradient of the running track were increased every 3 min to 2.7, 4, 5.5 and 6.8 km/h, and by 10°, 12°, 14° and 16°). The start and finish of the exercise during the treadmill stress test as well as exercise-provoked angina-like chest pain (epCP) episodes were marked on the micro-Digitrapper.

The obtained data were downloaded to a personal computer and analyzed using GASTROSOFT software. Standard pH-metric and manometric parameters were calculated[34]. The GASTROSOFT software also analyzed the relationships between chest pain and the type of esophageal abnormality (a decrease in esophageal pH, changes in esophageal pressure or peristaltic wave coordination). This analysis concerned a period of 2 min prior to and during chest pain episodes. Patients were classified as having "GER-related" chest pain when the SI, defined as a percentage of sCP episodes associated with acid reflux during 24-h esophageal pH-metry, was ⩾ 50%. Patients were classified as having "non-GER-related" chest pain if the percentage of sCP episodes during 24-h esophageal pH-metry and manometry associated with esophageal dysmotility was ⩾ 50% and the individual did not fulfill GER-related chest pain criteria. Esophageal dysmotility was classified following esophageal manometry parameters presented during chest pain or in periods of 2 min prior to its appearance, non-peristaltic contractions, or contractions with amplitude or duration exceeding 95% of their daily average value.

Apart from types of sCP and esophageal abnormalities appearing within 24-h esophageal examination, additional symptoms and esophageal pH-metric and manometric abnormalities occurring during the treadmill stress test were determined. Angina-like chest pain (retrosternal pressing) appearing during the treadmill stress test was termed exercise-provoked chest pain (epCP). Gastroesophageal acid reflux provoked by exercise (epGER) was defined as a decrease in esophageal pH to below 4 for more than 10 s during the exercise stress test. Exercise-provoked esophageal spasm (EPES) was diagnosed when the percentage of simultaneous contractions during the treadmill stress test exceeded 55%. Simultaneous contractions, according to gastrosoft software settings, were defined as a sequence of contractions with less than 0.25 s delay between adjacent transducers separated by 5 cm (a propagation speed higher than 20 cm/s). The value of the cut-off at the level of 55% originated from the work by Stein *et al*[35], who proposed such diagnostic criteria for diffuse esophageal spasm in 24-h manometry.

Ethics

The study protocol was approved by the local Bioethics Committee of Nicolaus Copernicus University in Toruń and the Collegium Medicum in Bydgoszcz, Poland. All subjects gave their informed consent prior to the start of enrolment procedures. All procedures have been conducted in compliance with the Declaration of Helsinki.

Table 2 Parameters for the clinical usefulness of exercise-provoked chest pain, exercise-provoked gastroesophageal reflux, and exercise-provoked esophageal spasm in the diagnosis of gastroesophageal reflux-related and non-gastroesophageal reflux-related spontaneous chest pain based on 24-h esophageal function examination (n = 111) (%)

Parameter	epCP for both sCP	epCP for GER-related sCP	epCP for non-GER-related sCP	epGER for GER-related sCP	EPES for non-GER-related sCP
Accuracy	28	42	57	44	48
Sensitivity	35	0	36	36	23
Specificity	80	83	83	92	84
PPV	64	0	64	44	59
NPV	55	89	61	89	53
LR+	1.75	0	2.1	4.5	1.4
LR-	0.81	1.2	0.77	0.7	0.92

epCP: Exercise-provoked chest pain; sCP: Spontaneous chest pain; EPES: Exercise-provoked esophageal spasm; GER: Gastroesophageal reflux; PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio.

Statistical analysis

Statistical analysis was conducted using a licensed version of statistical software STATISTICA PL 8.0 for Windows. The results were mainly presented as the mean ± SD or n (%). The normal distribution of variables was estimated using the Kolmogorov-Smirnov test. The comparison of demographic and clinical data between patients with and without significant coronary artery narrowing (Table 1) was made using an unpaired Student t-test (for quantitative variables) and the Fisher exact test for qualitative variables. In addition, the standard parameters of diagnostic test usefulness according to evidence-based medicine (EBM), e.g. accuracy, sensitivity, specificity, positive and negative predictive values, as well as the positive and negative likelihood ratios, were calculated. The diagnosis of GER-related and non-GER-related chest pain acted as a "gold standard" (reference point) for this analysis. According to such assumptions, the parameters of diagnostic test usefulness expressed the relationships between the occurrence of exercise-related disturbances (epCP, epGER, EPES) and diagnoses of GER-related and non-GER-related sCP in 24-h esophageal pH-metry and manometry. This means that they expressed the ability of exercise-related esophageal abnormalities to predict the presence of GER-related or non-GER-related sCP. Accuracy was defined as the proportion of subjects with and without spontaneous chest pain and the presence or lack of evaluated esophageal function disorder (e.g. EPES). This represented the ratio of patients with true positive and true negative results to the total number of subjects. Sensitivity, i.e. the percentage of true positive results, was defined as the proportion of subjects with a respective kind of sCP in 24-h esophageal examination (GER-related or non-GER-related) and the simultaneous presence of epCP, epGER or EPES during the treadmill stress test (Table 2). Specificity, i.e. the percentage of true negative

results, was defined as the proportion of asymptomatic subjects during 24-h esophageal pH-metry and manometry in whom evaluated exercise-related disorders (e.g. epCP, epGER and EPES) did not appear during the treadmill stress test (e.g. epGER and EPES) (Table 2). The positive predictive value (PPV) was defined as the percentage of subjects with the presence of an evaluated parameter (e.g. EPES) having chest pain during 24-h esophageal pH-metry and manometry (true positive/true + false positives). The negative predictive value (NPV) was defined as the percentage of patients without an evaluated parameter (e.g. epCP, epGER or EPES) in whom chest pain during 24-h esophageal pH-metry and manometry did not appear (true negative/true + false negatives). The positive diagnostic likelihood ratio (LR+) was defined as an odds ratio of likelihood that a patient with chest pain during 24-h esophageal pH-metry and manometry would have an evaluated disorder to the probability that an individual without chest pain would have this esophageal disturbance [LR+ = sensitivity/(1-specificity)]. However, a negative likelihood ratio (LR-) represented the odds ratio that the lack of an evaluated esophageal disorder (e.g. EPES) would be observed in subjects with chest pain during 24-h esophageal function monitoring compared with whether the same results would be observed in individuals with spontaneous chest pain; LR- = (1-sensitivity)/specificity.

RESULTS

Patients with and without significant coronary artery disease had a similar prevalence of estimated esophageal abnormalities (Table 1). During 24-h esophageal pH and pressure monitoring, 68/111 (61%) individuals were symptomatic and presented with sCP. Among them, 11/68 (16%) experienced GER-related sCP and in 57/68 (84%) non-GER-related sCP was diagnosed. These frequencies in patients both with and without significant coronary artery narrowing were similar.

In only 13/111 (12%), epCP not connected with signs of myocardial ischemia was observed and appeared significantly less frequently than sCP during 24-h esophageal pH-metry and manometry ($P = 0.0001$). The prevalence of epCP was not significantly greater in patients without CAD (Table 1). Chest pain during the stress test occurred in 6 subjects who did not show symptoms during 24-h pH-metry and manometry. This corresponded with 5% of all subjects and 14% (6/43) of individuals who did not report sCP during daily monitoring.

The monitoring of intraesophageal pH and pressure during the treadmill stress test revealed some exercise-provoked esophageal abnormalities, i.e. intraesophageal acidification, labeled epGER, in 16/111 (14%) of all subjects and EPES in 23/111 (21%) (Table 1). Of these patients, epGER was diagnosed in 4 (4%) and EPES in 13 (12%), who had no esophageal abnormalities (i.e. erosive esophagitis, pathological gastroesophageal acid reflux or diffuse esophageal spasm) in panendoscopy and in 24-h esophageal pH-metry and manometry. However, these esophageal disorders were not significantly related to chest pain presence during the treadmill stress test. Symptomatic epCP was noted in only 14% of epGER episodes ($P > 0.05$) and in 30% of EPES ($P > 0.05$).

In the next part of the analysis, the clinical usefulness of a short protocol of esophageal examination, limited only to treadmill stress test duration, was estimated and compared to the diagnostic efficacy of 24-h pH-metry and manometry, expressed by the diagnosis of GER-related or non-GER-related (dysmotility-related) sCP. This acted as the "gold standard". The occurrence of epCP, epGER and EPES during the stress test had only acceptable specificity as did the NPV value in the diagnosis of GER-related or non-GER-related sCP (Table 2). A separate analysis performed in patients both with and without significant coronary artery narrowing was conducted with similar results.

DISCUSSION

This study has addressed the question of whether it is possible to replace 24-h esophageal pH-metry and manometry with a short protocol of these examinations limited only to stress test duration in the diagnosis of noncardiac chest pain originating from the esophagus. In other words, this investigation estimated exercise as a provocative test offering a greater possibility of correlating symptoms with esophageal abnormalities and excluding the potential life-threatening state connected with myocardial ischemia. The obtained results met the assumed requirements only in part.

The main finding of this study was that diagnoses of exercise-related esophageal disorders, such as epCP, epGER and EPES, had high values of specificity and NPV (Table 2). This makes them useful in excluding rather than confirming an esophageal source of recurrent angina-like chest pain, non-responsive to PPI, in patients both with and without significant coronary artery narrowing. This means in practice that 24-h pH-metry and manometry would not offer any important information concerning the cause of chest pain, if a patient, non-responsive to empirical therapy with PPI, did not present retrosternal symptoms during a treadmill stress test (e.g. conducted during a cardiologic work-up). Similar conclusions prompted the diagnosis of epGER and EPES during exertional esophageal pH and pressure monitoring during a treadmill stress test. A recognition of epGER or epCP in patients non-responsive to PPI was weak in this study (LR+ > 2 or LR- < 0.5) or uncertain (LR+ < 2 or LR- > 0.5), regarding parameters in the prediction of GER-related and non-GER-related spontaneous chest pain appearance during 24-h esophageal function monitoring.

The next observation of this study, as well as the next argument against recommending 24-h esophageal pH and pressure monitoring substitution by their examination only during a treadmill stress test, was that chest pain appeared during the stress test significantly and several times less frequently than during the 24-h investi-

gation. This did not correlate with esophageal pH-metric and manometric abnormalities. This shows that esophageal monitoring during a treadmill stress test, although providing the possibility of diagnosing epGER in an additional 4% of patients and EPES in an extra 12% of subjects, did not increase the probability diagnosis of the origin of chest pain, mainly because of the low SI value. In addition, the outcome of epGER and EPES diagnosis was still obscure.

In the available papers, I did not find any analysis using EBM parameters of diagnostic test evaluation in patients with recurrent chest pain who were non-responders to PPI. However, it was reported that esophageal testing during exercise[9-16], dynamic position changing[17] and bending[18] made 24-h esophageal pH-metry more efficient in the detection of significant GER. Bovero *et al*[10] showed that the provocation of gastroesophageal acid reflux by exercise might improve the diagnostic efficiency of esophageal pH-metry. The clinically useful provocative effect of exercise on gastroesophageal reflux has also been reported by other authors[11-18]. Furthermore, Ravi *et al*[9], investigating the effect of treadmill use on esophageal motility, found that exercise decreased the esophageal wave amplitude in patients with GERD, nutcracker esophagus and diffuse esophageal spasm (DES). Unfortunately, the authors did not discuss the outcome of exercise on the effectiveness of esophageal motility, so they could reveal DES-like exertional motility disorders such as EPES. Some authors have shown one questionable role of esophageal motility disorders in noncardiac chest pain pathogenesis[35,36], mainly because of the personally-dependent overlapping of other noncardiac chest pain pathomechanisms, such as hypersensitivity or musculoskeletal disorders[4,33]. However, Adamek *et al*[20] have confirmed the role of esophageal spasm in noncardiac chest pain pathogenesis, reporting an increase in simultaneous contractions in patients with chest pain, both with and without significant coronary artery narrowing, in comparison to asymptomatic controls. Apart from the above mentioned discrepancies, in my opinion, my results might have a clinical importance. Firstly, they provide an analysis of the classic diagnostic procedures of noncardiac chest pain both in patients with and without significant coronary artery narrowing; unfortunately, in everyday praxis, the overlapping of causes of noncardiac chest pain in therapy-resistant patients with CAD is rarely recognized[3]. Secondly, they show the importance of angina-like chest pain analysis in the diagnosis, not only of cardiac but also noncardiac sources of chest pain. It is known that chest pain appearance during a treadmill stress test increases its clinical usefulness. My investigation showed that a lack of chest pain during a typical cardiological exercise test predicted the low diagnostic importance of 24-h esophageal pH-metry and manometry. This information may shorten the diagnostic process and prevent the performance of useless examinations and resource utilization because of the implied consideration of extraesophageal chest pain causes if a stress test during a cardiological work-up did not provoke chest pain. Thirdly, the tests showed that the newly-defined esophageal motility disorder of EPES with high specificity allowed the prediction of a lack of esophageal manometry usefulness in the diagnosis of non-GER-related chest pain. The influence of EPES diagnosis on the course of chest pain over a 2.7-year long follow-up will be discussed in other work.

This study, however, has certain limitations. The first results were from a small subject sample, but this was still greater than for the majority of works concerning diagnostic and therapeutic problems in patients with suspected noncardiac chest pain[4,9,10,20,21,36-50]. Secondly, diagnostic procedures were made "off-therapy", which was inconsistent with the majority of recommendations by Fass, Hirano and Sifrim suggesting chest pain investigation "on-PPI-therapy"[36,51,52]. However, a recent study considered the necessity of returning to such ("off-therapy") an esophageal function examination[7,53-55]. Thirdly, the reference points in the analysis of respective diagnostic test usefulness were subjective and susceptible to the effect of esophageal hypersensitivity, one being between the main noncardiac chest pain pathomechanisms[4,33,36]. On the other hand, the evaluation of relationships between symptoms and esophageal abnormalities is an acceptable method to increase the diagnostic yield of esophageal 24-h examinations[8]. However, a more reliable parameter for this purpose is SAP, not SI. Fourthly, esophageal pH-metry and 24-h manometry have recently been substituted by esophageal impedance with pH-metry and high resolution manometry[56,57] but, in particular, the latter does not seem to be useful in the diagnosis of esophageal function during a treadmill stress test.

In conclusion, the occurrence of angina-like chest pain, a decrease in esophageal pH to below 4, and an increase in simultaneous contraction percentage above 55% during a treadmill stress test has acceptable specificity and NPV to exclude an origin from the esophagus, both for GER-related and non-GER-related causes of recurrent chest pain, in comparison to the results obtained during 24-h esophageal function monitoring. However, less frequent chest pain appearance during a treadmill stress test than during 24-h esophageal function monitoring limits the clinical usefulness of this provocative examination to the diagnosis of previously unrecognized myocardial ischemia and exercise-provoked esophageal disorders such as epGER or EPES.

COMMENTS

Background

This article concerns a very important and still current problem in clinical praxis, which is the diagnosis of chest pain. The diagnostic procedures are often time-consuming, and their clinical yield is useful only 20%-60% of individuals. For this reason new diagnostic protocols are still being investigated, including provocative tests.

Research frontiers

The study was performed in a relatively small number of patients and needs confirmation. However, its results may be helpful both for cardiologists and gastroenterologists, especially as it was performed in patients who were unresponsive to proton pump inhibitors therapy, the first-line tool in the diagnosis

of gastroesophageal reflux-related chest pain. The shortcoming of it was in not using a more sensitive examination which might more securely differentiate between cardiac and esophageal exercise-provoked chest pain.

Innovations and breakthroughs

This study has shown that the asymptomatic course of the treadmill stress test predicted a low yield of esophageally-oriented diagnostic procedures for chest pain. In addition, this study showed a similar prevalence of esophageal abnormalities in patients with and without coronary artery disease.

Applications

The results of this study, after confirmation with a greater number of subjects, may change the strategy of chest pain diagnosis of suspected esophageal origin. The results imply a lack of usefulness of esophageal function monitoring in patients in whom a cardiological work-up did not provoke symptoms.

Peer review

The manuscript presents some interesting and novel results, however, the numbers are small. This is an original paper, which would be an asset to the journal.

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BRIEF ARTICLE

Effects of glutamine and curcumin on bacterial translocation in jaundiced rats

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Abstract

AIM: To investigate the effect of curcumin on bacterial translocation and oxidative damage in an obstructive jaundice model and compare the results to glutamine, an agent known to be effective and clinically used.

METHODS: Twenty-four female Wistar-Albino rats, weighing 200-250 g, were randomly divided into three groups (8 in each group). After ligation of the common bile duct in all animals, Group Ⅰ received oral normal saline, Group Ⅱ received oral glutamine and Group Ⅲ received oral curcumin for seven days. Blood samples via cardiac puncture, tissue samples (terminal ileum, liver and mesenteric lymph node) and peritoneal fluid were obtained from the animals at the time of death to investigate bacterial translocation and oxidative damage.

RESULTS: We observed that both glutamine and curcumin reduced bacterial translocation in blood, hepatocellular damage, plasma cytokine levels, oxidative tissue damage and apoptosis significantly compared to the control group. Additionally, glutamine showed protective effects on ileal epithelium and reduced villus atrophy.

CONCLUSION: On the basis of these findings, both curcumin and glutamine are thought to be effective in preventing or reducing bacterial translocation and oxidative damage in obstructive jaundice.

Key words: Obstructive jaundice; Bacterial translocation; Oxidative damage; Glutamine; Curcumin

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Karatepe O, Acet E, Battal M, Adas G, Kemik A, Altiok M, Kamali G, Koculu S, Cagatay A, Kamali S, Karahan S. Effects of glutamine and curcumin on bacterial translocation in jaundiced rats. *World J Gastroenterol* 2010; 16(34): 4313-4320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i34/4313.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i34.4313

INTRODUCTION

Obstructive jaundice is characterized by a disability in the secretion of bile into the intestinal system, accumulation of toxic bile salts and bilirubin in the tissues and signifi-

cant changes in systemic and hepatic functions[1]. Despite current modern diagnostic and therapeutic approaches, interventions in patients with biliary tract obstruction result in 10%-25% mortality and up to 56% major morbidity[2]. Biliary sepsis, wound infections, intra-abdominal abscess formation and renal failure are frequent complications in obstructive jaundice. Bacterial translocation and oxidative tissue damage have been emphasized as the leading causes of these complications in obstructive jaundice by numerous investigators[2-4]. Obstructive jaundice causes alterations leading to bacterial translocation both in the intestinal barrier and in the reticuloendothelial system. Some of these alterations may be listed as mucosal damage in the intestinal lumen due to lack of bile, apoptosis, bacterial overgrowth, motility disorder associated with oxidative stress and functional abnormalities in the tissue macrophages[2,5].

Glutamine, a non-essential amino acid, occupies a central role in numerous metabolic processes such as amino acid transport and nitrogen balance. It is the main energy source for rapidly proliferating cells such as enterocytes and lymphocytes. It has been reported not only to lower the rate of endotoxemia and translocation by preserving mucosal integrity but also to improve the immune system action against bacteria and endotoxins which succeed in passing the intestinal barrier[3,5-7].

Curcumin is a polyphenol derived from the herbal remedy and dietary spice turmeric. The antioxidant, anticancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies[8]. Gülçubuk *et al*[9] have declared that curcumin has positive effects on intestinal barrier function due to its anti-inflammatory properties and possibly can prevent bacterial translocation. We have previously shown positive effects of curcumin on oxidative damage and liver function in obstructive jaundice[10]. However, according to our knowledge, a comprehensive and comparative study regarding the effect of curcumin on bacterial translocation and oxidative damage in obstructive jaundice has not been performed yet.

The aim of this study was to compare the effects of curcumin with those of glutamine, a reliable control, on bacterial translocation and oxidative damage in an obstructive jaundice model and to evaluate the results with a review of the literature.

MATERIALS AND METHODS

Animals

Twenty-four healthy female rats weighing 200-250 g, housed in stainless steel cages under controlled temperature (22℃) and humidity and with 12-h dark/light cycles, were used in this study. Standard industrial rat feed containing 21% protein and fresh tap water were given *ad libitum* before and after operation. The experimental protocol was designed according to the ethical standards for animal use and approved by the local committee of animal use.

Surgical procedure and treatment

All procedures were performed under general anesthesia induced by intramuscular injections of ketamine hydrochloride 80 mg/kg (Ketalar flk; Pfizer, Istanbul, Turkey) plus 5 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey). The abdomen was shaved and soaked with Betadine solution. After a midline laparotomy of 1-2 cm, the common bile duct was identified, doubly ligated using 4/0 silk sutures and divided. Abdominal incisions were closed in two layers using 4/0 silk sutures. The animals were randomized into three groups (8 in each). Group Ⅰ was treated with normal saline 1 cc orally once daily after bile duct ligation. Group Ⅱ was treated with glutamine (Resource Glutamine powder 5 g; Nestle Health Care Nutrition, Germany) 200 mg/kg orally once daily after bile duct ligation. Group Ⅲ was treated with curcumin (Curcumin from *Curcuma longa*; Sigma Aldrich, Germany) 20 mg/kg orally once daily after bile duct ligation. Animals were regularly nourished and maintained for 7 d as described above. Glutamine was dissolved in distilled water and the solution was stirred immediately before use. Curcumin was suspended in distilled water and the suspension was stirred immediately before use. The re-laparotomy was performed through the old incision on postoperative 8th day under general anesthesia and subjects were sacrificed. Systemic blood via cardiac puncture, peritoneal fluid and tissue (terminal ileum, liver and mesenteric lymph node) samples were obtained to investigate bacterial translocation and oxidative damage. All procedures were performed aseptically using sterile instruments.

Microbiological examination

Systemic blood samples obtained via cardiac puncture and peritoneal fluid samples were cultured aerobically using BacTec Peds (BioMérieux, Durham, USA). Blood cultures were continuously monitored for 7 d. Positive cultures were plated out on appropriate media and species identified by Sceptor microdilution and standard bacteriological techniques. The mesenteric lymph node, liver and terminal ileum samples were removed and placed in pre-weighed sterile glass bottles containing sterile pre-reduced brain-heart infusion. The bottles were re-weighed and tissue homogenates were prepared in 2-mL brain-heart infusions using sterile mortars and pestles. A portion (0.1 mL) of homogenates was cultured on blood agar, eosin methylene blue agar. All the plates were examined after 24 and 48 h of incubation at 37℃.

Biochemical examination

Systemic blood *via* cardiac puncture and tissue (terminal ileum) samples were obtained from rats for biochemical evaluation. Tissues were washed with physiological serum for biochemical analysis, weighed and homogenized using the method of Sier *et al*[11]. Serum levels of cytokines tumor necrosis factor-α (TNF-α) (pg/mL) and interleukin-6 (IL-6) (pg/mL) were measured by immunoenzymatic enzyme-linked immunosorbent assay method (Quantikine High Sensitivity Human by R&D Systems, USA) according to the manufacturer's protocol. Minimum detectable concentrations were determined by the manufacturer to be 0.12 pg/mL and 0.03 pg/mL, respectively. Intra-assay

(2.6 for TNF-α and 1.6 for IL-6) and inter-assay (14 for TNF-α and 6.4 for IL-6) precision performances of the assay were determined on 20 replicates from the quality control data of the laboratory. Malondialdehyde (MDA) was determined spectrophotometrically by the thiobarbituric acid method. Aliquots of 0.2 mL of serum were mixed thoroughly with 0.8 mL of phosphate-buffered saline (pH 7.4) and 25 μL of butylated hydroxytoluene solution. The samples were placed on ice for 2 h after addition of 0.5 mL of 30% trichloroacetic acid. Then, samples were centrifuged at 2000 *g* at 25℃ for 15 min. After that, 1 mL of each supernatant was mixed with 0.075 mL of 0.1 mol/L ethylenediamine tetraacetic acid and 0.25 mL of 1% thiobarbituric acid in 0.05 mol/L sodium hydroxide (NaOH). Supernatant of each sample was kept in boiling water for 15 min and then cooled to room temperature. Finally, the absorbance of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm. The data regarding TBARS were expressed in mDA, using a molar extinction coefficient for MDA of 1.56×10^5/cm per mol/L and the results were expressed in nmol/L (range: 0.1-2.5). Serum nitric oxide (NO) levels were measured with Griess reagent as previously described[12]. The first step is the conversion of nitrate using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite to a purple azocompound. Protein interference was avoided by treatment of the reacted samples with zinc sulphate and centrifugation for 5 min at 10000 *g*; the azochromophore spectrophotometry was performed at 450 nm; sodium nitrate was used as the standard and results were expressed in mmol/L (range: 10-120 mmol/L). Myeloperoxidase (MPO) activity was measured as described previously[13]. In short, tissue homogenates were incubated with 0.5% hexadecyl-trimethylammonium bromide in 50 mol/L potassium phosphate buffer (pH 5.5), plus 0.026% orthodianisidine dihydrochloride substrate and 0.018% H_2O_2. The reaction kinetics were followed for 30 min at 450 nm in 96-well plates. The specificity of the reaction was checked with sodium azide (0.1 mmol/L). All samples were analyzed in duplicate and standardized using a homogenate of pooled human neutrophils, and MPO activity was expressed in arbitrary units (U/mg protein). The enzymatic activity of caspase-3 in tissue samples was measured as described previously[14]. Five 10 μm cryostat sections of tissues were suspended in a lysis buffer consisting of 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0), 40 mmol/L β-glycerophosphate, 50 mmol/L NaCl, 2 mmol/L $MgCl_2$, and 5 mmol/L ethylene glycol tetraacetic acid. After 10 min on ice, the cells were disrupted by 10 s of sonification followed by four cycles of freezing and thawing and stored at -80℃. Protein concentration was determined using the method described by Bradford[15]. For measurement of caspase-3 enzymatic activity, samples containing 15 μg protein were incubated with 2.5 nmol of the enzyme substrates DEVD-AMC (7-Amino-4-methylcoumarin, N-acetyl-L-aspartyl-L-glutamyl-L-valyl-l-aspartic acid amide) in a 100 mmol/L HEPES buffer (pH 7.25) containing 10% (w/v) sucrose, 0.1% (v/v) NP40, and 10 mmol/L DL-Dithiothreitol. During incubation at 37℃, fluorescent AMC was cleaved off by active caspases, corresponding with the level of caspase activity in the sample. The fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a FLUO star Optima plate reader. Calibration curves were constructed using free AMC. Caspase-3 activity was measured as pmolAMC/min per mg protein. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined according to Reitman and Frankel[16], whereas alkaline phosphatase (ALP) activity was estimated by the Belfield method[17]. Total bilirubin, and γ-glutamyl transferase (GGT) were determined using Diamond Diagnostic Kit as reported[18]. The results were reported as mean and standard deviation.

Histopathological examination

Liver and a 2-cm segment of terminal ileum samples were obtained at the final laparotomy. The bowel was stripped from its mesentery and the segment was opened along its length and rinsed in a cold solution. Specimens were fixed in 10% formalin in 0.15 mol/L phosphate buffer (pH 7.2), embedded in paraffin and then sections measuring 5 μm in thickness were cut. The specimens were stained with hematoxylin-eosin and examined under the light microscope (Olympus BX50, Japan). Histopathological examination was performed by an experienced pathologist who was not aware of the sample group. Ductular proliferation in liver samples was examined according to the modified scoring system used by Sheen-Chen *et al*[3]. This system, assessing ductular proliferation with seven different scores, was reduced to three scores since it was more appropriate for statistical assessment and number of subjects was fewer. Grade 1 (mild): Portal area involvement less than 50%; Grade 2 (moderate): Portal area involvement more than 50% or expansion of the portal tract; Grade 3 (severe): Presence of bridging in portal tracts. Apoptosis was also assessed in liver samples and reported as present or absent. Villus structures (villus length and width), lymphatic dilatation and sub-epithelial edema were examined to investigate mucosal damage in terminal ileum sections and reported as present or absent.

Statistical analysis

Findings obtained in the study were assessed using SPSS (Statistical Package for Social Sciences) for Windows 15.0 program. One-way Anova test was used in comparing parameters between groups and Tukey's HDS (Honestly Significant Difference) test was used in detecting the group causing variation when comparing qualitative data. Kruskal Wallis test was used in comparing parameters displaying abnormal distribution between groups and Mann Whitney *U* test was used in detecting the group causing variation. For comparison of qualitative data, χ^2 or Fisher exact test was used. Results were calculated as mean ± SD. $P < 0.05$ values were considered statistically significant.

RESULTS

Jaundice became apparent in all subjects on postoperative

Table 1 Biochemical results

	mean ± SD			*P*		
	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅰ-Ⅱ	Group Ⅰ-Ⅲ	Group Ⅱ-Ⅲ
T-Bil bilirubin	7.814 ± 0.855	7.303 ± 1.059	7.456 ± 1.037	> 0.05	> 0.05	> 0.05
ALT	285.714 ± 6.945	203.157 ± 5.777	230.5 ± 7.368	0.002	0.001	0.001
AST	277.571 ± 8.6	188.714 ± 5.908	227.25 ± 7.573	0.002	0.001	0.001
ALP	417.714 ± 13.865	373.714 ± 9.322	353.938 ± 141.502	0.002	0.042	0.015
GGT	410.429 ± 5.711	395.857 ± 4.337	402.375 ± 4.926	0.002	0.010	0.040
IL-6	1.087 ± 0.117	0.627 ± 0.147	0.984 ± 0.123	0.002	0.063	0.001
TNF-α	1.346 ± 0.21	0.53 ± 0.089	0.869 ± 0.104	0.002	0.002	0.001
MPO	0.537 ± 0.01	0.309 ± 0.038	0.468 ± 0.009	0.002	0.001	0.001
NO	149.286 ± 9.499	101.171 ± 3.396	135.625 ± 5.181	0.002	0.009	0.001
MDA	2.743 ± 0.196	1.753 ± 0.227	2.3 ± 0.379	0.002	0.011	0.015
CAS	35.741 ± 0.848	22.929 ± 1.533	30.445 ± 1.463	0.002	0.001	0.001

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase; IL-6: interleukin-6; TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; NO: Nitric oxide; MDA: Malondialdehyde; CAS: Caspase 3 activity.

day 3. Two of the rats (one in Group Ⅰ and one in Group Ⅱ) died during the experiment. The experiment was completed with 7 rats in Group Ⅰ, 7 rats in Group Ⅱ and 8 rats in Group Ⅲ.

Microbiological findings

Significant microbial growth was investigated in blood obtained by intracardiac puncture, samples of mesenteric lymph nodes, peritoneal fluid, and terminal ileum tissue. *Escherichia coli* (*E. coli*) was the most common bacteria detected (34%) among all positive cultures. Other detected bacteria were identified as *Enterococci, Klebsiella oxytoca, Streptococcus* spp and *Klebsiella pneumoniae*. Positive blood cultures were detected in 6 of 7 animals (85%) in Group Ⅰ, in 1 of 7 animals (14%) in Group Ⅱ, and in 2 of 8 animals (25%) in Group Ⅲ. The rates in groups Ⅱ and Ⅲ were determined to be significantly less than that of group Ⅰ (P = 0.029 and P = 0.041, respectively). The difference between Groups Ⅱ and Ⅲ was not statistically significant (P > 0.05). Positive cultures of mesenteric lymph node samples were detected in all animals (100%) in Group Ⅰ, in 3 of 7 animals (42%) in Group Ⅱ and in 7 of 8 animals (87%) in Group Ⅲ. There was no statistically significant difference among groups although positive cultures were fewer in Group Ⅱ. Positive cultures of peritoneal fluids were detected in 4 of 7 animals (57%) in Group Ⅰ, none of 7 animals (0%) in Group Ⅱ and in 4 of 8 animals (50%) in Group Ⅲ. These results were not statistically significantly different between groups (P > 0.05). Significant pathogens in terminal ileum samples were detected in all animals (100%) in Group Ⅰ, in 5 of 7 animals (71%) in Group Ⅱ and in 7 of 8 animals (87%) in Group Ⅲ. These results were not statistically significant either (P > 0.05).

Biochemical findings

ALT, AST, ALP, GGT, total bilirubin, IL-6 and TNF-α levels were measured in blood samples. MPO, NO, MDA levels and caspase-3 activity were measured in terminal ileum samples (Table 1). There was no significant difference in terms of serum total bilirubin values among groups and obstructive jaundice was detected in all subjects. ALT and AST levels, markers of hepatocellular damage, in Group Ⅱ were found to be significantly reduced compared to Group Ⅰ and Group Ⅲ (P = 0.002 and P = 0.001, respectively). Furthermore, these enzyme levels were found to be significantly reduced in Group Ⅲ when compared with Group Ⅰ (P = 0.001). ALP and GGT levels, markers of cholestasis, were found to be significantly lower (P < 0.05) in Groups Ⅲ and Ⅱ than in Group Ⅰ. When the results of Groups Ⅱ and Ⅲ were compared, ALP levels were lower in Group Ⅱ; whereas, GGT was found to be lower in Group Ⅲ and these results were statistically significant (P < 0.05). TNF-α levels were detected to be significantly decreased in Group Ⅱ when compared with Group Ⅰ and Group Ⅱ (P = 0.001 and P = 0.002, respectively). TNF-α levels detected in Group Ⅲ were also significantly lower than that of Group Ⅰ (P = 0.002). IL-6 levels detected in Group Ⅱ were significantly less than those in Group Ⅰ and Group Ⅲ (P = 0.002 and P = 0.001, respectively). Although the results were lower in Group Ⅲ they were not statistically significant when compared with Group Ⅰ (P > 0.05). MPO levels, a marker of tissue inflammation and neutrophil sequestration, were found to be significantly lower in Group Ⅱ than the other groups (P = 0.002 for Group Ⅰ and P = 0.001 for Group Ⅲ). MPO levels detected in Group Ⅲ were also lower than that of Group Ⅰ, which was statistically significant (P = 0.001). NO levels, an indicator of oxidative damage and MDA, an end product of lipid peroxidation and an index of oxidative stress, were determined to be significantly lower in Group Ⅱ than the other groups (P < 0.05). Additionally, values detected in Group Ⅲ were lower than those of Group Ⅰ and this was also statistically significant (P < 0.05). Caspase-3 activity, an apoptosis marker, was found to be significantly lower in Group Ⅱ than the other groups (P = 0.002 for Group Ⅰ and P = 0.001 for Group Ⅲ). The value detected in Group Ⅲ was lower than that of Group Ⅰ and this was also statistically significant (P = 0.001).

Histopathological findings

Ductal proliferation and apoptosis rates were measured

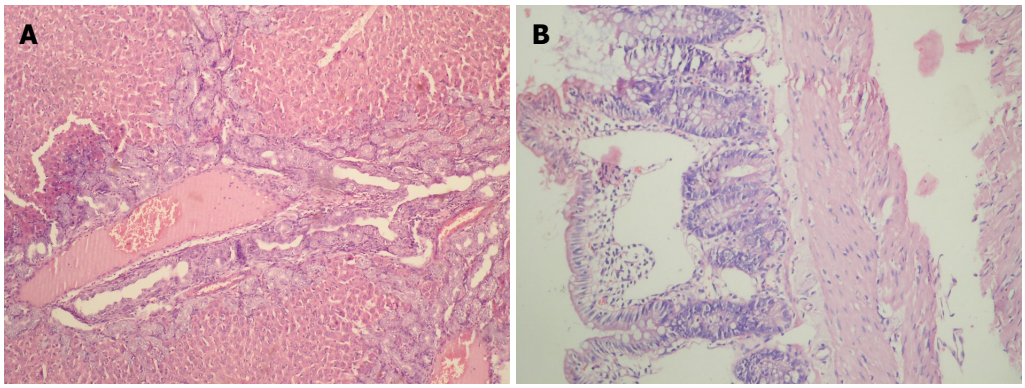

Figure 1 Histopathological view of liver and terminal ileum. A: Liver of a jaundiced rat, bridging of portal tracts, severe ductal proliferation, × 200; B: Terminal ileum of a jaundiced rat, shortening of villus length and severe lymphatic dilatation, × 200.

in the histopathological evaluation of liver sections (Figure 1A). Regarding the former, 14% mild, 57% moderate and 29% severe ductal proliferation was detected in Group Ⅰ, 43% mild, 14% moderate and 43% severe ductal proliferation was detected in Group Ⅱ and 38% mild and 62% severe ductal proliferation was detected in Group Ⅲ. Rates of apoptosis were determined to be 42% in Group I and Group Ⅱ and 62% in Group Ⅲ. No significant difference in terms of either ductal proliferation or apoptosis was found when the results of histopathological examination of liver samples were statistically compared among groups ($P > 0.05$). Villus height and width, lymphatic dilatation and subepithelial edema were evaluated in terminal ileum sections. When shortening in villus height was compared among groups, shortening in Group Ⅱ was significantly less than Group Ⅰ ($P = 0.02$). Shortening observed in Group Ⅱ was less than that of Group Ⅲ and shortening determined in Group Ⅲ was less than that of Group I but these differences were not statistically significant ($P > 0.05$). In the comparison of villus width, no significant difference was detected among groups, although it was found less frequently in Group Ⅱ ($P > 0.05$). Degree of lymphatic dilatation was observed to be significantly lower in Group Ⅱ than that of Group Ⅰ ($P = 0.02$). Lymphatic dilatation observed in Group Ⅱ was less than that of Group Ⅲ but this was not statistically significant. Lymphatic dilatation determined in Group Ⅲ was less than that of Group Ⅰ, but again this was not statistically significant ($P > 0.05$). No significant difference among groups in terms of subepithelial edema was detected ($P > 0.05$).

DISCUSSION

Bacterial translocation is the passage of bacteria or endotoxins from the gastrointestinal tract to extraintestinal sites, such as mesenteric lymph nodes, liver, spleen, and/or bloodstream. In a normal, healthy individual, gut-originated bacteremia and sepsis do not occur because the host has multiple defense mechanisms to prevent the bacteria and their products from crossing the mucosal barrier and spreading to systemic tissues. Under certain experimental and clinical circumstances, this intestinal barrier function becomes overwhelmed or impaired, resulting in bacterial translocation[19-21]. Current advances in the pathophysiology of intestinal failure in obstructive jaundice have shown that the breakdown of the gut barrier is multifactorial, involving disruption of immunologic, biological, mechanical, and biochemical barriers. Berg and Garlington[22] defined bacterial translocation as the passage of viable enteric bacteria through intestinal epithelial cells into the lamina propria and then to mesenteric lymph nodes, and possibly other tissues. Bacterial translocation was first defined as the passage of viable bacteria. However, at the present time either bacterial fragments or the translocation of bacterial products such as endotoxins from dead bacteria have been known to stimulate the immune system[23]. Under normal conditions, the gastrointestinal system keeps bacterial content in the intestinal lumen while absorbing nutrients. This is called "intestinal barrier function"[5]. Despite the number of bacteria present in the cecum, one cell beneath the mucosa is sterile. The intestinal barrier is both a functional and anatomical barrier to intestinal contents. The functional aspect is inherent in the modulation of tight junctional permeability and selective endocytosis of intestinal contents, while the anatomical barrier aspect is represented by the interconnected tight junctional/cell membrane system which effectively excludes large molecules and bacterial antigens. The first component of the intestinal barrier consists of intestinal microflora which have two functions, "bacterial antagonism" and "colonization resistance"[24,25]. The other components of the intestinal barrier are the physical barrier function of the mucosal epithelium, a mucus layer on the intestinal epithelium, blockade of epithelial adhesion sites by secreted IgA, and the preventive effects of intestinal peristalsis and intermittent desquamation of epithelial cells forming the mucosa. Despite these defense systems, translocated bacteria and bacterial products are neutralized in intestine-related lymphoid tissues (intraepithelial and lamina propria lymphocytes, lymphoid follicles, Peyer's patches and complexes of mesenteric lymph nodes), and the immune system (especially the reticuloendothelial system organs, such as the liver). In various studies bacterial translocation has been reported to develop in mechanical intestinal obstruction, hemorrhagic shock, sepsis,

endotoxemia, severe trauma, thermal injury, obstructive jaundice and cirrhosis[24,25]. The clinical importance of bacterial translocation was revealed in a study conducted by MacFie *et al*[26] in over 927 patients throughout 13 years. Bacterial translocation was detected in 130 patients (14%) in this study. Postoperative sepsis was seen more frequently (42.3% and 19.9%) in these patients. The authors reported ethical and methodological problems present in their study of bacterial translocation in humans and probably these problems are even bigger than described. According to our current knowledge, glutamine support, aggressive and targeted nutrition, adequate provision of visceral flow, appropriate use of antibiotics and selective intestinal decontamination are important objectives in restricting bacterial translocation[5].

Another important point in the pathophysiology of obstructive jaundice is the increased oxidative stress in the tissues. When the balance between the production of free oxygen radicals and antioxidant systems is impaired, oxidative stress leading to tissue damage occurs[26,27]. Increased intestinal oxidative stress which can cause intestinal damage and endotoxin translocation has been detected in rats with obstructive jaundice. In fact, obstructive jaundice causes oxidative stress in other organs such as the liver, kidneys, brain, heart and lungs. The development of tissue injury depends directly on the bile acids or occurs via macrophages; formation of oxygen free radicals as a result of systemic endotoxemia-induced xanthine oxidase, endotoxin-mediated systemic cytokine response, neutrophil chemotaxis of jaundice, increase of superoxide anion production and reduction of plasma levels of fat-soluble vitamins, particularly vitamin E, have been blamed for oxidative stress occurring in obstructive jaundice[28].

The effect of obstructive jaundice on bacterial translocation has been investigated in numerous clinical and experimental studies. Several changes have been shown to occur both in the intestinal barrier and in the reticuloendothelial system in obstructive jaundice. Bile and bile salts in the intestinal lumen are believed to have protective effects against bacterial translocation[23]. Luminal flow of bile salts has a regulatory effect on the intestinal flora and a direct detergent effect on endotoxins[25]. Furthermore, some trophic effects of pancreaticobiliary secretions on the intestinal mucosa have been identified[28]. Ogata *et al*[29] showed that oral administration of bile salts reduced absorption of endotoxin in rats with jaundice. Intestinal permeability increases with mucosal damage and as a consequence of changes in the intestinal flora a convenient media for bacterial translocation is prepared. Gatt *et al*[5] showed that reticuloendothelial system function was impaired subsequent to biliary tract obstruction and caused an increase in bacterial translocation. Reticuloendothelial system is defined as tissue macrophages. These are found chiefly in the liver, spleen, lung and bone marrow. They are responsible for cleaning up particulate materials such as bacteria, endotoxin, immune complexes and cell debris[3]. Kupffer cells in the liver are responsible for 80%-90% of the reticuloendothelial system activity. Assimakopoulos *et al*[28] detected increased intestinal oxidative stress which could cause intestinal damage and increased intestinal endotoxin translocation in an experimental model of rats with jaundice. Bacterial translocation and inflammatory responses in patients with obstructive jaundice were examined in a study performed by Ljungdahl *et al*[30] The results of this study which had few cases are as follows: bacterial translocation could not be detected in any of the patients. Elevated levels of preoperative endotoxin, TNF-α and IL-6 were measured in patients with jaundice. In these patients, the number of macrophages and apoptosis increased while T-lymphocyte count decreased in the mesenteric lymph nodes.

Treatment modalities and agents affecting the different pathophysiological steps described above have been investigated for many years, in order to reduce mortality and morbidity seen post-treatment or during the elapsed time until the definitive treatment of obstructive jaundice. Glutamine is one of the most important products surveyed for this purpose. Glutamine is the most common free amino acid in the body and has an important role in numerous metabolic events such as amino acid transport and nitrogen balance. It is the main food source of rapidly dividing cells such as enterocytes and lymphocytes. These cells have important roles in the intestinal mucosa barrier and the immune system. Numerous studies examining the effect of glutamine on bacterial translocation have been performed[6]. Glutamine reduces translocation not only by strengthening the intestinal barrier but also by reinforcing the immune system against bacteria and endotoxins successful in passing this barrier[3,6]. White *et al*[6] reported that glutamine regulated intestinal permeability, reduced bacterial translocation and even reinforced the immune system in rats with obstructive jaundice. Aldemir *et al*[7] showed that glutamine improved mucosal integrity and reduced bacterial translocation in the same model. In an experimental study performed by Margaritis *et al*[31], oral glutamine replacement was reported to reduce bacterial translocation, endotoxemia and apoptosis and to improve the ileal and liver histology in obstructive jaundice. Sheen-Chen *et al*[3] examined liver apoptosis in the obstructive jaundice rat model and determined that although glutamine replacement reduced liver apoptosis rate and ductal proliferation on day 3 of the experiment, the same effect could not be shown on day 7. In clinical studies, glutamine treatment was determined to have beneficial effects on bacterial translocation and sepsis. These effects can be listed as reduction in mucosal atrophy, rapid improvement in radiotherapy- and chemotherapy-induced mucosal damage, strengthening of intestinal and systemic immunity and decrease in length of hospital stay and infection rates in patients in intensive care. Use of glutamine before abdominal radiation has been shown to exert a protective effect on the intestinal mucosa, to increase intestinal glutamine metabolism and to decrease morbidity and mortality subsequent to total abdominal irradiation. Glutamine also shows this effect when administered after the radiation therapy. Glutamine also plays a critical role in synthesis of glutathione, a major antioxidant, which protects tis-

sues against free radical damage. The jejunal mucosal weight, DNA and nitrogen content increase and villus atrophy reduces significantly in glutamine-enriched total parenteral nutrition. Methotrexate-induced enterocolitis proceeds more slowly, and 5-fluorouracil-induced mucosal damage recovery occurs more rapidly, in patients fed on a glutamine-supplemented enteral diet[5,32]. In a study performed on rats by Kul *et al*[33] the positive effect of glutamine on oxidative stress both in a hypoxia-reoxygenation model and in healthy neonatal rats was reported. Glutamine supplementation has been suggested to prevent necrotizing enterocolitis in neonates.

In the present study, we examined the effects of glutamine and curcumin use on bacterial translocation and oxidative stress in rats with obstructive jaundice. Consistent with the literature, we detected the positive effects of glutamine use on these issues. Positive culture rates observed in the microbiological assays were less in all samples from the glutamine-treated group compared to those of the control and curcumin-treated groups. However, only the blood culture rates were statistically significant in comparison with the control group ($P = 0.029$). Moreover, shortening of villus height and lymphatic dilation were found to be significantly lower in the glutamine-treated group terminal ileum ($P < 0.05$).

Curcumin is a polyphenol derived from turmeric, which is used as a spice or herbal medicine. It is produced from the root of a plant, *Curcuma longa*. Dried roots of this plant have been used for thousands of years in Asian medicine[34]. Curcumin has been suggested to reduce inflammation which causes bacterial translocation by exhibiting an anti-inflammatory effect[9]. In the study performed by Shen *et al*[35], curcumin was shown to increase expression of antioxidant biomolecules and reduce neutrophil infiltration and reactive oxygen metabolites after ischemia-reperfusion injury in the liver. Treatment of rats with curcumin decreased total nitric oxide synthase activity after reperfusion. However, endothelial nitric oxide synthase activity was not affected. Moreover, curcumin has been shown to have positive effects on inflammatory damage and intestinal reperfusion injury in a recent experimental study by Karatepe *et al*[10].

In the present study, we examined the effects of curcumin on bacterial translocation and oxidative damage in an obstructive jaundice model and we compared it with glutamine, which is a reliable control and has been recently in clinical use. Microbiologically, positive culture rates were found to be less in all samples from the curcumin-treated group compared to those from the control group. However, only the rates of blood cultures were statistically significant ($P = 0.041$). No significant difference was observed when compared with the glutamine-treated group. All biochemical parameters (except ALP levels) of the glutamine-treated group were found to be lower in a statistically significant manner in comparison with those of the curcumin-treated group ($P < 0.05$). No statistically significant difference was detected in the histopathological examination of the samples obtained from the curcumin-treated group compared to the glutamine-treated and control groups ($P > 0.05$).

In conclusion, in the present study we detected positive effects of glutamine and curcumin on bacterial translocation and oxidative damage in rats with obstructive jaundice. Both glutamine and curcumin were observed to reduce bacterial translocation in blood, hepatocellular injury, serum cytokine levels, oxidative tissue damage and apoptosis rates significantly in comparison to the control group. However, more extensive comparative experimental and clinical studies are required before the clinical use of curcumin for this purpose and perhaps the combined use of glutamine with curcumin will be more effective.

COMMENTS

Background

Despite current modern diagnostic and therapeutic approaches, interventions in patients with biliary tract obstruction result in 10%-25% mortality and up to 56% major morbidity. Bacterial translocation and oxidative tissue damage have been emphasized as the leading cause of the complications in obstructive jaundice by numerous investigators.

Research frontiers

The antioxidant, anti-cancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies. Administration of glutamine has been shown to improve bacterial translocation and oxidative damage in obstructive jaundice but the exact role of curcumin in this issue is still unknown. In this study, the authors demonstrate that both curcumin and glutamine are effective in preventing or reducing bacterial translocation and oxidative damage in obstructive jaundice.

Innovations and breakthroughs

Obstructive jaundice causes alterations leading to bacterial translocation both in the intestinal barrier and in the reticuloendothelial system. Some of these alterations may be listed as mucosal damage in the intestinal lumen due to lack of bile, apoptosis, bacterial overgrowth, motility disorder associated with oxidative stress and functional abnormalities in the tissue macrophages. According to the authors' knowledge, a comprehensive and comparative study regarding the effect of curcumin on bacterial translocation and oxidative damage in obstructive jaundice has not been performed yet. Both glutamine and curcumin were observed to reduce bacterial translocation in blood, hepatocellular injury, serum cytokine levels, oxidative tissue damage and apoptosis rates significantly in comparison to the control group.

Applications

Curcumin can be used like glutamine in order to prevent bacterial translocation and oxidative damage observed in obstructive jaundice and to reduce mortality and morbidity observed in the elapsed time until definitive treatment or after treatment.

Terminology

Glutamine, a non-essential amino acid, has been reported not only to lower the rate of endotoxemia and translocation by preserving mucosal integrity but also to improve the action of the immune system against bacteria and endotoxins which succeed in passing the intestinal barrier. Curcumin is a polyphenol derived from the herbal remedy and dietary spice turmeric. The antioxidant, anti-cancer, anti-inflammatory and cytoprotective effects of curcumin have been demonstrated by numerous experimental and clinical studies.

Peer review

This is a well conceived and implemented experimental protocol which seeks to answer the question of the relative efficacy of glutamine and curcumin in the treatment or prevention of intestinal bacterial translocation in the setting of obstructive jaundice. The experimental protocol is well carried out and can be expected to extrapolate to human clinical situations in which obstructive jaundice is present.

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BRIEF ARTICLE

HCV genotype distribution and possible transmission risks in Lahore, Pakistan

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Abstract

AIM: To investigate the prevalence of hepatitis C virus (HCV) genotypes and their association with possible transmission routes in the general population of Lahore, as the data exclusively related to this city is limited.

METHODS: Complete data regarding patient's history, possible route of infection and biochemical tests was collected from the public hospital for 1364 patients. SPSS version 16 windows software was used for data analysis by univariate and multivariate techniques.

RESULTS: Age range ⩽ 40 years showed high prevalence of HCV infection. HCV genotype 3a was dominant (55.9%), followed by 1a (23.6%), 4a (12.5%), 3b (3.2%), untypable (2.5%), 4b (1.2%) and mixed type (1.2%). Blood transfusion, dental surgery and barber shops were the main risk factors for HCV transmission. Genotype prevalence was independent of age (P = 0.971) and gender (P = 0.122) while risk factors showed a significant association with age (P = 0.000) and genotypes (P = 0.000). We observed an independent association of risk factors and genotype 3a, while patients with genotype 1 and 4 were mostly infected due to dental surgery blood transfusion and barber shops. Risk factors of intravenous drug use and sexual exposure were exclusively found in ⩽ 40 years age group.

CONCLUSION: An increase in genotypes 1a and 4a suggest migration of people, possibly from Balochistan and the northern war-zone area. Government should focus on public education regarding infection routes.

Key words: Hepatitis C virus; Prevalence; Genotypes; Risk factors; Lahore

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver asso-

ciated diseases all over the world. An estimated 3% of the world's populations are chronically infected by HCV which is the main cause of liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) in a substantial number of patients[1,2]. It is an enveloped virus with a single-stranded, positive sense non-segmented RNA genome of approximately 9.6 kb that encodes a poly-protein of approximately 3000 amino acids[3]. To date, at least six major genotypes of HCV, each having multiple subtypes, have been identified worldwide[4]. The different genotypes are relevant to epidemiology, vaccine development, and clinical management of chronic HCV infection[5].

Genotype distribution has been identified in three different patterns[6]. One pattern is genetic diversity, in geographically different areas like West Africa with 1 and 2[7], Central Africa with type 4[8], and Asia with type 3 and 6[9]. The second distribution pattern involves subtypes in specific risk groups e.g. intravenous drug use (IVDU), where the subtype 3a is more common[10]. The third pattern of genotype distribution is the circulation of a single subtype in particular areas such as in Egypt with 4a and South Africa with subtype 5a[11]. Recently, a shift in genotype distribution, mostly comprising an increase of the prevalence of the genotypes 3a, 1a and 4, and decrease of prevalence of other genotypes has been seen in many countries like Serbia, Germany, France and Greece[12-15].

Almost 10 million people in Pakistan are living with HCV. The most prevalent genotype in Pakistan is 3a followed by 3b and 1a[16]. Few studies are available on the distribution of various HCV genotypes in individual cities of Pakistan[17,18]. Unfortunately, there is no national data collection system for evaluation of infection routes and their correlation with genotypes or patients' demographic data[19]. Lahore is the second largest metropolitan city of Pakistan with more than 7 million population[20]. Data exclusively related to HCV genotype-specific prevalence and route of infection in this city is limited. Although, Ijaz *et al*[18] showed a high prevalence of HCV 3a genotype (n = 80, 51.61%) followed by 3b (n = 43, 27.74%), 1b (n = 21, 13.54%), untypable (n = 5, 3.22%), mix 3a1b (n = 4, 2.58%), 3a1a (n = 1, 0.64%) and 3b1a (n = 1, 0.64%) in the population of Lahore city, their study utilized a small population size (n = 155) without any association to the mode of transmission[18].

The aim of the present study was to determine the frequency distribution of HCV genotypes, various risk factors prevalence with genotypes and age for its transmission in Lahore.

MATERIALS AND METHODS

Patients

Patients in this study were from Jinnah Hospital, Lahore, which is the only public facility that has HCV patient's testing service and is the 2nd largest hospital in the area. Therefore, patients visiting at this hospital can be regarded as representative of general population of the Lahore city. The data and samples were collected from March 2007 to September 2009 from the Department of Pathology, Jinnah Hospital, Lahore, Pakistan and data analysis was performed in collaboration with National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. In total 1364 adult patients (18-75 years) who were HCV RNA-positive based on HCV antibody (anti-HCV)-positive results were included in this study. A written informed consent was obtained from patients. A complete history with possible route and estimated time of infection, standard biochemical liver function tests and patient's contact information were collected. This study was approved by the Institutional ethics committee.

Viral investigations

HCV detection and genotyping was performed at the Department of Pathology, Jinnah Hospital, Lahore, Pakistan. RNA was extracted from 140 μL of serum samples using QIAamp viral RNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. cDNA was synthesized using Moloney murine leukemia virus (MmLV) followed by polymerase chain reaction (PCR) using primers derived from the 5'UTR non-coding region of HCV genome described by Chan *et al*[21]. For HCV RNA quantification, Qiagen HCV RG RT-PCR assay was used. Quantification was carried out with 10 μL of the extracted RNA on Rotor-gene Real-Time PCR machine (USA) using fluorescent probes to detect amplification after each replicating cycle as described by manufacturer protocol. The lower limit of detection for this assay is 1000 IU/mL HCV and genotyping was carried out using Invader HCV genotyping assay (Third wave technology, USA). Briefly, 100 ng of the HCV RNA was reverse transcribed to cDNA using 200 units of MmLV (Invitrogen, USA). From the amplified product, 2 μL were taken and the genotyping assay was performed for 12 different HCV types.

Statistical analysis

Data was analyzed using a statistical package SPSS version 16 for windows. The data is presented as mean and standard deviations, and categorical variables in absolute numbers and percentages. Student t-test and chi-square tests were applied to evaluate differences in proportions. A P value < 0.05 was considered significant. A multivariate analysis was used to identify variables associated within different genotypes. Bonferroni, Gabriel and LSD tests were performed to evaluate whether significant variables in the univariate analysis could predict differences among genotypes.

RESULTS

Age and gender specific prevalence of HCV

Of 1364 patients, 656 were male while 708 were female, with a median age of 36.8 ± 10.3 years (range 18-75 years). The age of infected patients was taken as a categorical as well as a continuous variable. Patients were divided in two age groups i.e. ≤ 40 years and > 40 years. Distribution of patients' gender in age groups is given in Table 1. It is clear from Table 1 that people of age group

Table 1 Patient data according to age and gender

Parameters	Age groups	
	≤ 40 yr (n = 931, 68.3%)	> 40 yr (n = 433, 31.7%)
Gender		
Male (n = 656)	468 (50.2%)	188 (43.3%)
Female (n = 708)	463 (49.8%)	245 (56.7%)
Age (yr)		
mean ± SD (36.8 ± 10.3)	37.77 ± 10.65	46.8 ± 12.38
Age range (18-75)	18-40	41-75

Table 2 Univariate logistic analysis of genotypes involved in hepatitis C virus infection according to age and gender

Variables	Type Ⅲ sum of squares	υ	Mean square	*F* value	*P* value
Sex	150.641	1	150.641	2.396	0.122
Age groups	0.084	1	0.084	0.001	0.971

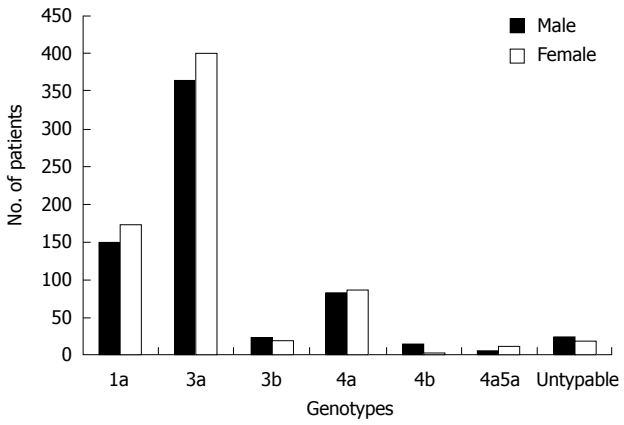

Figure 1 Frequency distribution of different genotypes in gender of patients. The prevalence of hepatitis C virus genotypes in gender was non-significant.

≤ 40 years (n = 931, 68.3%) were more affected with HCV in comparison to those in the older age group (> 40 years, n = 433, 31.7%).

Genotype distribution

Based on weighted analysis of patients infected with HCV genotypes 1, 3, 4, mix (4a5a) and untypable genotypes, the most commonly detected genotype in the study was genotype 3 (n = 806, 59.1%), with predominant subtype 3a (n = 763, 55.9%) and 3b (n = 43, 3.2%). Genotype 1 (n = 322, 23.6%,) was exclusively consisted of the subtype 1a, while genotype 4 (n = 186, 13.7%) comprised of the subtypes a (n = 170, 12.5%) and b (n = 16, 1.2%). Mixed inter-genotype 4a5a (n = 16, 1.2%) and untypable (n = 34, 2.5%) genotypes were also detected in 50 (3.66%) patients. The frequency distribution of genotypes revealed that patients of the age group ≤ 40 years were more affected with genotype 1a (71%) and 4a (68%) as compared to patients with the age group > 40 years. However, univariate analysis (Table 2) revealed that prevalence of genotype subtypes within age groups (P = 0.971) and gender (P = 0.122) of the patients were not statistically significant. Figure 1 illustrated the genotype distribution pattern in each gender of patients while Table 3 showed the prevalence of different genotype subtypes in different age groups.

Table 3 Hepatitis C virus genotype subtypes prevalence in age groups

Genotype subtypes	Computation	Age groups	
		≤ 40 yr	> 40 yr
1a	Count	230	92
	% within age groups	24.7%	21.2%
3a	Count	507	256
	% within age groups	54.5%	59.1%
3b	Count	32	11
	% within age groups	3.4%	2.5%
4a	Count	116	54
	% within age groups	12.5%	12.5%
4b	Count	13	3
	% within age groups	1.4%	0.7%
4&5	Count	11	5
	% within age groups	1.2%	1.2%
Untypable genotype	Count	22	12
	% within age groups	2.4%	2.8%

Table 4 Univariate logistic analysis of risk factors involved in hepatitis C virus infection according to age, gender and genotypes

Variables	Type Ⅲ sum of squares	υ	Mean square	*F* value	*P* value
Sex	0.014	1	0.014	0.004	0.950
Age	131.744	1	131.744	38.219	0.000
Genotype	457.878	6	76.313	22.139	0.000

Risk assessment for HCV infection

The possible routes of infection were determined by detailed questionnaire. Out of 1364 patients, the possible risk factors for 1183 (86.7%) patients were established, while 13.3% (n = 181) were unaware of their possible route of infection. The major route of HCV infection among patients resides within Lahore city was dental surgery (33.5%), followed by blood transfusion (22.6%), barber shops (12.4%), road accidents (8.4%), sexual exposure (5.9%) and IVDU (3.73%). Statistical analysis illustrated in Table 4 showed that risk factors were dependent on age (P = 0.000) and genotype (P = 0.000) and independent of gender (P = 0.950).

Age-specific distribution of the risk factors

The frequency distribution of different risk factors within age groups is illustrated in Table 5. Dental surgery was the cause of HCV infection in 458 of the 1364 patients (33.6%), and was found more frequently among patients ≤ 40 years (71%) than patients with age > 40 years. Infection due to blood transfusion, road accidents and barber shops was also prevalent in patients ≤ 40 years of age

Table 5 Distribution and multivariate analysis of risk factors in age groups

Risk factors	Computation	Age groups		95% CI		*P* value
		≤ 40 yr	> 40 yr	Lower	Upper	
Blood transfusion	Count	222	88	2.077	4.467	0.039
	% within age groups	23.8%	20.3%			
IVDU	Count	51	0	6.414×10^9	6.414×10^9	0.000
	% within age groups	5.5%	0.0%			
Sexual exposure	Count	79	0	9.014×10^9	9.014×10^9	0.000
	% within age groups	8.5%	0.0%			
Dental surgery	Count	325	133	2.068	4.209	0.610
	% within age groups	34.9%	30.7%			
Road accidents	Count	77	39	1.551	2.090	0.148
	% within age groups	8.3%	9.0%			
Barber shops	Count	95	74	1.016	2.363	0.042
	% within age groups	10.2%	17.1%			

IVDU: Intravenous drug use.

Table 6 Hepatitis C virus genotype prevalence within risk factors in Lahore - March 2007 - September 2009 (*n* = 1364) *n* (%)

Groups	Total No.	1a	3a	3b	4a	4b	Mix	Untypable
BT	310	51 (16.5)	202 (65.2)	13 (4.2)	23 (7.4)	4 (1.3)	4 (1.3)	13 (4.2)
≤ 40 yr	222	39 (17.6)	131 (59.0)	10 (4.5)	22 (9.9)	3 (1.4)	4 (1.8)	13 (5.9)
> 40 yr	88	12 (13.6)	71 (80.7)	3 (3.4)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)
IVDU	51	12 (23.5)	34 (66.7)	0 (0.0)	4 (7.8)	0 (0.0)	0 (0.0)	1 (2.0)
≤ 40 yr	51	12 (23.5)	34 (66.7)	0 (0.0)	4 (7.8)	0 (0.0)	0 (0.0)	1 (2.0)
> 40 yr	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
SE	79	14 (17.7)	53 (67.1)	0 (0.0)	10 (12.7)	1 (1.3)	0 (0.0)	1 (1.3)
≤ 40 yr	79	14 (17.7)	53 (67.1)	0 (0.0)	10 (12.7)	1 (1.3)	0 (0.0)	1 (1.3)
> 40 yr	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
DS	458	85 (18.6)	285 (62.2)	13 (2.8)	62 (13.5)	5 (1.1)	4 (0.9)	4 (0.9)
≤ 40 yr	325	69 (21.2)	173 (53.2)	13 (4.0)	58 (17.8)	5 (1.5)	3 (0.9)	4 (1.2)
> 40 yr	133	16 (12.0)	112 (84.2)	0 (0.0)	4 (3.0)	0 (0.0)	1 (0.8)	0 (0.0)
RA	116	30 (25.9)	68 (58.6)	4 (3.4)	11 (9.5)	1 (0.9)	1 (0.9)	1 (0.9)
≤ 40 yr	77	30 (39.0)	46 (59.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.3)
> 40 yr	39	0 (0.0)	22 (56.4)	4 (10.3)	11 (28.2)	1 (2.6)	1 (2.6)	0 (0.0)
BS	169	45 (26.6)	88 (52.1)	7 (4.1)	19 (11.2)	1 (0.6)	5 (3.0)	4 (2.4)
≤ 40 yr	95	19 (20.0)	64 (67.4)	7 (7.4)	0 (0.0)	0 (0.0)	3 (3.2)	2 (2.1)
> 40 yr	74	26 (35.1)	24 (32.4)	0 (0.0)	19 (25.7)	1 (1.4)	2 (2.7)	2 (2.7)
UN	181	85 (47.0)	33 (18.2)	6 (3.3)	41 (22.7)	4 (2.2)	2 (1.1)	10 (5.5)
≤ 40 yr	82	47 (57.3)	6 (7.3)	2 (2.4)	22 (26.8)	4 (4.9)	1 (1.2)	0 (0.0)
> 40 yr	99	38 (38.4)	27 (27.3)	4 (4.0)	19 (19.2)	0 (0.0)	1 (1.0)	10 (10.1)
Total	1364	322 (23.6)	763 (55.9)	43 (3.2)	170 (12.5)	16 (1.2)	16 (1.2)	34 (2.5)

BT: Blood transfusion; IVDU: Intravenous drug use; SE: Sexual exposure; DS: Dental surgery; RA: Road accidents; BS: Barber shops; UN: Unknown reasons.

(71.6%, 66.4% and 56.2%, respectively). Infection due to sexual exposure and IVDU was only observed among patients ≤ 40 years age. Our data revealed that infection due to dental surgery was more prevalent in both age groups (age group ≤ 40 years, 34.9%, n = 325; age group > 40 years, 30.7%, n = 133). The second most prevalent infection route in age groups was blood transfusion (age group ≤ 40 years, 23.8%, n = 222; age group > 40 years, 20.3%, n = 88), followed by barber shops (age group ≤ 40 years, 10.2%, n = 95; age group > 40 years, 17.1%, n = 74) and road accidents (age group ≤ 40 years, 8.3%, n = 77; age group > 40 years, 9.0%, n = 39). Multivariate analysis showed a significant association of IVDU (P = 0.000) and sexual exposure (P = 0.000) with age ≤ 40 years (Table 5) whereas route of infection due to dental surgery, blood transfusion, barber shops and road accidents was independent of age groups. Route of infection due to sexual exposure (n = 79, 8.5%) and IVDU (n = 51, 5.5%) was only observed in patients ≤ 40 years.

Prevalence of genotypes within risk factors

As risk factors showed a significant association with genotypes, the frequency distribution of various genotypes within risk groups (Table 6) revealed that subtypes 3a (65.2%) and 1a (16.5%) were found more frequently than subtypes 3b (4.2%), 4a (7.4%), 4b (1.3%), mixed (1.3%) and untypable (4.2%) in patients with a past history of blood transfusion. Moreover, subtypes 3a (62.2%), 1a (18.6%) and 4a (13.5%) were observed more frequently than subtypes 3b (2.8%), 4b (1.1%), mixed (0.9%) and un-

typable (0.9%) in patients infected due to dental surgery. In patients infected through barber shops and road accidents, genotype 3a (52.1% and 58.6%) and 1a (26.6% and 25.9%) were more prevalent than subtypes 3b (4.1% and 3.4%), 4a (11.2% and 9.5%), 4b (0.6% and 0.9%), mixed (3.0% and 0.9%) and untypable (2.4% and 0.9%) respectively. Patients with history of IVDU were mainly infected with genotype 3a (66.7%) and 1a (23.5%), while infection due to sexual exposure was predominantly genotype 3a (67.1%) followed by 1a (17.7%) and 4a (12.7%). These findings indicated that genotype subtype 3a is significantly dominant among all risk groups followed by 1a and 4a. Moreover our results revealed that the genotype with the highest frequency for risk factors is genotype 3a, the highest being in patients with a history of sexual exposure (53, 67.1%) followed by IVDU (34, 66.7%) and blood transfusion (65.2%). The second most frequent genotype was 1a with the highest frequency in patients possibly infected from barber shops (26.6%), subsequently; genotype 4a was the most frequent in patients experienced the dental surgery (13.5%). Patients (n = 5) with mixed (4a5a) and untypable genotypes (n = 13) were under risk due to barber shops and blood transfusion respectively.

Association of HCV risk factors with age and genotype

In patients infected due to blood transfusion, genotype 3a was more prevalent in age group > 40 years (80.7%) than ≤ 40 years (59%), while genotype 4a was more prevalent in age group ≤ 40 years (9.9%) than > 40 years (1.1%) (Table 6). The same prevalence order was observed in patients infected due to dental surgery where genotypes 1a (21.2%) and 4a (17.8%) were more prevalent in the age group ≤ 40 years than the age group > 40 years (12.0% and 3.0%, respectively) and genotype 3a was predominant in the age group > 40 years (84.2%) than the age group ≤ 40 years (53.2%). In patients infected due to barber shops, the prevalence of genotype 1a was higher in the age group > 40 years (35.1%) than the age group ≤ 40 years (20.0%), while genotype 3a was most prevalent in age group ≤ 40 years (67.4%) compared with age group > 40 years (32.4%).

DISCUSSION

HCV is an important cause of chronic liver disease and cirrhosis in Pakistan and accounts for significant morbidity and mortality. It is estimated that about 6% (10 million) of the Pakistani population is infected with HCV[19]. In the present study, we analyzed the relationship between distribution of HCV genotypes and mode of transmission of infection in different age groups and gender. Our results showed a non-significant prevalence of HCV distribution in gender and age which are in accordance to studies carried out by other groups who observed no relation between age, gender and HCV distribution[22,23]. When results were further analyzed keeping age as a categorical variable, people ≤ 40 years of age were more affected with HCV in comparison to those > 40 years of age in Lahore. These results are in conflict with previous data from Muhammad *et al*[24] in 2005 showing that the prevalence of HCV in Pakistan is greater in old age, however, our results agreed with the findings of Ali *et al*[25] that HCV prevalence observed was highest among an age group of 13-50 years and similar findings were observed by Shah *et al*[26]. This could be due to an increasing awareness and early diagnosis of HCV in urban area communities in Pakistan.

HCV shows considerable sequence diversity and sequence comparisons in different parts of the genome. This has led to the classification of the virus into a series of genotypes that show distinct geographical distribution across the world[27]. Genotyping is important because it provides information as to strain variation and potential association with disease severity and a guide about treatment duration and outcomes[28]. Our finding indicating a high prevalence of genotype 3a (55.9%) in Lahore city is consistent with others describing HCV 3a prevalence in Pakistan[17,18], however, an increased prevalence of genotype 1a and 4a (23.6% and 12.5%) found in our study is significantly higher than the previously reported HCV prevalence for these genotypes. Idress *et al*[17] in 2008 showed that the prevalence of HCV infection due to genotype 4 and 1 is increasing without an increase in the frequency of genotype 3 in various areas of Pakistan mainly NWFP (1a, 6.56% and 4, 2.30%) and Balochistan (1a, 25.80% and 4, 4.03%). They claimed the appearance of genotype 4 in Pakistan for the first time with 1.15% prevalence in the Punjab area which includes Lahore city. A shift in genotype distribution, mostly comprising an increase in the prevalence of the genotypes 3a, 1a and 4, and decrease in prevalence of other genotypes, is also found in many countries largely due to migration[12,23]. Our results indicated an increased prevalence of genotype 4 and 1 in Lahore city may suggest a recent migration of people, possibly from Balochistan and northern areas (active terrorist war-zone area). Although there is evidence of increasing population size for Lahore, recent reports highlighting migration data to the city of Lahore are not available[29]. These results suggest a possible disaster in HCV management for Pakistan in coming years due to poor response against current therapy for genotypes 4 and 1. Furthermore, genotype 4 is reported to be associated with liver cirrhosis[30], therefore, it may potentially lead to an increased risk of liver cirrhosis in Pakistan. The absence of genotype 1b, 2a and 2b in our study indicates that these are rarely present in our population. A similar frequency distribution pattern of genotypes in neighboring countries like India and Iran has been observed where genotype 3 is most prevalent and genotype 2 is very rare[22,31].

As described earlier there is no national database for collection of risk factors involved in HCV transmission, however, studies from different areas of Pakistan revealed that IVDU due to excessive use of unnecessary injections and reuse of needles, dental surgery by using unhygienic practice and unsafe blood transfusion are the major causes of HCV infection[19,32-35], while HCV transmission due to

sexual exposure and barber shaving was also reported in Pakistan[36,37]. Kuo *et al*[38] reported 93% HCV prevalence among IVDUs in Lahore. Although IVDU has been identified as a route of infection in Lahore, it was not significant in our HCV population due to denial by a large number of people. Our results indicate dental surgery to be a leading cause of HCV transmission followed by unsafe blood transfusion. Our results are also in agreement with the results from other countries reporting blood transfusion, unsafe injection, barber shops and dental instruments as the main routes of HCV infection[39].

Data analysis for finding an association of various HCV genotypes with possible routes of transmission indicated blood transfusion and dental surgery to be the leading cause in all genotypes while infection due to IVDU and sexual exposure was predominant in genotype 1 and 3. In the present study, genotype 3 had a high prevalence in patients with a history of blood transfusion and IVDU, similar to USA and Europe[19,34] while genotype 1 was most prevalent in patients infected from barber shops. Genotype 4 has been reported to be related with different routes of infection such as dental surgery, dialysis, barber shops[19,40] and we observed dental surgery as a major route of infection in genotype 4 followed by sexual exposure. Barber shops and blood transfusion were observed as possible routes of infection in mixed and untypable genotype, respectively. We observed a notable variation in the distribution of HCV subtypes in different risk groups within age groups. As patients infected due to blood transfusion and dental surgery were more frequent (57%) than infection from other risk factors, genotype 3a was more prevalent in the age group > 40 years, while genotype 1a and 4a were observed in the age group ≤ 40 years. These findings conclude 3a is the original subtype present in Lahore while the others were introduced at a later date by an increase in population movements like migration and change in mode of risk factors. A high prevalence of genotype 1a and 4a in the age group ≤ 40 years can confirm this epidemiological shift in new generation.

Our results also revealed that the routes of HCV transmission were significantly associated with age groups while risk factors like dental surgery, blood transfusion and road accidents were evenly distributed in both age groups. Sexual exposure was reported in 79 patients and all of them belonged to the age group ≤ 40 years. The major reason for infection due to sexual exposure was non-awareness of people about sexually transmitted diseases and low use of condoms[41]. However, sexual activity cannot be applied as an independent risk factor as Tong *et al*[42] suggested low transmission of HCV among spouses. Blood transfusion, the most commonly recognized transmission mechanism of HCV, showed a high prevalence (71.6%) for patients ≤ 40 years. This could be due to many reasons - mainly concealing HCV status of donors from their relatives during blood donation and improper screening of blood donors[43]. HCV prevalence due to barber shops was also significantly higher (56.2%) in patients ≤ 40 years. The possible reason could be due to the use of inadequately sterilized instruments, reuse of razors and other shaving kits and transfusion of contaminated blood also seen by others in Pakistan[31,35-37]. The use of illegal drugs by injection is rising especially in young people in Pakistan as the effect of IDVU is more satisfying and intense[19,33]. Although the number of patients with IDVU was small, 100% IVDU patients belonged to the age group ≤ 40 years.

In conclusion, this study used a large HCV population for the first time and reported the HCV genotype-specific prevalence with age and possible risk factors associated with its transmission in Lahore city of Pakistan. Genotype 3 was predominant and patients infected with genotype 4 are increasing along with genotype 1 due to possible inland migration in Pakistan. We observed a maximum prevalence for HCV in the age group ≤ 40 years (68.3%) with involvement of multiple routes of infections. Infection due to sexual exposure and IVDU was exclusively linked to ≤ 40 years age group. Our study revealed that at least 66% of HCV infections are associated with the health-care sector as most of the patients infected with HCV developed the infection as a result of blood transfusion, dental surgery and IVDU. For genotype 3, blood transfusion was the major infection route, while infection attributed to barber shops was associated with genotype 1. The most prevalent risk factor for genotype 4 was found to be dental surgery. This study also highlights future possible HCV disease complications due to an increase in genotypes 4 and 1 and also provides a basis for the government of Pakistan to implement higher health standards.

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COMMENTS

Background

Chronic hepatitis C virus (HCV) is one of the major causes of liver fibrosis, with distortion of the hepatic architecture and ultimate progression to cirrhosis. To date, at least six major genotypes of HCV, each having multiple subtypes, have been identified worldwide. The different genotypes are relevant to epidemiology, route of infection, vaccine development, and clinical management of chronic HCV infection.

Research frontiers

In Pakistan, more than 10 million people are infected with chronic HCV. The exact data about the relationship between HCV genotypes, epidemiological factors and route of infection is limited.

Innovations and breakthroughs

The frequency distribution of different HCV genotypes and subtypes and their association with various risk factors in Lahore, the second largest city of Pakistan was investigated in 1364 patients with chronic HCV. The maximum prevalence of HCV was found in the age group ≤ 40 years (68.3%). Genotype 3a was the predominant genotype followed by an increase in 1a and 4a as compared to previous reports suggesting a recent migration of people possibly from Balochistan and northern areas (active terrorist war-zone area). The main risk factors observed in patients infected with HCV were blood transfusion, dental surgery and intravenous drug use. Blood transfusion and barber shops were the major infection route, in genotype 3a and 1a respectively.

Applications

This article reported the prevalence of HCV genotypes in Lahore city. Additionally the relationship between different genotypes and subtypes with age, gender and different possible routes of infection was studied. Shifts in HCV genotype distribution needs to be paid more attention as genotype 1a and 4a are associated with severe cirrhosis.

Peer review

This article describes the prevalence of HCV in Lahore population. It is a good work and should be accepted for publication.

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BRIEF ARTICLE

Associated factors for a hyperechogenic pancreas on endoscopic ultrasound

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Abstract

AIM: To identify the associated risk factors for hyperechogenic pancreas (HP) which may be observed on endoscopic ultrasound (EUS) and to assess the relationship between HP and obesity.

METHODS: From January 2007 to December 2007, we prospectively enrolled 524 consecutive adults who were scheduled to undergo EUS. Patients with a history of pancreatic disease or with hepatobiliary or advanced gastrointestinal cancer were excluded. Finally, 284 patients were included in the analyses. We further analyzed the risk of HP according to the categories of visceral adipose tissue (VAT) and subcutaneous adipose tissue in 132 patients who underwent abdominal computed tomography scans.

RESULTS: On univariate analysis, age older than 60 years, obesity (body mass index > 25 kg/m^2), fatty liver, diabetes mellitus, hypertension and hypercholesterolemia were identified as risk factors associated with HP (P < 0.05). On multivariate analysis, fatty liver [P = 0.008, odds ratio (OR) = 2.219], male gender (P = 0.013, OR = 2.636), age older than 60 years (P = 0.001, OR = 2.874) and hypertension (P = 0.044, OR = 2.037) were significantly associated with HP. In the subgroup analysis, VAT was a statistically significant risk factor for HP (P = 0.010, OR = 5.665, lowest quartile *vs* highest quartile).

CONCLUSION: HP observed on EUS was associated with fatty liver, male gender, age older than 60 years, hypertension and VAT.

Key words: Endoscopic Ultrasound; Hyperechogenic pancreas; Obesity

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INTRODUCTION

Endoscopic ultrasound (EUS) has been an important tool for diagnosing gastrointestinal and pancreatobiliary disease since the 1980s[1]. EUS is particularly effective for evaluating patients with pancreatic disease, because EUS provides high resolution images of the pancreatic duct as well as the parenchyma. In recent years, EUS-guided fine-needle aspiration or trucut biopsy can be performed at the same time and this procedure enables tissue diagnosis.

Identification of hyperechogenic pancreas (HP) is not uncommon during EUS. However, the clinical significance of HP is still unclear. Fatty liver is associated with insulin resistance, dyslipidemia and obesity (especially central body fat distribution) and is considered a phenotype of metabolic syndrome[2-4]. Visceral fat is more important for metabolic syndrome and hepatic steatosis than subcutaneous fat owing to its steatogenesis and production of various cytokines[5,6]. The normal pancreatic echogenicity on ultrasound is equal to or slightly greater than that of the liver[7,8]. Pancreatic echogenicity is determined by fat deposited around the pancreas and within the septa transversing the normal pancreas[9]. However, the role of obesity as a risk factor for HP remains unclear. We hypothesized that HP is related to obesity in a similar way to its relationship with fatty liver. Many different methods have been used to quantify obesity, such as body mass index (BMI), waist circumference, waist-to-hip ratio, skin fold thickness and percentage of body fat. Among these methods, computed tomography (CT) is considered the gold standard not only for evaluating adipose tissue, but also for multi-compartment body measurements[10].

The aim of this study was to determine the incidence of HP in patients undergoing EUS and to identify the associated risk factors for HP on EUS.

MATERIALS AND METHODS

From January 2007 to December 2007, a total of 524 patients who underwent EUS were prospectively enrolled in the study. Pancreatic disease can alter the sonographic appearance of the pancreas, therefore patients with a history of or who showed the presence of pancreatic disease such as chronic pancreatitis were excluded (n = 156), and patients with hepatobiliary or advanced gastrointestinal cancer were also excluded from the study (n = 84). Finally, a total of 284 patients were included (Figure 1) and all EUS examinations were performed to evaluate subepithelial tumors. EUS examinations were performed using a radial echoendoscope (Olympus GF-UM2000 with 5 MHz and 7.5 MHz frequency transducers) by a single experienced endoscopist (Kim GH). Informed consent was obtained after the patients were given a complete description of the study. All the patients completed a questionnaire regarding their personal medical history, including alcohol intake and smoking. This study was approved by the Ethics Committee of Pusan National University Hospital.

During the study, we measured the levels of serum pancreatic enzymes, took clinical histories, conducted physical examinations, and performed blood analyses including blood sugar, total cholesterol and liver function tests. The degree of echogenicity of the pancreas was judged relative to the liver (or the kidney if the liver was hyperechogenic) (Figure 2). The patients' histories of alcohol consumption were obtained, and the term "nonalcoholic" was applied to men who consumed less than 30 g alcohol/d and to women who consumed less than 20 g alcohol/d. We further analyzed the risk of HP according to the categories of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) in 132 patients who underwent abdominal CT scanning for clinical purposes.

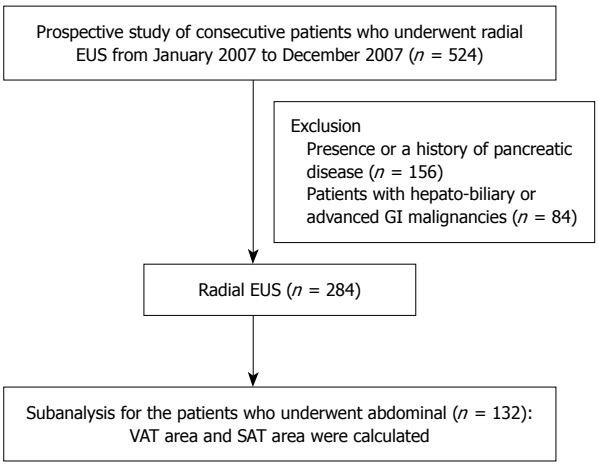

Figure 1 Flow chart indicating the progression from the initial assessment when first referred for endoscopic ultrasound to the final analysis. EUS: Endoscopic ultrasound; GI: Gastrointestinal; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue.

Laboratory investigations and assessment of the abdominal visceral fat area

The clinical characteristics of all subjects were prospectively evaluated, including gender, age, systolic blood pressure, diastolic blood pressure, BMI and routine blood values. These parameters were measured within 30 d of EUS. Hypertension was defined as systolic BP ⩾ 140 mmHg or diastolic BP ⩾ 90 mmHg[11]. Type 2 diabetes mellitus (DM) was defined as a fasting plasma glucose level ⩾ 126 mg/dL or if there were symptoms of hyperglycemia and the random venous plasma glucose level was ⩾ 200 mg/dL[12]. Hypercholesterolemia was defined when the serum total cholesterol level was above the reference value (more than 240 mg/dL at our hospital). The body mass index (BMI) was calculated as the body weight (kg) divided by the square of the standing height (m). The BMI was categorized into three levels according to the WHO criteria for the Western Pacific region[13]: normal weight-BMI < 23 kg/m^2, overweight-BMI ⩾ 23 kg/m^2 and ⩽ 25 kg/m^2 and obese-BMI > 25 kg/m^2. To determine the VAT and SAT on CT scans, adipose tissue area was calculated at the level of the umbilicus, with an attenuation that ranged from -50 to -250 Hounsfield units[2,14]. The subjects were examined in the supine position. The VAT was defined as intra-abdominal fat bound by the pa-

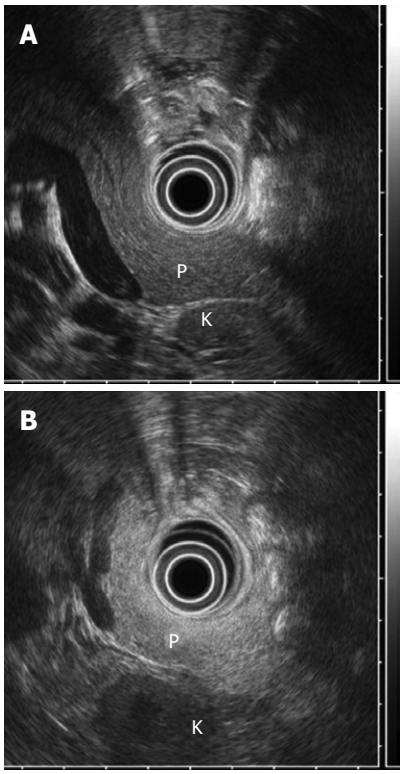

Figure 2 Echogenicity of the pancreas on endoscopic ultrasound. A: Normal echogenic pancreas; B: Hyperechogenic pancreas compared to kidney. P: Pancreas; K: Kidney.

rietal peritoneum or the transversalis fascia excluding the vertebral column and the para-spinal muscles, and the SAT was defined as fat superficial to the abdominal and back muscles. Using a cursor, the VAT was measured around the inner boundary of the abdominal wall muscles. A region of interest drawn around the external margin of the dermis was used to calculate the area of total adipose tissue (TAT). SAT was obtained by subtracting the VAT from the TAT (Figure 3)[2,15].

Statistical analysis

The data were expressed as means ± SD. The unpaired *t*-tests were used to compare the mean values of the characteristics between the HP group and the non-HP group. χ^2 tests were used for the nominal variables. Variables with a *P* value less than 0.25 on univariate analysis were included in a forward stepwise multiple logistic regression model. To detect the dose-response relationship, we used cut-off points to categorize the patients into quartiles for the SAT and VAT. Logistic regression analysis was used to estimate the crude and adjusted strength of the association between the positive and negative HP groups. All data analyses were performed using SPSS for Windows version 12.0 (SPSS, Chicago, IL, USA). A *P*-value less than 0.05 was considered significant.

RESULTS

Baseline characteristics of the study sample

The total number of subjects included was 284 (102 males and 182 females) and their mean age was 52.1 ± 12.2 years. Among the study subjects who underwent EUS, 11 were HP patients (38.7%). Thirty-four patients (33.1%) had fatty livers. There were 70 (24.6%) current smokers and 106 (37.3%) alcohol drinking patients. The mean BMI was 23.1 ± 2.9 kg/m^2. Among the 132 patients who underwent an abdominal CT scan, the mean SAT and VAT were 145.6 ± 49.3 and 71.7 ± 43.6 cm^2, respectively (Table 1).

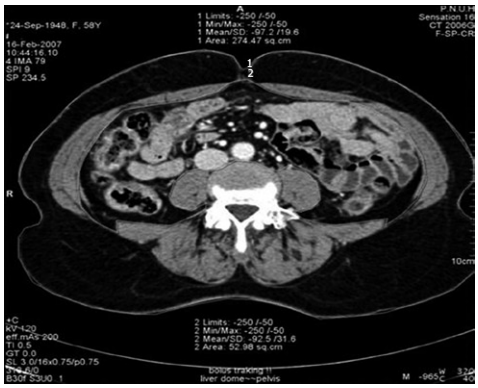

Figure 3 Calculation of the abdominal adipose tissue distribution using computed tomography scans. The total adipose tissue (TAT) area was obtained by applying an adipose tissue threshold to a region of interest (ROI) that was traced around the dermis (1). An ROI was traced around the inner margin of the abdominal wall muscles, and an adipose tissue threshold was applied to determine the area of visceral adipose tissue (VAT) in the ROI (2). The subcutaneous adipose tissue area was then obtained by subtracting the VAT from the TAT.

Table 1 Baseline characteristics of the study population (mean ± SD) *n* (%)

	Total patients (*n* = 284)
Gender (M:F)	102:182
Age (yr)	52.1 ± 12.2
Hyperechogenic pancreas positive	110 (38.7)
Current cigarette smoking	70 (24.6)
Current alcohol drinking	106 (37.3)
Hypertension (≥ 140/90 mmHg)	61 (21.5)
BMI (kg/m^2)	23.1 ± 2.9
Fatty liver	94 (33.1)
Diabetes mellitus	46 (16.2)
Hypercholesterolemia	46 (16.1)
CT measurement (*n* = 132)	
SAT area (cm^2)	145.6 ± 49.3
VAT area (cm^2)	71.7 ± 43.6

BMI: Body mass index; CT: Computed tomography; SAT: Subcutaneous adipose tissue; VAT: Visceral adipose tissue.

Comparison of patients with and without hyperechogenic pancreas

We analyzed the potential risk factors for HP. On univariate analysis, age older than 60 years, obesity (BMI more than 25 kg/m^2), fatty liver, type 2 DM, hypertension and hypercholesterolemia were the associated risk factors for HP ($P < 0.05$) (Table 2). On multivariate analysis, fatty liver [$P = 0.008$, odds ratio (OR) = 2.219], male gender ($P = 0.013$, OR = 2.636), age older than 60 years ($P = 0.001$, OR = 2.874) and hypertension ($P = 0.044$, OR = 2.037) were the significant associated risk factors for HP (Table 3).

Table 2 Comparison between the patients with and without hyperechogenic pancreas (mean ± SD) *n* (%)

	HP negative (*n* = 174)	HP positive (n = 110)	*P* value
Age (yr)	48.9 ± 12.0	57.2 ± 10.7	< 0.001
Male gender	69 (39.9)	33 (30.0)	0.099
BMI (kg/m^2)	22.4 ± 2.8	24.0 ± 2.7	< 0.001
Smoking	47 (27.0)	23 (20.9)	0.245
Alcohol	71 (40.8)	35 (31.8)	0.127
Type 2 DM	7 (4.0)	12 (10.9)	0.024
Hypertension	25 (14.4)	36 (32.7)	< 0.001
Hypercholesterolemia	20 (11.5)	26 (23.6)	0.007
Fatty liver	46 (26.4)	48 (43.6)	0.003

HP: Hyperechogenic pancreas; BMI: Body mass index; DM: Diabetes mellitus.

Table 3 Multivariate analysis of the clinical risk factors for hyperechogenic pancreas

	P value	OR	95% CI
Male gender	0.013	2.636	1.224-5.678
Age older than 60 yr	0.001	2.874	1.537-5.372
Obesity (BMI > 25 kg/m^2)	0.439	1.296	0.673-2.496
Smoking	0.612	1.227	0.557-2.701
Alcohol	0.435	0.773	0.405-1.475
Type 2 DM	0.646	1.304	0.420-4.055
Hypertension	0.044	2.037	1.018-4.072
Hypercholesterolemia	0.099	1.821	0.893-3.713
Fatty liver	0.008	2.219	1.226-4.016

BMI: Body mass index; DM: Diabetes mellitus; OR: Odds ratio; CI: Confidence interval.

A subanalysis was performed in patients who underwent abdominal CT (n = 132). On univariate analysis, VAT and SAT were significantly different between the groups (89.5 ± 47.8 cm^2 *vs* 59.8 ± 36.3 cm^2, respectively, P < 0.001 for VAT and 162.2 ± 55.7 cm^2 *vs* 134.5 ± 59.3 cm^2, respectively, P = 0.008 for SAT) (Table 4). On multivariate analysis, for patients who underwent abdominal CT, VAT was a statistically significant risk factor for HP after adjusting for age, gender, alcohol, smoking and BMI (P = 0.010, OR = 5.665, the lowest quartile *vs* the highest quartile) (Table 5).

DISCUSSION

EUS represents a major advance in gastrointestinal imaging technology. EUS of the pancreas is particularly useful, because the pancreas can be visualized either from the duodenum or from the stomach. EUS is less risky than endoscopic retrograde pancreatography, which is the traditional imaging test of choice and the gold standard for diagnosing chronic pancreatitis[16,17]. Having an understanding of the normal variations in the pancreatic parenchyma is crucial when evaluating pancreatic abnormalities. HP is not an infrequent finding during EUS, but the clinical significance of hyperechogenicity of the normal pancreas is not known. In this study, pancreatic echogenicity was compared with liver echogenicity to evaluate fat deposition in the pancreas. However, in fatty liver, liver echogenicity is not a good reference value for HP. Therefore, we used kidney parenchymal echogenicity if fatty liver was present[18]. A very high echogenicity of the pancreas could be a sign of chronic pancreatitis, which is often accompanied by dilatation of the pancreatic duct. A previous study showed that body weight and fatty infiltration have a significant influence on pancreatic echogenicity[19]. Hepatic steatosis (or fatty liver) is associated with obesity, old age, hyperlipidemia, hyperglycemia and hypertension[2,3,5]. Visceral adiposity is known to be more important than BMI for predicting the presence of hepatic steatosis[5].

Table 4 Comparison of adipose tissue between patients with and without hyperechogenic pancreas *n* (%)

	HP negative (*n* = 79)	HP positive (*n* = 53)	*P* value
VAT area (cm^2)			< 0.001
Quartile Ⅰ (< 35.2)	23 (29.1)	9 (17.0)	
Quartile Ⅱ (35.2-65.9)	25 (31.6)	8 (15.1)	
Quartile Ⅲ (65.9-94.0)	19 (24.1)	14 (26.4)	
Quartile Ⅳ (> 94.0)	12 (15.2)	22 (41.5)	
SAT area (cm^2)			0.008
Quartile Ⅰ (< 109.3)	24 (30.4)	9 (17.0)	
Quartile Ⅱ (109.3-139.7)	22 (27.8)	11 (20.8)	
Quartile Ⅲ (139.7-179.2)	18 (22.8)	16 (30.2)	
Quartile Ⅳ (> 179.2)	15 (19.0)	17 (32.1)	

HP: Hyperechogenic pancreas; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue.

At first, we assumed that HP in an otherwise normal pancreas would be associated with fatty liver and its associated risk factors, especially obesity. On multivariate analysis, fatty liver, age older than 60 years, male gender and hypertension were the significant risk factors for HP. The pancreas in older patients showed different changes, such as atrophy, fibrosis and fatty infiltration. A previous histopathologic study showed that after the age of 60 years, moderate to severe fat accumulation is typically evident in the acinar cells of the pancreas[20]. High echogenicity of the pancreas is a normal finding in older patients[21]. Another previous EUS study of the age-related changes of the pancreas showed that men had significantly greater odds for having abnormalities than did women (OR = 2.9, P = 0.01)[22]. Because the distribution of abdominal fat differs according to gender, the areas of subcutaneous fat are significantly greater in women than in men at the abdominal level[23-25]. Metabolic syndrome is more prevalent in men than in women up to the age of 60 and this is closely related to hepatic steatosis; our results may reflect such a profile[26,27].

Hepatic steatosis is usually prevalent in obese subjects and regional fat distribution associated with insulin resistance was found to be an important factor for hepatic steatosis in several studies[28]. The BMI reflects either the total body fat accumulation or the subcutaneous fat accumulation. Recent findings have shown that central obesity (visceral fat accumulation) may be a more important factor for hepatic steatosis than BMI[29]. CT is considered the gold standard not only for measuring adipose tissue, but also

Table 5 Multivariable analysis: Unadjusted and adjusted analyses for the relationships of the abdominal adipose tissue area with hyperechogenic pancreas, OR (95% CI)

	Unadjusted analysis	*P* value	Adjusted analysis[1]	*P* value
VAT area (cm^2)		0.015		0.010
Quartile Ⅰ (< 35.2)	Reference		Reference	
Quartile Ⅱ (35.2-65.9)	0.646 (0.202-2.059)		0.671 (0.214-2.365)	
Quartile Ⅲ (65.9-94.0)	1.311 (0.637-6.094)		1.997 (0.561-7.107)	
Quartile Ⅳ (> 94.0)	3.491(1.154-10.557)		5.665 (1.515-21.180)	
SAT area (cm^2)		0.414		0.960
Quartile Ⅰ (< 109.3)	Reference		Reference	
Quartile Ⅱ (109.3-139.7)	1.139 (0.374-3.471)		1.016 (0.316-3.271)	
Quartile Ⅲ (139.7-179.2)	1.97 (0.637-6.094)		1.353 (0.378-4.841)	
Quartile Ⅳ (> 179.2)	2.288 (0.720-7.268)		1.227 (0.301-4.992)	

[1]Adjusted for age, gender, smoking, alcohol, body mass index. VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue; OR: Odds ratio; CI: Confidence interval.

for performing multi-compartment body measurements[10]. Even so, there have been few studies on the relationship between HP and regional fat distribution as measured by CT. We performed a subgroup analysis on patients who underwent abdominal CT. This objective measure of visceral fat showed that VAT was an independent risk factor for HP after adjusting for age, gender, alcohol, smoking and BMI. Obesity is known to be accompanied by metabolic complications and is increasingly recognized as a risk factor for type 2 DM, dyslipidemia, atherosclerotic and cardiovascular disease. There is growing evidence that the regional distribution of adipose tissue appears to be an important indicator of metabolic and cardiovascular alterations, since only inconstant correlations between BMI and these disturbances have been found[30]. On multivariate analysis in the present study, although BMI was not a statistically significant risk factor, VAT was an independent, significant risk factor for HP. A possible reason for this is that VAT measured by CT scanning is more accurate for measuring visceral obesity than BMI[31].

Recently, two studies regarding fatty pancreas or hyperechogenic pancreas were reported[18,32]. In one study, the predictors of HP were found to be hepatic steatosis, alcohol use and increased BMI[32]. In the other study, fatty pancreas was associated with metabolic syndrome. In the latter study, visceral fat was also independently associated with fatty pancreas[18]. However, that study simply compared VAT area values between fatty pancreas and normal controls. In the present study, we subdivided SAT and VAT area into four quartiles to evaluate the relationship between adipose tissue area and HP.

In the present study, alcohol intake and cigarette smoking were not significant risk factors for HP. Another study has suggested that alcohol consumption and cigarette smoking affect the endosonographic appearance of the pancreas in a dose-dependent fashion[33]. Although an effort was made to gather precise information on drinking and smoking in our sample, underestimation of alcohol intake and cigarette smoking may explain this finding in our study.

Although we performed this study prospectively, there were some limitations. First, the control group (non-HP group) was not representative of the general healthy population because the sample was made up of patients who required EUS for the evaluation of a subepithelial tumor. Second, direct determination of the pancreatic fat and visceral fat in tissue was not conducted. Due to ethical considerations with regard to obtaining tissue specimens from disease-free subjects, we did not perform tissue biopsies. Third, quantitative analyses of the pancreatic parenchymal echogenicity were not performed. As we only compared pancreatic echogenicity with echogenicity of the liver or kidney, these comparisons might have been somewhat subjective. A future study that employs computer-assisted quantitative analysis may be warranted.

In conclusion, HP is correlated with hepatic steatosis, hypertension, male gender and age older than 60 years. VAT is positively correlated with HP regardless of BMI. Although it is unknown whether HP is a progressive condition, HP, and likewise fatty liver, may be one of the phenotypes of metabolic syndrome, which is characterized by obesity with visceral fat accumulation, DM, hyperlipidemia and hypertension. Further studies are needed to confirm this hypothesis.

COMMENTS

Background

A hyperechogenic pancreas (HP) is commonly found during endoscopic ultrasound (EUS). However, the clinical significance of HP is still unclear. Visceral fat is more important for metabolic syndrome and hepatic steatosis than subcutaneous fat owing to its steatogenesis and production of various cytokines. Pancreatic echogenicity is determined by fat deposited around the pancreas and within the septa transversing the normal pancreas. Yet the role of obesity as a risk factor for HP is unclear. The authors could assume that HP is related to obesity in a similar way to that of fatty liver.

Research frontiers

HP may is related to obesity in a similar way to that of fatty liver. Many different methods have been used to calculate a value for obesity, such as body mass index (BMI), waist circumference, waist-to-hip ratio, skin fold thickness and percentage of body fat. Among these methods, computed tomography is considered the gold standard not only for evaluating adipose tissue, but also for multi-compartment body measurements.

Innovations and breakthroughs

HP is correlated with hepatic steatosis, hypertension, male gender and age older than 60 years. Visceral adipose tissue is positively correlated with HP regardless of BMI.

Applications

Although it is unknown whether HP is a progressive condition, HP, and likewise fatty liver, may be one of the phenotypes for metabolic syndrome, which is characterized by obesity with visceral fat accumulation, diabetes mellitus, hyperlipidemia and hypertension.

Terminology

Hyperechogenic pancreas was observed when the degree of echogenicity of the pancreas relative to the liver (or the kidney if the liver was hyperechogenic) was higher.

Peer review

Choi *et al* have performed a prospective study in order to identify factors related to hyperechogenic pancreas on EUS. This is an interesting study as published data on "fatty" pancreas are not extensive.

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BRIEF ARTICLE

Limited endoscopic sphincterotomy plus large balloon dilation for choledocholithiasis with periampullary diverticula

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Abstract

AIM: To investigate the effectiveness and safety of limited endoscopic sphincterotomy (EST) plus large balloon dilation (LBD) for removing choledocholithiasis in patients with periampullary diverticula (PAD).

METHODS: A total of 139 patients with common bile duct (CBD) stones were treated with LBD (10-20 mm balloon diameter) after limited EST. Of this total, 73 patients had PAD and 66 patients did not have PAD (controls). The results of stone removal and complications were retrospectively evaluated.

RESULTS: There were no significant differences between the PAD and the control groups in overall successful stone removal (94.5% *vs* 93.9%), stone removal in first session (69.9% *vs* 81.8%), mechanical lithotripsy (12.3% *vs* 13.6%), and complications (11.0% *vs* 7.6%). Clinical outcomes were also similar between the types of PAD, but the rate of stone removal in first session and the number of sessions were significantly lower and more frequent, respectively, in type B PAD (papilla located near the diverticulum) than controls [23/38 (60.5%) *vs* 54/66 (81.8%), P = 0.021; and 1 (1-2) *vs* 1 (1-3), P = 0.037, respectively] and the frequency of pancreatitis was significantly higher in type A PAD (papilla located inside or in the margin of the diverticulum) than in controls (16.1% *vs* 3.0%, P = 0.047).

CONCLUSION: Limited EST plus LBD was an effective and safe procedure for removing choledocholithiasis in patients with PAD. However, some types of PAD should be managed with caution.

Key words: Endoscopic sphincterotomy; Large balloon dilation; Choledocholithiasis; Periampullary diverticula

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Kim HW, Kang DH, Choi CW, Park JH, Lee JH, Kim MD, Kim ID, Yoon KT, Cho M, Jeon UB, Kim S, Kim CW, Lee JW. Limited endoscopic sphincterotomy plus large balloon dilation for choledocholithiasis with periampullary diverticula. *World J Gastroenterol* 2010; 16(34): 4335-4340 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i34/4335.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i34.4335

INTRODUCTION

Although endoscopic sphincterotomy (EST) is the standard treatment for removing common bile duct (CBD) stones, it is associated with complications, including pancreatitis, bleeding, and perforation. Complications are primarily related to the indications for the procedure and applied endoscopic techniques, rather than age or general medical condition of the patients[1].

Staritz *et al*[2] introduced endoscopic papillary balloon dilation (EPBD) as an alternative method of removing bile duct stones. However, since Disario *et al*[3] reported a high frequency of pancreatitis and two deaths in an EPBD group, EPBD has seldom been used in removing bile duct stones. Recently, EPBD is primarily being used in patients with bleeding tendencies.

Since Ersoz *et al*[4] introduced large balloon dilation (LBD) after EST as an alternative technique in patients with bile duct stones that were difficult to remove with conventional methods, such as basket or balloon catheter extraction after EST, several studies have reported the safety and effectiveness of LBD after EST for removing bile duct stones[5-7].

Periampullary diverticula (PAD) are extraluminal mucosal outpouchings of the duodenum that arise within a radius of 2-3 cm from the ampulla of Vater[8]. PAD are found in 9% to 32.8% of patients who have undergone an endoscopic retrograde cholangiopancreatography (ERCP). The prevalence of PAD has a tendency to increase with age[8-15] and PAD occurred in up to 65% of elderly patients in some studies[16]. PAD are associated with an increased number of complications, which can be explained by a difficult technical approach during an ERCP; but many studies have reported conflicting results regarding the true impact of PAD on clinical outcomes[9-15].

Limited EST plus LBD can be a useful method for removing CBD stones in patients with PAD that are difficult to remove with conventional methods. However, the effectiveness and safety of limited EST plus LBD in these patients has not been evaluated. Therefore, we evaluated the clinical efficacy of limited EST plus LBD for removing CBD stones in patients with PAD.

MATERIALS AND METHODS

Patients

From August 2007 to August 2008, we enrolled consecutively 139 patients who had CBD stones ⩾ 10 mm in diameter and who underwent limited EST followed by large-diameter (⩾ 10 mm) balloon dilation for removal of bile duct stones. All patients were admitted to the hospital. Exclusion criteria included coagulopathy (international normalized ratio > 1.5), low platelet count (< 50 000/mL), anticoagulation or antiplatelet therapy, acute pancreatitis, septic shock, prior EST, Billroth Ⅱ anatomy or Roux-en-Y gastrojejunostomy, and combined intrahepatic bile-duct stones. Informed consent from all patients was obtained and the study was approved by the ethics committee of the internal review board of Pusan National University.

Methods

ERCPs were performed by experienced endoscopists who performed over 300 biliary interventions per year. Patients were placed under conscious sedation with midazolam and meperidine. After the side-viewing endoscope (JF-240 or TJF-240; Olympus Optical Co., Ltd., Tokyo, Japan) was advanced into the descending duodenum, 10 mg of cimetropium bromide was administered intravenously to reduce duodenal peristalsis. Selective cannulation of the bile duct was achieved by using a conventional catheter and a pull-type sphincterotome with or without a guidewire. If those attempts failed to yield deep bile duct cannulation after more than 10 min, and/or the pancreatic duct had been cannulated more than 3 times, a needle-knife fistulotomy (NKF) was used to gain access. The subsequent procedures were performed by Kang's methods[5]. Follow-up endoscopy was performed on the first or second day after the procedure to determine whether bleeding was present, if immediate bleeding after EST or balloon dilation was noted, and bleeding control therapy was administered. The results of stone removal and complications were retrospectively evaluated.

Definitions

Stone size, number, and diameter of CBD were documented on ultrasound, computed tomography, and ERCP. Stone size was estimated based on the relative diameters of the stone and the shaft of the endoscope, as measured on the cholangiogram.

Limited EST was defined as sphincterotomy performed until the upper margin of the cut portion was located at one third of major EST.

Two different types of PAD were classified according to the location of the major papilla with respect to the diverticulum: type A: papilla located inside or in the margin of the diverticulum; type B: papilla located near the diverticulum.

Serum amylase (reference range, 36-128 IU/L) and lipase (22-51 IU/L) concentrations were measured before and after (4 and 24 h, respectively) the procedure. A complete blood cell count and a liver function test were checked the next morning after the procedure.

Post-ERCP pancreatitis was defined as sustained abdominal pain for 24 h after the procedure and a serum amylase level increased by three-fold or more[1,17,18]. Hemorrhage was considered to be clinically significant only when there was clinical evidence of bleeding, such as me-

Table 1 Baseline characteristics of patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group), median (range)

Characteristics	PAD group	PAD subtypes			Control group	P^2 value
		Type A	Type B	P^1 value		
No. of patients	73	35 (47.9)	38 (52.1)		66	
M:F	36:37	19:16	17:21	NS	39:27	NS
Age (yr)	70 (40-89)	74 (54-88)	66.5 (40-89)	NS	64 (23-89)	< 0.001
CBD diameter (mm)	15 (10-30)	16 (10-26)	15 (10-30)	NS	15 (11-38)	NS
Size of stones (mm)	14 (10-33)	14 (10-30)	14 (10-33)	NS	12 (10-35)	NS
No. of stones	2 (1-20)	2 (1-9)	2 (1-20)	NS	2 (1-7)	NS
Distal CBD stricture	4	3	1	NS	1	NS
Needle-knife fistulotomy	7	3	4	NS	11	NS

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. CBD: Common bile duct; NS: Not significant.

Table 2 Outcome of limited endoscopic sphincterotomy plus large balloon dilation in patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group)

Characteristics	PAD group	Control group	P^2 value
Overall stone removal, *n* (%)	69/73 (94.5)	62/66 (93.9)	NS
Type A	33/35 (94.3)		NS
Type B	36/38 (94.7)		NS
P^1 value	NS		
Stone removal in first session, *n* (%)	51/73 (69.9)	54/66 (81.8)	NS
Type A	28/35 (80)		NS
Type B	23/38 (60.5)		0.021
P^1 value	NS		
No. of sessions, median (range)	1 (1-3)	1 (1-2)	NS
Type A	1 (1-3)		NS
Type B	1 (1-3)		0.037
P^1 value	NS		
Diameter of balloon dilation (mm), median (range)	13.5 (10-20)	12.5 (10-20)	NS
Type A	13.5 (10-20)		NS
Type B	13.8 (10-20)		NS
P^1 value	NS		
Mechanical lithotripsy required, *n* (%)	9/73 (12.3)	9/66 (13.6)	NS
Type A	5/35 (14.3)		NS
Type B	4/38 (10.5)		NS
P^1 value	NS		

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. NS: Not significant.

lena or hematemesis, together with a decrease of at least 2 g/dL in the hemoglobin level, or the need for blood transfusion for stabilization of vital signs[1,17,18].

Statistical analysis

For the statistical analysis, the χ^2 test and Fisher's exact test was used for categorical variables and the Student *t* test or ANOVA test for continuous variables. Analyses were performed with SPSS 12.0 (SPSS Inc., Chicago, IL). A *P* value < 0.05 was considered statistically significant. Continuous variables are expressed as the median (range).

RESULTS

A total of 139 patients (median age, 68 years old; 76 men, 63 women) with CBD stones underwent limited EST plus LBD. Seventy-three patients (median age, 70 years old; 36 men, 37 women) had PAD (PAD group) and 66 patients (median age, 64 years old; 39 male, 27 female) did not have PAD (control group). There were no differences between the two groups regarding baseline characteristics, except age (70 years *vs* 64 years, $P < 0.001$) (Table 1).

In the PAD group, type A PAD comprised 35 patients (47.9%) and type B PAD comprised 38 patients (52.1%). There were no differences between the two types regarding baseline characteristics (Table 1).

The rate of overall stone removal and stone removal in first session did not differ significantly between the PAD and the control groups [overall, 69/73 (94.5%) *vs* 62/66 (93.9%); and first session, 51/73 (69.9%) *vs* 54/66 (81.8%), respectively] (Table 2). Failure of complete stone clearance occurred in 8 patients, 4 from each group. The major causes in 7 patients were capture failure with mechanical basket due to multiple, impacted, or large stones (3 patients in the PAD group, 4 patients in the control group) and one patient (PAD group) had large stones above

Table 3 Complications of limited endoscopic sphincterotomy plus large balloon dilation in patients with common bile duct stones who also had periampullary diverticula (periampullary diverticula group) or did not have periampullary diverticula (control group) *n* (%)

Complications	PAD group	Control group	P^2 value
Pancreatitis	7/73 (9.6)	2/66 (3.0)	NS
Type A	5/35 (14.3)		0.047
Type B	2/38 (5.3)		NS
P^1 value	NS		
Hemorrhage	0/73 (0)	1/66 (1.5)	NS
All complications	7/73 (9.6)	3/66 (4.5)	NS
Type A	5/35 (14.3)		NS
Type B	2/38 (5.3)		NS
P^1 value	NS		

[1]Comparing between subgroups of periampullary diverticula (PAD); [2]Comparing PAD group with control group. NS: Not significant.

the stricture. These patients had a biliary stent placed to ensure biliary drainage and were treated by percutaneous transhepatic cholangioscopy with electrohydraulic lithotripsy (7 patients) or open surgery (1 patient).

The groups had a similar frequency of mechanical lithotripsy [9/73 (12.3%) *vs* 9/66 (13.6%)]. Large-sized stones (> 15 mm) were the main indication for mechanical lithotripsy.

Between the types of PAD, there were no significant differences in the overall stone clearance, the stone removal in the first session, or the use of mechanical lithotripsy. However, when comparing each type of PAD with the controls, the rate of stone removal in first session and the number of sessions in type B PAD were significantly lower and more frequent, respectively, than controls [23/38 (60.5%) *vs* 54/66 (81.8%), $P = 0.021$ and 1 (1-2) *vs* 1 (1-3), $P = 0.037$, respectively] (Table 2).

The overall frequency of short-term complications was similar between the PAD and control groups [7/73 (9.6%) *vs* 3/66 (4.5%)] (Table 3). The frequency of pancreatitis did not differ significantly between the PAD and control groups [7/73 (9.6%) *vs* 2/66 (3.0%)]. Pancreatitis occurred in 5 and 2 patients with type A and B PAD, respectively. Pancreatitis related to NKF only occurred in one control patient. In comparing each type of PAD with controls, pancreatitis was significantly higher in type A PAD than controls [5/31 (14.3%) *vs* 2/66 (3.0%), $P = 0.047$]. All pancreatitis cases were clinically mild and they were treated conservatively. Clinically significant hemorrhage occurred in one patient in the control group. Immediate bleeding occurred after 10 mm balloon dilation and was controlled by endoscopic treatment. The next day, melena occurred and a blood transfusion was performed. Active bleeding was not found in a follow-up endoscopy, but blood clots appeared in the major papilla. Hemorrhage did not occur in any patient in the PAD group. Perforation and cholangitis did not occur in any patient.

DISCUSSION

The majority of CBD stones are removed by EST and conventional methods, but 10%-15% may be difficult to remove by conventional methods. The main reasons for failure are a difficult approach to the bile duct (PAD, Billroth Ⅱ anatomy, Roux-en-Y gastrojejunostomy), large (> 15 mm) stones, and impacted stones[19,20].

Conflicting results have been reported regarding the true impact of PAD on the technical success and complications of ERCP[9-15]. Many studies have reported recently that PAD does not make a difference to the success and complication rates of ERCP[14,15,21]. However, clinical outcomes associated with the technical success of selective cannulation of the bile duct or EST may be influenced by PAD. Boix *et al*[15] classified PAD into three types, according to the position of the major papilla. That study concluded that the presence or type of PAD did not significantly influence the difficulty of deep cannulation, but they did not evaluate the association between the types of PAD and the technical difficulties in removing CBD stones.

After the first study from Ersoz *et al*[4] demonstrating the technique of EST plus LBD, several studies established this procedure as an effective and safe treatment for removing CBD stones[5-7]. However, there have been few studies about the effectiveness and safety of EST plus LBD in patients with PAD. Three recent studies reported clinical outcomes of EST plus LBD in patients with PAD[22-24]. Two studies reported similar results for stone removal (84% *vs* 87.5%, 93.8% *vs* 89.2%) and complications (8.3% *vs* 18.8%, 3.1% *vs* 10.8%) between the PAD and control groups[22,23]. These studies suggested that minor EST with LBD in patients with PAD was a safe treatment modality for removing CBD stones. Another study compared minor EST plus EPBD with EST alone in patients with PAD and found similar outcomes in terms of overall stone clearance (100% *vs* 100%), stone clearance at first attempt (78% *vs* 72%), and the use of mechanical lithotripter (12% *vs* 21%)[24]. However, complications were rare in the EST plus EPBD group compared to EST alone (4% *vs* 21%, $P < 0.005$). The authors suggested that minor EST plus EPBD was safer than EST alone for removing bile duct stones in patients with PAD. However, these studies had limitations due to being published in abstract form and having a small number of patients.

In the current study, the rates of overall stone removal and the stone removal in first session did not differ significantly between the PAD and control groups (94.5% *vs* 93.9% and 69.9% *vs* 81.8%, respectively). The overall success rate of stone removal was similar to those of previous studies (84%-100%)[5,23,24]. The stone removal in first session rate in the PAD group was lower than that of the control group and of previous studies (81.8%-95%)[5,7,22], although this was not statistically different. This finding might be attributed to an older age group (median ages, 70 years old *vs* 62 years old, $P < 0.001$); elderly patients tend to have cardiopulmonary instability or poor general condition, thus they are less able to tolerate the procedure for long. The high prevalence of multiple stones in the PAD group might also have influenced the poor result, though this was not significantly different from controls.

Finally, the PAD condition might reduce the potential aggressiveness of the procedure by the endoscopist due to the consideration of possible complications.

The outcomes of stone removal were not different between the types of PAD. However, the rate of stone removal in the first session in type B PAD was lower compared to type A PAD, although the difference was not statistically significant. In comparing each type of PAD with controls, the rate of stone removal in first session and the number of sessions in type B PAD was significantly lower and more frequent, respectively, than controls, [23/38 (60.5%) *vs* 54/66 (81.8%), $P = 0.021$; and 1 (1-2) *vs* 1 (1-3), $P = 0.037$, respectively]. This finding might be attributed to a higher prevalence of multiple stones in type B PAD compared to controls and type A PAD, although this difference was not statistically significant.

The frequency of mechanical lithotripsy was similar between the PAD and control groups (12.3% *vs* 13.6%) and was similar to results with other studies (8.0%-12%)[5,6,24]. Again, no differences were found between the types of PAD in the frequency of mechanical lithotripsy.

These results suggest that the success rate for the clearance of bile duct stones and the use of mechanical lithotripsy were influenced by the number and size of the stones rather than the presence or type of PAD.

Generally, the length of EST is shorter in patients with PAD than in those without PAD due to the weakness of the sphincter of choledochus and risk of perforation in patients with PAD. For similar reasons, the diameter of the balloon may be influenced by the position of the major papilla in PAD; there was a tendency to use a smaller sized balloon in PAD compared to controls. However, in the current study, there was no difference in balloon diameters between the PAD and control groups or between the types of PAD. Although the precise reasons are not clear, PAD itself had no influence on the diameter of balloon.

In the current study, the overall rates of complication were not significantly different between the PAD and control groups (9.6% *vs* 4.5%). However, pancreatitis in the PAD group occurred more frequently than in other studies (4%-8.3%)[5-7,23,24]. Nevertheless, all pancreatitis cases were clinically mild and they were treated conservatively. The rate of pancreatitis was not statistically different between the types of PAD. However, the frequency of pancreatitis in type A PAD was significantly higher than in controls (14.3% *vs* 3.0%, $P = 0.047$). In the current study, the cause of more frequent pancreatitis in type A PAD is not clear, but it may be related to the presence of type A PAD. Firstly, the cannulation of the bile duct in type A PAD is generally more difficult than in type B PAD or in controls due to more frequent cases of poorly detectable papilla or more difficult prediction of the direction of bile duct in type A PAD. These features may lead to induction of pancreatitis because of the unnecessary injection of contrast medium or manipulation of the pancreatic duct, but the frequency of NKF due to difficult cannulation was not significantly different among the groups in our study. Secondly, because EST before LBD was performed to prevent post-ERCP pancreatitis by induction of separation between the pancreatic and biliary orifices, the more frequent pancreatitis in type A PAD may be related to injury of the pancreatic duct during balloon dilation due to less separation between the pancreatic and biliary orifices after EST compared to the control group and type B PAD group. Clinically significant hemorrhage did not occur in any patient in the PAD group.

In conclusion, limited EST plus LBD was equally successful and had similar complication rates in the PAD and control groups for the clearance of CBD stones. Therefore, this procedure is effective and safe for removing CBD stones in patients with PAD. Nevertheless, this procedure requires caution in some types of PAD for successful stone removal and prevention of complications. Larger and prospective studies are needed to evaluate clinical outcome in the presence of different types of PAD due to the retrospective nature and relatively small sample sizes in this study.

COMMENTS

Background

Endoscopic sphincterotomy (EST) plus large balloon dilation (LBD) is a useful method to remove common bile duct (CBD) stones, but the effectiveness and safety of this procedure is not well known in patients with periampullary diverticula (PAD) which are reportedly associated with difficulties and complications during associated procedures. We conducted this trial to evaluate the effectiveness and safety of limited EST plus LBD for removing CBD stones in patients with PAD.

Research frontiers

The majority of CBD stones are removed by EST and conventional methods, but 10%-15% may be difficult to remove by conventional methods. The main reasons for failure are a difficult approach to the bile duct (PAD, Billroth II anatomy, Roux-en-Y gastrojejunostomy), large (> 15 mm) stones, and impacted stones. In addition, PAD are associated with an increased number of complications, which can be explained by a difficult technical approach during an ERCP. However, conflicting results have been reported regarding the true impact of PAD on the technical success and complications of ERCP. Therefore, LBD after EST in some patients with PAD may be ineffective and complicated.

Innovations and breakthroughs

After the first study by Ersoz *et al* demonstrating the technique of EST plus LBD, several studies established this procedure as an effective and safe treatment for the removal of bile duct stones; but there have been few studies about the effectiveness and safety of EST plus LBD in patients with PAD. Three recent studies reported clinical outcomes of EST plus LBD in patients with PAD, but these studies had limitations due to being published in abstract form and having a small number of patients.

Applications

Limited EST plus LBD was equally successful and had similar complication rates in the PAD and control groups for the clearance of CBD stones. Therefore, this procedure is effective and safe for removing CBD stones in patients with PAD.

Terminology

Limited EST was defined as sphincterotomy performed until the upper margin of the cut portion was located at one third of major EST. Two different types of PAD were classified according to the location of the major papilla with respect to the diverticulum: type A: papilla located inside or in the margin of the diverticulum; type B: papilla located near the diverticulum. LBD was defined as the diameter of the balloon used for dilation being 10 to 20 mm.

Peer review

Kim *et al* have performed a retrospective study in order to evaluate the effectiveness and safety of limited EST plus LBD for removing CBD stones in patients with PAD. This paper is interesting and it could be valuable for other researchers.

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BRIEF ARTICLE

Pathophysiological significance of gallbladder volume changes in gallstone diseases

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Abstract

AIM: To study the pathophysiological significance of gallbladder volume (GBV) and ejection fraction changes in gallstone patients.

METHODS: The fasting GBV of gallstone patients with acute cholecystitis (n = 99), chronic cholecystitis (n = 85) and non-gallstone disease (n = 240) were measured by preoperative computed tomography. Direct saline injection measurements of GBV after cholecystectomy were also performed. The fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects who received health examinations were measured by abdominal ultrasonography. Proper adjustments were made after the correction factors were calculated by comparing the preoperative and postoperative measurements. Pathological correlations between gallbladder changes in patients with acute calculous cholecystitis and the stages defined by the Tokyo International Consensus Meeting in 2007 were made. Unpaired Student's t tests were used. $P < 0.05$ was deemed statistically significant.

RESULTS: The fasting GBV was larger in late stage than in early/second stage acute cholecystitis gallbladders (84.66 ± 26.32 cm^3, n = 12, *vs* 53.19 ± 33.80 cm^3, n = 87, P = 0.002). The fasting volume/ejection fraction of gallbladders in chronic cholecystitis were larger/lower than those of normal subjects (28.77 ± 15.00 cm^3 *vs* 6.77 ± 15.75 cm^3, $P < 0.0001$)/(34.6% ± 10.6%, n = 65, *vs* 53.3% ± 24.9%, n = 53, $P < 0.0001$).

CONCLUSION: GBV increases as acute cholecystitis progresses to gangrene and/or empyema. Gallstone formation is associated with poorer contractility and larger volume in gallbladders that contain stones.

Key words: Gallbladder volume; Pathophysiology; Gallbladder ejection fraction; Gallstone formation; Acute cholecystitis

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INTRODUCTION

Gallbladder volume (GBV) can reflect clinical and therapeutic implications, physiological and functional status, and possibly pathophysiological mechanisms of gallstone diseases. In 1880, Courvoisier and Terrier's law stated that large painless, palpable gallbladder was mostly due to malignant obstruction of the bile duct[1]. In 1963, Maingot found that tensely swollen, tenderly palpable gallbladder in acute cholecystitis was due to stone impaction in the cystic duct[2]. In 1989, Masclee noted, in one third of patients with cholesterol gallstones, increased GBV and delayed gallbladder emptying were present at the postprandial phase[3-5]. In 2000, Portincasa demonstrated in a comparative study that patients with cholesterol gallstones showed significantly larger fasting volume and postprandial volume of gallbladders than did controls and patients with pigment stones at each time point during a 2-h study[6]. He proposed that larger GBV predisposes to bile stasis.

However, the differences and changes in GBV among different age groups in non-gallstone subjects, and their correlations with the formation of gallstones have not been reported previously. Also, the correlations between GBV changes at different stages in the pathophysiological development of acute cholecystitis in gallstone patients are not clear. Furthermore, we wished to investigate the possible reciprocal correlation between GBV and gallbladder contractibility.

The aims of this study were to study the pathophysiological significance of gallbladder volume and ejection fraction changes in gallstone patients by comparing the GBV among gallstone patients with acute cholecystitis, chronic cholecystitis, contracted gallbladder and non-gallstone subjects, and by comparison between gallstone patients with chronic cholecystitis and non-gallstone subjects with different ages and sex. Furthermore, to investigate the correlations between gallbladder physiological changes and the development of gallstones, we further investigated the differences in gallbladder contractility between gallstone patients with chronic cholecystitis and normal subjects.

MATERIALS AND METHODS

Between October 1, 2007 and September 30, 2008, 99 gallstone patients with acute cholecystitis, 85 gallstone patients with chronic cholecystitis, and five patients with gallbladder contraction were admitted to Chung Shan Medical University Hospital to undergo cholecystectomy.

Diagnostic criteria

Acute cholecystitis: The clinical imaging findings of acute cholecystitis in abdominal ultrasonography include thick gallbladder wall (> 3 mm), stones in the gallbladder, distended gallbladder with pericholecystic fluid, and sonographic Murphy's sign with tenderness over the gallbladder from the ultrasound transducer. The pathological features of acute cholecystitis include leukocyte infiltration throughout the tissues, local flattening and denuded mucosal folds, and tissue edema. If untreated or treated late, localized regions of necrosis (gangrene) or abscess formation (empyema) can be found.

Chronic cholecystitis: The pathological features of chronic cholecystitis include subserosal fibrous tissues, lymphocytes, plasma cells, macrophages found beneath the columnar epithelium, and Rokitansky-Aschoff sinus in the muscular layer.

Gallbladder contraction: Gallbladder contraction is known to result from long-standing chronic cholecystitis. Anatomical characteristics include severe adhesions and complete coverage by greater omentum, severe pericholecystic fibrosis, thickening of gallbladder walls (usually thicker than 5 mm), loss of elasticity, obscure anatomy at triangle of Calot, dense adhesions to the duodenum, transverse colon and common hepatic duct, and small GBV (usually < 10 mL).

Non-gallstone controls: We randomly chose 240 patients who received abdominal computed tomography (CT) for non-gallstone reasons between July 1, 2008 and July 31, 2008.

Demographic data: The age, sex, associated diseases and previous operations for all patients were recorded. The procedures included preoperative CT measurement of gallbladder diameter, and directly measured gallbladder diameter. Directly measured GBV by the saline-filling method in patients with gallstones and acute and chronic cholecystitis were also recorded. The CT measurements of non-gallstone controls were also recorded. The GBV was calculated from the gallbladder lengths and short axis diameters by the equation shown below.

GBV measurements and calculations: We measured the lengths and short axis diameters of the fasting gallbladder of patients from their preoperative CT films, measured the lengths and short axis diameters directly after their removal in the operating room, and recorded their lengths and short axis diameters measured by the pathologist postoperatively. We then calculated the GBV according to the equation: GBV (cm^3) = $4/3 \times \pi \times r1 \times r2 \times r3/\cos\theta$, where r1, r2 and r3 represent the three greatest radii of the gallbladder ellipsoid and θ is the angle between the z axis of the gallbladder and the z axis of the transverse cross sectional CT imaging.

We also measured directly the GBV after the gallbladders were removed. After withdrawing the gallbladder bile with a syringe, the volume of normal saline injected

into the intact gallbladders was added to the volume of gallbladder stones measured after opening the GB walls. Comparing the GBV obtained by direct measurement and calculations using the various techniques, correction factors for different measuring methods were obtained to modify the calculated GBV, thus minimizing the measurement bias.

Rationale for GBV equation[7]

The gallbladder is a pear-shaped organ located under the liver, which stores bile. It can be approximated to an ellipsoid shape. The volume of an ellipsoid can be obtained as follows[8]: Claim area for ellipse is π r1 r2: given an ellipse $x^2/r1^2 + x^2/r2^2 = 1$. Since an ellipse is symmetric to the x and y axes, Area = $4\int^{r1}_{0} r2\ (1 - x^2/r1^2)^{1/2}\ dx = 4r2/r1\int^{r1}_{0} (r1^2 - x^2)^{1/2}\ dx$. (Let $x = r1\sin\theta$, then $dx = r1\cos\theta d\theta$, where $-\pi/2 < \theta < \pi/2$) $= 4r2/r1\int^{\pi/2}_{0} (r1^2 - r1^2\sin^2\theta)^{1/2}\ r1\cos\theta\ d\theta = 4r1r2\int^{\pi/2}_{0} \cos^2\theta d\theta = 4r1r2\int^{\pi/2}_{0} 1/2\ (1 + \cos2\theta)\ d\theta = 2\ r1r2\ [\theta + 1/2\sin^2\theta]^{\pi/2}_{0} = \pi\ r1r2 = >$ For a slice of ellipse of the ellipsoid parallel to the yz plane, the radius parallel to the z axis is $g(z) = r1\ (1 - z^2/r3^2)^{1/2}$. Similarly, its radius parallel to the y axis is $h(z) = r2\ (1 - z^2/r3^2)^{1/2}$. Thus, the volume V of the ellipsoid $= \int^{r3}_{-r3}\pi\ g(z)\ h(z)dz = \int^{r3}_{-r3}\pi\ r1\ (1 - z^2/r3^2)^{1/2}\ r2\ (1 - z^2/r3^2)^{1/2}\ dz = \pi\ r1\ r2\int^{r3}_{-r3} (1 - z^2/r3^2)\ dz = \pi\ r1\ r2\ [z - z^3/3r3^2]^{r3}_{-r3} = 4/3\pi\ r1r2r3$ #.

Correction factors for GBV

θ: The gallbladder does not always lie in the same plane, therefore, the measured GBV is corrected by multiplying a correction factor $1/\cos\theta$, where θ is the angle between the z axis of the gallbladder and the z axis of the transverse cross sectional CT imaging and $0° \leqslant \theta < 90°$.

c = 0.82 ± 0.40 (*n* = 65): CT measurement correction factor = (Direct measurement calculated GBV)/(CT measurement calculated GBV).

p = 0.85 ± 0.42 (*n* = 65): Pathology measurement correction factor = (Pathology measurement calculated GBV)/(Direct measurement calculated GBV).

s = 0.88 ± 0.36 (*n* = 65): Shueh-Ding correction factor = (Direct injection measurement GBV)/(Direct measurement calculated GBV).

Direct measurement GBV = Direct measurement calculated GBV × 0.88 (s, Shueh-Ding correction factor).

u = 0.90 (*n* = 65): Ultrasound (US) measurement correction factor = (Direct measurement calculated GBV)/(US measurement calculated GBV).

Direct injection measurement GBV = Direct measurement calculated GBV × s = CT measurement calculated GBV × cs (cs = 0.72 = 0.82 × 0.88) = US measurement calculated GBV × us (us = 0.79 = 0.9 × 0.88).

Substudy on gallbladder contractility: To investigate and compare the gallbladder contractility between patients with gallstones and chronic cholecystitis and normal subjects, and the possible reciprocal correlation between GBV and gallbladder contractility, we designed and conducted a subsequent second study. Between October 1, 2008 and June 30, 2009, we measured the fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects, by abdominal ultrasonography.

Study protocol: Subjects came to the hospital after an overnight fast.

They were reminded the night before the study not to eat or drink after supper. Their adherence to this instruction was confirmed by performing a history taking to make sure that the subjects did adhere to the investigator's instructions, including nothing by mouth after the previous supper. Gallbladder size was determined in the fasting state. The subjects then drank a standard liquid meal with a total 420 kcal: 30% as fat, 15% as protein, and the rest as carbohydrate. The postprandial gallbladder size was measured and GBV was calculated 90 min after the meal[9].

Ultrasound technique: The abdominal sonography was performed by the same investigator (Huang SM) with techniques modified from the literature[9]. The hand-held transducer was placed in a sagittal longitudinal projection in the right-upper quadrant and rotated until the largest longitudinal dimension of the gallbladder was obtained on the oscilloscope screen. The image was frozen, measured by electronic calipers, and photographed. The transducer was then rotated 90° and the largest short axis through the gallbladder was obtained. Occasionally, deep inspiration was required, but all patients were successfully examined. We used three dimensions - length, lateral and antero-posterior diameters - to calculate the GBV, which was modified empirically as described above.

The ejection fraction (EF) of the gallbladder was calculated by the following equation: EF (%) = [Fasting GBV (mL) - Postprandial GBV (mL)]/[Fasting GBV (mL)] × 100.

Statistics: We compared the GBV measurements and patient age using Student's unpaired *t* test, and the χ^2 test to compare the sex differences. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data

We found a male predominance in the acute cholecystitis group and a female predominance in the chronic cholecystitis group: male/female (M/F) = 53/46 and 32/53, respectively ($P = 0.04$ by χ^2 test). There was no sex difference between patients in the chronic cholecystitis group and the non-gallstone group (M/F = 32/53 *vs* 117/123, $P = 0.10$). There was no age difference among patients in the acute cholecystitis group, the chronic cholecystitis group and the non-gallstone group (57 ± 17 years, 53 ± 15 years and 54 ± 18 years, $P = 0.09$ and 0.64, respectively). Associated diseases and previous operations undergone for all patients and normal subjects included cerebral vascular accident (3), diabetes mellitus

Table 1 Demographic data and gallbladder volume in gallstone and non-gallstone subjects

Variables diagnosis	M/F	Mean age (yr)	Mean GBV (cm^3) (*n*)	SD (cm^3)	*P*-value (95% CI[1])
Acute Cholecystitis	[D]53/46[2]	57 ± 17	[A]57.00 (99)	41.20	0.0004[3] (7.73-26.35)
Chronic Cholecystitis	32/53	53 ± 15	[B]28.77 (85)	15.00	< 0.0001[4] (8.14-15.86)
Contracted Gallbladder	1/4	44 ± 21	6.15 (5)	2.52	
Non-gallstone	[E]39/41	54 ± 18	[C]16.77 (240)	15.75 -	

[1]95% CI of gallbladder volume (GBV) difference; [2]*P* value D *vs* E = 0.04, between gender of acute and chronic cholecystitis; [3]*P* value A *vs* B: between GBV of acute and chronic cholecystitis; [4]*P* value B *vs* C: between chronic cholecystitis and non-gallstone subjects. SD: Standard deviation.

Table 2 Incidence of gallstones and gallbladder volume of non-gallstone subjects at different ages

Patients age periods (*n* = 85)	Incidence of gallstone[1] (%)	Non-gallstone Mean GBV (cm^3) (*n*)	Non-gallstone SD (cm^3)	*P* value
Age ≤ 20	0	[A]6.33 (9)	2.75	0.007[2]
20 < Age ≤ 40	15	[B]16.84 (35)	11.04	0.30
40 < Age ≤ 60	42.5	[C]15.12 (106)	7.55	0.0003[3]
60 < Age ≤ 80	42.5	[D]19.83 (81)	9.99	0.76
80 < Age	0	18.73 (9)	10.32	

[1]*P* = 0.0027 by Fisher's exact test; [2]*P* value A *vs* B: between Age ≤ 20 and 20 < Age ≤ 40; [3]*P* value C *vs* D: between 40 < Age ≤ 60 and 60 < Age ≤ 80. GBV: Gallbladder volume; SD: Standard deviation.

(4), pneumonia (1), hyperlipidemia (1), hypertensive cardiovascular diseases (5), status/post exploratory laparotomy (2) and coronary artery disease (5) in the gallstones patients with acute cholecystitis; diabetes mellitus (5) and manic-depressive psychosis (1) and status/post gastrectomy/vagotomy (2) in the gallstones patients with chronic cholecystitis; and liver tumor (2), liver cysts (5), diabetes mellitus (9), hypertensive cardiovascular diseases (10) and status/post exploratory laparotomy (5) in the non-gallstone group. The GBV in the diabetic and non-diabetic non-gallstone patients did not differ significantly (16.18 ± 2.79 cm^3, $n = 9$ *vs* 16.79 ± 9.43 cm^3, $n = 231$, $P = 0.85$). The GBV in the s/p gastrectomy/vagotomy and non-s/p gastrectomy/vagotomy, non-gallstone patients did not differ significantly either (19.85 ± 3.41 cm^3, $n = 2$ *vs* 16.74 ± 9.30 cm^3, $n = 238$, $P = 0.64$). Thirty patients in the acute cholecystitis group underwent laparoscopic cholecystectomy. They comprised 30.3% (30/99) of the acute cholecystitis patients. Seventy-seven patients in the chronic cholecystitis group underwent laparoscopic cholecystectomy. They comprised 90.6% (77/85) of the chronic cholecystitis patients. The remaining 77 patients underwent open cholecystectomy.

The fasting GBV in gallstone patients with acute cholecystitis ($n = 99$), chronic cholecystitis ($n = 85$), gallbladder contraction ($n = 5$) and non-gallstone subjects ($n = 240$) was 57.00 ± 41.20 cm^3, 28.77 ± 15.00 cm^3, 6.15 ± 2.52 cm^3 and 16.77 ± 15.75 cm^3, respectively (Table 1). The average fasting GBV was larger in gallstone patients with acute cholecystitis than that with chronic cholecystitis ($P < 0.0001$). In turn, the average fasting GBV was larger in gallstone patients with chronic cholecystitis than in non-gallstone subjects ($P < 0.0001$, Table 1).

In non-gallstone subjects, there was a significant difference in the fasting GBV between two age groups, i.e. age ≤ 20 years *vs* 20-40 years (6.33 ± 2.75 cm^3, $n = 9$ *vs* 16.84 ± 4.04 cm^3, $n = 35$, $P = 0.0070$) and age 40-60 years *vs* 60-80 years (15.12 ± 7.55 cm^3, $n = 106$ *vs* 19.83 ± 9.99 cm^3, $n = 81$, $P < 0.0003$) (Table 2). The fasting GBV had a tendency to increase with age. In accordance with this tendency, the occurrence of gallstones with chronic cholecystitis also increased with age ($P = 0.0027$ by Fisher's exact test, Table 2).

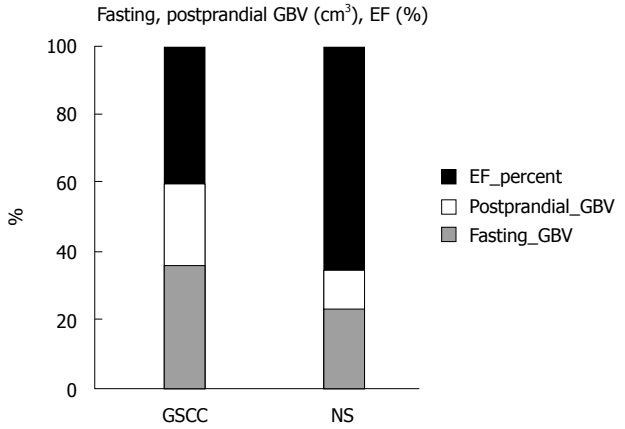

Figure 1 Histogram of the mean fasting, postprandial gallbladder volume and ejection fraction in gallstone patients with chronic cholecystitis and normal subjects, measured by abdominal ultrasonography. GBV: Gallbladder volume; EF: Ejection fraction; GSCC: Gallstones with chronic cholecystitis; NS: Non-stone.

In the second substudy, the average fasting/postprandial GBV in patients with gallstones and chronic cholecystitis was 31.44 ± 16.46 cm^3/20.50 ± 6.20 cm^3. The average gallbladder EF of patients with gallstones and chronic cholecystitis was 34.6% ± 10.6% ($n = 65$). The average fasting/postprandial GBV in normal subjects was 19.22 ± 11.30 cm^3/9.03 ± 3.63 cm^3. The average EF of normal gallbladder was 53.3% ± 24.9% ($n = 53$). The differences in the average fasting, postprandial GBV and EF were statistically significant (all three $P < 0.0001$, Figure 1, Table 3). Also, the differences in GBV between fasting and postprandial states in patients with gallstones and chronic cholecystitis and normal subjects were statistically significant (both $P < 0.0001$).

The fasting GBV was larger in late stage than early/second stage acute cholecystitis in gallstone patients (84.66 ± 26.32 cm^3, $n = 12$, *vs* 53.19 ± 33.80 cm^3, $n = 87$, $P = 0.002$, Table 4). The average fasting GBV was larger (28.77 ± 15.00 cm^3 *vs* 6.77 ± 15.75 cm^3, $P < 0.0001$) and the average EF was lower (34.6% ± 10.6%, $n = 65$, *vs*

Table 3 Mean fasting, postprandial gallbladder volume and ejection fraction in gallstones patients and normal subjects

	Fasting mean GBV ± SD (cm^3) (*n*)	Postprandial mean GBV ± SD (cm^3) (*n*)	EF ± SD (%) (*n*)
GS	[A]31.34 ± 16.46 (65)	[B]20.50 ± 6.20[1] (65)	[C]34.6 ± 10.6 (65)
Normal subjects	[D]19.24 ± 11.30[2] (53)	[E]9.03 ± 3.63[2] (53)	[F]53.3 ± 24.9 (53)
P-value (95% CI)	< 0.0001[3] (A-D, 6.93-17.47)	< 0.0001[4] (B-E, 9.56-13.48)	< 0.0001[5] (C-F, 11.9-25.5)

[1]*P* value A *vs* B < 0.0001, A-B 95% CI: 6.62–15.26; [2]*P* value D *vs* E < 0.0001; D-E 95% CI: 6.98-13.44; [3]*P* values A *vs* D: between fasting gallbladder volume (GBV) of chronic cholecystitis and normal subjects; [4]*P* values B *vs* E: between postprandial GBV of chronic cholecystitis and normal subjects; [5]*P* values C *vs* F: between ejection fraction (EF) of chronic cholecystitis and normal subjects. SD: Standard deviation; GS: Gallstones.

53.3% ± 24.9%, $n = 53$, $P < 0.0001$, Table 3) in gallstone patients with chronic cholecystitis, compared with those in non-gallstone and normal subjects.

DISCUSSION

From clinical observations, male patients with gallstone disease seem to have a higher threshold for pain caused by biliary stones than their female counterparts. Perhaps this is one of the reasons that underlay the male preponderance seen in the acute calculous cholecystitis group in this study.

Our study showed that the fasting GBV increased in two age groups in non-gallstone subjects, i.e. from age 20 to 40 years and 60 to 80 years; especially the former period. In accordance with this differential increase in GBV in specific periods, the occurrence of gallstones reached its plateau between 40 and 80 years of age (from 15% before 40 years old to 42.5% after 40 years, $P = 0.0027$, Table 2).

The gallbladder is a hollow visceral elastic organ. Its actual maximal fasting volume under *in vivo* physiological conditions is difficult to measure. Measuring methods include *in vivo* ultrasonography, nuclear scintigraphy, CT and *in vitro* post-cholecystectomy saline-filling measurement. hepatobiliary iminodiacetic acid scintigraphy is seldom used clinically except to demonstrate cystic duct obstruction in acute cholecystitis patients, due to its isotope radioactivity. On the other hand, *in vitro* post-cholecystectomy saline-filling measurement is the most accurate gold standard for measuring GBV. In contrast, abdominal ultrasonography is the most convenient examination for measuring GBV. However, the accuracy and precision depend greatly on the operators' individual experiences. CT represent the more uniform, constantly used and easier method for diagnosis of gallstone diseases and for measuring GBV. These are the reasons why we adopted abdominal ultrasonography, CT and *in vitro* post-cholecystectomy saline-filling measurement in the present study.

Table 4 Mean gallbladder volume in acute calculous cholecystitis group: simple *vs* with gangrene/abscess

Variable GS patients	Mean GBV ± SD (cm^3) (*n*)	[1]*P*-value A *vs* B (A-B, 95% CI)
Simple ACC	[A]53.19 ± 33.80 (87)	0.002 (12.28-52.66)
With gangrene/abscess	[B]84.66 ± 26.32 (12)	

[1]*P* values, A *vs* B: mean gallbladder volume (GBV) of patients with simple acute calculous cholecystitis (ACC) *vs* ACC with gangrene/abscess. SD: Standard deviation; GS: Gallstones.

GBV can have clinical and therapeutic implications and reflect pathophysiological mechanisms of gallstone diseases. The clinical and therapeutic implications of increased GBV lie in the cystic duct obstruction in acute calculous cholecystitis. The dilatation of the gallbladder is due to stones being trapped in the spiral valve of Heist. The onset of painful dilatation of the gallbladder is acute and is caused by the stones irritating the gallbladder mucosa, and the pressure exerted by the dilated gallbladder on the visceral nerve endings distributed over the entire gallbladder wall. Early dilatation of the gallbladder due to undrained mucus secretions also predisposes to compromised local blood circulation to the mucosa and the gallbladder wall, which leads to impaired absorption of water and electrolytes, which further aggravates the dilatation[10-12]. GBV can usually reach up to 3.4 times the normal size. The vicious cycle of undrained mucus secretions and impaired absorption of water and electrolytes, coupled with chemical irritation by lysozyme, cytokines and chemokines, sometimes causes gangrene and/or perforation in 15% and 1.5%, respectively, of patients with acute calculous cholecystitis[2].

In our study, the fasting GBV was larger in gallstone patients with acute cholecystitis than with chronic cholecystitis (Table 1). Furthermore, local gallbladder wall necrosis/abscess was associated with further increases in GBV (Table 4). It is possible that gallbladder decompression might prevent gangrene/empyema and perforation and make it easier to manipulate the gallbladder during cholecystectomy.

Gallbladder contraction is known to result from long-standing chronic cholecystitis and has the smallest GBV. The anatomical characteristics make it a difficult challenge to perform laparoscopic cholecystectomy safely.

The fasting GBV was larger in gallstone patients with chronic cholecystitis than that in non-gallstone subjects (28.77 ± 15.00 cm^3, $n = 85$, *vs* 16.77 ± 15.75, $n = 240$, $P < 0.0001$), which indicated possible pathophysiological mechanisms of weaker gallbladder contractility in gallstone patients with chronic cholecystitis, which was confirmed in our second study. In 2000, Portincasa proposed that larger GBV predisposes to bile stasis[6].

Weaker gallbladder contractility in gallstone patients with chronic cholecystitis might provide a suitable micro-milieu for cholesterol monohydrate micro-crystals

to grow into larger gallbladder stones, by preventing them from expulsion from the gallbladder lumen, when the gallbladder contracts in response to cholecystokinin (CCK) secreted from duodenum I cells stimulated by proteins and lipids in ingested food[8].

In our second study, we measured fasting and postprandial GBV of 65 patients with gallstones and chronic cholecystitis and 53 healthy subjects, by abdominal ultrasonography and calculated the gallbladder EF. The average EF of patients with gallstones and chronic cholecystitis was lower than that of the healthy subjects. The difference in EF was statistically significant (Table 3).

Developmental stages of acute cholecystitis

From our observations on the GBV changes during the development of acute cholecystitis from a chronic inflammatory state, we hypothesize that there are three stages based on GBV and pathological changes. These correspond to the three stages of severity of acute cholecystitis proposed by Miura *et al*[13] at the Tokyo International Consensus Meeting in 2007.

Early (clinical) stage

In the early (clinical) stage of acute cholecystitis, which corresponds to mild (grade Ⅰ) severity in the Tokyo consensus meeting, the mechanical effect of cystic duct obstruction causes an increase in GBV (from 28.77 ± 15.00 cm^3, n = 85, to 54.16 ± 35.17 cm^3, n = 44, P < 0.0001). No acute inflammatory leukocyte infiltration is observed, but chronic inflammatory reactions, such as subserosal fibrous tissues, and infiltration of lymphocytes, plasma cells, and macrophages beneath the columnar epithelium and Rokitansky-Aschoff sinus in the muscular layer are present, as revealed by pathological examinations. The early (clinical) stage of acute cholecystitis comprises about 44.4% (44/99). Early laparoscopic cholecystectomy is suggested by the Tokyo guidelines.

Second (pathological) stage

In the second (pathological) stage of development, which corresponds to the moderate severity (grade Ⅱ) stage of the Tokyo guidelines, the GBV does not change significantly. However, pathological findings of acute immunoreactive and inflammatory responses appear in the gallbladder. These include leukocyte infiltration throughout the tissues, local flattening and denuded mucosal folds, and tissue edema. It comprises about 43.4% (43/99). Early laparoscopic or open cholecystectomy is suggested by the Tokyo guidelines. If a patient has serious local inflammation that makes early cholecystectomy difficult, then percutaneous or operative drainage of the gallbladder is recommended. Elective cholecystectomy can be performed after improvement of the acute inflammatory process.

Late (complicated) stage

If the condition goes untreated or is unresolved by treatment, the late (complicated) stage ensues, which corresponds to the severe (grade Ⅲ) organ dysfunction stage of the Tokyo guidelines. In this stage, GBV further increases (Table 4). Besides the pathological findings of acute immunoreactive and inflammatory responses, local gallbladder wall necrosis (gangrene), abscess and/or even perforations occur. Organ dysfunctions appear. It comprises about 12.2% (12/99). Appropriate organ support in addition to medical treatment for patients with organ dysfunction is suggested by the Tokyo guidelines. Management of severe local inflammation by percutaneous gallbladder drainage and/or cholecystectomy is needed. Biliary peritonitis due to perforation of the gallbladder is an indication for urgent cholecystectomy and drainage. Elective cholecystectomy may be performed after improvement of the acute illness by gallbladder drainage[13].

Zhu *et al*[14] have found that the amount of gallbladder CCK receptor is lower in patients with gallstones who have poor gallbladder contraction. Choi has found that FGF15 knockout mice have a gallbladder that is completely devoid of bile, and administration of recombinant FGF15 or FGF19 restores the GBV[8,15]. Whether gallstone patients with poor gallbladder contraction and increased GBV have lower levels of CCK receptor and/or lower FGF19 gene expression is worth further investigation.

Different kinds of diet predispose humans to different gallstone diseases. At present, we have no data concerning how the diet consumed by the gallstone patients was different from that consumed by the normal population. If the diet differs, it is worthwhile investigating whether the diet predisposes the patients to gallstone diseases through its effect on GBV.

There is a limitation to this study. The gallstone patients were not subgrouped into those with cholesterol and pigment stones. Therefore, the findings of Masclee[3-5] and Portincasa[6] could not be investigated in this study.

In summary, we found that the fasting GBV increased in two periods in non-gallstone subjects, i.e. from age 20 to 40 years and 60 to 80 years. In accordance with these age preferences, the occurrence of gallstones reached its peak between 40 and 80 years of age. Moreover, we found that gallstone formation was associated with poorer gallbladder contractility and larger fasting and postprandial GBV Also, GBV increased as acute cholecystitis progressed. Therefore, gallbladder decompression is mandatory to prevent gangrene and/or empyema of gallbladders.

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COMMENTS

Background

Gallbladder volume (GBV) changes can have clinical and therapeutic implications, and reflect physiological and functional status, and possibly pathophysiological mechanisms of gallstone diseases. However, the differences and changes in GBV among different age groups in non-gallstone subjects and their correlations with the formation of gallstones have not been reported previously. Also, the correlations between GBV changes in different stages in the pathophysiological development of acute cholecystitis in gallstone patients are not

clear. Furthermore, the authors wished to investigate the possible reciprocal correlation between GBV and gallbladder contractility. The methodology used to measure *in vivo* GBV is hardly reported in the literature.

Research frontiers

The methods the authors developed in this study can provide a reliable method to measure the *in vivo* GBV in a way that can be adjusted to approximate the direct measurements by the saline injection method. In this study, the authors found that GBV increased as acute cholecystitis progressed to gangrene and/or empyema. The fasting volume of gallbladders was larger in late stage than in early/second stage acute cholecystitis. This forms the pathophysiological basis for the guidelines suggested by the Tokyo International Consensus Meeting in 2007.

Innovations and breakthroughs

This is believed to be the first study to report the differential increase in GBV in specific age groups, which coincides with and partly explains the high occurrence rate of gallstones, which reached their plateau between 40 and 80 years of age. This is also believed to be the first study to demonstrate one of the pathophysiological mechanisms that underlie the development of acute calculous cholecystitis, which form the basis for the severity staging system in the Tokyo International Consensus Meeting.

Applications

An interesting area of future investigation would be to apply the authors' measurements and gallbladder emptying assessment to patients without gallstone disease but with right upper quadrant pain. By contributing to the authors' understanding of how acute calculous cholecystitis develops, this study could represent a rationale for therapeutic decompression intervention, by drainage or straight cholecystectomy, in the treatment of patients with acute calculous cholecystitis.

Peer review

The reviewer found that this was an interesting study that was performed well. Also, the reviewer wonders what the diet was like in the normal and diseased populations, because that would have an effect on GBV. This is a good contribution to a topic that has not been described previously.

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BRIEF ARTICLE

Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: A meta-analysis

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Abstract

AIM: To find evidences about whether NOD1/CARD4 insertion/deletion polymorphism is associated with inflammatory bowel disease by meta-analysis.

METHODS: We surveyed the studies on the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease in PubMed. Meta-analysis was performed for genotypes GG/T *vs* T/T, GG/GG *vs* T/T, GG/T + GG/GG *vs* T/T, GG/GG *vs* T/T + GG/T, and GG allele *vs* T allele in a fixed/random effect model.

RESULTS: We identified 8 studies (6439 cases and 4798 controls) in Caucasian populations using PubMed search. We found no association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease, Crohn's disease, and ulcerative colitis. Stratification of cases by age showed that NOD1/CARD4 insertion/deletion polymorphism was associated with inflammatory bowel disease in younger age group at onset (< 40 years) (GG *vs* T: OR = 0.68, 95% CI: 0.50-0.93, P = 0.02; GG/T + GG/GG *vs* T/T: OR = 0.71, 95% CI: 0.59-0.85, P = 0.0003).

CONCLUSION: This meta-analysis demonstrates an association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease in the younger age group at onset (< 40 years) in Caucasian populations.

Key words: NOD1; CARD4; Genetic polymorphisms; Inflammatory bowel disease; Meta-analysis

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Lu WG, Zou YF, Feng XL, Yuan FL, Gu YL, Li X, Li CW, Jin C, Li JP. Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: A meta-analysis. *World J Gastroenterol* 2010; 16(34): 4348-4356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i34/4348.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i34.4348

INTRODUCTION

The inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a com-

mon relapsing condition characterized by both gastrointestinal and systemic manifestations and is responsible for a significant morbidity in both adults and children. Clinical symptoms of IBD include weight loss, abdominal pain, as well as diarrhea accompanied by blood, and disease progression is often accompanied by an increase in granulomas and activated monocytes, which produce significant amounts of eicosanoids and cytokines[1,2]. The etiology of IBD most likely involves a complex interaction of genetic, environmental and immunoregulatory factors[3].

The normal gut consists of an epithelial barrier, the mucosal immune system, and a number of stromal/supportive cells. The external environment comprises native mucosal microbiota, potential pathogenic microorganisms, abundant food antigens and allergens, all of which are encountered mainly at the vast surface areas of mucosal membranes, and forms the most important source of stimulation of the entire immune system. The induction of preventive and protective immune responses to mucosal infectious agents and to inert food antigens and environmental allergens that would limit their absorption, is usually the most emphasized functional aspect of the mucosal immune system[4]. Dysfunctional innate immune response seems important in the pathogenesis of IBD[5]. By means of genome-wide scans, numerous IBD susceptibility loci have been identified[6]. Specific single gene defects have been discovered, including mutations in the leucine rich region (LRR) of the nucleotide-binding oligomerization domain 2 (NOD-2) gene, also known as CARD-15 (caspase activation and recruitment domain 15)[7,8]. The identification of the NOD2 is a breakthrough in IBD genetics, which heralded extensive analyses of signaling pathways of the innate immune system implicated in the pathogenesis of IBD[9,10].

Innate immunity depends on the specific recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs). The NOD protein is a family of intracellular PRRs. After the intracellular PRRs recognized PAMPs, the pro-inflammatory pathways would be activated[11-13]. NOD1 (also known as CARD-4) is a host cytosolic signaling PRR, and acts as a cytosolic receptor for the diaminopimelate (DAP)-containing GlcNAc-tripeptide muropeptide found mostly in Gram-negative bacterial peptidoglycans[14]. NOD1/CARD4 signaling leads to activation of NF-κB, and plays an important role in innate immunity[15]. In 1903, Sutton[16] explained that dominance and recessiveness were features of "chromatin entities" rather than morphological characters. In other words, dominance and recessiveness are properties of genetic information resulting in a certain function rather than the function itself. This means that certain polymorphisms and mutations in NOD1/CARD4 may result in dysfunctional innate immune response during bacterial recognition with direct implications for IBD pathogenesis. Genome-wide scans for IBD linkage demonstrated a susceptibility locus on chromosome 7p14, and the same locus where the NOD1/CARD4 gene is located[17]. Therefore, NOD1/CARD4 gene is a perfect candidate for predisposition to IBD.

NOD1/CARD4 insertion/deletion polymorphism (rs6958571) was identified by Hysi *et al*[18]. Since its discovery in 2005, this polymorphism has attracted widespread attention. A number of case-control studies were conducted to investigate the association of this polymorphism with human IBD[19-25]. However, these studies reported conflicting results. There are several possible explanations for this, such as small sample size, ethnic background, uncorrected multiple hypothesis test, and publication bias.

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis for the estimation of genetic effects[26]. The aim of the present study was to investigate the association of NOD1/CARD4 insertion/deletion polymorphism with human IBD, using a meta-analysis.

MATERIALS AND METHODS

Identification of eligible studies

Available articles were identified through a literature search using the keywords "nucleotide-binding oligomerization domain 1" or "NOD1" or "caspase activation and recruitment domain 4" or "CARD4" and "polymorphism" in the PubMed database. Additional literature was collected from cross-references within both original and review articles. We only recruited data from the wholly published paper, but not from meeting or conference abstracts. No language restrictions were applied. A study was included in the current meta-analysis if (1) it was published up to December 2009; and (2) it was a case-control study. We excluded the studies containing overlapping data and the family-based studies because our analysis was based on linkage considerations. When there were multiple publications from the same population, only the largest study was included. When a study reported the results on different subpopulations, we took it as a separate study.

Additionally, an independent PubMed search was done (by Lu WG and Feng XL) by the same method. The abstracts were reviewed independently by two investigators (Yuan FL and Gu YL) to determine if they met the eligibility criteria for inclusion. References in the studies were reviewed (by Jin C, Li X and Li CW) to identify additional studies. If discrepancies occurred, a third investigator (Li JP) did an additional assessment.

Data extraction

If original genotype frequency data was unavailable in relevant articles, a request for additional data was sent to the corresponding authors. In addition, two investigators (Feng XL and Li JP) independently extracted the data with the standard protocol and the result was reviewed by a third investigator (Zou YF). Discrepancies were resolved

Table 1 Characteristics of the studies included in the meta-analysis

ID	Study	Yr	Ethnic group	Diseases	Sample size (frequency of GG allele, %)		OR (95%CI) for GG *vs* T allele	Hardy-Weinberg equilibrium of genotype control
					Case	Control		
1	Hancock *et al*[19]	2008	Caucasian	CD	594 (27.1)	1024 (24.6)	1.130 (0.960-1.330)	0.010
2	Cantó *et al*[20]	2007	Caucasian	CD	97 (21.7)	50 (31.0)	0.615 (0.357-1.060)	0.147
3	Henckaerts *et al*[21]	2007	Caucasian	IBD	1052 (24.5)	280 (25.4)	0.952 (0.768-1.180)	0.751
				CD	809 (24.8)		0.973 (0.780-1.214)	
				UC	222 (22.9)		0.878 (0.656-1.175)	
4	Van Limbergen *et al*[22]	2007	Caucasian (Scottish)	IBD	1079 (26.1)	1233 (26.4)	0.984 (0.863-1.089)	0.261
				CD	515 (26.4)		1.003 (0.850-1.182)	
				UC	537 (26.0)		0.985 (0.837-1.160)	
5	Van Limbergen *et al*[22]	2007	Caucasian (Swedish)	IBD	632 (25.3)	277 (23.3)	1.112 (0.880-1.406)	0.741
				CD	244 (24.7)		1.086 (0.817-1.444)	
				UC	388 (25.5)		1.129 (0.875-1.456)	
6	Franke *et al*[23]	2006	Caucasian	IBD	961 (22.4)	841 (21.5)	1.055 (0.900-1.235)	0.958
				CD	633 (21.1)		0.983 (0.822-1.174)	
				UC	332 (24.4)		1.181 (0.955-1.460)	
7	Tremelling *et al*[24]	2006	Caucasian	IBD	1360 (25.2)	758 (25.7)	0.975 (0.844-1.126)	0.580
				CD	641 (24.9)		0.964 (0.812-1.144)	
				UC	665 (25.4)		0.991 (0.837-1.173)	
8	McGovern *et al*[25]	2005	Caucasian	IBD	664 (26.6)	335 (31.8)	0.777 (0.634-0.952)	0.195
				CD	358 (24.4)		0.694 (0.548-0.878)	
				UC	306 (29.1)		0.880 (0.693-1.117)	

OR: Odds ratio; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

through discussion among our research team. From each study, we extracted the first author's name, year of publication, source of publication, racial ancestry, type of diseases, the number of cases and controls, and the available genotype and allele frequency information from the NOD1/CARD4 insertion/deletion polymorphism.

Meta-analysis methods

Allele frequencies at the NOD1/CARD4 insertion/deletion polymorphism from the respective studies were determined by the allele counting method. A χ^2 test was used to determine if the observed frequencies of genotypes conformed to Hardy-Weinberg equilibrium expectations.

We examined the relationship between the allele and susceptibility to IBD (GG *vs* T), and the genotypes. The following genotype contrasts were included: GG/T + GG/GG *vs* T/T, GG/GG *vs* T/T + GG/T, GG/GG *vs* T/T, and GG/T *vs* T/T. The contrast of GG/T + GG/GG *vs* T/T genotypes corresponds to a dominant genetics effect of the GG allele. The contrast of GG/GG *vs* T/T + GG/T genotypes corresponds to a recessive genetics effect of the GG allele. The odds ratio (OR) and its 95% confidence interval (95% CI) were estimated for each study. The heterogeneity between studies was assessed by the Chi-square test based Q-statistics[27]. A significant Q-statistics ($P < 0.10$) indicated the heterogeneity among studies, and then the result of the random effect model was selected. Otherwise, the result of fixed effect model was selected. We also measured the effect of heterogeneity using the formula: $I^2 = 100\% \times (Q\text{-}df)/Q$[28].

Finally, the pooled OR was obtained by Mantel-Haenszel method in the fixed effect model and by DerSimonian and Laird method in the random effect model[29,30]. The pooled OR was performed, weighting the individual OR by the inverse of their variance. The significance of the pooled OR was determined by the Z test.

Evaluation of publication bias

Publication bias was investigated with the funnel plot. Funnel plot asymmetry was further assessed by the method of Egger's linear regression test[26]. Analyses were performed using the software Review Manager 4.2 (Cochrane Collaboration, http://www.cc-ims.net/RevMan/relnotes.htm/) and Stata version 10 (StataCorp LP, College Station, Texas, USA). A P value less than 0.05 was considered statistically significant, and all the P values were two sided.

RESULTS

Characteristics of eligible studies

Characteristics of studies included in the current meta-analysis are presented in Table 1[19-25]. There were 46 papers relevant to the searching words. Through the screening of the abstract, 19 of these articles were excluded (5 were reviews; 4 were not conducted in humans; 10 did not explore NOD1/CARD4 gene polymorphisms), leaving 27 studies for full publication review. Of the 27 studies, 13 without focusing on IBD, were excluded, leaving 14 studies[19-25,31-37] for more detailed assessment. Seven of them were excluded (one was a family-based study; one was a duplicate report; and 5 did not study the NOD1/CARD4 insertion/deletion polymorphism)[31-37]. As a result, 7 studies were included in the current meta-analysis (Figure 1). One of the eligible studies contained data on two dif-

Table 2 Meta-analysis of association between NOD1/CARD4 insertion/deletion polymorphism and inflammatory bowel disease

Polymorphism	Disease	Sample size		*n*	Test of association				Test of heterogeneity		
		Case	Control		OR (95%CI)	*Z*	*P* value	Model	χ^2	*P* value	I^2 (%)
GG *vs* T	Overall	12878	9596	8	0.98 (0.90-1.07)	0.39	0.70	R	12.60	0.08	44.4
	CD	7782	9596	8	0.96 (0.86-1.07)	0.78	0.43	R	14.49	0.04	51.7
	UC	4900	7448	6	1.01 (0.92-1.09)	0.14	0.89	F	5.12	0.40	2.3
	IBD onset < 40	1486	4752	4	0.68 (0.50-0.93)	2.38	0.02	R	8.30	0.04	63.9
GG/T + GG/GG *vs* T/T	Overall	6439	4798	8	1.00 (0.98-1.08)	0.01	0.99	F	10.69	0.15	34.5
	CD	3891	4798	8	0.97 (0.86-1.10)	0.42	0.67	R	12.36	0.09	43.4
	UC	2450	3724	6	1.00 (0.90-1.11)	0.06	0.95	F	4.64	0.46	0.0
	IBD onset < 40	743	2376	4	0.71 (0.59-0.85)	3.65	0.0003	F	4.83	0.18	37.8
GG/GG *vs* T/T + GG/T	Overall	6439	4798	8	0.95 (0.81-1.11)	0.62	0.53	F	12.02	0.10	41.8
	CD	3891	4798	8	0.91 (0.76-1.09)	0.99	0.32	F	11.74	0.11	40.4
	UC	2450	3724	6	1.03 (0.83-1.27)	0.23	0.81	F	4.89	0.43	0.0
	IBD onset < 40	743	2376	4	0.61 (0.28-1.35)	1.22	0.22	R	7.98	0.05	62.3
GG/GG *vs* T/T	Overall	4033	3020	8	0.93 (0.74-1.17)	0.63	0.53	R	12.59	0.08	44.4
	CD	2442	3020	8	0.88 (0.68-1.14)	0.96	0.34	R	12.96	0.07	46.0
	UC	1623	2312	6	0.97 (0.78-1.20)	0.32	0.75	F	3.04	0.69	0.0
	IBD onset < 40	500	1435	4	0.52 (0.22-1.21)	1.52	0.13	R	8.84	0.03	66.0
GG/T *vs* T/T	Overall	2816	2098	8	0.95 (0.80-1.12)	0.64	0.53	F	10.85	0.15	35.5
	CD	1685	2098	8	0.92 (0.76-1.11)	0.92	0.36	F	9.94	0.19	29.6
	UC	1093	1648	6	1.03 (0.83-1.28)	0.27	0.79	F	4.67	0.46	0.0
	IBD onset < 40	290	1112	4	0.94 (0.64-1.37)	0.33	0.74	F	5.95	0.11	49.6

R: Random effect model; F: Fixed effect model; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

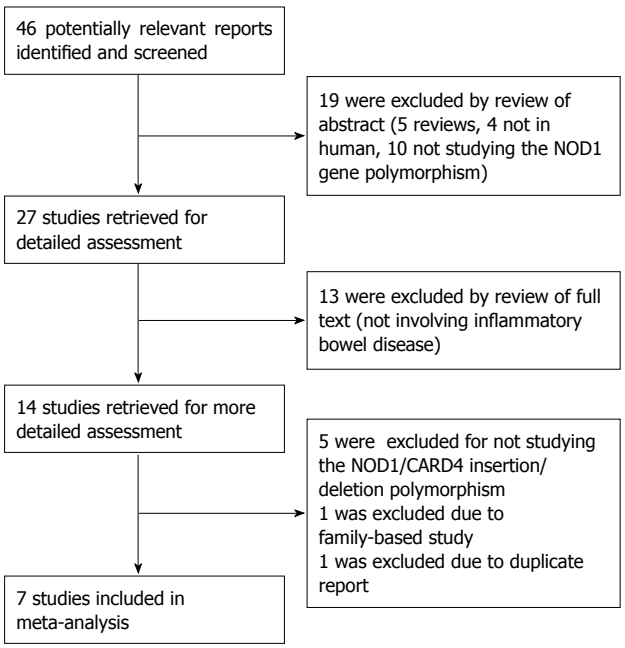

Figure 1 Study selection in Medline.

ferent subpopulations and we treated it independently[22]. Finally, a total of 8 separate studies were considered in the current meta-analysis (Table 1).

We got the data from the corresponding author of the study by Franke *et al*[23]. Thus, the allele and the genotype frequencies of the NOD1/CARD4 insertion/deletion polymorphism were extracted from all the eligible studies. The 8 separate studies were conducted in Caucasian populations. Of these, 8[19-25] were conducted in patients with CD and 6[21-25] were conducted in patients with UC. Meanwhile, 4 studies[20,22,24,25] showed stratified data of cases by age (IBD onset at < 40 years). The results of Hardy-Weinberg equilibrium test for the distribution of the genotype in control populations are shown in Table 1. Only one study belonged to Hardy-Weinberg equilibrium among the eligible studies[19]. The distribution of the genotype in the overall control population was consistent with Hardy-Weinberg equilibrium ($P = 0.240$).

Meta-analysis

The summary of the meta-analysis for the NOD1/CARD4 insertion/deletion polymorphism and IBD is shown in Table 2.

Overall effects

The *Q*-test of heterogeneity was not significant and we conducted analyses using fixed effect models except in the contrasts of GG *vs* T and GG/GG *vs* T/T. We found no association between NOD1/CARD4 insertion/deletion polymorphism and IBD in the overall population (GG *vs* T: OR = 0.98, 95% CI: 0.90-1.07, $P = 0.70$; GG/T + GG/GG *vs* T/T: OR = 1.00, 95% CI: 0.98-1.08, $P = 0.99$; GG/GG *vs* T/T + GG/T: OR = 0.95, 95% CI: 0.81-1.11, $P = 0.53$; GG/GG *vs* T/T: OR = 0.93, 95% CI: 0.74-1.17, $P = 0.53$; GG/T *vs* T/T: OR = 0.95, 95% CI: 0.80-1.12, $P = 0.53$).

Subgroup analyses

We performed group-specific meta-analysis of CD, UC and IBD onset in the populations aged < 40 years.

Analysis of CD population: The *Q*-test of heterogeneity was significant and we conducted analyses using random effect models except in the contrasts of GG/GG *vs* T/T + GG/T and GG/T *vs* T/T. No association of

Table 3 Egger's linear regression test to measure the funnel plot asymmetry[1]

Comparisons	Y axle intercept: a (95%CI)				
	GG *vs* T	GG/T + GG/GG *vs* T/T	GG/GG *vs* T/T + GG/T	GG/GG *vs* T/T	GG/T *vs* T/T
Overall	-1.92 (-5.57-1.72)	0.96 (-2.65-4.58)	2.38 (-0.87-5.64)	2.38 (-1.00-5.77)	2.32 (-0.71-5.36)
CD	-2.62 (-6.54-1.29)	1.60 (-2.38-5.59)	2.79 (-0.20-5.78)	2.89 (-0.31-6.09)	2.51 (-0.29-5.33)
UC	-0.39 (-6.54-5.75)	0.57 (-5.25-6.39)	-0.57 (-7.13-5.97)	-0.43 (-7.00-6.13)	-0.57 (-6.89-5.74)
IBD onset at < 40 yr of age	-2.16 (-8.80-4.47)	1.49 (-4.16-7.15)	1.86 (-4.04-7.77)	1.94 (-4.40-8.29)	1.63 (-3.48-6.75)

[1]All $P > 0.05$. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

NOD1/CARD4 insertion/deletion polymorphism was found with CD (GG *vs* T: OR = 0.96, 95% CI: 0.86-1.07, $P = 0.43$; GG/T + GG/GG *vs* T/T: OR = 0.97, 95% CI: 0.86-1.10, $P = 0.67$; GG/GG *vs* T/T + GG/T: OR = 0.91, 95% CI: 0.76-1.09, $P = 0.32$; GG/GG *vs* T/T: OR = 0.88, 95% CI: 0.68-1.14, $P = 0.34$; GG/T *vs* T/T: OR = 0.92, 95% CI: 0.76-1.11, $P = 0.36$).

Analysis of UC population: The Q-test of heterogeneity was not significant and we conducted analyses using fixed effect models in the UC population. No association of NOD1/CARD4 insertion/deletion polymorphism with UC was discovered (GG *vs* T: OR = 1.01, 95% CI: 0.92-1.09, $P = 0.89$; GG/T + GG/GG *vs* T/T: OR = 1.00, 95% CI: 0.90-1.11, $P = 0.95$; GG/GG *vs* T/T + GG/T: OR = 1.03, 95% CI: 0.83-1.27, $P = 0.81$; GG/GG *vs* T/T: OR = 0.97, 95% CI: 0.78-1.20, $P = 0.75$; GG/T *vs* T/T: OR = 1.03, 95% CI: 0.83-1.28, $P = 0.79$).

Analysis of IBD onset in a population aged < 40 years: The Q-test of heterogeneity was significant and we conducted analyses using random effect models except in the contrasts of GG/T + GG/GG *vs* T/T, and GG/T *vs* T/T. We found an association between NOD1/CARD4 insertion/deletion polymorphism and IBD in a younger age group at onset (< 40 years) when examining the contrasts of GG *vs* T, and GG/T + GG/GG *vs* T/T (GG *vs* T: OR = 0.68, 95% CI: 0.50-0.93, $P = 0.02$; GG/T + GG/GG *vs* T/T: OR = 0.71, 95% CI: 0.59-0.85, $P = 0.0003$), and the forest plots are shown in Figure 2. However, the association was not found when the contrasts of GG/GG *vs* T/T + GG/T, GG/GG *vs* T/T and GG/T *vs* T/T were examined (GG/GG *vs* T/T + GG/T: OR = 0.61, 95% CI: 0.28-1.35, $P = 0.22$; GG/GG *vs* T/T: OR = 0.52, 95% CI: 0.22-1.21, $P = 0.13$; GG/T *vs* T/T: OR = 0.94, 95% CI: 0.64-1.37, $P = 0.74$).

Evaluation of publication bias

Funnel plot asymmetry was assessed by the method of Egger's linear regression test. If there was asymmetry, the regression line would not run through the origin. The larger its deviation from zero, the more pronounced the asymmetry. The results of Egger's linear regression test are shown in Table 3. It was shown that there was no publication bias (all $P > 0.05$). For the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in the group of younger age at onset (< 40 years), the Egger's linear regression test provided no evidence of publication bias (GG *vs* T: $t = -1.40$, $P = 0.296$; GG/T + GG/GG *vs* T/T: $t = 1.14$, $P = 0.373$) (Figure 3A). Figure 3B shows that the distribution of the ORs from individual studies in relation to their respective standard deviation was symmetric in funnel plot.

DISCUSSION

The identification of NOD2/CARD15 as a CD susceptibility gene makes its homologous gene NOD1/CARD4 a potential candidate gene for predisposition to IBD[8,38,39]. NOD1/CARD4 is the founding member of the Nod-like receptors (NLRs) family, and is expressed in large and small bowel[18]. It plays an important role in colonic epithelial defenses against intracellular organisms, such as enteroinvasive *E. coli* and *Shigella flexneri*[40,41]. The presence of bacterial flora is essential for IBD to develop in animal models[42]. Antibiotics and fecal diversion are effective therapies for CD[43,44]. NOD1/CARD4 has been mapped to chromosome bands 7p14-p15 (UniGene Cluster Hs 19405), a region which was previously reported to contain an IBD susceptibility locus[17]. Thus, NOD1/CARD4 appeared to be a good candidate for IBD. Recently, many studies have been conducted to test the association of NOD1/CARD4 insertion/deletion polymorphism with IBD, but the association trends observed have been variable with several studies showing an association while others do not[19-25]. It is, therefore, necessary to perform a comprehensive meta-analysis to assess the importance of the NOD1/CARD4 insertion/deletion polymorphism for IBD pathogenesis.

In the present study, we retrieved 8 studies, including 6439 cases and 4798 controls, to evaluate the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in Caucasian populations. Our meta-analysis did not detect the association of NOD1/CARD4 insertion/deletion polymorphism with IBD, CD, and UC in the overall population. However, we did find a significant genetic association between NOD1/CARD4 insertion/deletion polymorphism and IBD in the group of younger age at onset (< 40 years), and GG allele was a protective allele for IBD pathogenesis. As far as we know, this is the first meta-analysis carried out so far which aimed at investigating the association of NOD1/CARD4 insertion/deletion polymorphism with IBD.

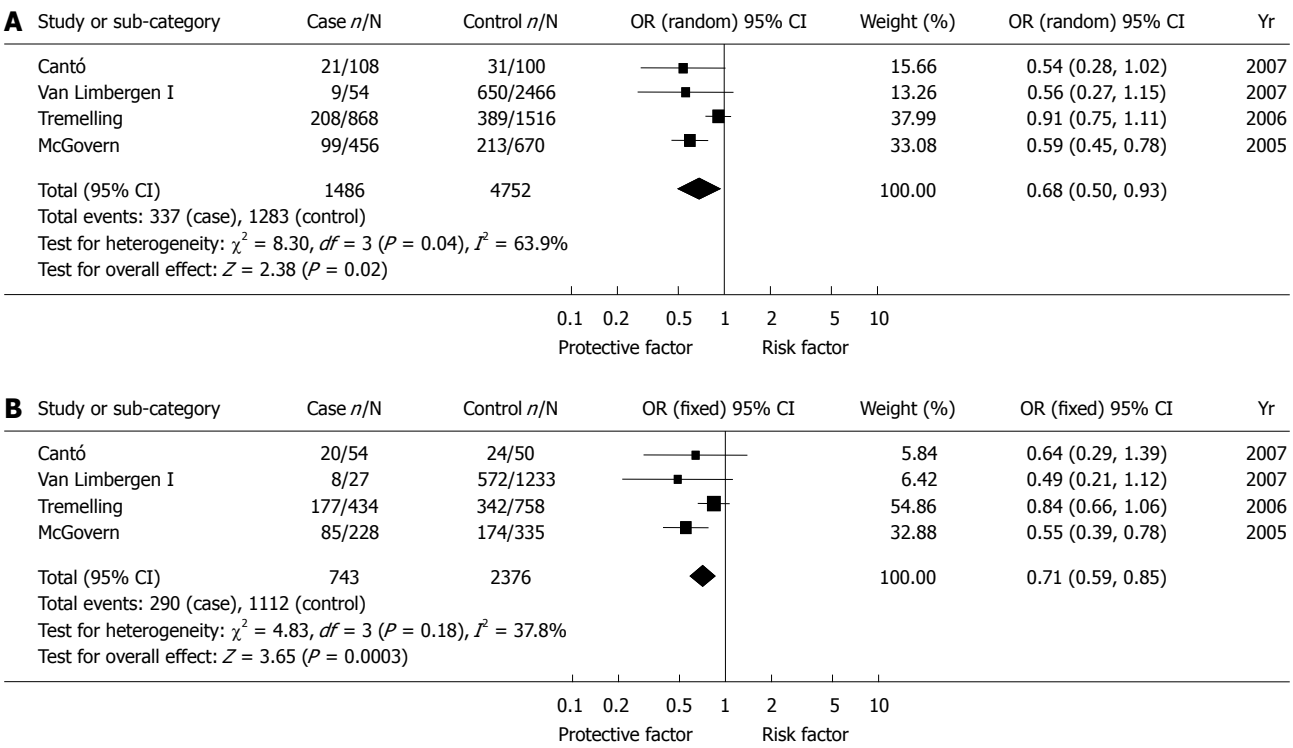

Figure 2 Forest plots for meta-analysis of positive results. Inflammatory bowel disease onset at < 40 years of age. A: GG *vs* T; B: GG/T + GG/GG *vs* T/T.

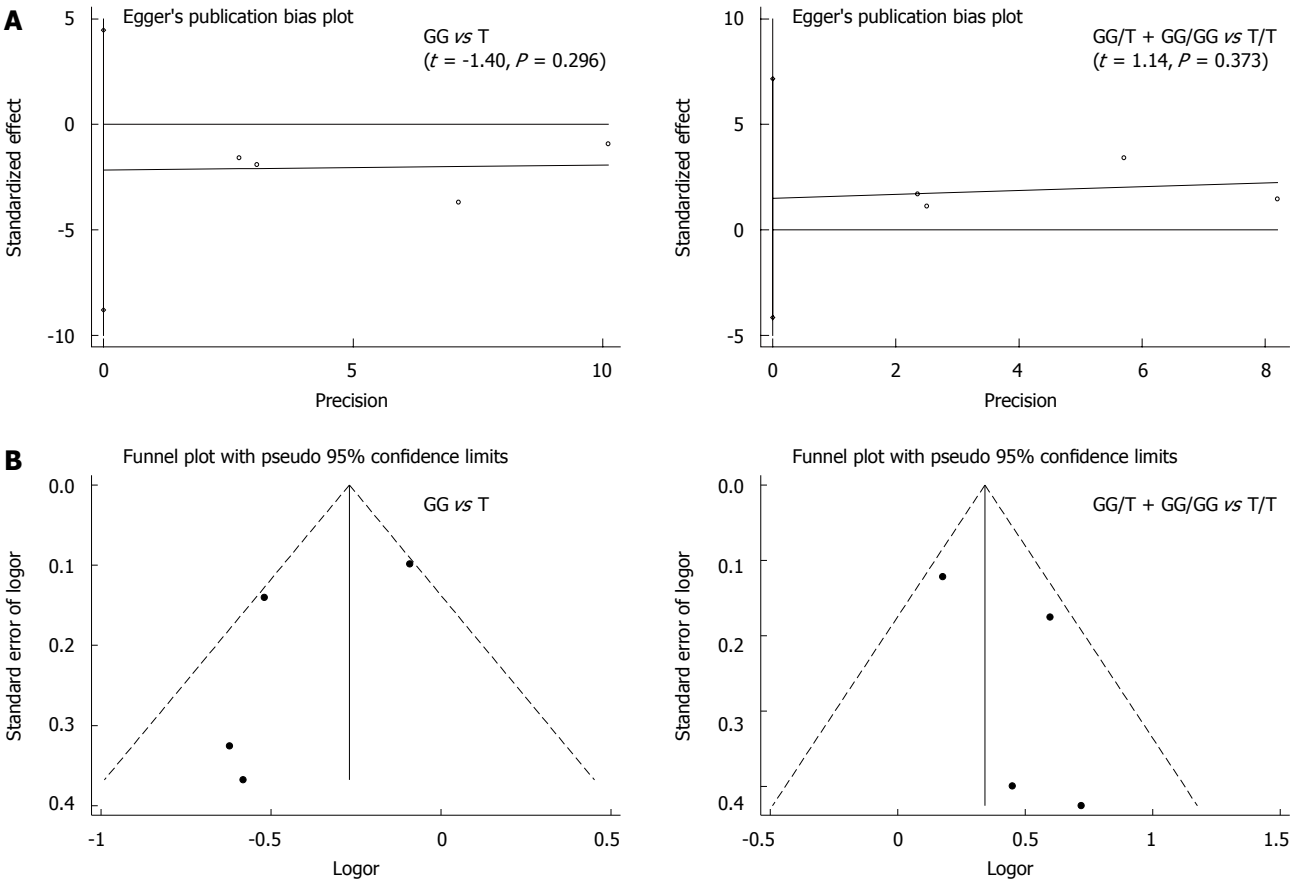

Figure 3 Egger's linear regression test for publication bias of positive results (A), funnel plots for meta-analysis of positive results; inflammatory bowel disease onset at < 40 yeas of age (B).

In our study, we found that the NOD1/CARD4 GG allele decreased the risk of IBD in the group of younger

age at onset (< 40 years), indicating that this locus is important in determining the susceptibility to IBD. The result is not surprising, since NOD1, similar to NOD2, is involved in the recognition of intracellular bacterial PAMPs[45]. The two molecules share structure and functional similarities. NOD1/CARD4 insertion/deletion polymorphism is located at the beginning of intron IX[18]. Hysi *et al*[18] firstly demonstrated an effect of this polymorphism on the binding of an unidentified nuclear protein. Hysi *et al*[18] demonstrated the presence of different isoforms of NOD1 transcripts. A recent study showed that some of these isoforms resulted in disruption of the LRR region critical for NOD1 mediated bacterial sensing[46]. Therefore, although noncoding, this polymorphism may affect immune response with direct implications for IBD pathogenesis either by altered binding of a cis/trans activating protein, resulting in abnormal gene expression, or by the generation of functionally significant splice variants. However, to date, the detailed functions of this polymorphism are still unclear. Further studies on the function of NOD1/CARD4 insertion/deletion polymorphism are required. Of course, the association may result from the direct effect of the polymorphism itself, or through linkage disequilibrium with another functional polymorphism in the structural part of the gene or in regulatory regions. Additionally, the association of NOD1/CARD4 insertion/deletion polymorphism with IBD was only detected in the contrasts of GG *vs* T and GG/T + GG/GG *vs* T/T, indicating that the GG allele of this polymorphism may have a dominant effect on risk for IBD.

Some limitations of this study should be discussed. Firstly, the current meta-analysis only included the wholly-published studies, not the meeting or conference abstracts. Thus, publication bias may have occurred, even though the use of a statistical test did not show it. Secondly, significant heterogeneity between studies was detected in the current meta-analysis, which may distort the analysis. However, it is not a major problem because IBD itself is heterogeneous, and different populations may contribute to the heterogeneity. Thirdly, these results should be interpreted with caution because the population from six countries was not uniform. Fourthly, the analysis in IBD onset in the population aged < 40 years only included four studies (743 cases and 2376 controls), and more studies based on a larger sample size, case-control design and stratification by age are still needed in the future research. Finally, meta-analysis remains retrospective that is subject to the methodological deficiencies of the included studies. Therefore, we minimized the likelihood of bias by developing a detailed protocol before initiating the study by performing a meticulous search for published studies and by using explicit methods for study selection, data extraction and data analysis.

In conclusion, our study demonstrates the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease in the younger age group at onset (< 40 years) in Caucasian populations.

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COMMENTS

Background

Recently, many studies have been conducted to prove the association of NOD1/CARD4 insertion/deletion polymorphism with inflammatory bowel disease (IBD), but the association trends observed have been variable with several studies showing an association while others do not. It is, therefore, necessary to perform a comprehensive meta-analysis to assess the importance of the NOD1/CARD4 insertion/deletion polymorphism for IBD pathogenesis.

Research frontiers

The etiology of IBD most likely involves a complex interaction of genetic, environmental and immunoregulatory factors. The identification of the NOD2 is a breakthrough in IBD genetics, which heralded extensive analyses of signaling pathways of the innate immune system implicated in the pathogenesis of IBD. NOD1/CARD4 signaling leads to activation of nuclear factor-κB, and plays an important role in innate immunity. Certain polymorphisms and mutations in NOD1/CARD4 may result in dysfunctional innate immune response during bacterial recognition with direct implications for IBD pathogenesis.

Innovations and breakthroughs

The authors collected 8 studies (6439 cases and 4798 controls) in Caucasian populations to evaluate whether NOD1/CARD4 insertion/deletion polymorphism is associated with IBD by meta-analysis. They found the association of NOD1/CARD4 insertion/deletion polymorphism with IBD in the younger age group at onset (< 40 years) in Caucasian populations.

Applications

The authors found that the NOD1/CARD4 GG allele decreased the risk of IBD in the younger age group at onset (< 40 years), indicating that this locus is important in determining the susceptibility to IBD. The association of NOD1/CARD4 insertion/deletion polymorphism with IBD was only detected in the contrasts of GG *vs* T and GG/T + GG/GG *vs* T/T, indicating that the GG allele of this polymorphism may have a dominant effect on risk for IBD.

Terminology

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis.

Peer review

This is a very interesting meta-analytic study dealing with an important topic in IBD.

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BRIEF ARTICLE

Standard triple, bismuth pectin quadruple and sequential therapies for *Helicobacter pylori* eradication

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Abstract

AIM: To compare the effectiveness of standard triple, bismuth pectin quadruple and sequential therapies for *Helicobacter pylori* (*H. pylori*) eradication in a randomized, double-blinded, comparative clinical trial in China.

METHODS: A total of 215 *H. pylori*-positive patients were enrolled in the study and randomly allocated into three groups: group A (n = 72) received a 10-d bismuth pectin quadruple therapy (20 mg rabeprazole *bid*, 1000 mg amoxicillin *bid*, 100 mg bismuth pectin *qid*, and 500 mg levofloxacin *qd*); group B (n = 72) received the sequential therapy (20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, in 5 d, followed by 20 mg omeprazole *bid*, 500 mg tinidazole *bid*, 500 mg clarithromycin *bid*, for another 5 d); group C (n = 71) received a standard 1-wk triple therapy (20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, 500 mg clarithromycin *bid*). After all these treatments, 20 mg omeprazole *bid* was administrated for 3 wk. *H. pylori* status was assessed by histology, 13C-urea breath test and rapid urease test at baseline and 4-6 wk after completion of treatment. Ulcer cicatrization was assessed by gastroscopy. χ^2 test (P < 0.05) was used to compare the eradication rates and ulcer cicatrisation rates among the three groups.

RESULTS: The eradication rate was 83.33% (60/72) in group A, 88.89% (64/72) in group B, and 80.56% (58/71) in group C. The ulcer cicatrisation rate was 86.44% (51/59) in group A, 90.16% (55/61) in group B, and 84.91% (45/53) in group C. The sequential therapy yielded a higher eradication rate and ulcer cicatrisation rate than the standard triple and bismuth pectin quadruple therapies. Statistically, the eradication rate of group B was significantly different from groups A and C (P < 0.05), but the difference of ulcer cicatrisation rate and side effects was not statistically significant among the three groups (P > 0.05). The three protocols were generally well tolerated.

CONCLUSION: The sequential therapy has achieved a significantly higher eradication rate, and is a more suitable first-line alternative protocol for anti-*H. pylori* infection compared with the standard triple and bismuth pectin quadruple therapies.

Key words: *Helicobacter pylori*; Sequential therapy; Triple therapy; Bismuth pectin quadruple therapy; Eradication rate

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INTRODUCTION

Helicobacter pylori (H. pylori) infection habitually causes chronic active gastritis, which significantly enhances the risk for intestinal metaplasia in the stomach, and it is undoubtedly involved in gastric carcinogenesis. Moreover, *H. pylori* also play a crucial role in the pathogenesis of peptic ulcer and mucosa-associated lymphoid tissue lymphoma, including peptic ulcer complications, such as bleeding or stenosis[1-4]. According to the Maastricht 2 guidelines, the first-line treatment for *H. plori* eradication is the triple therapy using a proton-pump inhibitor (PPI) *bid*, 1 g amoxicillin *bid*, and 500 mg clarithromycin *bid*. In the case of penicillin allergy, 500 mg metronidazole *bid* is substituted for amoxicillin. After a decade of clarithromycin-based treatment and continued widespread use of long-acting macrolides in general practice, 10%-15% of *H. pylori* strains are resistant *de novo* to clarithromycin[3]. As a result, the failure rate is around 20% for the triple therapy (PPI plus amoxicillin plus clarithromycin)[4,5]. When the first-line *H. pylori* eradication treatment fails, a second-line treatment of quadruple therapy, with a PPI *bid*, colloidal bismuth subcitrate *qid*, 500 mg metronidazole *tid*, and 500 mg tetracycline *qid*, is recommended. Some recent studies have compared the efficacy of the triple *vs* quadruple therapy, and a meta-analysis has assessed these studies[6]. Eradication rates were not significantly different among patients receiving triple or quadruple therapy. The eradication rates in the patients receiving either triple or quadruple therapy in this study were almost similar to those obtained previously[4,7,8].

Clarithromycin and metronidazole resistance has increased substantially in recent years, and there has been a corresponding decrease in the eradication rate for *H. pylori* infection in most Western countries[4]. In China, recent nationwide multi-center studies have demonstrated that clarithromycin resistance increased to 27.6%, and metronidazole resistance is extremely common, the average resistance rate being 75.6%. Furthermore, combined clarithromycin-metronidazole cross-resistance was found in 85.1% of clarithromycin-resistance *H. pylori* strains. Eradication rates in most Western countries and China have declined to unacceptable levels. Therefore, Antibiotic resistance is the main cause of failure in *H. pylori* eradication and bata-lactamase produced by resistant *H. pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy[1].

Sequential therapy is a latest protocol for *H. pylori* eradication suggested by De Francesco *et al*[9]. Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination (e.g. 40 mg pantoprazole with 1 g amoxicillin *bid*) followed by 5 d of two more antibiotics plus a PPI (e.g. 500 mg clarithromycin and 500 mg tinidazole plus 40 mg pantoprazole *bid*). A subgroup data-analysis in a large, prospective, controlled multi-center study showed that the eradication rate of this "new" treatment was significantly higher than that of the clarithromycin-based treatment (82% *vs* 44%, $P < 0.0155$). Child, adult and elderly patients receiving this "new" treatment achieved a high eradication rate and had less adverse reactions[10,11].

To our knowledge, no data are available about the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China. The present study aimed to compare the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy; to further test whether the 10-d sequential therapy is able to increase the eradication rate compared with the 7-d standard triple therapy and 10-d bismuth pectin quadruple therapy; to observe the adverse reactions; and to evaluate the reliability, safety and efficacy of this treatment in China.

MATERIALS AND METHODS

Patients

This is a prospective, parallel, open-label, randomized study. The study population consisted of patients with dyspepsia defined as having pain or discomfort in the upper abdomen.

A total of 215 patients infected with *H. pylori* were enrolled. The patients were screened from 15 322 patients who underwent gastroscopy at the Endoscopy Center of Weihai Municipal Hospital from January 1, 2005 to December 31, 2009. Patients enrolled in the present study had not been previously treated for *H. pylori* infection. Patients were excluded if they were taking PPI, H2-receptor antagonists, bismuth preparations or antibiotics 4 wk before the study. Pregnant women, patients with antibiotic allergy or severe diseases of organs, neoplasm, serious complications of ulcers, and hepatic impairment or kidney failure were not enrolled. All the participants signed informed consent form.

H. pylori infection was assessed at entry. All patients underwent endoscopy with biopsies for histology (two samples from the antrum and two from the corpus) and a rapid urease test (one sample from the antrum) (CP-test, China). Patients were diagnosed to be *H. pylori*-positive if both tests were positive. Biopsy specimens were histologically detected for *H. pylori* by hematoxylin and eosin stain. In patients diagnosed with ulcers by gastroscopy, the diameter of ulcers must be between 5 mm and 3 cm, and number of ulcers must be no more than 2 in stomach and/or duodenum, except for those with a history of peptic ulcer before present illness. The post-eradication assessment was undertaken 4-6 wk after completion of the treatment (after the subsequent 3-wk course of PPI) using a 13C-urea breath test (Infai, Sofar, Italy). Citric acid (1.5 g) as a test meal and 13C-urea (75 mg) as a water solution were given to the patients after collection of a baseline sample by blowing through a disposable plastic straw into a 20-mL container; an additional breath sample was collected 30 min later. The breath samples were considered positive if there was a greater than five per 1000 of $13CO_2$ difference over baseline, according to the manufacturer's recommendations. Meanwhile, all patients

underwent endoscopy with biopsies for histology (two samples from the antrum and two from the corpus) and a rapid urease test (one sample from the antrum), and the healing of ulcers *vs* pre-therapy was observed.

Therapeutic regimens

In the center, patients were randomly assigned using a computer generated list to one of the following treatments: Group A (n = 72): A 10-d triple therapy with 20 mg rabeprazole *bid*, 1000 mg amoxicillin *bid*, 100 mg bismuth pectin *qid*, and 500 mg levofloxacin *qd*; Group B (n = 72): A sequential therapy with 20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, for 5 d, followed by 20 mg omeprazole *bid*, 500 mg tinidazole *bid*, 500 mg clarithromycin *bid*, for another 5 d; and Group C (n = 71): A standard triple therapy with 20 mg omeprazole *bid*, 1000 mg amoxicillin *bid*, 500 mg clarithromycin *bid*.

For each regimen, the PPI was prescribed at 30 min before meals, but all antibiotics were given after meals. Patients were asked to return to assess the compliance and estimate the adverse reactions at the end of the treatment. Side effects were evaluated using a structured questionnaire by personal interview.

Statistical analysis

The sample size was calculated based on available data in the literature. By hypothesizing a 95% eradication rate for the sequential regimen[12] and 80% for either the 7-d standard triple, 10-d sequential therapy or 10-d bismuth pectin quadruple therapy[13], it was calculated that all patients per treatment arm were needed to find a statistically significant difference with a level of $P < 0.05$ and a power of 0.85. The eradication rates and their 95% CIs were calculated for each treatment regimen. For all other variables, χ^2, Fisher's exact test and Student's t test were used as appropriate, and $P < 0.05$ was considered significant. The difference in the eradication rates among the three treatments was estimated. Before pooling those estimates, a Fisher's exact test was applied to investigate the heterogeneity between the differences.

RESULTS

Eradication rates

Two hundred and thirteen patients with *H. pylori* were enrolled in the study. As shown in Table 1, the three patient groups did not differ in age, sex, gastritis distribution and location and number of peptic ulcers in gastric mucosa. All patients completed the treatment. *H. pylori* infection was successfully cured in 60/72 (83.33%) with a 10-d bismuth pectin quadruple therapy, in 64/72 (88.89%) with the sequential therapy, and in 58/71 (80.56%) with the 7-d standard triple therapy, respectively. As shown in Table 2, the eradication rates achieved by the sequential therapy were significantly higher than that by both the 10-d bismuth pectin quadruple therapy and 7-d standard triple therapy, with significant differences ($P < 0.05$). The ulcer cicatrisation was successfully cured in 86.44% by the 10-d bismuth pectin quadruple therapy, in 90.16% by the sequential therapy, and in 84.91% by the 7-d standard triple therapy. As shown in Table 1, although the sequential therapy tended to give better results in eradication rates when compared with the 10-d bismuth pectin quadruple therapy and 7-d standard triple therapy, no statistically significant difference was found ($P > 0.05$).

Table 1 Demographic and clinical characteristics of patients at entry into each treatment group

Patient characteristics (n)	Group A	Group B	Group C
Number of patients	72	72	71
Sex (M/F)	31/41	35/37	34/37
Age (yr), mean ± SD	45 ± 10	47 ± 13	43 ± 15
Antral gastritis	58	61	57
Pangastritis	17	15	19
Intestinal metaplasia	19	21	17
Duodenitis	11	13	10
Gastric ulcer	40	42	39
Duodenal bulb ulcer	13	12	10
Compound ulcers	6	7	4

Table 2 Eradication and ulcer cicatrisation rates in each treatment group

	Group A	Group B	Group C
Eradication rate (%)[a]	83.33 (60/72)	88.89 (64/72)	80.56 (58/71)
Ulcer cicatrisation rate (%)[b]	86.44 (51/59)	90.16 (55/61)	84.91 (45/53)

[a]$P < 0.05$, Group B *vs* Group C, Group A; [b]$P > 0.05$, Group B *vs* Group C, Group A.

Compliance and side effects

Compliance with the therapy was good (greater than 95% of prescribed drugs). Six patients (16.67%) treated with the 10-d bismuth pectin quadruple therapy complained of side effects (three with abdominal discomfort, two with abdominal pain, four with nausea/vomiting, two with parageusia and one with glossitis). Fourteen patients (19.44%) receiving the sequential therapy reported side effects (five with abdominal pain, one with constipation, two with parageusia, three with nausea/vomiting and three with pruritus). Eleven patients (15.49%) receiving the 7-d standard triple therapy complained of side effects (one with diarrhea, four with abdominal pain, one with parageusia, one with glossitis and three with nausea/vomiting). No statistically significant difference in the incidence of side effects was found among the three groups ($P > 0.05$). All side effects were self-limiting after the therapy was ended.

DISCUSSION

Since antibacterial activity of the majority of antibacterials decreases under intragastric low pH and the slime layer may prevent the drugs penetrating fully into the depth of the biofilm, *H. pylori* is not easily eliminated and can develop resistance to antimicrobial drugs. It is extremely important that a protocol with a high eradication rate should

be selected to ensure a successful eradication of *H. plori* in the treatment of peptic ulcers. At present, triple therapies suggested by either Canadian or European guidelines are the most preferred first-line protocols in clinical practice[2,14]. The proposal is being used by 85%, 84% and 67% of primary-care physicians in Italy, Israel and the United States, respectively[7,15,16]. However, the eradication rates substantially decreased by the triple therapy in several countries. Indeed, a success rate of less than 80% has been found in several European and Asian countries, the United States and Canada[17-25]. Eradication rate was extremely low (25%) in a recent study[26]. The resistance to clarithromycin and/or metronidazole is the primary cause of the descending *H. pylori* eradication rate[8,27,28]. In order to reinforce the curative effect of the standard triple therapy, some scholars suggest that the duration of the treatment may be extended to 14 d. One meta-analysis suggests that the 14-d triple therapy can increase the *H. pylori* eradication rate by 12% compared with the 7-d therapy, but the expenditures increase simultaneously. Therefore, it is imminent to seek a new eradication strategy.

Sequential therapy is a recent proposal for *H. pylori* eradication suggested by Zullo *et al*[29]. De Francesco found that double drugs administration for 14 d and subsequent triple drugs for 7 d significantly increased the eradication rate (97.3%) compared with the proposal of converse administration (81.6%, triple drugs administration for 7 d and subsequent double drugs for 14 d). It suggests that the sequence of antibiotic administration affects the *H. pylori* eradication. Zullo *et al*[30], Sánchez-Delgado *et al*[31] and Zullo *et al*[32] further simplified this proposal, and named it sequential therapy. Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination (40 mg pantoprazole and 1 g amoxicillin *bid*) followed by 5 d of two more antibiotics plus a PPI (500 mg clarithromycin and 500 mg tinidazole plus 40 mg pantoprazole *bid*). Subgroup data analysis in a large, prospective, controlled multi-center study showed that the eradication rate of this "new" treatment was significantly higher compared with the clarithromycin-based treatment (82% *vs* 44%, $P < 0.0155$). Child, adult and elderly patients receiving this "new" protocol all achieved a high eradication rate and had less adverse reactions[10,11].

This study compared the effectiveness among sequential therapy, triple therapy, and Bismuth Pectin quadruple therapy for *H. pylori* eradication. *H. pylori* was eradicated effectively in all groups, with a success rate of over 80% that was consistent with the standards of Maastricht and other guidelines. Sequential therapy reached an eradication rate of 88.89%, with significant differences compared with other therapies ($P < 0.05$), but the healing rate of ulcers was not significantly different ($P > 0.05$) among the three groups. It was basically the same as the previous publications. Sequential therapy (omeprazole, clarithromycin, amoxicillin plus tinidazole are administrated in sequence for 10 d) has several advantages: the treatment duration is appropriately increased. Amoxicillin acts on the cell wall of bacteria in the first 5-d treatment to prevent clarithromycin pathway formation, thus increasing the sensitivity of the bacteria to clarithromycin, and effectively avoiding the *collateral resistance to* clarithromycin. Omeprazole, clarithromycin plus tinidazole are administrated for the remaining 5-d treatment. Clarithromycin acts on bacterial nucleic acid, restrains protein synthesis, stabilizes in acid environment, and increases the synergetic effects of the drugs and the cure rate of *H. pylori* infection.

Resistance to metronidazole and clarithromycin is the main reason for treatment failure of eradicating *H pylori*[33].

Early documents generally demonstrated that *H. pylori* primary resistance to clarithromycin is very low and usually not more than 10%. But for the past a few years, following the wide use of clarithromycin, *H. pylori* resistance to clarithromycin has gradually increased, so did the nitroimidazoles, and cross-resistance has also appeared. In China, the recent nationwide multi-center studies[34] have demonstrated that the resistance to clarithromycin increased to 27.6%, and resistance to metronidazole reached 75.6%. Furthermore, cross-resistance to metronidazoles appeared in 85.1% of clarithromycin-resistant *H. pylori* strains. It suggests that *H. pylori* resistance to clarithromycin and metronidazoles is an extremely serious problem. As resistance to clarithromycin also increased in Western countries, there has been a corresponding decrease in the eradication rate for *H. pylori* infection[4]. A study in Italy[35] presented that in the past 15 years, resistance to clarithromycin doubled from 10.2% in 1989-1990 to 21.3% in 2004-2005. But the resistance rate to metronidazole in adult is 10%-50% in Western countries and 77%-95% in developing countries[36]. The eradication rate of the sequential therapy in this study (88.89%) is lower than in Western countries (over 90%). According to the known mechanism of the proposal, it only improves the *H. pylori* sensitivity and prevents collateral resistance to clarithromycin. As resistance to metronidazole is extremely common in China, it has decreased the *H. pylori* eradication rate of the protocol.

Therefore, antibiotic resistance is the main cause of failure in *H pylori* eradication and beta-lactamase produced by resistant *H. pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy[1].

In short, the 10-d sequential therapy is significantly dominant compared with standard triple and bismuth pectin quadruple therapy, and adverse effects are not significantly different ($P > 0.05$). Therefore, the sequential therapy is a better choice of treatment for *H. pylori* eradication. But further researches are needed to formulate the strategies of sequential therapy and probe into the exact mechanism of eradicating *H. pylori*.

COMMENTS

Background

Clarithromycin and metronidazole resistance has increased substantially in recent years, and there has been a corresponding decrease in the eradication

rate for *Helicobacter pylori* (*H. pylori*) infection in most Western countries and China. Sequential therapy is a recent protocol for *H. pylori* eradication.

Research frontiers

To the authors' knowledge, no data are available about the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China. The present study aimed to compare the efficacy of a 7-d standard triple therapy, a 10-d bismuth pectin quadruple therapy and a 10-d sequential therapy in China.

Innovations and breakthroughs

The results denote that the 10-d sequential therapy is significantly dominant compared with standard triple and bismuth pectin quadruple therapy, and adverse effects are not significant different. The eradication rate of the sequential therapy in this study (88.89%) is lower than in Western countries (over 90%). As resistance to metronidazole and clarithromycin is extremely more common in China than in Western countries, it has decreased the *H. pylori* eradication rate of the therapy.

Applications

Sequential therapy is a better choice of treatment for *H. pylori* eradication in China. It may be suggested as the first-line protocol for eradicating *H. pylori*. Therefore, it may increase the eradicate rate, decrease the resistance to antibiotics, then decrease the prevalence of *H. pylori*-related diseases. However, the strategies of sequential therapy need further studies to fit for the situation of China.

Terminology

Sequential therapy refers to the idea of adding more antibiotics to the treatment regimen, but giving them in sequence rather than giving all 4 drugs together. Typically, this involves an initial 5-d therapy with a benign combination of drugs, followed by 5 d of two more antibiotics plus a proton-pump inhibitor.

Peer review

This is an interesting study that provides further strong support for sequential therapy being superior to other regimens. The study appears to have been designed well, but some details of the design and study structure are absent.

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CASE REPORT

Therapy-refractory gastrointestinal motility disorder in a child with c-kit mutations

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Abstract

Constipation and fecal impaction are frequent and distressing complaints in pediatric gastroenterology. Especially in neurologically handicapped children, treatment of severe forms of slow-transit constipation (STC) can be difficult. In the majority of cases, STC is of unknown etiology. However, in recent years, there is growing evidence that interstitial cells of Cajal (ICCs), which serve as electrical pacemakers and generate spontaneous electrical slow waves in the gastrointestinal tract, might play an important role in the pathophysiology of STC. It remains unclear whether morphological ICC alterations seen in affected patients are based on congenital developmental anomalies, or whether they are a consequence of long-term constipation with secondary damage of the gastrointestinal nervous system. To the best of our knowledge, we present the first case of a patient with histological alterations in ICC morphology who displayed multiple alterations of c-kit at the level of mRNA. The protein encoded by c-kit is the receptor tyrosine kinase Kit (CD117), which is crucial for development and function of ICCs. Therefore, these findings provide a new explanation for congenital alterations of ICC development that result in gastrointestinal motility disorders.

Key words: Slow-transit constipation; Interstitial cells of Cajal; c-kit

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INTRODUCTION

Chronic constipation is one of the most common complaints of patients in pediatric gastroenterology. Most cases can be classified as a functional disorder and are usually managed by dietary modifications and administration of oral laxatives.

Nevertheless, during childhood, some patients develop severe forms of slow-transit constipation (STC), associated with prolonged bowel passage and resistance

to medical treatment. Clinical symptoms can include low stool frequency, lack of urge to defecate, abdominal distention, bloating, and abdominal discomfort[1]. Although the pathophysiology has not yet been fully understood, visceral neuropathies[2], dysregulation of neurotransmitters[3], degeneration of enteric neurons[4], and alterations of interstitial cells of Cajal (ICCs)[5] are possible etiological mechanisms that have been discussed in the literature[6].

ICCs form a network that is widely distributed in all layers of the gastrointestinal tract. Myenteric ICCs are located to the myenteric plexus and integrate between the enteric nervous system and the muscle components of the bowel. In recent years, there has been growing evidence that ICCs serve as electrical pacemakers and generate spontaneous electrical slow waves in the gastrointestinal tract[7]. Slow waves organize gut contractions into phasic contractions that are the basis for peristalsis and segmentation. Important new insights have been gathered by studies on animals with loss-of function mutations in the c-kit signaling pathway[8,9]. The protein encoded by c-kit is the receptor tyrosine kinase Kit (CD117). The activation of Kit is crucial for development and function of the ICC phenotype in the gastrointestinal tract. Functional studies in c-kit-knock-out mice and in animals with specific mutations that modify the binding of the receptor ligand stem cell factor (SCF) have shown grossly underdeveloped networks of ICCs[10] with significantly reduced gastrointestinal motility[9]. Recognition of these physiological properties in animals has led to studies of ICCs in several human gastrointestinal motility disorders, especially in cases of idiopathic megacolon and STC. Nowadays, there is no doubt that damage or dysfunction of ICCs results in gut dysmotility.

Here, we present a new case of a patient with specific genetic alterations of the receptor tyrosine kinase Kit that led to abnormal ICC architecture and function in the gut, with severe chronic constipation.

CASE REPORT

The male patient was born to a healthy woman with an inconspicuous family history at the 36th gestational week. His birth weight and height were below the 3rd percentile. Vital signs were normal at birth (Apgar score 9/10/10). Physical examination showed cleft palate, low-set deformed ears, a flat nasal bridge, brachycephalus, strabismus, and oblique eye fissures with epicanthic skin folds. Laboratory investigations at birth revealed no abnormal findings. Chromosomal analysis did not detect any abnormalities. Developmental delay became evident by the end of the first year, accompanied by a significant failure to thrive. The patient neither learned to speak nor to walk unassisted. Spoon-feeding was possible, but was supported by a percutaneous gastrostomy.

At the age of 14 years, a malignant melanoma on the left lower leg was diagnosed and subsequently excised. In recent years, the patient developed recurring episodes of abdominal pain and chronic constipation, without evidence of stool-withholding behavior. Laboratory tests demonstrated no electrolyte abnormalities, anemia, hypothyroidism, or evidence of celiac disease or colitis. We used different laxatives without much benefit. By the age of 16 years, the patient had developed a severe megacolon and mechanical assistance for defecation was needed daily. Hinton's test showed considerably extended intestinal transit time. Although usually not the first choice in treating patients with slow transit, double-barreled ileostomy construction was performed to avoid further complications. During the procedure, we took intestinal biopsies from the ileum and sigmoid colon for histopathological analysis. After surgery, no laxative therapy was needed. Colonic diameter and the patient's complaints were significantly reduced.

Histological examination of the intestinal biopsies revealed abnormal morphology and an extremely low density of c-kit-positive ICCs in the colon (Figure 1). Immunohistochemistry showed normal staining of mast cells and neurons in the gut mucosa and submucosa. To investigate whether the lack of ICCs might be due to functionally relevant genetic alterations in the synthesis of the tyrosine kinase Kit, we used mast cells of the patient as easy-to-obtain Kit-expressing cells. Multiple genetic alterations of Kit were detected at the level of mRNA in mast cell progenitors, which resulted in changes of the deduced amino acid sequence (Figure 2): E270K, insertion of intron 20 between exons 20 and 21, which created a stop codon at amino acid position 935; and six isoforms of transcripts of c-kit, which are due to alternative pre-mRNA splicing (ins Q252; del GNNK510-513; del S715).

However, the examination of genomic mast cell DNA for the presence of another well known mutation of c-kit, the gain-of-function mutation D816V, which is found in > 80% of cases of systemic mastocytosis, was negative. In bone marrow examination, normal hematopoietic tissue was found, and in laboratory work-up, normal values for tryptase in serum and histamine metabolites in urine were detected. The absence of any hematopoietic abnormalities in our patient suggests appropriate secretion of SCF.

DISCUSSION

In patients with idiopathic megacolon and STC, it has been proposed that colonic dysmotility might result from alterations of neuronal cells, smooth muscle cells and/or ICCs in the gastrointestinal tissue. By now, histopathological and functional abnormalities of all three final effectors of gastrointestinal sensomotoric function have been reported in the literature. However, it still remains unclear whether these changes are primary, secondary or merely epigenomic[11]. Only a few specific histopathological abnormalities have been found and described to date[12]. Concerning ICCs, there have been two studies of patients with idiopathic megabowl[5,13] and four studies of patients with idiopathic constipation with decreased ICC density[6,14-16]. By contrast, another study of 63 patients with megacolon has shown no consistent alterations in colonic ICC histology[17].

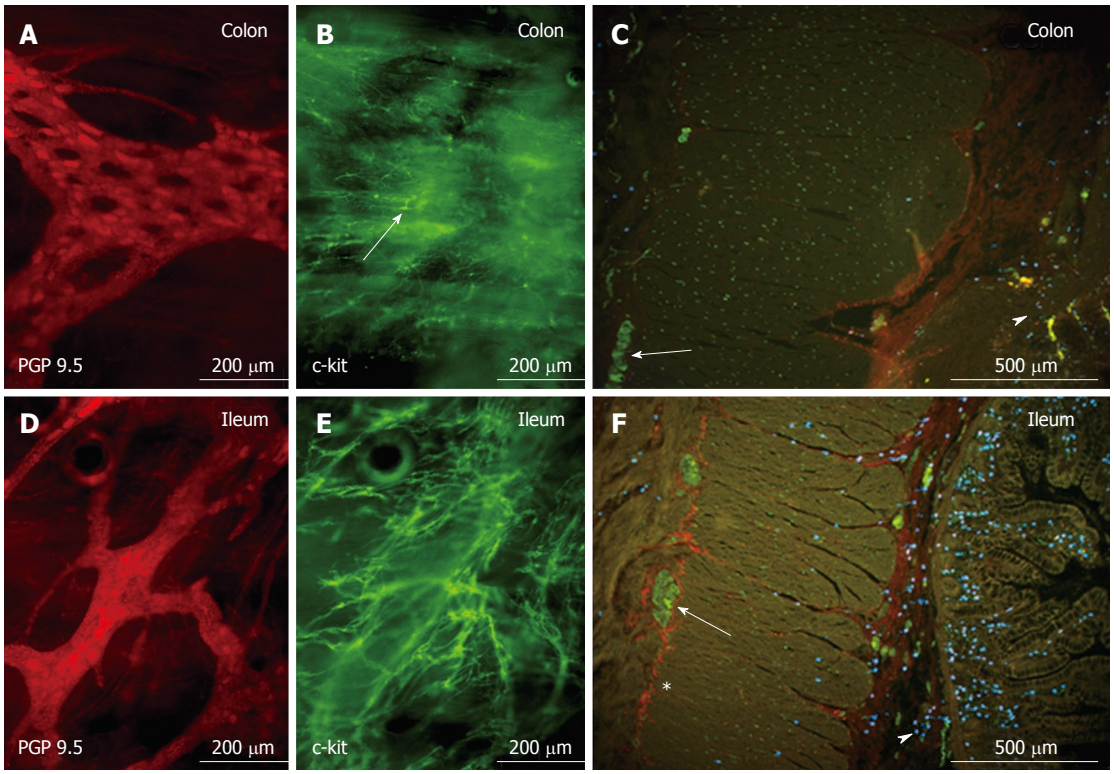

Figure 1 Whole mount preparation of the myenteric plexus of the patient's colon (A and B) and ileum (D and E), which shows abnormal morphology (arrow in B) and extreme low density of c-kit-positive interstitial cells of Cajal in the colonic biopsy specimen (B), while a dense interstitial cells of Cajal network with normal cell morphology was present in the ileum (E). Also remarkable are the holes in the colonic ganglia (A), which are very uncommon in young patients. D shows normal morphology and density of ganglia and protein gene product (PGP)-positive myenteric neurons in the ileum. Cross-sections of colon (C) and ileum (F) illustrate the normal appearance of myenteric interstitial cells of Cajal (ICCs) (*c-kit, red) in the ileum, but the complete absence of myenteric ICCs in the colon. (A and D: staining for the neuronal marker PGP 9.5, red; PH164, The Binding Site, Birmingham, UK; B and E: staining for c-kit as ICC marker, green; PC34, Oncogene, Boston, MA, USA; C and F: PGP-positive neurons (green) in a ganglion (arrow), mast cells in mucosa/submucosa (arrowhead), stained by a tryptase antibody, blue; MAB1222, Chemicon, Schwalbach, Germany.

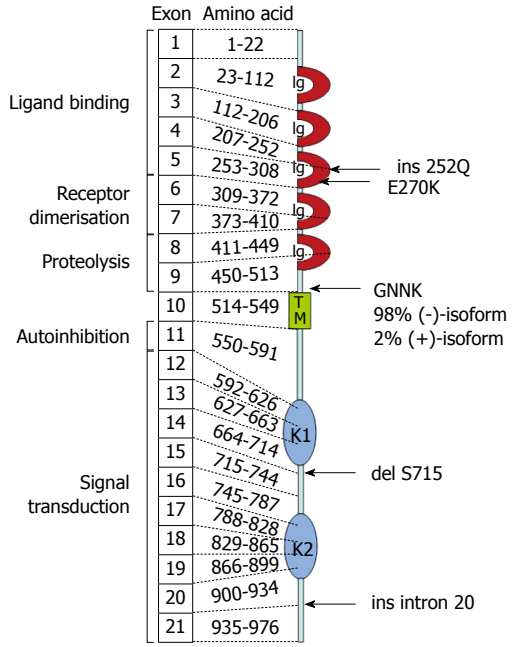

Figure 2 Schematic representation of the molecular and functional structure of the tyrosine kinase Kit (CD117), which indicated the patient's mutations in the deduced amino acid sequence. Details are discussed in the text (Ig, immunoglobulin-like domain; TM, transmembranous domain; K1, ATP-binding region of kinase domain; KI, kinase-insert-region; K2, region of phosphorylation of kinase domain).

In our patient, the biopsy specimen showed only a few ICCs in the ileum and complete absence of ICCs in the sigmoid colon, which is similar to single patients reported by Sabri *et al*[18] and Kenny *et al*[19]. However, in our patient, mutational analysis of Kit revealed multiple specific genetic alterations at the level of mRNA, which could have resulted in a loss of function of the Kit protein. In particular, mRNA that contains the premature stop codon at amino acid position 935 can be rapidly degraded via nonsense-mediated mRNA decay[20]. Accordingly, synthesis of the corresponding Kit protein would be reduced. The functional relevance of the six isoforms of Kit (Figure 2) cannot be assessed on the basis of the present data. In particular, the two GNNK-isoforms differ markedly in their functional activities, with the GNNK(-) isoform showing tumorigenic potential[21,22], which was almost exclusively detected in our patient. The point mutation E270K is probably only of minor relevance for the functional activity of Kit, because E270 is not highly conserved between species.

To date, animal studies have already shown that point mutations in the tyrosine kinase Kit correlate with abnormal intestinal contractions *in vitro*[23]. Accordingly, we suggest that the genetic alterations of Kit in our patient led to loss of protein function and to alterations in ICC architecture, and therefore, reduced bowel peristalsis. This

hypothesis provides a novel intriguing explanation for congenital interference with ICC development in cases of STC.

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CASE REPORT

Gastrojejunostomy followed by induction chemotherapy for incurable gastric cancer with outlet obstruction

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Abstract

A 72-year-old male gastric cancer patient with outlet obstruction underwent laparoscopic exploration. The examination disclosed intraperitoneal free cancer cells with no overt peritoneal, lymphatic, or hepatic metastasis. The patient underwent laparoscopy-assisted gastrojejunostomy (LAGJ) and started chemotherapy with S-1 plus cisplatin on postoperative day 13. Three course of the chemotherapy shrank the tumor markedly. Then, the patient underwent gastrectomy with a curative intent. Laparotomy revealed no intraperitoneal free cancer cells, and microscopically complete resection was achieved. The patient received S-1 chemotherapy as postoperative adjuvant treatment for 1 year, and is still alive with no evidence of peritoneal recurrence. LAGJ followed by S-1 plus cisplatin is one of the optional treatments that should be considered for patients with outlet obstruction as it may widen opportunities for potentially curative resection.

Key words: Gastric cancer; Outlet obstruction; CY1; Laparoscopy-assisted gastrojejunostomy; S-1 plus cisplatin

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INTRODUCTION

In gastric cancer, intraperitoneal free cancer cells, described as CY1 in the Japanese Classification of Gastric Carcinoma (JCGC), 2nd English Edition, are one of the incurable factors[1]. The term "CY1" means histologically remnant tumors and gastric cancer with CY1 is diagnosed as stage Ⅳ and associated with poor prognosis. It has been reported that the survival time of gastric cancer patients with CY1 but not with overt peritoneal and other incurable metastases (P0CY1) is almost the same as that of patients with gross peritoneal metastases[2]. However, several reports have revealed that S-1, an oral agent consisting of tegafur, gimeracil, and oteracil potassium at a molar ratio 1:0.4:1[3], might improve prognosis in gastric cancer patients with P0CY1[4,5]. Furthermore, the recent phase Ⅲ clinical trial named SPIRITS trial demonstrated that S-1 plus cisplatin prolongs the survival time of patients with advanced or recurrent gastric cancer compared to S-1 alone[6].

Gastric cancer with outlet obstruction (GCOO) is a type of advanced cancer arising from the distal third of

the stomach. GCOO is associated with not only food intake inability but also metastatic disease[7]. In particular, Japanese patients with GCOO are faced with an oncology-specific issue, namely the patients cannot receive the most promising chemotherapy with S-1 plus cisplatin for incurable gastric cancer because of the inability to ingest S-1 capsules. In such patients, palliative gastrectomy is commonly selected and chemotherapy with S-1 plus cisplatin is subsequently prescribed, if possible. However, the SPIRITS trial also revealed that a considerable number of patients administering S-1 plus cisplatin suffered from severe toxic events and withdrew from the treatment[6]. Palliative gastrectomy seems to be unsuitable for inducing patients with incurable GCOO swiftly to the highly effective chemotherapy with S-1 plus cisplatin.

To solve such a practical problem, we have devised a pioneering therapeutic strategy to facilitate early induction and reliable continuation of S-1 plus cisplatin as an induction treatment for GCOO patients with P0CY1. The strategy consists of two steps. The first step is laparoscopy-assisted gastrojejunostomy (LAGJ) to allow the patient to ingest food and induce chemotherapy with S-1 plus cisplatin, the second step is gastrectomy for complete resection of the tumor and postoperative chemotherapy using S-1 alone.

Here, we present a successfully treated patient with incurable GCOO under our new therapeutic strategy.

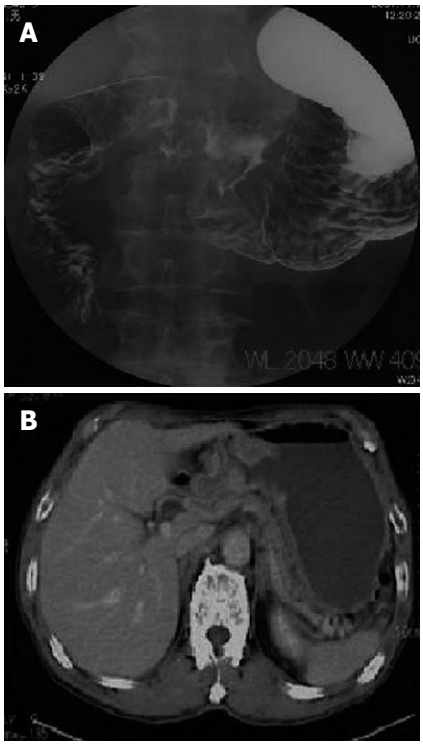

Figure 1 Gastrografin meal study revealing a tumor with ulceration at the gastric antrum, causing gastric outlet obstruction (A), and abdominal computed tomography scan showing remarkable thickness of gastric wall with no evidence of direct invasion to the pancreas head, and lymph node metastasis or distant metastasis (B).

CASE REPORT

A 72-year-old male suffering from GCOO was referred to our hospital. A gastrografin meal study revealed a type 3 tumor existing at the gastric antrum and causing gastric outlet obstruction (Figure 1A). Abdominal computed tomography scan showed neither lymph node metastasis nor distant metastasis (Figure 1B). Laboratory tests revealed that all the data were within normal limits.

Considering the high possibility of existing peritoneal metastases, we conducted laparoscopic exploration. Laparoscopic examination disclosed P0CY1, and we decided to conduct our new therapeutic strategy for GCOO with P0CY1. Laparoscopic examination was immediately converted into LAGJ with the intention of early induction of chemotherapy with S-1 plus cisplatin and the subsequent radical surgery was planned, that would enable potentially curable resection. The LAGJ was made in a Roux-en Y fashion in an antecolic manner (Figure 2A). We partitioned the stomach using a linear stapler, creating a small tunnel at the lesser curvature. Anastomosis was made between the distal stump of the proximal stomach and the jejunum. We located the partition at the upper part of the stomach, with the intent that cutting the tunnel would be a single procedure in reconstruction of the next surgery to be performed after chemotherapy (Figure 2B). The patient recovered swiftly and chemotherapy with S-1 plus cisplatin was started on postoperative day 13. The daily dose of S-1 was 120 mg/body (3 wk on and 2 wk off). Cisplatin (90 mg/body) was given intravenously on day 8 of S-1 administration. The patient received 3 cycles of the chemotherapy at the outpatient clinic without significant adverse events.

After 3 courses of the chemotherapy, examination revealed that the tumor shrank markedly although the gastric outlet obstruction still remained. The patient underwent laparotomy with a curative intent. Surgical exploration revealed that there was no metastasis to the peritoneum or the liver, and lavage cytology was negative. The tumor was excised with distal gastrectomy and D2 lymph node dissection (Figure 2C). Reconstruction was not necessary because we could retain the proximal gastrojejunostomy as a reconstruction route, which was previously made at the LAGJ.

Grossly, the resected specimen had a shallow depressed lesion at the antrum, which appeared to be only fibrosis (Figure 3). Histopathological examination revealed that the fibrotic change generated from the chemotherapy extended widely and live cancer cells were found throughout the whole gastric wall. Out of the 34 dissected lymph nodes, cancer metastasis was found in a single lymph node at station No. 7. The gastric cancer was finally diagnosed as T3, N2, H0, P0, CY0, M0, stage ⅢB, based on the JCGC, 2nd English Edition.

The patient was discharged on postoperative day 8 and underwent subsequent postoperative chemotherapy with S-1 alone for 12 mo. The patient is still alive and shows no evidence of peritoneal recurrence 20 mo after the initial surgery.

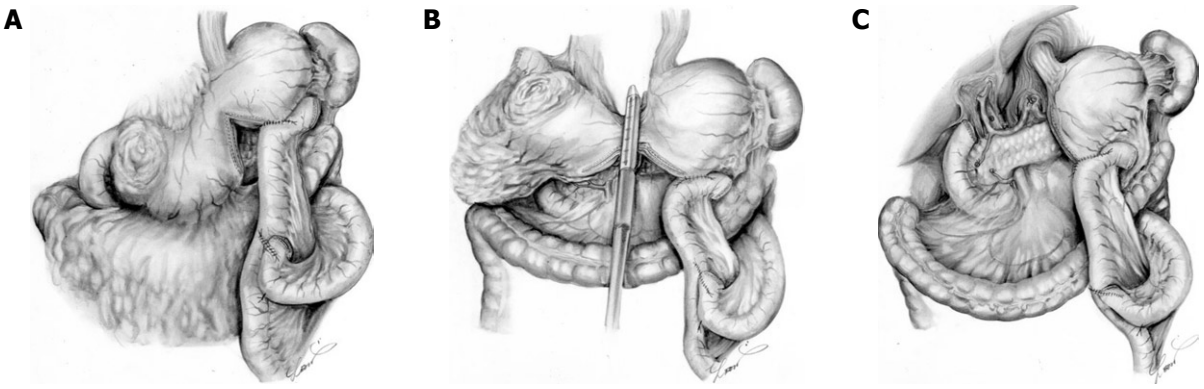

Figure 2 Laparoscopy-assisted partitioning gastrojejunostomy conducted in a Roux-en Y fashion in an antecolic manner (A), cutting the tunnel as a single procedure in reconstruction of the second surgery after chemotherapy (B), and the completely excised tumor with distal gastrectomy and D2 lymph node dissection (C).

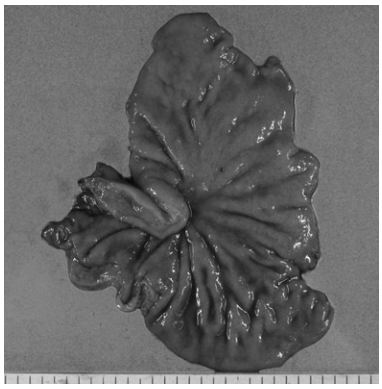

Figure 3 Resected specimen showing a grossly shallow depressed lesion as only fibrosis at the antrum with live cancer cells found in the fibrotic tissue throughout the whole gastric wall at histopathological examination.

DISCUSSION

We presented a treated patient suffering from unresectable GCOO. The patient was treated with our new therapeutic strategy for GCOO with P0CY1. He underwent LAGJ and was induced swiftly to chemotherapy with S-1 plus cisplatin as an induction treatment, followed by potentially curative gastrectomy. We reported the present case because we believe in its importance in devising a novel therapeutic strategy for GCOO with P0CY1.

In Japan, it is considered important to facilitate immediate induction and reliable continuation of S-1-based chemotherapy for the treatment of unresectable and recurrent gastric cancer. In particular, S-1 plus cisplatin, demonstrating a response rate (RR) of 54% and a median survival time (MST) of 13 mo in the SPIRITS trial[6], is recommended as the first-line chemotherapy. Recent clinical trials in other countries, such as the V325 study and the REAL-2 trial, demonstrated that RRs of docetaxel, cisplatin, and 5-fluorouracil (DCF) and epirubicin, oxaliplatin, and capecitabine (EOX) are 37.5% and 47.9%, MSTs of DCF and EOX are 9.2 and 11.2 mo, respectively[8,9]. S-1 plus cisplatin is more favorable than the other regimens tested in Western countries, and is the first choice of treatment for unresectable and recurrent gastric cancer in Japan.

There are two possible options for induction to S-1 plus cisplatin for unresectable GCOO. First, palliative gastrectomy is initially done, followed by chemotherapy with S-1 plus cisplatin. Second, methods other than gastrectomy, such as bypass surgery or metallic stent insertion to enable patients to ingest food and S-1 capsules, are performed with S-1 plus cisplatin subsequently prescribed[10,11]. There is a major problem in the first option, i.e. the feasibility of S-1 plus cisplatin after recent gastrectomy has not been established yet. S-1 plus cisplatin is a toxic regimen and the SPIRITS trial demonstrated that more than 30% of the patients assigned S-1 plus cisplatin suffered from grade 3 to 4 anorexia and myelosuppression, and withdrew from the trial because of toxic events[6]. It is possible that patients who undergo palliative gastrectomy for GCOO fail in immediate induction and reliable continuation of chemotherapy with S-1 plus cisplatin. In contrast, the second option possibly enables patients to more immediately receive chemotherapy with S-1 plus cisplatin and more reliably continue it. In the present case, we conducted LAGJ to induce immediate ingestion of food and S-1 capsules, considering that LAGJ was reported to minimally suppress the patients' immune function and enable earlier recovery of bowel movement[12]. In fact, the present patient started S-1 plus cisplatin on day 13 after LAGJ and did not suffer from severe toxicities.

In the present case, we chose S-1 plus cisplatin as the induction treatment for gastric cancer with P0CY1, and CY1 were eventually eliminated. CY1 are one of the most chemosensitive lesions among the metastases. Nakagawa *et al*[13] revealed that 61% of patients who receive preoperative chemotherapy have no free cancer cells at the time of the second surgery. Satoh *et al*[14] reported that CY1 observed by staging laparoscopy can be eliminated by preoperative chemotherapy with S-1 plus cisplatin in 7 of 10 patients. Based on these favorable data, we intended to perform curative gastrectomy for the present patient and were actually able to do so. However, whether this result contributes to survival benefits is unclear. Satoh *et al*[14] also

described that 4 of 7 responders to preoperative chemotherapy for CY1 remain free from peritoneal metastasis. Our patient presented with no signs of peritoneal recurrence even though he initially had stage Ⅳ gastric cancer. However, whether the therapeutic strategy adopted in the present case truly provides survival benefits or not, needs a longer follow-up of the patient.

Chemotherapy with S-1 plus cisplatin is suitable for gastric cancer with P0CY1. Thus, LAGJ followed by induction chemotherapy with S-1 plus cisplatin is a possible strategy if the patient has GCOO and complies strictly with the regimen.

In conclusion, LAGJ, followed by induction chemotherapy with S-1 plus cisplatin and subsequent gastrectomy with curative intent, is one of the relevant strategies for GCOO with P0CY1.

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS