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Contents

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Contents

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EDITORIAL

Current position of vasoconstrictor and albumin infusion for type 1 hepatorenal syndrome

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Abstract

Spontaneous bacterial peritonitis (SBP), refractory ascites, hepatorenal syndrome (HRS), hyponatremia and hepatic encephalopathy are complications which frequently happen during a clinical course of decompensated cirrhosis. Splanchnic and peripheral vasodilatation, increased intrarenal vasoconstriction and impaired cardiac responsive function are pathological changes causing systemic and hemodynamic derangement. Extreme renal vasoconstriction leads to severe reduction of renal blood flow and glomerular filtration rate, which finally evolves into the clinical feature of HRS. Clinical manifestations of type 1 and type 2 HRS come to medical attention differently. Patients with type 1 HRS present as acute kidney injury whereas those with type 2 HRS will have refractory ascites as the leading problem. Prompt diagnosis of type 1 HRS can halt the progression of HRS to acute tubular necrosis if the combined treatment of albumin infusion and vasoconstrictors is started timely. HRS reversal was seen in 34%-60% of patients, followed with decreasing mortality. Baseline serum levels of creatinine less than 5 mg/dL, bilirubin less than 10 mg/dL, and increased mean arterial pressure of over 5 mmHg by day 3 of the combined treatment of vasoconstrictor and albumin are the predictors of good response. Type 1 HRS can be prevented in some conditions such as albumin infusion in SBP, prophylactic antibiotics for upper gastrointestinal hemorrhage, albumin replacement after large volume paracentesis in cirrhotic patients with massive ascites. The benefit of albumin infusion in infection with primary source other than SBP requires more studies.

Key words: Albumin; Acute kidney injury; Hepatorenal syndrome; Cirrhosis; Vasoconstrictor

Core tip: Type 1 hepatorenal syndrome (HRS), which presents as acute kidney injury, is an uncommon, but critical problem in decompensated cirrhosis. The most common precipitating factor is infection especially spontaneous bacterial peritonitis. The combined regimen of albumin and vasoconstrictor is the pharmacotherapy

of choice for type 1 HRS based on pathogenic mechanisms of peripheral and splanchnic vasodilatation. Prompt treatment with the combined regimen can lead to HRS reversal in 34%-60% of patients. Type 1 HRS can be prevented in cirrhotic complications such as albumin infusion for spontaneous bacterial peritonitis, large volume paracentesis with albumin replacement, and prophylactic antibiotics for upper gastrointestinal hemorrhage.

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DEFINITION AND TYPES OF HEPATORENAL SYNDROME

In 2004, the Acute Dialysis Quality Initiative workgroup developed a consensus definition and classification for a new terminology of acute renal failure which was termed acute kidney injury (AKI) to reflect the entire spectrum of acute renal failure[1]. In 2007, the AKI Network group proposed a revision of the criteria of AKI and redefined AKI as an absolute increase in serum creatinine level of $\geqslant$ 0.3 mg/dL, or an increase in serum creatinine level > 1.5 times from baseline, or a reduction in urine output < 0.5 mL/kg per hour for > 6 h[1]. AKI that develops in patients with decompensated cirrhosis can be triggered by volume depletion, infection or nephrotoxic acute tubular necrosis, primary renal parenchymal diseases, obstructive nephropathy and hepatorenal syndrome (HRS).

Since the first definition and the diagnostic criteria of HRS were established in 1994, HRS has been widely accepted as a reversible functional renal failure[2]. The International Ascites Club met in 2007 and set up a consensus on a new definition, diagnostic criteria and recommendation for the treatment of HRS[2]. HRS is divided into two types: type 1 and type 2 HRS[2,3]. Type 1 HRS is typified by rapid deterioration of renal function as defined by a rising of serum creatinine level to over 2.5 mg/dL in less than 2 wk, and is characteristically presented as AKI[2-5]. Type 2 HRS runs a slower progressive clinical course with a median survival of 6 mo, and is classically associated with refractory ascites[2-5]. Patients with decompensated cirrhosis or acute on chronic liver failure who suffer from type 1 HRS would present with a critical illness, carrying a grave prognosis and having high mortality[5,6]. The consensus emphasized on the potential reversibility of type 1 HRS as a functional renal failure and the role of spontaneous bacterial peritonitis (SBP) as an important precipitating factor of HRS[2,3,5]. Contrasting with a previous consensus, this new definition gives an importance to HRS which was precipitated by SBP without ongoing shock. The panel emphasized that the treatment for HRS should be started early in the clinical course of SBP-precipitated HRS[2,4,5].

Incidences of HRS in patients with decompensated cirrhosis who develop ascites was equal to 18% after 1 year, and could go up to 39% after 5 years[4]. Apart from SBP, bacterial infection with primary sources of infection other than SBP, upper gastrointestinal hemorrhage, large volume paracentesis without albumin administration, and severe acute alcoholic hepatitis have been reported to precipitate type 1 HRS[4,5].

PATHOGENESIS OF HRS

While liver failure in decompensated cirrhosis progresses, splanchnic and peripheral arterial vasodilatation become markedly prominent with the development of hyperdynamic circulation[2,4,5]. Increased local release of vasodilators, especially nitric oxide, is the principal cause of the vasodilatation[2,4,5,7,8]. Proinflammatory cytokines release after the occurrence of SBP aggravates splanchnic and peripheral vasodilatation[3,7,8].

Relative reduction of intravascular pressure from peripheral and splanchnic vasodilatation stimulates renin-angiotensin, aldosterone and sympathetic nervous system, causing the release of systemic and renal vasoconstrictors[2,4,5,7,8]. Intense renal vasoconstriction from vasoconstrictive hormones leads to markedly low renal perfusion and a significant reduction of glomerular filtration rate[5,7-9]. Suboptimal cardiac function as a consequence of impaired systolic and diastolic stimuli responses is an intensifying pathogenic mechanism in decompensated cirrhosis with type 1 HRS[2-5,7-9].

TREATMENT OF TYPE 1 HRS

Liver transplantation is the definite treatment for type 1 and type 2 HRS[3]. From a recent retrospective study in decompensated cirrhotic patients with type 1 HRS who underwent liver transplantation, type 1 HRS resolved in 75.8% of patients after transplantation[10]. The predictor of HRS non-reversal at post-liver transplant was the duration of pre-transplant dialysis[10]. However, because of scarcity of liver grafts and the complexity of patient conditions, the first line treatment of type 1 HRS, which should be considered, are vasoconstrictors plus albumin infusion[2,4,5,7,8]. Vasoconstrictors such as terlipressin help to improve hepatic and renal hemodynamics, decreasing hepatic venous pressure gradient and portal venous blood flow, and raising mean arterial pressure[11]. Terlipressin should be initiated at 0.5-1 mg every 4-6 h[9]. The dose of terlipressin can be increased every 2 d if there is no clinical response, to a maximum of 12 mg/d[2,5]. Terlipressin should be discontinued if there is no response seen for reduction of serum creatinine level after 3 d[2,5]. Treatment duration should be expanded until type 1 HRS resolves, or having received the combined treatment for a maximum of 14 d[2,5]. Albumin should be started at 1 g/kg on the

first day, and the dose can be increased to a maximum of 100 g if there is no or inadequate clinical response, followed by 20-40 g/d on the following days[2,5]. From a previous study, the complete response rate could reach 60% in patients with type 1 HRS after the combined pharmacotherapy[2]. The renal dysfunction was reversible in 34%-60% of patients and the recovery of renal function was sustainable in 70%-80% of patients after the treatment was discontinued[4,12-14]. After the completed treatment of terlipressin and albumin, type 1 HRS rarely recurs, if it is reversal[15]. Baseline serum levels of creatinine < 5 mg/dL and bilirubin < 10 mg/dL, and an increase mean arterial pressure ⩾ 5 mmHg on day 3 are predictive factors of good response to terlipressin and albumin[15-18]. From the results of meta-analysis studies, besides the benefit in HRS reversal, the combined treatment is helpful in reducing mortality[13,19]. A meta-analysis showed that the treatment of type 1 HRS with terlipressin led to increased incidence of cardiovascular events including cardiac arrhythmia, myocardial ischemia, intestinal infarction and hypertension comparing to the control groups (14% *vs* 0%)[13]. When terlipressin was compared with other vasoconstrictor drugs such as norepinephrine, there was no difference in term of the efficacy in HRS reversal between both drugs[14,20,21]. However, adverse events during the course of type 1 HRS treatment were less often seen in the type 1 HRS patients who were treated with norepinephrine[20,21]. The cost of norepinephrine is cheaper than terlipressin, but the drug requires continuous intravenous infusion under close monitoring of vital signs in intensive care units. A recent randomized controlled trial revealed that terlipressin plus albumin was more effective in HRS reversal than midodrine and octreotide plus albumin[22].

PREVENTION OF TYPE 1 HRS

In decompensated cirrhotic patients who developed SBP, albumin infusion (1.5 g/kg at the first diagnosis of infection together with 1 g/kg at 48 h later), comparing to a control group, can decrease the development of type 1 HRS (10% *vs* 33%) and hospital mortality (10% *vs* 29%)[5,23]. For type 1 HRS with ongoing infection, treatment with antibiotics alone can reverse type 1 HRS in only 33% of treated patients[24]. The patients who did not have HRS reversal were associated with poorer prognosis and higher mortality than those who were in the treatment response group[24]. Thus, for type 1 HRS precipitated by infection, early management with vasoconstrictors plus albumin infusion along with antibiotics tends to be more useful and logical than treatment with antibiotics alone. A proof of concept study was recently reported[25]. Early treatment with terlipressin and albumin is effective and safe in type 1 HRS patients with sepsis[25,26]. Renal improvement is seen in two-thirds of decompensated cirrhotic patients with sepsis and type 1 HRS after the combined treatment of terlipressin and albumin[25].

For cirrhosis with infection with primary sources other than SBP, albumin infusion in combination with antibiotics have more advantages in improving renal and circulatory function than using antibiotics alone[27]. The use of albumin infusion in patients with infection other than SBP tended to reduce the incidences of HRS and improve survival[27].

Antibiotic prophylaxis in decompensated cirrhosis with upper gastrointestinal bleeding was showed to reduce bacterial infection and mortality[8]. Albumin replacement (8 g per 1 liter of ascites removal) after large volume paracentesis prevents renal and electrolyte impairment and the activation of endogenous vasoactive hormones[28,29]. The use of pentoxifylline in severe acute alcoholic hepatitis is useful in reducing renal dysfunction and type 1 HRS[8].

CONCLUSION

Type 1 HRS is a critical condition affecting patients with decompensated cirrhosis. It is an infrequent event, but has a grave prognosis. Management of type 1 HRS requires very rapid diagnosis according to the consensus criteria of the International Ascites Club (2007) and exclusion of other causes of AKI. The role of renal biomarkers for early diagnosis of type 1 HRS is currently under investigation. Unless the diagnosis of type 1 HRS is made promptly and the combined treatment of albumin and vasconstrictors is started quickly, the complete response of acute renal dysfunction may not be achieved, and poor outcomes with high mortality can be the consequences. The advantage of early treatment of vasoconstrictors plus albumin is fully supported from the study of sepsis-related type 1 HRS. Until now, data has not confirmed the benefits of terlipressin over norepinephrine or other vasoconstrictive drugs. The choice of vasoconstrictors depends on the suitability and availability of the medications and medical facilities.

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REVIEW

Primary biliary cirrhosis: From bench to bedside

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Abstract

Primary biliary cirrhosis (PBC) is a chronic non-suppurative destructive intrahepatic cholangitis leading to cirrhosis after a protractive non cirrhotic stage. The etiology and pathogenesis are largely unknown and autoimmne mechanisms have been implicated to explain the pathological lesions. Many epitopes and autoantigens have been reported as crucial in the pathophysiology of the disease and T and B cells abnormalities have been described, the exact pathways leading to the destruction of small intrahepatic ductules are mostly speculative. In this review we examined the various epidemiologal and geoepidemiological data as well as the complex pathogenetic aspects of this disease, focusing on recent *in vivo* and *in vitro* studies in this field. Initiation and progression of PBC is believed to be a multifactorial process with strong infuences from the patient's genetic background and by various environmental factors. The role of innate and adaptive immunity, including cytokines, chemokines, macrophages and the involvement of apoptosis and reactive oxygen species are outlined in detailed. The current pathogenetic aspects are presented and a novel pathogenetic theory unifying the accumulated clinical information with *in vitro* and *in vivo* data is formulated. A review of clinical manifestations and immunological and pathological diagnosis was presented. Treatment modalities, including the multiple mechanisms of action of ursodeoxycholate were finally discussed.

Key words: Primary biliary cirrhosis; Innate immunity; Adaptive immunity; Ursodeoxycholate; Chemokines; Macrophages; Cytokines

Core tip: Primary biliary cirrhosis (PBC) is a chronic non-suppurative destructive intrahepatic cholangitis leading to cirrhosis of largely unknown pathophysiology. We examined the epidemiological and geoepidemiological data as well as the pathogenetic aspects of PBC. A novel pathogenetic theory unifying the accumulated clinical information with *in vitro* and *in vivo* data is formulated. A review of clinical manifestations and immunological

and pathological diagnosis was presented. Treatment modalities, including the multiple mechanisms of action of ursodeoxycholate are discussed.

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INTRODUCTION

Primary biliary cirrhosis (PBC), also known as chronic non-suppurative destructive intrahepatic cholangitis is characterized by a gradual destruction of small intrahepatic bile ducts. Addison *et al*[1] in 1851 published the first report of a patient with obstructive jaundice without evidence of large bile duct abnormalities. Dauphinee *et al*[2] in 1949 first used the term PBC but it was Ahrens *et al*[3] in 1950 that reported on a group of older women with progressive jaundice, pruritus, and hepatosplenomegaly. The basic histological description as chronic intrahepatic non-suppurative destructive cholangitis was reported by Rubin *et al*[4] in 1965.

Autoimmune mechanisms have been implicated to explain the disease evolution of a condition which is in fact a member of the wider vanishing bile duct syndrome. Many epitopes and autoantigens have been reported as crucial in the pathophysiology of the disease and T and B cells abnormalities have been described, the exact pathways leading to the destruction of small intrahepatic ductules are mostly speculative. However the underlying pathways leading to liver damage are still under investigation. In this review, we examine epidemiological, pathogenetic and clinical characteristics of PBC. Finally we compile the clinical information and the novel data gathered from *in vivo* and *in vitro* studies that are presented in this review in order to propose a unifying hypothesis for the pathogenesis of this complex disease.

EPIDEMIOLOGY

There is a large variation of the incidence and prevalence of PBC worldwide (Table 1). Moreover there is a tendency to increase over the years. Thus, studies from the region of Victoria in Australia demonstrated that in an earlier population-based survey a prevalence of 19.1 per million was reported[5] while ten years later in the same region a 10-fold higher incidence was reported[6]. In a review of 37 older and more recent published studies the incidence of PBC ranged from 0.7 to 49 per million per year and the prevalence was estimated to range from 6.7 to 402 per million, with a tendency for higher values in the most recent studies[7].

A prospective study performed in 2006-2007 in France shows that the incidence was 9 per million per year, with an estimate of 36 per million in women over 45 years with a prevalence of 207 per million[8], according to a recent systematic review the prevalence of PBC ranges from 1.9 to 40.2 per 100000 inhabitants[9].

Although it is usually stated that the disease is more prevalent in Northern Europe (*e.g.*, England and Scotland)[10,11] or the Northern United States (*e.g.*, Minnesota)[12], we recently reported a prevalence of 365 cases/million population in the island of Crete indicating that in South Europe the disease is highly prevalent with a figure among the higher published in the world[13].

Spatial and geographic differences in prevalence of PBC has been reported in several studies[14-16]. In the North East of England there was a clear spatial distribution of patients with clusters of cases identified in several urban areas (up to 13 cases/km^2). No demographic or geographical factors could explain this variation, although the presence of unidentified environmental factor(s) was suggested[17]. Geographic clustering of PBC has also been reported in coastal First Nations of British Columbia, where disease has been recorded to be as high as 1 in 4 within generations of well-characterized multiplex families[18]. In our own study we identified loci of very high prevalence within the homogeneous population of the island of Crete. Prediction map identified areas of high risk (11 patients per 50 km^2) in the Eastern part, while low risk areas (122 patients per 50 km^2) were located in the Western part of the island[13].

It should be noted that all epidemiological estimations may represent only the tip of the iceberg since it has been reported and confirmed that the incidence of antimitochondrial antibody (AMA) positivity without liver disease is two times higher than the incidence of AMA positivity with liver disease[19,20]. The significance of these findings is unclear. Possibly they do represent a hidden reservoir of future overt PBC or alternatively this is only an immunological abnormality without clinical consequences. If one adds to these observations the very long and often asymptomatic course of the disease, it is clear that epidemiological studies in PBC have many problems as presented in a recent review[21].

PATHOGENESIS

As with many other diseases of unclear etiology it is stated that PBC is an autoimmune disease associated with genetic and environmental factors. Both, autoimmunity[22] and environmental factors[23,24] have been emphasized while several genetic associations in particular with recent genome-wide analyses[25-27] have been published. However this is a very general description that may fit to other diseases as distant to PBC as inflammatory bowel disease, rheumatoid arthritis or Hashimoto's thyroiditis. We will try therefore to delineate these complex association and propose an unifying model to accommodate most aspects of the mechanism leading to the disease.

Table 1 Indicative studies and metanalysis reporting incidence and prevalence of primary biliary cirrhosis

Ref.	Year of publication	Country/region	Period	Incidence (/10^6)	Prevalence (/10^6)
Watson *et al*[5]	1995	Australia/Victoria	1974-1991		19.1
Metcalf *et al*[11]	1997	United Kingdom/Newcastle	1987-1994	14.0-32.0	180.0 (1987) 240.0 (1994)
James *et al*[10]	1999	United Kingdom	1987-1994		201.9 (1987) 334.6 (1994)
Kim *et al*[12]	2000	United States/Minnesota	1975-1995	27.0	402.0
[1]Prince *et al*[7]	2003	Multiple		0.7-49.0	6.7-402.0
Sood *et al*[6]	2004	Australia/Victoria	1990-2002		51.0
Corpechot *et al*[8]	2008	France	2006-2007	9.0	207.0
[1]Boonstra *et al*[9]	2012	Multiple		3.3-58.0	19.1-402.0
Koulentaki *et al*[13]	2014	Greece/Crete	1990-2010	20.9	365.0

[1]Metanalysis.

PBC AS A GENETIC DISEASE

Epidemiological reports have shown that PBC is a heritable condition with a well proven familial prevalence. Family history of the disease has been reported to vary between 1.33% and 9.00%[9]. In our disease population familial PBC was found to be a high 9.9%. This may reflect the fact that in many parts of the island of Crete societies are relatively close and marriages between distant relatives are common[28]. The relative sibling risk in PBC has been estimated to be 10.5, a figure very close to other classical autoimmune disorders[29]. The existence of a first degree relative with the disease is an independent risk factor disease appearance, with an odds ratio of 6.8-10.7[30,31]. Moreover in monozygotic twins a concordance rate of 0.63 for PBC has been reported, the highest reported among autoimmune diseases[32]. All these data do imply that a strong genetic background participate to the pathophysiology of PBC.

Most gene associations are derived from human leukocyte antigen (HLA) associations. HLA data in European populations has been produced from GWAS and iCHIP studies[33] as for example in a large Italian cohort[34]. Data on HLA alleles were also provided from iCHIP studies by Liu *et al*[35] and Juran *et al*[36]. When these studies are combined it seems that in European populations, PBC has been associated with either risk haplotypes (DRB1*08:01-DQA1*04:01-DQB1*04:02 and DRB1*04:04-DQB1*03:02) or protective haplotypes (DRB1*11:01-DQA1*05:01-DQB1*03:01 and DRB1*15:01-DQA1*01:02-DQB1*0602). In the Italian study, the HLA associations were driven by their DRB1 alleles, either the risk alleles DRB1*08 and DRB1*14, or the protective allele DRB1*11.

Apart from HLA associations, genes related to the regulation of components of the immune system appear to be involved in PBC. Candidate genes like SPIB are implicated in the development of plasmacytoid dendritic cells and natural killer (NK) cells, or to the development of dendritic cells, like IRF5 and IRF8[37-39]. Genes that regulate innate immune responses have also been associated with PBC. Thus negative regulators of antigen presenting cells, like RPS6KA4 and DENND1B participating in the signal pathways by pattern recognition receptors and TNFRSF1A respectively are among candidate genes[40-43]. IRF5 in particular is an important mediator since it is also involved in macrophage polarization and Th1-Th17 differentiation and therefore might be a significant link between innate and adaptive immune response[44]. Genes that regulate adaptive immune response have also been associated with PBC like those that drive activated CD4+ve Th0 cells to differentiate into Th1 cells by IL-12 interacting with IL-12R.

Four GWAS and two iCHIP studies of PBC have been recently published[25,27,35,36,45-47].

GWAS studies of PBC from Japan confirmed the genetic heterogeneity of PBC[27]. Risk loci significant in Europeans were not associated with PBC in Japan, including 1p31 (the IL-12RB2 locus, P 1/4 0.054), 3q25 (the IL-12A locus, P 1/4 0.342) and 19q13 (the SPIB locus, P 1/4 0.180). By contrast, other loci like 9p32 (TNFSF15) and 11q23 (POU2AF1) important for the Japanese, were not identified in European populations[25,45,46]. Two excellent reviews on PBC genetics have been recently published[48,49].

PBC AND ENVIRONMENTAL FACTORS

Despite extensive studies of specific genes only weak associations were established in PBC. Environmental factors may therefore be equally important. The presence of geographical disease clusters provides evidence for the participation of largely unknown environmental factors in the pathogenesis of PBC. These factors have been recently reviewed[50,51]. A recent geoepidemiological study from Crete with a rather homogeneous and stable population has shown a large heterogeneity in the time-spatial distribution which together with the suggested route of disease spread strongly indicates the importance of environmental factors[13].

Infections, through the mechanism of molecular mimicry, are strong candidate environmental factors in the pathogenesis of PBC. A possible candidate epitope is the mycobacterial hsp65 sharing a common motif with the PDC-E2 antigen[52]. The same motif has been identified in Lactobacillus delbrueckii as the target of IgG3 antibodies in PBC sera[53]. This motif, namely the PDC-E2 221-226 is a candidate common epitope in the

model of molecular mimicry. However another pathogen seems to be the most likely candidate in molecular mimicry. Reports have been focused on the intestinal commensal proteobacterium *Novosphingobium aromaticivorans* (*N. aromaticivorans*), as an etiological factor in many cases of human PBC[54,55]. *N. aromaticivorans* was found in fecal samples from 25% of patients with PBC as well as in controls. PDC-E2 from this bacteria has high homology to human PDC-E2, and AMAs in serum samples from patients with PBC have a 1000-fold stronger reaction against *N. aromaticivorans* PDC-E2 than against *Escherichia coli* PDC-E2[56,57]. Based on these clinical studies the group of Gershwin has reported on a mouse model of a liver disease closely resembling human PBC, initially triggered by this bacterium[58]. The pathology of liver lesions and the presence of anti-PDC-E2 antibodies were related to the mouse genetic background, the liver persistence of Novosphingobium and the liver infiltration by NKT-cells activated by Novosphingobium glycosphingolipids. The interaction of glycosphingolipids of the Novosphingobium with NKT-cells may be the link between an environmental pathogen and the immune reactions that have been described in PBC. A previously unidentified, infection with Novosphingobium might also explain the reported redistribution of NKT-cells from the blood to the livers of PBC patients and the biliary epithelial expression of CD1d[59,60].

Other infective factors associated with PBC include lipopolysaccharide (LPS), lipoteichoic acid, *Helicobacter*, β-retrovirus, *P. acnes*, *E. coli* and *Chlamydia*. The evidence however for their implication is not as strong as for the previous infectious agents[61].

Non microbial environmental factors seem to be related to disease progression or disease severity. Thus familiar occurrence of disease, smoking, urinary tract infections or other autoimmune conditions seem to be detrimental while oral contraceptives might have a protective role[31].

There might be a link between genetics and the influence of environmental factors**.** The mitochondrial autoantigen PDC-E2 contains lipoic acid; PDC-E2 captures electrons to alternate oxidation and reduction of a disulfide bond within lipoic acid. In genetically susceptible individuals, drugs or chemicals that modify this disulfide bond might lead to loss of tolerance to PDC-E2. This protein is well conserved throughout evolution, so loss of tolerance could also result from an immune response against similar epitopes in bacteria (molecular mimicry)[58]. In addition to bacteria, chemical xenobiotic agents might induce PBC[14]. Some halogenated organic compounds can attach to specific epitopes of PDC-E2 and induce production of antibodies that have higher affinities for modified mitochondrial epitopes than for normal PDC-E2. One such agent is 2-octynoic acid, which is a component of many cosmetics like nail polish; *in vivo* and *in vitro* data indicate its role in autoimmune biliary disease[62,63].

Non-obese diabetic mice injected with bovine serum albumin-conjugated 2-octynoic acid had high titers of AMAs and developed histologic features of PBC, including liver granulomas and infiltration of portal tracts with CD8+ve T lymphocytes[64]. However in our population of PBC patients no association of the disease with the use of nail polish was found. This might be due to either a different specific genetic background of our patients or to the lack of significant quantities of octynoic acid in the nail polish brands used by our patients[28].

PBC AND HUMOURAL IMMUNITY

A characteristic of PBC is considered to be the loss of tolerance to a mitochondrial antigen, the PDC-E2, a member of the 2-oxoacid dehydrogenase complexes (2-OADC). The antigenic epitopes are located within the inner lipoyl-binding domain overlapping amino acids 212-226[65-67]. Earlier reports based on the inhibition of 2-OADC enzymatic activity by AMA and the presence in the portal tracts of B cells producing anti-PDC antibodies,suggested a pathogenetic role for AMAs[68]. The best evidence for their pathogenetic role is the presence of secretory IgA anti-PDC in patients saliva, bile and urine of patients which inhibit the enzyme activity[69-71]. It is however possible that AMAs might indirectly implicated in pathogenesis through apoptotic biliary bodies that activate innate responses[72]. Moreover, AMAs is not a specific finding in PBC, since they are occasionally present in other autoimmune and non-autoimmune liver diseases. Therefore loss of tolerance to PDC-E2 alone does not seem to be sufficient to cause autoimmune cholangitis[73,74]. Against the pathogenetic role of AMAs is also the presence of the clinical condition of the so called autoimmune cholangitis or better AMA-negative PBC, a disease clinically and histologically identical to PBC but without detectable AMA. The present evidence does not favour the role of AMA in the pathogenesis of PBC[75].

There have been other antibodies that, although they are not pathogenetically associated, they influence the progress or the severity of the disease like the autoantibodies against the nuclear pore proteins gp210 and p62[76]. The combination of anti-nuclear envelope antibody (ANEA) and anti-gp210 identified a subgroup of patients with increased fibrosis and inflammation and was associated with poor prognosis in our group of PBC patients[77]. There is recent evidence to support deregulated B-cell immune responses in patients with PBC[78].

INNATE AND ADAPTIVE IMMUNITY IN PBC (TLR RESPONSE AND T CELL RESPONSES)

Adaptive CD4+ve and CD8+ve T-cells and innate responses have been implicated in PBC pathogenesis, following environmental insults in genetically susceptible

individuals[72,79,80]. The role of bacterial and viral factors has already been discussed. Biliary epithelial cells express molecules of the toll-like receptor (TLR) family that recognize pathogen-associated molecular patterns (PAMPs) thus triggering the innate immune responses. It is also suggested that cholangiocyte damage might result from a combined dysregulation of TLRs and of peroxisome proliferator-activated receptor-gamma (a negative regulator of intracellular signaling) resulting in Th1-predominant cytokine effects. Moreover, CD4-positive, IL-17 secreting Th17 cells are important for the development of inflammation as tey infiltrate biliary ductules and are directly related to the biliary innate responses to PAMPs[81-85].

In PBC, besides IL-17$^+$ cells infiltrating damaged bile ducts, hepatic NK-cells active against biliary epithelial cells are found, but their role in the loss of immune tolerance reported in PBC has not been elucidated[86,87]. Earlier reports have demonstrated the presence of CD4 and CD8 T-cells reactive against the human PDC in PBC livers during the earliest disease states[88-92].

CD4$^+$ CD28-ve cells are markedly increased as intraepithelial lymphocytes within damaged bile ducts and may therefore participate in the bile duct damage[93]. The residues 159-167 of PDC-E2 seem to be important in the pathogenesis since HLA DR4*0101-restricted T cell epitope, spanning residues 159-167 have been identified[94-98]. while CD8 T cells from PBC livers are cytotoxic against PDC-E2 159-167 pulsed autologous cells[99].

An interesting model implicating T cells in the pathogenesis of PBC is a mouse manipulated for T cell-restricted expression of a dominant-negative (dn) form of transforming growth factor-beta receptor Ⅱ (TGFbR Ⅱ). A PBC-like disease, not identical to the human condition develops which further connects the T cell role with the involvement of two cytokines namely TGFb and IL-12[100].

IL-12 consists of the two p35 and p40 subunits, while TGFb signaling is essential in the development of the pro-inflammatory Th17 cells[101]. Further data from this model indicate that it is the p40 subunit which is fundamental for development of bile duct damage[102]. The importance of IL-12 signaling and the PBC-like disease of the dnTGFbRII mice is further supported by genetics relating the IL-12 pathway with human PBC as mentioned in the genetics section[45].

In PBC, as already mentioned, T cells, particularly CD8 cytotoxic cells infiltrating the liver are mandatory for bile duct damage[97]. However in the CD25-negative mouse model, lack of CD8 cells attenuates but does not abolish bile ductular destruction, indicating that an additional mechanism of destruction is also present in PBC[103]. Equally important is the role of T regulatory cells. Animal experiments indicate that deficiency of regulatory T cells (Tregs) results in the development of AMA +ve, autoimmune bile ductular lesions anti[104]. Moreover a reduction of Tregs has been found in the portal tracts of PBC patients[105].

TLRS AND MACROPHAGES IN PBC

Kupffer cells are the resident liver macrophages and constitute approximately 80% of body macrophages. Kupffer cells respond to all TLR ligands by increasing the production of tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-12, IL-18, and IL-10. Liver DCs express all members of the TLR family, although TLR5 expression is low. Hepatic pDCs activated by TLR7 and TLR9 ligands, produce TNF-α, IL-6, IL-12, and IFN-α[106,107]. Based on the reported connection between the biliary tract and the intestinal lumen, it is possible that intestinal-derived bacterial products could contribute to PBC progression[61,84,108]. In response to ligands for TLR2, TLR3, TLR4, TLR5, and TLR9, PBMCs from PBC patients produce increased amounts of pro-inflammatory cytokines compared to PBMCs from healthy subjects[109]. In PBC patients, high expression of TLR9 leads to increased intracellular immunoglobulin M and AMAs production by B cells[110,111]. Furthermore, a number of TLR9 SNPs may cause B cells to produce higher amount of intracellular immunoglobulin M[112]. High expression of TLR3 and type Ⅰ interferon in macrophages surrounding portal tract and hepatocytes has been reported in the livers of PBC patients[113]. Liver macrophages produce IFN-α through TLR3 signaling leading to synergistically enhanced LPS-induced NK-cell cytotoxicity to autologous BECs. Furthermore, in PBC patients liver NK-cell cytotoxicity is higher compared to controls, when these cells are incubated with poly I:C and LPS-primed liver macrophages[86].

Mice immunized with 2-octynoic acid have been found to develop a type of autoimmune cholangitis with AMAs, similar to human PBC, but did not develop liver fibrosis. CD8 T cell infiltration, inflammatory cytokine expression and liver fibrosis were induced by Poly I:C treatment in this mouse model of PBC, thus further supporting the impact of TLR3 signaling in PBC[114].

CYTOKINES IN PBC

Earlier reports have demonstrated the predominance of a Th1 cytokine profile in PBC with a parallel relative decline of Th2 prevalence[115-117]. Overall, the data suggest that the IL-12 cytokine pathway and downstream JAK-STAT signaling is significant in PBC pathogenesis. The interaction of IL-12 and IL-12 receptor leads to the production of one more candidate gene in PBC, the STAT4 transcription factor that activates of Th1 cytokines production. More experimental data concerning the specific role of IL-12 signaling are needed in PBC and animal models. Interestingly, early onset of biliary cirrhosis has been reported in an IL-12-deficient child but the immediate relevance of this finding to PBC is unclear[118].

Another important cytokine possibly involved in PBC pathogenesis is TGF-β as mentioned before. We have demonstrated that the serum profile of the 3 TGF-β isoforms is different in the liver disease groups studied. An increase in TGF-β3 is a characteristic of

PBC irrespective of stage and therefore it may be pathogenetically important. Results from the hepatic vein samples indicate that the liver seems to be the source of the TGF-β abnormalities[119]. Plasma levels of IL-6 are decreased in PBC[120] but monocytes from PBC patients secrete more IL-1 and IL-6 after *in vitro* challenge with TLR ligands[109] and increased liver expression of IL-6 was described in PBC[117].

CHEMOKINES IN PBC

As mentioned before, many mechanisms of biliary epithelium death have been proposed, including various cytotoxic T cells subpopulations and NKT-cells[82,83,121-124]. Additional destructive mechanisms involve the activation of TNF, CD 40, and Fas receptors[125]. All these mechanisms however depend on the recruitment of these cells in the area of destruction. Chemokines are a discrete class of cytokines that regulate leukocyte recruitment, positioning and retention in tissues. Epithelial cells produce the chemokine Fractalkine (CX3CL1) and its receptor CX3CR is expressed in CD8+ve and CD4+ve T cells[126]. An overexpression of fractalkine has been reported in injured bile ducts of PBC, while CD4+ve and CD8+ve lymphocytes expressing CX3CR1 predominate in portal tracts and within the biliary epithelium. Other chemokine receptors like CXCR3 and CCR5 are mostly expressed on Th1 cells[124,127]. In early PBC Th1 cells positive for the CXCR3 densely surround the damaged bile ductules. As a consequence the ratio of CXCR3-/CCR4-positive lymphocytes (Th1/Th2) is significantly higher in early compared to late stage PBC[128]. In more recent reports the significance of two other chemokines, namely the IFN-γ-inducible protein 10 (IP-10, CXCL10) and monokine induced by IFN-γ (MIG, CXCL9) has been stressed. These are produced by macrophages, *i.e.*, the Kupffer cells in the liver and are also implicated in the recruitment of Th1 cells *via* the same receptor CXCR3. Alternatively the sinusoidal endothelial cells may be another site of their production[129,130]. MIG and IP-10 are also secreted by biliary epithelial cells and activated stellate cells[131,132]. Increased plasma IP-10 and MIG levels have been reported in PBC patients accompanied by an increase in their mRNA in the liver tissue. Most importantly increased plasma levels were also found in their first degree relatives[133]. The third CXC chemokine I-TAC was not detected.

We recently reported a significant increase of plasma MIG and IP-10 compared to normal controls. The CXCR3A variant mRNA was found in PBLs from all PBC patients as well as in normal controls. However the CXCR3B variant mRNA was expressed in 4/20 (19%) normal controls and all 20 PBC patients. These data suggest a possible pathogenetic implication of MIG and IP-10 chemokines and their receptor in the pathogenesis of PBC[134]. Recent papers demonstrated that these chemokines and their receptors are also involved in the hepatic recruitment of Tregs and the pro-inflammatory Th17 and Tc17+ lymphocytes[135,136].

There is also evidence that IP-10 is a pro-fibrotic factor participating in the interactions between hepatic stellate cells and NK cells which play a central role in the regulation of hepatic stellate cells activity and fibrosis. IP-10 blockade might be a future target for treatment of liver fibrosis[137].

THE ROLE OF APOPTOSIS IN PBC

Experimental and clinical evidence suggests that apoptosis is the most important mechanism of biliary epithelial cell damage. Apoptotic biliary epithelial cells cells identified by the TUNEL reaction are frequently described in PBC liver tissue[138]. Apoptosis markers have been demonstrated within the portal tracts in liver specimens from PBC patients[139,140] including a reduced expression of the anti-apoptotic protein bcl-2[141]. Cells responsible for apoptosis induction are the cytotoxic CD8+ve T-cells. Biliary epithelia, unlike other cell types, have a rather unique characteristic considering the fate of the pyruvate dehydrogenase complex during apoptosis. Glutathione fails to bind to the lysine-lipoyl residue of the dehydrogenase complex and thereby the autoreactive epitope is not cleaved[142].

PDC-E2 is a ubiquitous protein present in mitochondria of nucleated cells; biliary epithelial cells translocate intact PDC-E2 to apoptotic bodies and create an apotope, which is immunoreactive. PDC-E2 remains intact and immunogenic during biliary epithelial cell apoptosis; This allows biliary PDC-E2 to be exposed to dendritic cells and its epitopes to be presented to T cells in draining lymph nodes, leading to production of AMAs[72,80,143]. However in established transgenic mice that constitutively express PDC-E2, aberrant expression of PDC-E2 in the cytoplasm of BECs was not followed by biochemical, serological or histological features of PBC. The results indicated that PDC-E2 expression BECs is not sufficient for the initiation of autoimmunity. Additional factors may be required to establish a model of PBC[144].

ROLE OF REACTIVE OXYGEN SPECIES IN PBC

There are few data on the significance of reactive oxygen species (ROS) in bile ductular damage in PBC. Biliary epithelial cells express low levels of glutathione-S-transferase with a resultant decrease of intracellular glutathione. On the other hand, lipid peroxidation seem to be increased as reflected by increased perinuclear expression of 4-hydroxynonenal[145].

Other antioxidants including vitamin E, retinol, alpha-tocopherol, total carotenoids, lutein, zeaxanthin, lycopene, alpha and beta-carotene are reduced in PBC[146-148]. Total serum antioxidant capacity (measured with chemiluminescence) is reduced in PBC patients[149]. In contrast, we have reported increased levels of corrected total antioxidant capacity in PBC[150] a fact that

may reflect a compensatory but probably not sufficient increase to counteract an increased ROS production.

In vitro studies further support the role of ROS as an effector mechanism in bile duct damage in PBC. Ursodeoxycholic acid (UDCA), the current drug of choice in PBC treatment, is a potent ROS scavenger, inhibiting mitochondrial oxidative stress and lipid peroxidation in a dose-dependent manner[151-153]. Indirect evidence for the role of ROS comes from the rat bile duct-ligated model. Lipid peroxidation is a late event in the bile ligated model and a correlation seems to exist between lipid peroxidation and the activation of inflammatory cells[154,155]. In this model overproduction of Free radicals is due to activation of NF-κB resulting in increased production of the proinflammatory cytokines TNF-α, IL-6 and IL-1b[156]. Moreover, *in vitro* experiments have shown that retention of several bile acids like taurochenodeoxycholic acid and taurocholic acid lead to hepatocyte damage producing hydroperoxide by mitochondria[157,158]. The same bile acids induce hepatocyte apoptosis in a time and concentration-dependent manner due to ROS generation by mitochondria[159]. These findings are relevant to human PBC because increased bile acid retention is a feature of at least late PBC.

A further connection of increased ROS and apoptosis of biliary epithelia has been provided by Celli *et al*[160] They demonstrated, that the reduction in the intracellular level of the antioxidant molecule glutathione leads to increased degradation of the anti-apoptotic bcl-2 protein and therefore to an increase in biliary epithelial apoptosis.

CURRENT VIEWS ON THE PATHOGENESIS OF PBC

Every effort to elucidate the complex pathogenetic mechanisms leading to the development of PBC should account for two facts that are critical in the disease pathophysiology. First, the main PDC auto-antigen is an inner mitochondrial membrane component and therefore any attempt of the immune cells to contact its epitopes should overcome three different membranes. This problem has been partly answered by the unique characteristic of biliary epithelial cell apoptosis and the retention of immunologically active antigens within the apoptotic blebs as mentioned before. Moreover apoptotic blebs does not seem to be an early phenomenon. The initiation event in a genetically predisposed individual still is not known. Second, PBC initially affects only the small intrahepatic bile ductular cells, while the antigen is widely distributed in many tissues including the extrahepatic bile ducts.

Pathogenetical models proposed so far consider as the crucial event the break down of T cell self tolerance to PDC epitopes. The generation of antibodies against PDC by themselves is not enough to damage the biliary ductules,as already mentioned[144]. The molecular mimicry model of self-tolerance breakdown was proposed as a more logical approach compared with the rather obscure concept of autoimmunity[161].

According to this theory,bacterial or retroviral infections, probably trigger an immune attack leading to apoptosis of epithelial cells. This T-cell attack is mediated by TLR interaction with a micro-organism epitope cross-reacting with a self-PDC epitope. The retroviral etiology of PBC has been reviewed in detail[75].

Endotoxins and PAMPs are potent activators of the immune response. These bacterial products are normally cleared by the liver and eliminated in bile[162]. Lipid A is dephosphorylated and inactivated through the action of alkaline phosphatase in bile[163]. Lipid A, the immunogenic constituent of gram-negative bacterial LPS, and other PAMPs accumulate in hepatocytes and biliary epithelium, thus contributing to small bile duct inflammation[82,84,164,165]. PBC patients exhibit a strong immune response to LPS[166,167]. The loss of tolerance to bacterial products could be at least partly secondary to cholestasis and, more specifically, to impaired alkaline choleresis or a defect in transporters involved in the generation of ductal choleresis[168].

Apart from bacterial antigens viruses have been investigated as triggering agents in PBC.Thus viral particles have been described within biliary epithelial cells of PBC patients, and antibodies against retroviruses have been found in their sera[169,170]. In a cohort of patients genetic material of a human beta retrovirus (95% homologous to the mouse mammary tumour virus) has been identified in the lymph nodes of 75% of patients[171].

Epidemiological evidence may be interpreted as supportive of viral implication in the pathogenesis of PBC. Description of PBC clusters among individuals who emigrated from low PBC to high PBC prevalence areas and PBC cases among unrelated individuals living in the same house are some of these. Furthermore, in patients treated with tacrolimus PBC recurrence after liver transplantation occurs earlier and is mere severe, while the contrary is observed with cyclosporine administration[172-174]. However the retroviral etiology of PBC remains debatable[175].

In accordance with the molecular mimicry model, Jones described an alternative pathogenetic model where virals or bacterial epitopes with homology to PDC are the initial trigger[176]. Self-PDC reactive T cells escape negative selection in the thymus, because of the low affinity of their T cell receptor towards the the complex of self-peptide and major histocompatibility complex (MHC). An interesting feature of this model is that the state of activation of APC may determine the efficacy of antigen presentation and promote tolerance breakdown[109].

As mentioned before,several other cells of the innate immune system are implicated in the pathophysiology of PBC. Granulomas common in PBC, increased levels of polyclonal IgM, hyper-responsiveness to CpG oligodeoxynucleotides and increased numbers of NK cells are related to innate responses. Bacterial and viral

epitopes induce innate immune responses through binding to TLRs. TLRs were described in cultured human biliary epithelial cells[109-111,177]. These findings make the inclusion of innate immunity mandatory in any pathogenetic model of PBC.

Another finding connects senescence and shortened telomere length as a unique feature of damaged BEC in PBC. Significantly decreased telomere length in the affected biliary epithelial cells compared to histologically normal BEC in both PBC patients and controls has been reported Immunohistochemistry confirmed cellular senescence. These changes are not inconsistent with the retroviral hypothesis, since viruses can directly damage the DNA in susceptible individuals[178].

PATHOGENESIS OF PBC: A UNIFYING HYPOTHESIS

A significant increase of endothelins, particularly ET2 (and to a lesser extent of ET1) both in peripheral blood and in the hepatic vein of PBC patients from the early stages of the disease has been reported in our earlier study[179]. We then proposed a new hypothesis for the pathogenesis of PBC. Here we modify our previous hypothesis to incorporate new data that have emerged since our previous publication[180]. We consider that a primary defect is the overproduction by the liver endothelial cells of ET2 (and to a lesser extent ET1). This defect might be genetically controlled. It is possible that the initiating event in genetically susceptible patients is the effect of either a retrovirus or a pathogen like *N. aromaticivorans* or PAMPs. The presence of scavenger receptor type B capable of internalizing foreign antigens has been described in endothelial cells. A strongly antigenic component of gram positive bacteria namely lipoteicholic acid, has been described in endothelial cells of PBC patients[181]. ET2 may induce Kupffer cells to produce pro-inflammatory cytokines, such as IL-1 and IL-6. This has been already described for mouse peritoneal macrophages[182]. On the other hand, PAMPs acting on the TLRs of the Kupffer cells may lead to the production of TNF-α and further augmentation of IL-1 and IL-6. ET2 has a similar peptide homology with CXC chemokines and through ETB receptor on macrophages is a strong chemoattractant of these cells[183]. Indeed macrophages comprise 30% of the cells infiltrating portal areas and are mostly found around damaged bile ducts[184]. Electron microscopy verifies the presence of activated macrophages that differentiate into epithelioid cells near bile epithelial cells[185].

Many cells at the periphery of epithelioid granulomas are MCP2 and MCP3 positive and almost 60% co-express CD68, a finding compatible with their macrophage origin[186]. A possible link with the progression to cirrhosis in stage 3 and 4 PBC is the observation that Kupffer cells and myofibroblasts are increased and closely associated in periseptal and periportal areas. This might be an indication of an interaction between Kupffer and stellate cells leading to fibrosis[187].

Endothelins also cause contraction of stellate cells thus helping in the early appearance of portal hypertension in PBC[188]. In agreement with this hypothesis are findings from our group indicating that disease specific antibodies against liver sinusoidal cells circulate in PBC[189]. In addition we reported that polymorphisms related to endothelial cells were associated with PBC. Both eNOS intron4 VNTR and eNOS exon7 G894T SNP were related to increased risk in PBC while endothelin-1 rs2071942 "A" and rs5370 "T" alleles had a tendency for association with disease progression[190].

Bile ducts are supplied with blood only from hepatic arteries; the epithelial cells receives blood from a network of capillaries originating from the terminal branches of the hepatic artery. It is called the peribiliary vascular plexus (PVP) and drains into the sinusoids. This specific vascular supply, is responsible for the extensive involvement of the interlobular bile ducts in ischemic injury[191]. Reduced perfusion of this network can cause extensive bile duct damage[192].

There is evidence that endothelins (ETs) and nitric oxide are the principal regulators of circulation of the PVP[193]. We therefore propose that patients with PBC an initiating damage of the biliary epithelium is caused by ischaemia due to ET2 induced vasoconstriction observed from the ealy stages PBC. This finding was disease specific since it was not observed in other chronic liver disease patient with or without cirrhosis[179]. Ischaemia might then lead to apoptosis of bile ductular cells a mechanism already reported in epithelial destruction of PBC. As mentioned earlier, biliary epithelial cells undergo a unique apoptosis with an antigenically active PDC complex shed from mitochondria into the cytoplasm as early as 6 h after induction of apoptosis possibly through the action of caspase 3[194]. These immunoreactive epitopes, are then taken up by peribiliary antigen presenting cells expressing MHC Ⅱ (this uptake might be either genetically regulated or alternatively be induced by proinflammatory cytokines[195]. This is a mechanism that could explain the proposed similarities between PBC and graft *vs* host disease (GVHD)[196-198] which is also attributed to endothelial cell injury[199]. Interestingly in graft *vs* host disease observed after small bowel transplantation, increased levels of ET1 have been histochemically demonstrated in endothelial and epithelial cells some days before GVHD, strongly suggesting a pathogenetic significance[200]. An additional feature of this model is that increased secretion of chemokines IP-10 and MIG possibly by endothelial or Kupffer cells is an early event that leads to accumulation of immune cells and also inactivation of NKT-cells by IP-10 with resultant reduced apoptosis of liver sinusoid endothelial cells[137].

A possible role for TGF-β abnormalities have also been incorporated in this model. It is TGF-β1 that modulates FoxP3 expression and the regulatory activity of CD4 cells[201]. Could it be that TGF-β3 influences the development of Tregs? Differences in TGF-β isoforms

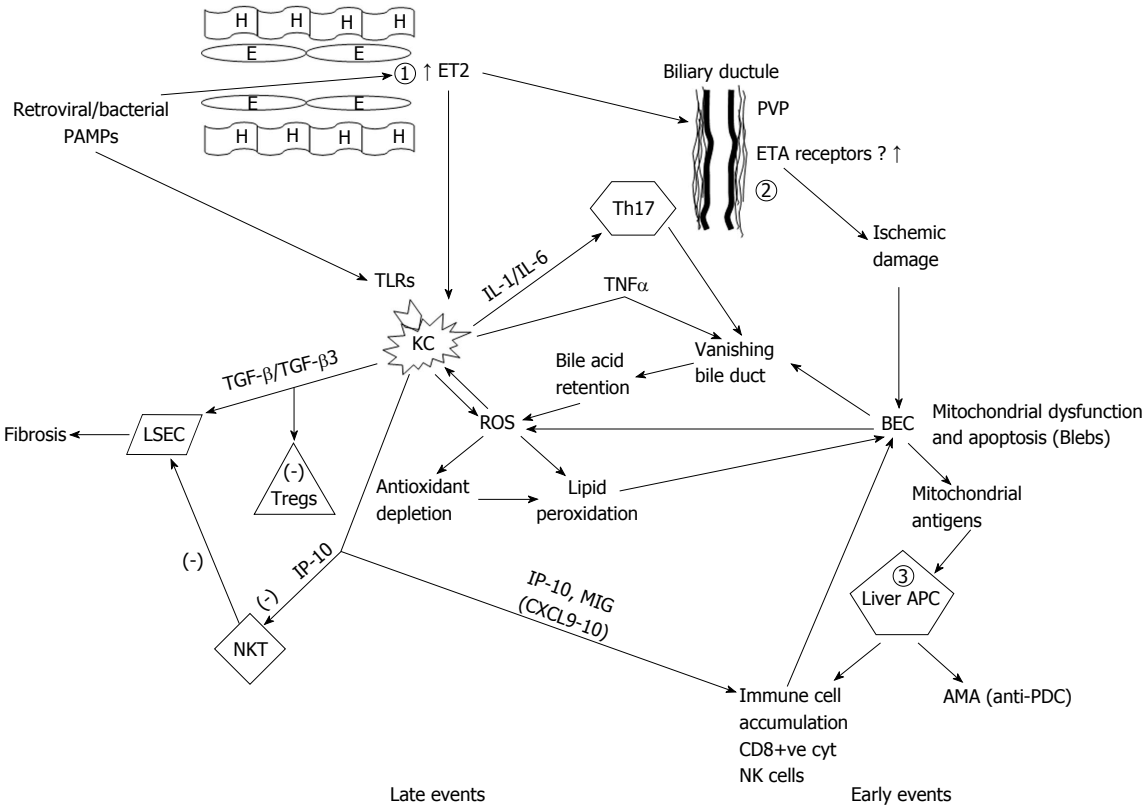

Figure 1 Liver sinusoidal endothelial cells and Kupffer cells are mandatory in the pathogenesis of primary biliary cirrhosis with early and late events in the unifying model. The fundamental early defect in primary biliary cirrhosis (PBC) is the overproduction of endothelin 2 (ET2) by endothelial cells (1) possibly driven by a retrovirus or other microbial pathogens in genetically predisposed individuals. ET2 is a chemoattractant for Kupffer cells and also causes contraction of stellate cells leading to early portal hypertension. ET2 leads to ischemic damage of biliary epithelial cells (BEC) (2) through constriction of the peribiliary vascular plexus with resultant BEC mitochondrial dysfunction and eventually apoptosis leading to the vanishing bile duct syndrome. Apoptotic blebs containing immunologically active mitochondrial antigens possibly generated through caspase cleavage, are presented by hyper-responsive liver dendritic cells (APC) (3) and lead to immune cell accumulation and antimitochondrial antibody production. The second fundamental defect occurring later in the disease process is the production of reactive oxygen species (ROS) driven by both bile acid accumulation as a result of the vanishing bile ducts and generation by Kupffer cells through ingestion of BEC apoptotic bodies. ROS drives the accumulated Kupffer cells to produce more ROS and pro-inflammatory cytokines. Moreover, a relative antioxidant insufficiency allows ROS to cause lipid peroxidation and further BEC apoptosis and mitochondrial dysfunction. CXCL chemokines produced by endothelial and Kupffer cells drive the accumulation of various immune cells while IP-10 inhibits apoptosis of LSCs induced by natural-killer T (NKT)-cells and thus favors the fibrosis induced by transforming growth factor (TGF)-β produced by Kupffer cells. However an increased TGF-β3 production may also explain the reduced Tregs found in PBC. In this model BECs are destroyed by other mechanisms in addition to the initial ischemia. ROS damage, NK cell killing induced by interferon alpha (IFNα) production by Kupffer cells, cytotoxicity by CD8+ve cells and T-helper 17 (Th17) cell, are also implicated. All these lead to the development of the vanishing bile duct syndrome characteristic of PBC. H: Hepatocyte; E: Endothelial cell; ET2: Endothelin 2; PVP: Peribiliary vascular plexus; PAMP: Pathogen-associated molecular patterns; ETA: Endothelin-A; TLR: Toll-like receptors; IL: Interleukin; TNFα: Tumor necrosis factor alpha; KC: Kupffer cells; TGF-β: Transforming growth factor beta; BEC: Biliary epithelial cells; ROS: Reactive oxygen species; LSEC: Liver sinusoid endothelial cells; Tregs: Regulatory T-cells; IP-10: Interferon gamma-induced protein 10; APC: Antigen presenting cells; MIG: Monokine induced by gamma interferon; NKT: Natural killer T-cells; Anti-PDC: Anti-pyruvate decarboxylase antibodies; AMA: Antimitochondrial antibodies.

may lead to a deranged balance between Tregs and Th17 cells. We have also proposed that these findings may be the causative link leading to Treg and/or Th17 deregulations reported in PBC. The presence of increased local levels of TGF-β3 (and the concomitant relative lack of TGF-β1) in conjunction with increased levels of IL-6 may shift the balance towards an increased activity of the proinflammatory Th17 cells adding to the CD8 cytotoxic lymphocytes destructive mechanism, while at the same time leading to functionally defective Treg. The increased presence of TGF-β3 instead of TGF-β1 could be a further mechanism that favors Th17 activity since one can postulate that TGF-β3 could either be associated with functionally defective Tregs or could directly favor the increase of Th17. Further evidence for such a hypothesis is provided by a report that mice with mutation of the gene encoding the FoxP3 transcriptional factor developed AMA positivity and features resembling PBC with increased levels of IL-17 and IL-23, cytokines associated with Th17 cells[104].

The proposed model requires that additional aspects have to be verified in future. An important point is that the increased concentration of ET2 we reported was also observed in the systemic circulation.The vasoconstriction and the consequent ischemic injury therefore ought to be present in other organs away from the liver and PVP. This liver selectivity might be due to increased expression or increased affinity of ET receptors for ET2 in the PVP of PBC patients, also genetically determined, but this explanation requires further research.

Our proposed model is diagrammatically presented in Figure 1 and pathophysiological abnormalities are subdivided into early and late events. This hypothetical

model has several advantages. An interaction between innate and adaptive immunity in the pathogenesis of PBC is described. The initiating event involves innate immunity while adaptive immunity causes perpetuation of the disease even after disappearance of the initiating event. It also integrates the role of infective agents whether retroviral or bacterial and the similarity of PBC with GVHD. Interaction of endothelial and Kupffer cells with stellate cells allows for the development of fibrosis and cirrhosis.

In this model AMA production is a secondary phenomenon rather than pathogenetically related to bile ductular damage. Therefore the existence of AMA negative PBC is also explained as well as the presence of many other autoantibodies both disease and non-disease specific. Most importantly, it is recurrence with the idea of a complex genetical predisposition as it offers many levels for genetic control as outlined before. Candidates are endothelial cells (and possibly Kupffer cells), the perivascular plexus and ET receptor expression and affinity and peribiliary antigen presenting cells that might be genetically hyper-responsive. Furthermore some of them may be estrogen dependent, thus explaining the extreme female prevalence of the disease, but this requires further research.

CLINICAL MANIFESTATIONS

The typical PBC is characterized by a gradual long term disappearance of small intrahepatic bile ductules and a parallel long term increase in fibrosis, resulting in biliary cirrhosis over a period of 10-20 years. Ten percent to twenty percent of patients, are presented with fluctuating or persistent biochemical and histological evidence of auto-immune hepatitis. These patients have a more rapid progression of liver fibrosis and liver failure. A small minority of patients, develop a very rapid onset of vanishing bile duct syndrome with serious cholestatic jaundice and quick progress to cirrhosis in less than 5 years[202,203]. At diagnosis, many patients are asymptomatic diagnosed at a routine check by an increase in cholestatic liver enzymes. Approximately 30% of them will remain asymptomatic for many years. The rest will develop symptoms within the next 4 years. The commonest symptom at presentation is fatigue followed by pruritus (21% and 19% respectively)[204-206]. Severe fatigue impairs quality of life and appears in up to 80% of PBC during the course of the disease but it is not correlated with disease severity[207,208].

However symptoms of autonomic dysfunction like orthostatic hypotension, excessive daytime somnolence and sleep disturbance have been reported[209]. The pathophysiological mechanisms of chronic fatigue in PBC are not clear. Treatment is particularly difficult. UDCA is often ineffective while liver transplantation can not ameliorate the symptom. The second commoner symptom is pruritus in 20% to 70% of patients and can be debilitating at times[210]. The severity of cholestasis and the histological stage of the disease do not correlate with the intensity of pruritus. In our population pruritus is extremely rare at any stage of the disease for unknown reasons. The pathophysiology is obscure and the molecules that may be responsible for itching in cholestasis have not been identified. Proposed causative agents include bile salt metabolites, progesterone metabolites, histamine and endogenous opioids but the existing evidence for the implication of these agents is weak[211,212].

Cutaneous manifestations with various degrees of severity are common in PBC patients. We have described fungal infections of the skin even in the earlier histological stages. Plantar mycoses, onychomycoses, and interdigital mycoses were more frequent in PBC patients compared to normal controls. Dermographism and melanosis were also very common. In as many as 38.7% of patients the presenting symptom was a dermatologic lesion[213].

An increased portal pressure defined as the difference between the wedge and free pressure in the hepatic veins is frequently found in PBC. A reliable predictor of survival is the stability or improvement of this pressure gradient[214,215]. Cirrhosis is present only at stage 4, but variceal hemorrhage has been described early in the disease due to early development of nodular regenerative hyperplasia[216].

A relative lack of intestinal bile acids leads to malabsorption of calcium and vitamin D and hence to osteoporosis. This is usually a late finding. Decreased osteoblastic and increased osteoclastic activity have been incriminated in the progress of osteoporosis in PBC patients[217,218].

Regarding breast cancer, both increased risk[219,220] and equal risk[221] compared to a healthy women have been reported in PBC patients. The incidence of hepatocellular carcinoma in advanced PBC is more or less similar to other types of cirrhosis and is more frequent in male patients. The incidence of hepatocellular carcinoma (HCC) amongst patients with a diagnosis of PBC is estimated at 0.36 per 100 person years. An increasing risk for HCC has been observed in the most advanced stages of PBC[222-224]. As with other autoimmune diseases PBC has been reported to be associated with many immune-mediated diseases. Thyroid abnormalities are often reported in PBC, sometimes preceding its diagnosis[225]. Other autoimmune diseases develop in approximately 33% of patients with PBC (for example scleroderma, Hashimoto's disease, Raynaud syndrome, and Sjögren's syndrome), as do cholestasis or pruritus during pregnancy[30,31,226-228].

Dyslipidemia was found in 69.4% of our patients, in 60% of their first degree relatives and in 40.9% of the study controls.Multivariate analysis confirmed the association with the presence of at least another autoimmune disease while a cancer history was mere frequently reported in patients than in controls (P = 0.033)[28]. Coeliac disease is far more common in PBC compared to inflammatory bowel diseases and has been reported in up to 6% of PBC patients[229].

However this is not true in all populations and criteria for diagnosis should always include duodenal and small bowel biopsies. In our series of patients a very high proportion of false positive results of celiac disease associated antibodies was found. Twenty-one percent of PBC patients and 35% of patients with AMA negative PBC had anti-gliadin antibodies while the corresponding numbers for anti-transglutaminase were 10% and 18% respectively. By contrast anti-reticulin and anti-endomysial antibodies were not detected. Duodenal and small bowel biopsies performed in two thirds of patients with either AMA positive or AMA negative PBC patients with at least one antibody present, failed to confirm histologicaly even a single case of celiac disease and therefore in our patient population an increased risk for celiac disease could not be demonstrated. In accordance with our findings a high prevalence of false-positive anti-gliadin and anti-tissue transglutaminase antibodies has been reported in chronic liver disease[230].

DIAGNOSIS

Presence of serum AMAs, elevations of cholestatic enzymes mainly of alkaline phosphatase and increased levels of IgM immunoglobulin in serum, are the criteria used for diagnosis of PBC. The M2 subclass of AMAs is considered highly specific for PBC. The presence of two criteria indicate a probable diagnosis, but a definitive diagnosis requires all three[231,232].

Histology compatible with PBC, although usually not necessary for diagnosis, is mandatory for identification of the histological stage of the disease.

Various other autoantibodies are frequently found in PBC. Most prominent are the antinuclear antibodies (ANAs) reported in 30% of PBC patients[233-235]. The so-called "multiple nuclear dots" ANA pattern corresponds to the Sp100 and Sp140 antigens, pro-myelocytic leukemia nuclear body proteins and small ubiquitin-like modifiers[236,237] is considered specific for PBC. A second immunofluorescence pattern of AMAs, the "nuclear membrane" (rim) pattern, corresponds to ANEAs, such as gp210 and nucleoporin p62[238,239]. Earlier reports indicated that the anti-gp210 antibodies are specific for PBC and their presence is associated with more severe and active disease[76,240]. An increased expression of gp210 on the nuclear envelope of bile ductules and portal mononuclear cells and peri-portal hepatocytes was reported by Nakamura *et al*[241] who also confirmed their association with active disease. We also reported that 46.9% of patients with PBC have detectable ANEAs and 21% of them had detectable anti-gp210 antibodies in a subgroup of PBC patients with histologically advanced disease with poor prognosis[77].

The role of serum surrogate markers in PBC diagnosis is doubtful to say the least. We recently reported on simple biochemical markers of liver fibrosis in patients with PBC and other chronic liver diseases. Significantly increased levels of hyaluronan and collagen Ⅳ, were found in all chronic liver diseases compared to controls, but laminin was increased only in HBV and HCV cirrhosis. Serum Hyaluronan was significantly increased in late PBC compared to early stage disease (154.5 ng/mL; 95%CI: 55.3-764.4 and 54.5 ng/mL; 95%CI: 27.3-426.9 respectively, $P < 0.05$). The areas under the curve for late PBC were relatively high for hyaluronan and collagen Ⅳ (0.74 and 0.70 respectively) and low for leptin and laminin (0.63 and 0.59 respectively). Moreover hyaluronan had 96% sensitivity and 90% Negative Prognostic Value for late stages of PBC. However no single measurement could differentiate between early and late stage PBC. Serum hyaluronan nonetheless proved to be a useful serum marker for sequential follow up studies in PBC[242]. Other surrogate markers like APRI, Forns, Fibroindex and FIB-4 do not offer any advantage over the single biochemical measurements we used[243].

Other non invasive techniques, namely transient elastography, supersonic shear imaging and acoustic radiation force impulse elastography have been tested in patients with PBC, in an effort to establish advanced fibrosis[244,245]. It is doubtful however that their use can replace the gold standard of liver biopsy particularly in early PBC. The evolution of fibrosis over the years might be a field where these techniques can assist in the follow up of these patients.

PATHOLOGY

PBC is histologically classified into 4 stages according to Ludwig[246] or Scheuer classification[247]. In early stages, lesions mostly progress in small interlobular bile ductules. Swelling and degeneration of their epithelium finally leading to necrosis is a prominent feature of early PBC[248]. There is extensive infiltration of the portal tracts with mononuclear cells, including macrophages and eosinophils mostly around affected bile ductules. In some cases granulomas or aggregates of epithelioid cells are found either in portal tracts or within the lobule. Granulomas can be found in any histological stage. Occasionally mild Kupffer cell hyperplasia or infiltration of sinusoids by lymphocytes can be found. A histological finding associated with disease progression is the presence of periportal hepatitis. In later stages, the injured bile ducts gradually disappear probably through apoptosis and fibrosis and ultimately cirrhosis develop. A useful finding in diagnosis is an excessive accumulation of copper within hepatocytes present from the early stages. As already mentioned a liver biopsy may not be necessary for diagnosis.This is true but staging of the disease still requires a liver biopsy since neither serum surrogate markers nor elastography can replace an adequate liver tissue sample. In particular elastography, although relative simple to perform, can only assess fibrosis and not the other parameters a liver biopsy can provide. Moreover the distinction between stages Ⅱ and Ⅲ in the METAVIR scale is extremely difficult[243,244].

Kakuda *et al*[249] analyzed the usefulness of a new histological classification system in predicting

retrospectively the clinical outcome of patients with PBC. The prognostic performance of this system, which was developed a few years ago by this Japanese team[250-252] was compared to those of the Scheuer's and Ludwig's classical systems. They concluded that, while the place of liver biopsy in PBC remains clearly limited for diagnostic purposes, an accurate histological examination of the liver based on the evaluation of several individual lesions of prognostic relevance may be useful to predict the long-term prognosis and the outcome under UDCA treatment.

NATURAL HISTORY BEFORE UDCA TREATMENT

The introduction of the UDCA in the treatment of PBC has altered the natural course of the disease. Before UDCA, an increasing serum bilirubin after a relatively stable phase, was an ominous sign and death was the final event after a few months[253]. Bilirubin above 2 mg/L, was associated with a median survival of 4 years and for levels over 6 mg/L, it was only 2 years. At a multicenter European study of 236 patients, (54% on stage 1-2 and 19% on stage 3), were followed up for a period of 4 years. Almost half of the patients developed histologically-proven cirrhosis[254]. In another large study of 770 patients from the Northeast England liver failure appeared in 15% of them within a 5-year follow up[255]. Very high rates of histological deterioration was reported in three large 4 years follow up studies of untreated patients with a median 2 years for advanced fibrosis to develop and the probability of remaining in the early stage of only 29% (95%CI: 15%-52%)[254,256]. In a additional report of 256 patients prospectively studied for 5.6 years the appearance of esophageal varices was 31%[257].

TREATMENT OF PBC

Medical management in PBC is aimed at two specific goals; first treatment of symptoms (jaundice, fatigue and pruritus) and complications (ascites, metabolic bone disease, hypercholesterolemia, malabsorption, anemia and vitamin deficiencies) resulting from chronic cholestasis. The second aim focuses on suppressing the underlying pathogenetic process that revolves around the destruction of intralobular bile ducts[258].

Treatment PBC patients is not disease specific and virtually has not changed over the last 25 years. Standard treatment is the long term administration of the secondary bile acid UDCA. The first trial looking at UDCA in PBC was conducted by Poupon *et al*[259] in 1987 and demonstrated dramatic improvement in liver biochemistries in PBC patients receiving 12-15 mg/kg per day of UDCA. Several independent cohort analyses showed that survival in UDCA-treated PBC patients (13-15 mg/kg per day) was significantly longer than expected from natural history models of the disease[260-262]. The effect of UDCA on survival has been studied with either observational studies or Markov models. Two hundred and sixty-two patients who received 13-15 mg/kg UDCA daily were followed up for 1-20 years (mean 8 years). Transplantation free survival rates were 84% and 66% at 10 and 20 years. By contrast, there was a significantly high probability of death or liver transplantation in patients treated in the late stages of the disease with a relative risk of 2.2[263].

We also reported similar results in our cohort of patients with the favourable outcome more prominent when UDCA was commenced in histological stage 1-2 of the disease[264]. Although these comparisons are indirect, they further support a sustained beneficial effect of UDCA on survival in PBC patients. Withdrawal of UDCA even after more than 6 years of treatment was followed by immediate biochemical rebound[265].

In addition to survival, a large number of randomized controlled trials were performed to assess the effect of UDCA on progression of fibrosis. In these trials the dose of UDCA administered was usually 13-15 mg/kg per day[266-271]. In an early report, UDCA treated patients were followed for 6 years and compared with untreated patients. There was a significant reduction of cirrhosis development (13% *vs* 49%)[272]. Similar results were reported in another study where treated patients had a 5-fold lower rate of advancement of fibrosis or development of cirrhosis. In a 4-year follow up 76% of the treated patients were still in early stages as compared to only 29% of placebo controls[273]. UDCA was equally effective in reducing the rate of esophageal varices appearance in a 4-year study of 180 patients. Only 16% of patients developed varices compared to 58% on the placebo arm[274].

On the other hand, a recent Cochrane systematic review of 16 randomized trials of 1447 patients in total has concluded that UDCA has a significant effect on reducing jaundice, ascites and liver biochemistries but had no significant effect on mortality or liver transplantation[275] in accordance with earlier meta-analyses[276,277]. However, other meta-analyses that excluded short duration trials (< 2 years) and trials using suboptimal UDCA doses,less than 13 mg/kg per day confirmed in fact the beneficial effect on transplant-free survival of UDCA (13-15 mg/kg per day) in early stage PBC patients[278].

Not all patients respond to UDCA treatment. Age is associated with response while male sex is a predictor of non-response to UDCA[279]. Biliary enrichment of UDCA by at least 40% is usually regarded as a surrogate of a therapeutic response; it rarely exceeds 50% with standard doses, but can reach as high as 65%-75% with very high doses (30 mg/kg per day)[280]. Several studies tried to define the best way to assess if a response to UDCA therapy has occurred in patients with PBC. The Barcelona criteria suggests that a decrease of 40% in serum alkaline phosphatase (ALP) level is a predictor of favourable response to UDCA[262]. Corpechot *et al*[281], looking at the efficiency of several combinations of threshold lab biochemistries in predicting outcomes

of 292 patients with PBC established the Paris Ⅰ criteria (ALP < 3 time the upper limit of normal (ULN), aspartate aminotransferase (AST) < 2 ULN and bilirubin < 1 mg/dL after 1 year of UDCA). A transplant-free survival rate of 90% at ten years was observed in patients fulfilling these criteria and only 51% for those who did not[281]. The same group recently established the better validated Paris Ⅱ criteria which defined the best biochemical response in early stage (histological stage 1-2) as ALP and AST < 1.5 ULN, with a normal bilirubin[282]. The suboptimal therapeutic response evaluated by different biochemical criteria has been recently reviewed by Corpechot[283]. Normalization of all biochemical abnormalities is achieved in 20% of patients after 2 years treatment with UDCA[284].

The biochemical response to UDCA should be monitored every 3 to 6 mo. Improvement in liver tests may be observed as early as the first month, and 90% of the improvement usually occurs within the first year. In patients with non-advanced disease, the maximum response is observed after 3 years and maintained in the long-term despite slight and marginal increases in bilirubin and aspartate aminotransferase[285].

Today there is a more or less general agreement that a biochemical response to a dose of 13-15 mg/kg per day of UDCA delays progression of disease in the majority of patients. Major changes in the natural history of PBC have been achieved, mainly resulting in decreased rates of liver transplantation and prolonged survival due to earlier diagnosis of the disease and hence timely intervention with UDCA[214,262,271,281,286,287].

An increasing use of UDCA is accompanied by a similar decrease in the number of patients transplanted[288,289]. UDCA has very few adverse effects even after many years of treatment. A slight weight gain not exceeding an average of 2 kg, is reported by many patients particularly those who have stopped smoking. It is possible that an alteration of bile acid pool composition might lead to signaling through the TGR5 pathway and subsequent weight gain[290]. Administration of UDCA in patients with pruritus may in fact cause deterioration of pruritus and it is recommended that a low dose of 200-400 mg/d should initially be given with a gradual increase in 4-8 wk up to the usual daily dosage. UDCA is not approved for use during the first trimester of pregnancy, although embryotoxicity has not been reported in humans. A number of retrospective studies found no serious side-effects in women with PBC who started gestation during UDCA therapy and who continued the treatment throughout their pregnancy[291,292]. Breast feeding with UDCA treatment is also not approved, but was incidentally reported to cause no detrimental effects in the newborn[293].

Despite the favourable response of most patients to UDCA, there is a number of patients with incomplete response to the drug. Various combinations with additional drugs have been tested. Recent studies aimed at combining other agents to UDCA have shown promise. Bezfibrate has proved to have an additional anticholestatic effect in combination with UDCA[294,295]. Two meta-analyses showed different results. A Cochran meta-analysis concludes that the addition of bezafibrate offers no advantage over UDCA alone[296], while favorable results were reported in a very recent meta-analysis[297].

Immunosuppressants such as colchicine or methotrexate demonstrated a sustained clinical response. The addition of methotrexate was reported to improve both liver biochemistry and histology[298,299] although some studies reported no effect from this combination[300-302]. Furthermore, in a 10-year study, the addition to UDCA of either methotrexate or colchicines offered no survival advantage to that predicted by the Mayo model. However pre-cirrhotic patients taking methotrexate showed no progression therefore verifying that the drug is a useful addition to UDCA at least in some patients[303,304].

Non responders could be treated with a combination of UDCA and budesonide but there is not a general consensus for that treatment. In newly diagnosed PBC patients a combination of UDCA and budesonide improves liver biochemistry and histology but whether this is accompanied by a clear survival benefit remains to be established[305,306].

Chrorambucil has also been tried. Despite improvement in some outcome measures such as serum bilirubin levels in PBC patients treated with chlorambucil, a Cochrane systemic review concluded that evidence is inadequate for the usage of chlorambucil in PBC patients. Bone marrow suppression was a serious side-effect with this drug[307].

Tetrathiomolybdate was also investigated and the agent was efficacious for the management of PBC. Only biochemical endpoints were used without a proof of histological improvement. Hence, as the authors suggest longer clinical trials to examine transplant-free survival and histological progression are needed[308].

Novel treatment options have also been suggested for non-responders to UDCA; B cell depletion using rituximab was investigated with promising results that require verification by additional studies[309-311].

The T cell receptor, cytotoxic T lymphocyte antigen 4 (CTLA-4) acts as a co-stimulatory molecule in T-cell activation which in turn leads to biliary damage. Recent evidence supports the utilization of an optimized course of therapy by means of CTLA-4 Ig to potentially aid in the therapy of PBC patients[312]. However the most promising new treatment since the introduction of UDCA is a derivative of chenodeoxycholic acid, obeticholic acid (OCA). The effect of obeticholic acid is mediated through its action in bile acid homeostasis, acting as a potent farnesoid X receptor (FXR) ligand[313,314]. FXR is the key nuclear receptor for homeostasis and entero-hepatic circulation of bile acids. Obeticholic acid is approximately 100 times more potent FXR activator than chenodeoxycholic acid, the strongest natural activator of FXR known so far[315]. Activation of FXR promotes bile acid secretion by activating bile acid transporters in the apical membrane of hepatocytes together with a

parallel reduction in bile acid uptake at the basolateral membrane. FXR agonists also reduce bile acid synthesis modulating the transcription of cholesterol 7-alpha-hydroxylase, the rate-limiting enzyme in bile acid synthesis, *via* induction of the suppressor protein small heterodimer partner and activation of the fibroblast growth factor 19 (FGF-19) enterohepatic signaling, which mediates bile acid feedback regulation[316,317].

Since OCA and UDCA exert their secretagogue effects on bile acids through distinct mechanisms, there is a potential for synergistic therapeutic effects. Experimental evidence indicates that OCA has also antifibrotic properties consistent with FXR agonist effects *in vivo*[318,319]. Clinical evaluation of OCA is currently ongoing, but phase-Ⅱ efficacy and safety data in PBC have already been reported. One hundred sixty-five patients with persistently high ALP levels (> 1.5 × ULN) were randomized to receive either a placebo or OCA at 10, 25 or 50 mg once daily for 12 wk while maintained on the usual UDCA treatment Results were initially presented as abstracts and very recently as a full paper[320,321]. Seventy percent of patients demonstrated a 25% reduction in ALP in the three OCA arms compared with a 3% change from baseline in the placebo group was reported. In addition, levels of aminotransferases, gamma-glutamyltransferase (GGT) as well as C4 sterol used as a surrogate marker of bile acid synthesis and the primary bile acids cholic acid and CDCA, were all significantly decreased while FGF-19 concentrations increased[322]. Biochemical improvement was maintained for an additional 12 mo open label extension of the initial trial. However an 85% incidence of pruritus with a dose response relationship was also observed but only 13% were forced to withdraw from the extended trial due to severe pruritus.

In another study, 59 UDCA-naive patients were randomized to either a placebo or OCA at 10 or 50 mg once daily as monotherapy for 12 wk[323]. A 50% reduction in ALP levels from baseline compared with no change in the placebo group was noted. Similar results were reported with aminotransferases, GGT and IgM levels significantly decreased in the OCA groups, but pruritus was seen in > 70% of patients and led to treatment discontinuation in up to 38% of cases.

MODE OF ACTION OF UDCA

Endogenous primary bile acids cholic acid and CDCA in both serum and bile are significantly decreased by the oral administration of UDCA, with a parallel marked increase in UDCA concentration, which then accounts for up to 50% of the circulating bile acid pool[324,325]. UDCA interrupts the enterohepatic circulation of endogenous bile acids increasing therefore their fecal elimination[326-328]. It also facilitates the endogenous secretion of bile acids into bile by promoting insertion and possibly activation of two bile carriers (the bile salt export pump bile salt export pump and the conjugate export pump, multidrug resistance-associated protein 2) into the canalicular membrane of the hepatocyte. UDCA also protects hepatocytes from apoptosis induced by bile acid. This is attributed to inhibition of mitochondrial membrane permeability transition, and stimulation of a survival pathway[329]. Another mechanism by which UDCA probably acts is the restoration of ductal expression of the anion exchanger 2, which is thought to compensate for defective biliary alkaline secretion thus contributing to the reduction of cytotoxicity of endogenous bile acids against cholangiocytes (the so called "biliary HCO3-umbrella hypothesis")[330]. Enhancement of defenses against oxidative stress is also a mechanism by which UDCA protects cholangiocytes and hepatocytes. In chronic cholestasis there is an excessive production of peroxide and hydroxynonenal protein adducts which is probably counteracted by an UDCA-induced enhancement of liver glutathione levels and the upregulation of J-glutamylcysteine synthetase and methionine S-adenosyltransferase[151,331-335]. In experimental animals[336] and patients with PBC[337,338], additional oxidative stress protective mechanisms, namely Nrf2-mediated hepatocellular transport, detoxification and anti-oxidative stress systems, have been induced by UDCA. UDCA may also exert hepatic as well as systemic anti-apoptotic effects. *In vitro* UDCA and tauroursodeoxycholic acid can prevent apoptosis caused by different agents like hydrophobic bile acids, ethanol, TGFb1, Fas ligand and okadaic acid in both hepatic and non-hepatic cells[339]. In patients with PBC, UDCA also exerts anti-apoptotic properties in cholangiocytes and so may inhibit the availability of mitochondrial antigens in dendritic cells and macrophages[340]. The mechanisms involved in the anti-apoptotic properties of UDCA include targeting of mitochondrial function and integrity, reduction of endoplasmic reticulum stress, and interactions with various survival signals in the cAMP, Akt, NF-κB, MAPK and PI3K signaling pathways[341-344].

We have demonstrated in HepG2 cells that UDCA modulates caspase activation and apoptosis in a concentration-dependent, while activation of the caspase cascade does not always correlates with increased apoptosis. We therefore concluded that serum UDCA concentrations should adjusted through dosage modifications to achieve the required effect[345].

UDCA is an extraordinary molecule since apart from the action mentioned above it seems likely, that it exerts anti-inflammatory[346] and immunological protective effects as well. UDCA strongly reduced MHC class Ⅰ expression in the liver in PBC[347]. In several experimental models, UDCA exerted immunosuppressive properties by interfering with B-and T-cell functions. UDCA inhibited CpG-induced immunoglobulin production by B cells[348] cytokine (IL-2, IL-4, IFN-J) release by mononuclear cells when challenged with LPS[349] and intrahepatic TNF-α and MIP-2 in the concanavalin A model of liver damage in experimental animals[350]. These findings suggest that UDCA modifies TLR TLR4 and TLR9 signaling pathways, therefore reducing inflammation[351]. The anti-inflammatory properties of

UDCA might, in addition, be mediated by activation of the vitamin D receptor in cholangiocytes to promote the secretion of antimicrobial peptides in human bile[352,353].

UDCA administration in PBC blunts the accumulation of endotoxin in bile and suppresses the immune reaction against lipid A[164,166,167]. The mechanisms involved in UDCA-induced tolerance to endotoxin are both a reduced intestinal absorption of gut-derived endotoxin[354] and protection against endotoxemia by enhancing the transport of LPS across hepatocytes from blood to bile[355]. LPS is inactivated in alkaline bile by alkaline phosphatase as it becomes less active in a lower pH milieu[163].

In many autoimmune mediated liver diseases an important pathogenetic role in the initiation and perpetuation of the hepatocyte injury is played by the NKT-cells[58]. UDCA corrects NKT-cell activity by inhibiting prostaglandin E2 production in PBC. Since, as mentioned before, a reduced function of NKT-cells induced by IP-10 inhibits liver stellate cell apoptosis, restoration of NKT activity is evidently favourable in PBC[356].

Two additional mechanisms have been proposed by us to further explain the beneficial effect of UDCA in PBC. We have suggested that an increase in ET2 observed from the early stages of the disease may be the initiative mechanism causing ischemic damage to cholangiocytes. UDCA treatment caused a significant reduction of ET2, possibly interrupting the early detrimental effect of ET2[179]. A second mechanism may involve the CXCL chemokines. As mentioned before, PBC patients have increased levels of the chemokines IP-10 and MIG both in plasma and the liver[133]. These chemokines are important for the attraction of cells cytotoxic for cholangiocytes.

We recently reported a significant serum decrease of these chemokines after UDCA administration. Moreover we demonstrated using flow cytometry a significantly lower CXCR3 expression in normal controls (13.5%) compared to PBC (37.2%), which was decreased (28.1%, $P < 0.01$) after UDCA treatment, a finding that might indicate a new additional mechanism of action for UDCA[134].

TRANSPLANTATION

Liver transplantation is the only curative treatment of PBC which is a very frequent indication for liver transplantation in the United States[288]. The indications are the same with any other cirrhosis and include refractory ascites, hepatic encephalopathy, and portal hypertension leading to hepatorenal syndrome, spontaneous bacterial peritonitis. However in PBC there are two additional indications unique in this disease. These are intractable pruritus and debilitating fatigue.

Liver transplantation improves long term survival in PBC patients. An earlier study of 161 PBC patients showed a significant improvement ($P < 0.01$) of outcomes when expected patient survival through utilization of the Mayo natural history model was compared to actual survival. Moreover the 2 year survival was 74% for transplanted patients as compared to 31% for non-transplanted patients[357]. These survival figures have been considerably improved in recent years[358].

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REVIEW

Antioxidants in liver health

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Abstract

Liver diseases are a worldwide medical problem because the liver is the principal detoxifying organ and maintains metabolic homeostasis. The liver metabolizes various compounds that produce free radicals (FR). However, antioxidants scavenge FR and maintain the oxidative/antioxidative balance in the liver. When the liver oxidative/antioxidative balance is disrupted, the state is termed oxidative stress. Oxidative stress leads to deleterious processes in the liver and produces liver diseases. Therefore, restoring antioxidants is essential to maintain homeostasis. One method of restoring antioxidants is to consume natural compounds with antioxidant capacity. The objective of this review is to provide information pertaining to various antioxidants found in food that have demonstrated utility in improving liver diseases.

Key words: Antioxidant; Oxidative stress; Naringenin; Quercetin; Curcumin; Resveratrol; Silymarin; Coffee; Liver diseases

Core tip: The objective of this review is to provide an evidence-based update of antioxidants present in food and to describe their benefits on liver diseases.

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INTRODUCTION

The liver is the main organ that metabolizes xenobiotics and endogenous molecules to maintain metabolic homeostasis in the organism. Therefore, the liver is a target of many insults that result in dysregulated hepatic homeostasis and lead to hepatic diseases[1,2]. The liver is composed of the following cells types: hepatocytes, Kupffer cells, liver sinusoidal endothelial cells, pit cells, and hepatic stellate cells (HSC)[3]. Cirrhosis is caused by liver injury from a variety of etiological factors and is the end stage of progressive fibrosis[4]. Oxidative stress plays an important role in the establishment of fibrosis and subsequently in cirrhosis[5]. Therefore, the use of molecules with antioxidant properties has

been proposed as a treatment for fibrosis and cirrhosis caused by oxidative stress.

LIVER DISEASES

Liver diseases are a major medical problem worldwide. There are numerous liver diseases caused by different insults, and the disease type depends on the location of development. The main causes of liver disease are viral and parasitic infections in regions such as Africa and Asia. Alcohol abuse is the most important cause of liver diseases in Europe and America. However, viral hepatitis has increased recently[6]. Cirrhosis is likely the most important liver disease, and it is characterized by the accumulation of extracellular matrix proteins (including collagens Ⅰ, Ⅲ and Ⅳ) and distortion of the hepatic architecture[7].

OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between the production of free radicals (FR) and the antioxidant defenses[8]. The overproduction of pro-oxidants causes deleterious events in the cell such as lipid peroxidation, oxidative DNA damage, and protein damage[9]. FR are defined as atoms or molecules with one or more unpaired electrons. FR can exist as radical cations or radical anions[10] and are usually unstable and highly reactive because they can react with nearby molecules and abstract electrons. Oxygen can reduce and generate reactive oxygen species (ROS) by the interaction with transition metals or by the excitation of electrons secondary to the addition of energy[9]. Oxidative stress contributes to fibrogenesis by increasing harmful cytokines such as transforming grown factor-β (TGF-β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α)[2]. Furthermore, TGF-β increases ROS production in endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts[3].

MAIN SOURCES OF FR IN BIOLOGICAL SYSTEMS

There are several pathways to produce FR, the principal source in the body is own metabolism into the cell; however, this is not the only mechanism to induce oxidative stress. The environment plays an important role in the production of FR, ROS and reactive nitrogen species (RNS), for example, air pollution, UV irradiation, X-rays and gamma-rays[10]. The production of ROS can be induced by endogenous or exogenous substances. The most important endogenous sources are cytochrome P450 metabolism, peroxisomes, microsomes, metal-catalyzed reactions, neutrophils, eosinophils and macrophages during inflammation, and mitochondria-catalyzed electron transport reactions in the complexes Ⅰ and Ⅲ[11,12]. Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes, consequently, mitochondria generates approximately 2-3 nmol of superoxide/min per mg of protein, indicating that this organelle is the most important physiological producer of ROS and hydrogen peroxide (H_2O_2)[12]. However, there are other sources of superoxide anion (O_2^-) like xanthine oxidase, an enzyme that belongs to molybdenum iron-sulphur flavin hydroxylases, which is widely distributed among species and is present in several tissues in mammals. This enzyme plays an important role in the hydroxylation of purines, particularly, by the oxidation of hypoxanthine to xanthine, then from xanthine to uric acid. In both reactions, molecular oxygen is reduced, forming O_2^- in the first reaction and H_2O_2 in the second[11]. Another endogenous source of ROS generation is during inflammation, by macrophages and neutrophils. Activated macrophages trigger an increase in oxygen uptake, resulting in the formation of O_2^-, nitric oxide (NO) and H_2O_2[13]. In neutrophils, nicotine adenine dinucleotide phosphate [NAD(P)H] oxidase generates O_2^- that is required for the respiratory burst necessary for bacterial destruction, also nonphagocytic NAD(P)H oxidases produce O_2^- in a range of 1%-10%[14]. Cytochrome P450 enzymes are another pathway of ROS production during the breakdown or uncoupling of the P450 catalytic cycle. Microsomes generate 80% of the H_2O_2 at hyperoxia sites, and peroxisomes produce H_2O_2 but not O_2^- under physiological conditions, the liver is the major organ where peroxisomes contribute with the overall H_2O_2 production[11]. Meanwhile, RNS like NO are synthetized by nitric oxide synthases (NOSs), which metabolizes arginine to citruline in a five-electron oxidative reaction, resulting in the formation of NO[15]. Cells from the immune system can produce also NO in the oxidative burst triggered during inflammation processes. In the extracellular environment, NO can react with oxygen and water then to form nitrate and nitrite anions, also the NO and O_2^- can react together and lead to a more reactive FR called peroxynitrite anion ($ONOO^-$) that can cause lipid peroxidation and DNA fragmentation[16].

ANTIOXIDANTS

Antioxidants are molecules that in low concentrations can prevent or delay the oxidation of an oxidizable substrate[17]. Antioxidants are present in our body and exist in several foods. Antioxidants have a high affinity for FR and scavenge these molecules to protect our health. Compounds with antioxidant properties donate electrons to FR to reduce their reactivity and maintain the cellular pro-oxidant/antioxidant balance. There are many types of molecules with antioxidant activity. Natural compounds have been studied extensively and are relevant to many illnesses including liver diseases (Table 1).

CURCUMIN

Curcumin is also known as diferuloylmethane or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepadieno-

Table 1 Different antioxidants found in food (clinical effects and relevance)

Antioxidant	Main clinical effects	Clinical relevance
Curcumin	Antioxidant Anti-fibrotic Anti-inflammatory Anti-microbial Wound healing Anti-carcinogenic	No studies available in human hepatic disorders
Resveratrol	Antioxidant Anti-inflammatory Anti-carcinogenic Lipid modulation Antifibrotic	Current data are contradictory, more clinical studies are needed
Coffee	Antinecrotic Antifibrotic Antioxidant Anticholestatic Chemoprotective	Inverse relationship between coffee-cirrhosis has been demonstrated, but it is necessary to do more basic research and prospective clinical trials
Quercetin	Chelation of transition metal ions Anti-carcinogenic Cardioprotective Bacteriostatic Antioxidant Antifibrotic Anti-inflammatory Anti-apoptotic Anti-aggregatory Vasodilating	No studies available in human hepatic disorders
Silymarin	Antioxidant Antifibrotic Anti-inflammatory Anti-carcinogenic Immunomodulation	Silymarin has been shown to be effective, but it is necessary to do more clinical trials focused on survival rates of patients with cirrhosis
Naringenin	Antioxidant Hypocholesterolemic Anti-estrogenic Hypolipidemic Antihypertensive Anti-inflammatory Antifibrotic Anti-carcinogenic Anti-atherogenic	No studies available in human hepatic disorders
Green tea	Anti-inflammatory Anti-arthritic Antimicrobial Antioxidant Neuroprotective Antidiabetic Antiangiogenesis Anti-carcinogenic	More clinical studies are needed

3,5-Dione. It is obtained from the rhizomes of Curcuma longa and has several pharmacological properties including strong antioxidant, anti-fibrogenic, anti-inflammatory, anti-microbial, and anti-carcinogenic actions in addition to wound healing effects[18,19]. Approximately, intake of turmeric in the Indian diet is of 2-2.5 g in a 60-kg individual, this is equal to 60-100 mg of curcumin daily. The Food and Drug Administration classified turmeric as a generally recognized as safe (GRAS). Toxicity assays on animals proved that curcumin is safe even at high doses. However, some species like mice and rats with prolonged high-dose intake of turmeric are susceptible to hepatotoxicity[20]. ^{3}H-curcumin was found to be poorly absorbed in the rat intestine[21]. It is metabolized into curcumin glucuronide and curcumin sulfonate[22]. When curcumin is administrated intraperitoneally, it is metabolized to tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol[23]. Despite curcumin has low bioavailability when is administered orally, Arcaro *et al*[24] (2014) used piperine, an inhibitor of hepatic and intestinal concomitantly with curcumin. They had shown that both 90 mg/kg of curcumin and 20 mg/kg of piperine had antidiabetic and antioxidant effects. Nevertheless, coadministration of curcumin and piperine did not change the antidiabetic and antioxidant activity of curcumin. Additionally, when the dose of piperine was increased to 40 mg/kg this abrogated the beneficial effects of curcumin. Contrary, Sehgal *et al*[25] proved the effect of piperine in curcumin in mitigating benzo(a)pyrene toxicity in liver. They found that pretreatment of 100 mg/kg

of body weight of curcumin protects against a single dose of benzo(a)pyrene; however, the coadministration with piperine produced a better effect than curcumin alone, suggesting an enhancer activity by piperine. Curcumin has demonstrated hepatoprotective actions on acute and chronic liver injury[26]. Both of types of liver injuries necrosis, oxidative stress, and an inflammatory state[27]. In 2007, Reyes-Gordillo *et al*[28] demonstrated that curcumin inhibits the increase in cytokines such as TNF-α, IL-1β and IL-6. Additionally, curcumin reduces oxidative stress induced *via* carbon tetrachloride (CCl_4) metabolism by inactivating the nuclear factor-κB (NF-κB) pathway. Moreover, curcumin can elicit its hepatoprotective effect interacting with Fe^{3+} and Cu^{2+}. In a study performed by Jiao *et al*[29], they suggest that curcumin could be an iron chelator because they found that transferrin receptor 1 and iron regulatory proteins, indicators of iron depletion, increased in response of curcumin. In agreement, Bernabé-Pineda *et al*[30], reported that when cyclic voltammograms are in basic media, that a chemical reaction has taken place between curcumin and specially Fe^{3+}. On the other hand, curcumin has been tested in liver[31], but its chelating Cu^{2+} behavior has not been investigated; however, Baum *et al*[32] in 2004 tested the interaction of curcumin with Cu^{2+} and Fe^{2+}, they reported that two molecules of curcumin bind to ion Cu^{2+} or Fe^{2+}. In a study performed by Li *et al*[33], it was found that curcumin increases the levels of glutathione (GSH) and heme oxygenase-1 (HO-1), as well as, nuclear factor-erythroid 2-related factor 2 (Nrf2) proteins, suggesting another way to prevent oxidative stress by curcumin. In agreement, Charoensuk *et al*[34] have shown that curcumin increased the levels of mRNA and protein of Nrf2 and HO-1 and gene expression of NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCL), activating transcription factor-3, peroxiredoxin 3 (Prdx3) and Prdx6, so increasing the antioxidant system in the cell. Curcumin also has demonstrated to increase the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)[35,36] activity. Another mechanism of action of curcumin is by interacting with enzymes or genes implicated in liver cirrhosis. Hassan *et al*[37] proved effect of curcumin by modulating miRNA 199 and 200 that are the main miRNA associated to liver fibrosis. They showed that miRNA 199 and 200 were increased by the administration of CCl_4. However, curcumin restored these miRNAS to their basal levels. Finally, curcumin has shown that in low concentrations, it inhibits the activity of CYP2E1 and its protein levels in alcohol-induced liver damage, thus inhibiting the metabolism of alcohol for this pathway[38]. However, other studies have shown that curcumin does not have an effect on CYP2E1 activity in the liver[39-41].

RESVERATROL

The phytoalexin resveratrol (3,5,4'-trans-trihydroxystilbene) is a polyphenol found in the skin of red grapes, red wine, peanuts and berries[42]. This compound possesses several beneficial activities including antioxidant, anti-inflammatory, anti-carcinogenic, and anti-fibrogenic properties, in addition to affecting lipid modulation[43]. The rate of absorption of resveratrol is approximately 75% after an oral administration[44]. Resveratrol is metabolized to resveratrol sulfate and in low concentrations to resveratrol glucuronide[45] *via* enzymes of phase Ⅱ through UDP-glucuronosyltransferase (UGT) or sulfotransferase (ST)[46]. These results were based on *in vitro* experiments with hepatocytes treated with resveratrol. Conversely, *in vivo* experiments performed in rats demonstrated that the enterohepatic recirculation plays an important role in the overall systematic exposure to resveratrol when it was administered in aglycone or glucuronide form[47]. Resveratrol has been reported as a compound well tolerated in clinical trials[48]. Nevertheless, in a study performed by Crowell *et al*[49] in an animal model, resveratrol at the highest dose used (3000 mg/kg body weight/day for 4 wk) produced renal toxicity and reduced final body weights and food consumption as well as other markers of tissue lesions. However, no histological effects in the liver were observed, despite of the clinical chemistry changes and increased liver weight. On the other hand, Williams *et al*[50] reported not toxicity caused by high-purity trans-resveratrol at different times of exposure and doses. They used 700 mg/kg body weight/day for 90 d as the higher dose and time of exposure, not finding any adverse effect. In 2007, Chávez *et al*[43] demonstrated that resveratrol decreased the cytokine TGF-β and prevented hepatic fibrosis and NF-κB translocation to the nucleus following chronic use of CCl_4. Resveratrol has antioxidant capacity and protects against ethanol-induced lipid peroxidation[51], toxicity by acetaminophen (APAP)[52], and oxidative stress in animal models of cholestasis[53]. It has been suggested that the OH groups play an important role in the antioxidant activity of resveratrol[6]. A study performed by blocking OH group methylation showed that resveratrol and trimethylated resveratrol afford some degree of protection, but the latter possesses the best protective effects[54]. One explanation of this phenomenon considers that the half-life of resveratrol is very short[55] and that trimethylated resveratrol may act as a prodrug and increase the protective effect of resveratrol. However, resveratrol could be less potent than trimethylated resveratrol[6]. Another hepatoprotection mechanism of resveratrol is by activating genes related to antioxidant system or inhibiting enzymes. A study performed by Cheng *et al*[56] suggest that resveratrol can activate extracellular signal-regulated kinase (ERK) signaling pathway, which in turn can enhance the activation and translocation of Nrf2 to the nucleus, therefore, elevating the expression of HO-1 and glyoxalase. According to the previous study, Bagul *et al*[57] have shown that resveratrol was able to elevate the translocation of Nrf2 the nucleus, thus suggesting an alternative pathway to protect from oxidative stress. Resveratrol has been reported to decrease acetylation

of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) and increasing its activity by the activation of the protein deacetylase sirtuin 1 (SIRT1), thus improving mitochondrial function and protecting against metabolic disease[58]. Price *et al*[59] found that resveratrol activates AMP-activated protein kinase (AMPK) in mice treated with moderate dosage and increase nicotinamide adenine dinucleotide (NAD^+) levels, also increasing the levels of PGC1-α. AMPK has been shown to augment SIRT1 by increasing NAD^+ levels in an indirect way, while this protein deacetylates the AMPK liver kinase B1, leading to phosphorylation and activation of AMPK. Zhu *et al*[60] have shown that, after administration of resveratrol in mice, the antioxidant system was increased (SOD, GPx, and GSH) in liver tissue as well as the levels of SIRT1 and p-AMPK were upregulated. Resveratrol has also shown to inhibit the activity of CYP2E1 in microsomes of rat liver[61], and to significantly inhibit the activity of this P450 isoform in a model of APAP-induced liver injury[62] and in DEN-induced hepatocarcinogenesis model[63]. The only clinical double-blind study performed to determine the resveratrol hepatoprotective effect demonstrated that a 500 mg resveratrol dose for 12 wk caused a significant reduction in inflammatory cytokines, serum cytokeratin-18, NF-κB activation, liver alanine aminotransferase (ALT), and hepatic steatosis grade compared to the placebo group in patients with non-alcoholic fatty liver disease (NAFLD)[64]. Contrary to this study, a clinical trial performed by Chachay *et al*[65] in 2014 showed that resveratrol did not significantly improve any characteristics of NAFLD compared to the placebo group. Interestingly, the results showed increased hepatic stress and elevated levels of ALT and aspartate aminotransferase (AST) in the liver. Based on these data, additional clinical trials are needed to determine the actual hepatoprotective effect of resveratrol[66].

COFFEE

Coffee is a mixture of several different molecules including carbohydrates, lipids, vitamins, alkaloids, nitrogenous molecules, and phenolic compounds[67]. Coffee is possibly the most popular drink worldwide[68]. However, the amount of antioxidants in different bean roasts can vary[69]. The three major compounds present in coffee are caffeine, diterpene alcohols (cafestol and kahweol), and chlorogenic acid[70]. Coffee consumption has been associated with the reduction of several chronic diseases[71]. This result may be due to the pharmacological properties that have anti-necrotic, anti-fibrotic, anti-cholestatic, chemoprotective, and antioxidant functions[72]. Caffeine is the best-known active component of coffee, and it is absorbed rapidly after an oral ingestion (5 min) and reaches its peak blood levels after 30 min. When caffeine is consumed in high amounts produced side effects. Recommendations from Health Canada in 2013, stipulated that the caffeine intake per day for children should not exceed 2.5 mg/kg of body weight. Additionally, tachycardia and arrhythmia typically arise when more than 200 mg of caffeine are ingested[73]. Worthley *et al*[74] have given 250 mL of a sugar-free energy drink to 50 young people, this drink contained about 80 mg of caffeine, they have observed that caffeine increased the blood pressure compared with controls. Moreover, other kind of sickness have been reported for caffeine consumption such as cardiovascular diseases, a negatively impact in cognition, perpetual memory and learning[73]. Smith *et al*[75] in 2002, reported that the intake of 300 mg of caffeine increased anxiety and tension. Also, caffeine triggered hallucinatory experiences in people who drink 300 mg of coffee (about 7 cups per day). Patients with panic disorders were more sensitive to caffeine[73]. The half-life of caffeine is approximately 5-6 h[76,77]. Caffeine is almost completely metabolized in the liver. The principal metabolite is paraxanthine, which results from the activity of CYP1A2. However, this enzyme produces other metabolites such as theobromine, theophylline, and 1,3,7-trimethyluric acid. Other enzymes such as CYPs 1A1 and 2E1 participate in caffeine metabolism[78]. An important property of caffeine is that it easily passes through the blood-brain barrier[79]. The absorption rate of chlorogenic acid is 33% of the dose in both, rats and humans. Several metabolites have been identified in human plasma and urine, and these metabolites include polyphenolic acids, glycine conjugates, sulfates and glucuronides of hydroxycinnamic acid, and hydrogenated hydroxycinnamic acid[80]. Many studies have shown an inverse relationship among coffee consumption and liver cirrhosis[71]. In the prospective study performed by Klatsky *et al*[81], the authors demonstrated an inverse coffee-cirrhosis relationship for the first time. In 1994, Corrao *et al*[82] performed a study and identified a dose-response relationship between coffee intake and cirrhosis. The data show that the odds ratio for liver cirrhosis decreases from 1.0 for people who do not drink coffee to 0.47, 0.23, 0.21, and 0.16 for 1, 2, 3 or 4 cups of coffee daily, respectively. Furthermore, the study showed that the caffeine *per se* did not show any relationship with cirrhosis by testing other drinks with caffeine. Although coffee is beneficial to liver health, the study failed to demonstrate a causative role of coffee in preventing liver injury. Thus, additional basic research and controlled prospective studies are needed. Arauz *et al*[72] in 2013 demonstrated that coffee has a beneficial effect on liver injury caused by chronic administration of thioacetamide (TAA). Coffee prevented cholestasis and necrosis measured by the enzymes γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase, and ALT. Human trials demonstrated the same results[83]. This inverse relationship was particularly high in heavy alcohol drinkers[84]. Conversely, liver injury inhibits caffeine metabolism, and people with liver diseases may experience adverse consequences after drinking coffee. Furthermore, it is important to distinguish between former coffee drinkers and nondrinkers in future epidemiological studies[71,85]. Moreno *et al*[86] in 2011 and

Arauz *et al*[72] in 2013 demonstrated in murine models that coffee prevents experimental liver cirrhosis in two models of liver injury using carbon tetrachloride and thioacetamide. Both studies showed that coffee reduced the expression of the profibrogenic cytokine TGF-β. The study by Arauz *et al*[72] measured the expression of connective tissue growth factor (CTGF), which has been suggested as an important downstream modulator of TGF-β that increases its profibrogenic response. This finding is consistent with the significant upregulation of extracellular matrix in fibrotic liver[72]. Cavin *et al*[87] have reported coffee as an inductor of GST, aldo-keto reductase, GSH, HO-1, glutathione-S-transferase P1, that are enzymes involved in the detoxification process. Also, they suggest that a possible mechanism of chemoprotection of coffee is by stimulation of Nrf2 pathway. In another study, coffee was able to elevate mRNA levels of NQO1 and glutathione-S-transferase A1 in liver and small intestine also, UDP-glucuronosyltransferase 1A6 and GCL catalytic (GCLC) were increased in small intestine. Further, the same group reported that this induction was bigger in mice possessing Nrf2 in contrast with Nrf2 knockout mice[88].

QUERCETIN

Quercetin is also known as 3,3',4',5,7-penta-hydroxyflavone. It is a flavonol found in natural products, especially in apples and onions[89]. Quercetin is known to have biological effects including chelation of heavy metals, anti-carcinogenic, cardioprotective, bacteriostatic, anti-inflammatory, and antioxidant properties[90], in addition to functioning as a hepatoprotective agent[91]. The normal intake of quercetin is less than 5-40 mg/d. However, people who eat the peel of food with high amounts of quercetin may consume 200-500 mg/d[90]. In 2004, high purity quercetin used in foods was GRAS in the range of 0.008%-0.5% or 10-125 mg/serving[90]. Bors *et al*[92] in 1990 showed the characteristics that an antioxidant must have to exert an effective activity. These characteristics include the presence of ortho-dihydroxy or catechol groups in the B-ring, a 2,3-double bond of the C-ring, and OH substitution on positions 3 and 5 of the C-ring and A-ring, respectively[92]. The quercetin ring presents all of these features. The structure of flavonoids can interact with both FR and metal ions like Fe^{3+} and Cu^{2+}, therefore showing chelating properties. In a study performed by Mira *et al*[93], it was shown that quercetin was capable of reducing Fe^{3+} and Cu^{2+}, due to its 2, 3-double bond and the catechol group in the β-ring. Furthermore, its ability to reduce the Cu^{2+} seems to be dependent of hydroxyl groups. After oral intake, quercetin is rapidly absorbed and peaks at approximately 30 min[94] before it is metabolized by glucuronidation and sulfation by the UGT and ST, respectively. Furthermore, the addition of O-methylation in the position 3' or 4' of the catechol group in the B-ring results in isorhamnetin (3'-O-methylquercetin) and tamaraxetin (4'-O-methylquercetin) by catechol-O-methyl transferase. These processes begin in the intestine, and the compounds are released into the lumen before conjugation in the liver by the same enzymes. However, other tissues such as the kidneys can also metabolize quercetin[94-97]. Quercetin has shown hepatoprotective properties in rats treated chronically with CCl_4 for 8 wk by preventing the expression of profibrogenic genes including TGF-β, CTGF, and collagen-1α (Col-1α). Therefore, quercetin reduces the fibrogenic process and liver enzymes associated with a significant reduction of activated HSC and inhibition of NF-κB. Conversely, quercetin increased the gene expression and improved the activity of SOD and CAT, in addition to activating metalloproteinases 2 and 9 (MMP2 and MMP9)[91]. Pavanato *et al*[98] in 2003 used the same hepatotoxin for 16 wk and observed that quercetin improves the hepatic liver enzymes AST, ALT, inducible NOS (iNOS) expression, and collagen content and reduces lipid peroxidation. de David *et al*[99] showed similar results using TAA hepatotoxin and found that quercetin inhibited the change in the p-ERK 1/2 pathway and significantly prevented the increase in apoptosis by regulating the Bax/Bcl-2 ratio[99]. In a study performed by Granado-Serrano *et al*[100] in HepG2 cells, they found that quercetin modulated Nrf2 and p38, it was dependent on the concentration used and the time of exposure, quercetin rapidly activated Nrf2 by up-regulating its phosphorylation, consequently, translocation to the nucleus and binding to antioxidant response element (ARE), also increased GSH content and expression of GPx. However, when the time of exposure is larger, this effect was blocked by quercetin which, in turn activated p38-MAPK *via*. Therefore suggesting that Nef2-ARE acts as a sensor and responds to a chemical. However, Tanigawa *et al*[101] reported that quercetin possesses an enhanced effect in the ARE binding activity and Nrf2-mediated transcription activity in HepG2 cells. Moreover, quercetin apart from up-regulating expression of Nrf2 mRNA and protein, also stabilized Nrf2 protein inhibiting its proteasomal degradation and reduced the levels of kelch-like ECH-associated protein 1 (Keap1) through the formation of a modified Keap1. On the other hand, a study performed by Ji *et al*[102] showed that quercetin does not possesses an enhanced activity in mRNA expression of Nrf2 or Keap1. However, they suggested that quercetin could interact with Keap1 and fill the binding site of Nrf2 in Keap1, thus inhibiting its interaction and inducing the transcriptional activation of Nrf2. Quercetin has shown to suppress the activity of CYP2E1 when ethanol over activated it and induces HO-1 in hepatocytes[103]. According with this findings, in a non-alcoholic steatohepatitis (NASH) model, quercetin was able to decrease by 2-fold CYP2E1 activity compared with NASH group[104]. On the other hand, quercetin effect was inhibited by CYP2E1 compared with a control measuring by HPLC in rat liver microsomes[105]. Currently, there are no clinical studies available on quercetin

hepatoprotection[106].

SILYMARIN

Silymarin is a natural substance derived from Silybum marianum, also known as Milk thistle or Saint Mary's thistle[6]. Silymarin has been reported as a safe compound in acute doses in animal models due to its lack of side effects. In contrast, in a clinical trial, thousands of patients suffered mainly mild gastrointestinal disorders by silymarin consumption[107]. In other clinical trial, El-Kamary *et al*[108] (2009) no side effects were reported in 105 patients using 140 mg of silymarin. The range of doses used in literature is from 280 to 800 mg/kg of body weight/day. After oral administration, the silymarin peak plasma concentration is reached at approximately 6-8 h. Silymarin has poor bioavailability (23%-47%). The metabolites of silymarin are conjugated in the liver by UGT and ST (phase Ⅱ reactions)[109]. Among the hepatoprotective effects of silymarin, it is known that silybin, the major constituent of silymarin, has iron-chelating properties[110,111]. Silymarin has also been probed as iron chelator in children with β-thalassemia with iron overload[112]. In a study performed by Najafzadeh *et al*[113], they suggest that hepatoprotective effect of silymarin in iron-overload induced hepatotoxicity was due to an iron-chelator activity but no studies have been made proving the chelating properties *per se* of silymarin in liver diseases. Silymarin has hepatoprotective properties against several hepatotoxins such as CCl_4. Silymarin can prevent oxidative stress, fibrosis, cirrhosis, and lipid peroxidation by modulating the content of phosphatidylethanolamine[114]. Thus, it improves liver enzyme activities and protects against the harmful increases in cholesterol/phospholipids and sphingomyelin/phosphatidylcholine ratios in the membrane. This effect was associated with a decrease in Na^+/K^+ and Ca^{2+}-ATPase activities induced by CCl_4. However, silymarin does not reverse well-established cirrhosis[115-119]. Kim *et al*[120] showed that silymarin increases nuclear translocation of Nrf2 in activated HSC, however, expression of other molecules related to a detoxifying effect have not been measured. Also, silymarin has been reported to increase the activity of antioxidant enzymes like SOD, GPx[121] and CAT[122]. A clinical trial examining silymarin in a complex with phosphatidylcholine found reduced levels of the liver enzymes, ALT and γ-GTP, and serum bilirubin levels in a dose-dependent manner in patients with hepatitis caused by virus infection or alcohol abuse[123]. Another clinical study showed similar results when silymarin was administered alone[124]. In patients with cirrhosis, silymarin administration for 41 mo significantly increased the survival rate compared to a placebo group[125]. However, in the study performed by Parés *et al*[126], silymarin showed no effect on survival rate in the clinical course in alcoholic patients with liver cirrhosis.

NARINGENIN

Naringenin is also known as 5,7,4′-thihydroxyflavanone. Naringenin is a flavanone found in citrus fruits and tomatoes[127]. In a study performed recently, Yang *et al*[128] reported that naringenin does not cause deleterious effects in beagle dogs, the maximum time of exposure was 180 d and with doses varying of 20, 100, or 500 mg/kg body weight/day. Also, Surampalli *et al*[129], showed that naringenin was harmless upon exposure to rat gastrointestinal epithelium in doses ranging from 1 mmol/L to 100 mmol/L, thus suggesting naringenin as a safe compound. Naringenin has many pharmacological properties including hypolipidemic, anti-hypertensive, anti-inflammatory, antioxidant and anti-fibrotic functions[127]. Flavonoids are absorbed in the aglycone form rather than in the glycoside form like quercetin. The glycoside form of naringenin is cleaved in the small intestine before absorption, which results in sulfate and glucuronide metabolites in the small intestine wall and liver[127,130] by UGT and ST. Mira *et al*[93] showed that naringenin has shown capacity of reduction of the Fe^{3+} and Cu^{2+} but in less potential than quercetin. Chtourou *et al*[131] found that naringenin prevents the depletion of SOD, CAT, GPx and GSH. Conversely, naringenin also prevented the increase in lipid peroxidation, ALT and AST. Additionally, expression of the following genes was also affected in an NAFLD rat model induced by a high cholesterol diet: pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, EGF-like module-containing mucin-like hormone receptor-like 1, iNOS, NF-κB, MMP2 and MMP9[131]. Similar results were obtained by Yen *et al*[132] using naringenin alone and a naringenin-loaded nanoparticle system (NARN). Both treatments exhibited antioxidant and hepatoprotective activities. The treatments also inhibited the activation of caspases 3, and 8. However, NARN was more effective as a hepatoprotector and antioxidant than free naringenin because it also inhibits caspase 9 during CCl_4-induced hepatotoxicity in rats[132]. In a study performed by Goldwasser *et al*[133] it was found that naringenin activates peroxisome proliferator-activated receptor alpha (PPARα), then decreasing the levels of very low density lipoprotein production without causing lipid accumulation in hepatocytes, in a hepatitis C virus (HCV) model. Similar results were found by Cho *et al*[134], who have shown that naringenin intake causes a significant depletion in the amount of total triglycerides and cholesterol in plasma and liver of rats. Also, naringenin-fed animals showed an increment in PPARα protein expression in liver. Goldwasser *et al*[133] found that the flavonoid regulates the activity of PPARγ and liver X receptor alpha (LXRα), by activating the ligand-binding domain of PPARα and PPARγ, while inhibiting LXRα, thus modulating different genes related to fatty acid oxidation and lipogenesis. Han *et al*[135], found that a pretreatment with naringenin-7-O-glucoside increased NQO1, ERK and phosphorylation and translocation of Nrf2 to the nucleus in H9c2 cardiomyocytes, as well as, upregulating the

mRNA expression of GCLC and GCL modifier[135], thus inducting endogenous antioxidant enzymes. Similar findings was reported by Esmaeili *et al*[136], they showed that naringenin was capable of attenuating CCl_4-induced liver injury by downregulating TNF-α, iNOS and cyclo-oxigenase-2, both protein and mRNA, as well as by increasing Nfr2 and HO-1 expression. Motawi *et al*[137] suggested that naringenin could be another example of CYP2E1 inhibitor, they probed it, in rat liver microsomal assay in co-administration with simvastin, and such inhibition of CYP2E1 is another *via* to improve antioxidant defenses[137]. There are currently no studies available in human hepatic disorders[138].

GREEN TEA

Camellia sinensis, also known as green tea, is a worldwide consumed beverage. Its beneficial effects on health are due in part to its antioxidant, anti-inflammatory, anti-arthritic and anti-angiogenic effects. Moreover, green tea is a mixture of polyphenols (the major class of active compounds) including catechins (also known as flavan-3-ols) which constitute about 30% (mass fraction) of green tea leaves; the major catechins in green tea are (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-gallate, (-)-gallocatechin, (-)-gallocatechin gallate and (-)-epigallocatechin-3-gallate (EGCG). Flavonoids like quercetin, kaempferol and myricetin; methylxanthine alkaloids such as caffeine, theophylline and theobromine, and phenolic acids (gallic acid, chlorogenic acid and caffeic acid)[139,140]. EGCG is the most abundant catechin and represents up to 50% of total polyphenols and possesses the strongest antioxidant capacity, therefore, it is considered the main biological active compound[140]. On the other hand, green tea does not only exert its antioxidant properties by polyphenols, L-theanine is the primary amino acid in green tea and represents 1%-2% of the leaf dry weight, it is synthetized in the roots of green tea and is concentrated in the leaves. L-theanine chemical structure is similar to glutamic acid, the latest is a precursor of GSH. Studies have shown that L-theanine protects the cell maintaining the levels of GSH in cancer and neurotixicity diseases[141]. The intake of green tea can be considered safe when its consumption does not exceed 1-2 cups/d. Nevertheless, hepatotoxicity has been attributed to the intake of green tea when it is used for weight control; furthermore[140]. Pérez-Vargas *et al*[141] found that L-theanine prevented the increased expression of NF-κB and down-regulated IL-1β and IL-6 and the cytokines TGF-β and CTGF induced by carbon tetrachloride. Moreover, the expression of the corresponding mRNAs decreased accordingly. On the other hand, L-theanine promoted the expression of IL-10 and the fibrolytic enzyme metalloproteinase 13 (MMP13). In a study performed by Yu *et al*[142] they have shown that EGCG ameliorates liver inflammation, necrosis and fibrosis and suppressed the expression of TNF-α, IL-1β, TGF-β, MMP9, α-SMA, and Col-1α1. Similar results were obtained in HSC cell line LX-2, where EGCG was capable of suppressing TGF-β1, Col-1α1, MMP2, MMP9, TIMP1, and α-SMA. Moreover, Bin Dajem *et al*[143] used the aqueous extract of green tea in a Schistosoma mansoni-infected mice model to investigate its effect on the oxidative stress, antioxidant system and liver pathology induced by the parasite. They found that green tea extract suppressed the oxidative stress by decreasing the lipid peroxides. However, failed to enhance the antioxidant system and to reverse alterations in the liver such as necrosis. In a study performed by Higashi *et al*[144] they found that EGCG modulates the growth of HSC activated cells by Rho-signaling pathways and induces the phosphorylation of ERK 1/2, c-Jun kinase and p38, suggesting a mechanism of its anti-fibrotic capacity. In a cisplatin-induced nephrotoxicity model in rats, EGCG increased the levels of Nfr2, HO-1, SOD, CAT, GPx and GSH[145]. In clinical trials, green tea has shown protective effects against various kinds of cancers, including premalignant prostate, esophageal, colon, rectum and pancreatic cancers[146]. Nevertheless, in hepatocellular carcinoma, green tea did not have any protective effect[147]. In a study performed by Halegoua-De Marzio *et al*[148] they have shown, after a single oral dose of green tea (400 mg), in patients with cirrhosis induced by HCV, that it is safe and well tolerable by all patients, therefore suggesting the use of green tea in the treatment of cirrhosis in the future. However, more clinical studies related to the beneficial effects on liver diseases are needed.

The information shown above represents some of the antioxidants uses in different kind of experiments in animals and clinical trials. However, it is difficult to say which of these antioxidants possess the best hepatoprotective properties since they have different chemical structures and antioxidant potency, then its scavenger capacity is not the same. Moreover, other parameters need to be considered, such as the bioavailability, and pharmacokinetics. We focus our hepatoprotective ranking mainly based on the chemical structure showed in Figure 1. We suggest that silymarin has the best hepatoprotective effect because is a mixture of flavonolignans including silybin, isosilybin, silydianin, silychristin, isosilychristin and the flavonoid taxifolin. In addition, silybinin is composed of 2 diasteroisomeric compounds (silybin A and silybin B) in a 1:1 ratio[149]. Flavonoids in its structure have different forms to stabilize FR including hydroxyl phenolic groups, double bonds and sometimes a catechol group[92]. Therefore, silymarin seems to be the best choice referred to hepatoprotective effect. Green tea is another mixture of polyphenols, as mentioned earlier, containing catechins, flavonoids and methylxanthine alkaloids. Nevertheless its data referred to hepatoprotection is lower than silymarin, for these reason we decided ranked green tea in the second place. The antioxidant property of EGCG is related of its hydroxyl phenolic groups, that maybe acts mainly from hydrogen atoms transfer or single electron transfer reactions. This groups are presented in the B- and D-rings of

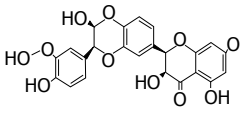
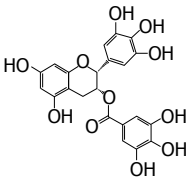
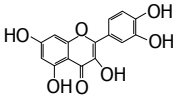
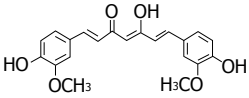
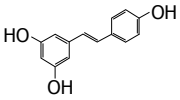
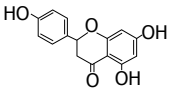
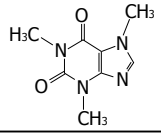
Antioxidant	Efficacy on hepatoprotective effect	Structure
Silymarin[1]	The highest	
Green tea[1]	Lower than silymarin	
Quercetin	Lower than green tea	
Curcumin	Lower than quercetin	
Resveratrol	Lower than curcumin	
Naringenin	Lower than resveratrol	
Coffee[1]	The lowest	

Figure 1 Comparison between hepatoprotective effect-related antioxidant capacities. [1]The structure presented is based on the most studied compound. For silymarin we show the structure of silybin, in the case of green tea the structure of (-)-epigallocatechin-3-gallate, and in the case of coffee the structure of caffeine.

EGCG[150]. Quercetin, as mentioned above, is a flavonoid that have all the elements to exert a magnificent hepatoprotective effect related to its structure showing a catechol group in the B ring, substitution of hydroxyl phenolic groups in the A and C ring and a double bond in the position 2-3 of the C ring[92]. Curcumin has been used in the treatment of experimental liver diseases since 1970 and shows a powerful antioxidant capacity and immunomodulatory properties. However, it does not have the same structure of flavonoids, showing two hydroxyl phenolic groups and a heptadiene linkage two methoxyphenol rings. Ak *et al*[151] suggest that keto form of curcumin, the heptadienone linkage between the two methoxyphenol rings, contain a carbon atom that can donate a hydrogen, therefore, stabilizing FR. We considered that its capacity of stabilize FR is lower than quercetin. Resveratrol possesses hydroxyl phenolic groups and a system of conjugated double bonds that can donate electrons to FR. Resveratrol has two phenolic rings: monophenol and diphenol. Gülçin[152] suggests that subtraction of hydrogen atom is easily in the monofenol ring. Naringenin is another flavonoid with lower antioxidant capacity than quercetin, shows a hydroxyl phenolic group in its structure in the A ring. However, it does not have the catechol group or the double bond[92]. Also, Cao *et al*[153] suggest that in flavonoids the hydroxyl substitution is relevant in the $ORAC_{OH^{\cdot}}$ activity. Caffeine has double bonds in its structure. Chu *et al*[154] reported that pure caffeine had very low $ORAC_{OH^{\cdot}}$ values, whereas, crude caffeine had higher values than pure caffeine. We considered that caffeine has the lowest antioxidant activity of all the compounds showed; therefore coffee has the lowest antioxidant capacity.

CONCLUSION

Investigations of antioxidants show that compounds in food are candidates for the treatment of several diseases because they improve the antioxidant system in the body, especially when the disease involves oxidative stress. This review describes antioxidants that can be investigated for experimental and clinical trials and will be used for the treatment of liver diseases such as liver cirrhosis. Curcumin, quercetin, and naringenin are effective in the treatment of experimental liver injury, and they can be studied in clinical trials. Green tea have been shown to protect against different kinds of cancer in clinical trials, except in hepatocellular carcinoma. Conversely, there are no clinical trials investigating resveratrol, coffee, and silymarin. However, the data are poor or contradictory, and it is necessary to perform more clinical trials to use these antioxidants for the

treatment of liver diseases in patients.

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ORIGINAL ARTICLE

Basic Study

Nociceptin effect on intestinal motility depends on opioid-receptor like-1 receptors and nitric oxide synthase co-localization

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Abstract

AIM: To study the effect of the opioid-receptor like-1 (ORL1) agonist nociceptin on gastrointestinal (GI)

myenteric neurotransmission and motility.

METHODS: Reverse transcriptase - polymerase chain reaction and immunohistochemistry were used to localize nociceptin and ORL1 in mouse tissues. Intracellular electrophysiological recordings of excitatory and inhibitory junction potentials (EJP, IJP) were made in a chambered organ bath. Intestinal motility was measured *in vivo*.

RESULTS: Nociceptin accelerated whole and upper GI transit, but slowed colonic expulsion *in vivo* in an ORL1-dependent manner, as shown using [Nphe^1]NOC and AS ODN pretreatment. ORL1 and nociceptin immunoreactivity were found on enteric neurons. Nociceptin reduced the EJP and the nitric oxide-sensitive slow IJP in an ORL1-dependent manner, whereas the fast IJP was unchanged. Nociceptin further reduced the spatial spreading of the EJP up to 2 cm.

CONCLUSION: Compounds acting at ORL1 are good candidates for the future treatment of disorders associated with increased colonic transit, such as diarrhea or diarrhea-predominant irritable bowel syndrome.

Key words: Nociceptin; Gastrointestinal transit; Opioid-receptor like-1; Electrophysiology; Expression

Core tip: Here we aimed to study the effect of the endogenous opioid-receptor like-1 (ORL1) agonist nociceptin on gastrointestinal (GI) myenteric neurotransmission and motility. We observed that nociceptin reduces excitatory and inhibitory neurotransmission in motorneurons and interneurons and the overall effect on the GI tract may depend on co-localization of ORL1 receptors with nitric oxide synthase. Since ORL1 activation, unlike other opioid receptor-active drugs, is not associated with central side effects, compounds acting at the ORL1 may be good candidates for the future treatment of disorders associated with increased colonic transit, such as diarrhea or diarrhea-predominant irritable bowel syndrome.

Sibaev A, Fichna J, Saur D, Yuece B, Timmermans JP, Storr M. Nociceptin effect on intestinal motility depends on opioid-receptor like-1 receptors and nitric oxide synthase co-localization. *World J Gastrointest Pharmacol Ther* 2015; 6(3): 73-83 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i3.73.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i3.73

INTRODUCTION

The heptadecapeptide nociceptin, also known as orphanin FQ, is the only known endogenous agonist at the opioid-receptor like-1 (ORL1) receptor[1,2]. From a structural perspective nociceptin is closely related to the dynorphin family, but differs from other endogenous opioids[1-5]. Although the ORL1 receptor, alternatively named nociceptin or opioid-4, is a G protein-coupled receptor that shares significant (> 60%) sequence homology to the classical opioid binding sites (μ, δ, κ)[4,6-8], it remains the only target for nociceptin. Furthermore, the effects of nociceptin are not blocked by naloxone, which would further argue against grouping the ORL1 with classical opioid receptors.

ORL1 mRNA is widely distributed in the central and peripheral nervous systems, and in peripheral organs. In the gastrointestinal (GI) tract ORL1 was localized by molecular and immunohistochemical methods. In the guinea pig colon nociceptin is co-localized with calretinin, substance P and enkephalin and, to a small extent, also with nitric oxide synthase (NOS) and VIP immunoreactivity, suggesting that it may be localized in excitatory motorneurons, as well as in descending interneurons[9].

The GI tract is one site where opioids show actions and opioids are known to slow motility[10-13], by reducing peripheral neurotransmitter release[14,15] or by blocking enteric neurotransmission at multiple sites within the peristaltic reflex[10,16-21].

Over the last decade, organ bath experiments revealed that nociceptin induces contractions by reducing inhibitory neurotransmission and causes relaxation by reducing cholinergic neurotransmission[22-24]. More complex preparations disclosed that nociceptin reduces the excitatory components of the peristaltic reflex and thus slows GI motility[25]. Depending on the model used colonic transit was increased or reduced and it seems that timing and route of administration of nociceptin injections may also influence the direction of the results[26,27]. Many of the previously published results are conflicting and it is intriguing that the majority of these studies characterized nociceptin, but failed to shed some light on the involvement of ORL1. Some of the confusion may be caused since previous *in vivo* studies used different ORL1 agonists; however, ORL1 antagonists were never employed *in vivo*, thus probing an ORL1 involvement of the observed effects.

The aim of our study was to investigate the endogenous nociceptin system in attempt to elucidate the role and function of ORL1 receptors in the GI tract. Based on the effects of nociceptin on the GI motility observed *in vivo*, localization and co-localization of the peptide and its receptor were examined in the mouse ileum and colon. Electrophysiology was employed to investigate whether cholinergic excitatory junction potentials (EJPs), purinergic fast excitatory and inhibitory junction potentials (fIJPs) and nitrergic slow IJPs (sIJPs) are changed by nociceptin, if nociceptin has effects on long-distance neurotransmission and motorneurons and interneurons are involved. Our results shed new light on the endogenous nociceptin system physiology, what may have important clinical implications in future.

Table 1 Antisera used for immunohistochemistry

Antisera (host)	Source	Dilution
Primary antisera		
ORL1 (KOR-3) (H-85) (rabbit)	Santa Cruz Biotechnology (sc-15309)	1:50
ORL1 (KOR-3) (N-19) (goat)	Santa Cruz Biotechnology (sc-9759)	1:50
Nociceptin (guinea-pig)	Abcam (ab10276)	1:100-200
NOS (rabbit)	Euro Diagnostica (B 220-1)	0.389
NOS (guinea pig)	Euro Diagnostica (B-GP 225-1)	0.736
PGP 9.5 (guinea pig)	Chemicon (AB5898)	0.389
Secondary antisera and streptavidin complexes		
FITC-conjugated donkey anti rabbit	Jackson ImmunoResearch (711-095-152)	0.111
Cy3-conjugated donkey anti rabbit	Jackson ImmunoResearch (711-165-152)	0.215
Biotinylated donkey anti rabbit	Jackson ImmunoResearch (711-065-152)	0.111
Biotinylated donkey anti goat	Jackson ImmunoResearch (705-065-147)	0.111
Biotinylated donkey anti goat	Jackson ImmunoResearch (706-065-148)	0.111
FITC-conjugated donkey anti goat	Jackson ImmunoResearch (705-095-147)	1:50
FITC-conjugated donkey anti guinea pig	Jackson ImmunoResearch (706-095-148)	1:50
Cy3-conjugated donkey anti guinea pig	Jackson ImmunoResearch (706-165-148)	0.215
Cy3-conjugated Streptavidin	Jackson ImmunoResearch (016-160-084)	0.215
FITC-conjugated Streptavidin	Jackson ImmunoResearch (016-010-084)	1:100
		Streptavidin FITC: Jackson ImmunoResearch 016-010-084; 1:100

ORL1: Opioid-receptor like-1; NOS: Nitric oxide synthase; FITC: Fluorescein isothiocyanate.

MATERIALS AND METHODS

Animal use for these studies was approved by the Animal Care Committee of the Government of Bavaria/ Germany and the University of Calgary Animal Care Committee and all experiments were performed in accordance with institutional Animal Ethics Committee guidelines.

In vivo motility studies

Whole gut transit and colonic expulsion test was performed as described previously in non-fasted male mice[28,29].

To test the involvement of ORL1 receptors, co-administration studies with selective antagonist [NPhe^1]-NOC [100 pmol, intravenous (*iv*)] were performed. To interfere with the expression of the ORL1 protein, a phosphorothioate antisense oligodeoxynucleotide AS ODN, synthesized by the UCDNA Synthesis Lab (Calgary, AB, Canada) was used. The AS ODN sequence (5′-C*A *G*G*C*A*C*T*C*G*A*T-3′, where an asterisk marks the phosphorothioate linkage) was chosen to selectively target exon 4 of ORL1, but not the ORL1 alternative variant. The control consisted of a mismatched sequence (MM ODN), in which four bases were switched (5′-C*G*G*G*T*A*C*G*C*G*C*T-3′, where bold indicates the mismatch). ODN solutions were prepared in sterile water immediately prior to use. Mice received either AS or MM ODN at a dose of 5 μg per animal (*i.p.*), injected every 12 h over 2 d (in total 4 injections). Twelve hours after last injection the animals were used to test colonic bead expulsion. Treatment with AS or MM ODN did not alter the normal behaviour of mice.

RNA isolation and reverse transcriptase - polymerase chain reaction

Total RNA from mouse brain, longitudinal muscle-myenteric plexus layer (LMMP) and mucosa of ileum and LMMP and mucosa of colon was reversely transcribed in complementary DNA as described before[30,31]. mRNA expression of mouse ORL1 was tested using specific primers [mORL1 (S): 5′-CTGCCTCGTCATGTATGTCATC-3′; mORL1 (AS): 5′-GGAAGATGCAGATGGCAAATACA-3′]. As control, we used GAPDH [mGAPDH (S): 5′-GCTGAACGG GAAGCTCACTG-3′; mGAPDH (AS): 5′- GCTGTTGAAGTCG CAGGAGAC-3′].

Immunohistochemistry

Cryosections and whole mounts of mouse colon and ileum were used for immunohistochemistry. Immunohistochemical incubations were carried out as detailed previously[25]. Antisera and streptavidin complexes are listed in Table 1. Negative controls and specificity controls were performed as described previously[25].

Tissue preparation for electrophysiological experiments

Tissue preparation was performed as described previously[25].

Intracellular electrical recording

Intracellular recordings of circular colonic smooth muscle cells were performed as detailed previously[32,33]. Neurons were stimulated with single electrical pulses (15 V; 0.3 ms duration) using single or multiple platinum electrodes as described previously[32,33].

Membrane potentials were recorded as described previously[25].

Drugs

Hexamethonium, atropine, tetrodotoxin (TTX) methylene blue were obtained from Sigma (Irvine, United Kingdom); [NPhe^1]NOCNH$_2$ ([NPhe^1]NOC) and nociceptin were from Biotrend (Köln, Germany). Drugs were

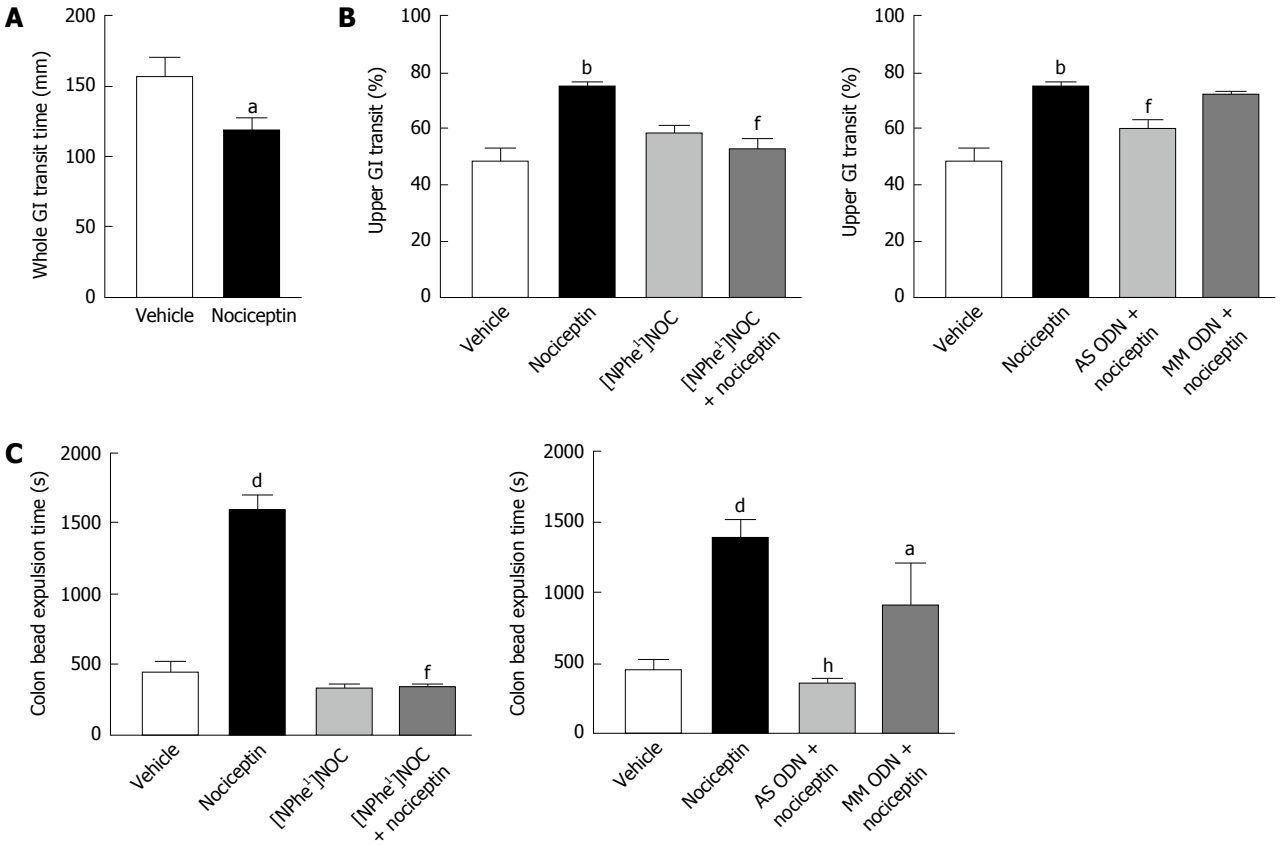

Figure 1 ***In vivo*** **effects of nociceptin in the gastrointestinal tract in mice.** A: Nociceptin (100 pmol, *iv*) accelerated whole gastrointestinal transit; B: Nociceptin (100 pmol, *iv*) accelerated upper gastrointestinal transit. This effect was absent in animals pretreated with [NPhe^1]NOC (100 pmol, *iv*) at a dose that had no effects when given alone and in the AS ODN-treated mice; C: Nociceptin (100 pmol, *iv*) slowed colonic bead expulsion. This effect was absent in animals pretreated with [NPhe^1]NOC (100 pmol, *iv*) at a dose that had no effects when given alone and in the AS ODN-treated mice. Data are mean ± SEM of n = 5-8 mice/group. $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$, *vs* vehicle-treated mice; $^fP < 0.01$, $^hP < 0.001$ *vs* nociceptin alone. AS and MM ODN, antisense and mismatched deoxynucleotide, respectively. GI: Gastrointestinal; *iv*: Intravenous.

added to the bath in microliter volumes and appropriate control experiments were performed with vehicles to exclude vehicle-induced effects. For the *in vivo* experiments drugs were first dissolved in DMSO and then further diluted in saline.

Data analysis and statistics

Data analysis and statistics was performed as described previously[25].

RESULTS

Nociceptin accelerates whole and upper gastrointestinal transit, but delays colonic expulsion in an ORL1-dependent manner

As shown in Figure 1A and B, nociceptin (100 pmol, *iv*) significantly increased the passage through whole and upper GI tract. The effect of nociceptin on upper GI transit time was blocked by the selective ORL1 antagonist, [NPhe^1]NOC (100 pmol, *iv*) and the pretreatment with AS ODN for two days. On the contrary, a significant delay in bead expulsion time was observed in mice that received nociceptin *iv* (100 pmol) (Figure 1C). [NPhe^1]NOC alone (100 pmol, *iv*) did not change colonic bead expulsion, but significantly reversed the effects of nociceptin, confirming the involvement of ORL1. Blocking ORL1 by *i.p.* treatment of mice with AS ODN over 48 h abolished the inhibitory effect of nociceptin (nociceptin 100 pmol, *iv*: 1383 ± 146.8 s *vs* AS ODN + nociceptin 100 pmol, *iv*: 373 ± 33.6 s, $P <$ 0.001) (Figure 1C). The repeated administration of MM ODN produced a minor decrease of the nociceptin action in the mouse colon.

ORL1 mRNA is expressed in mouse ileum and colon

To verify the mechanisms underlying the differential effect of nociceptin based on the site of action, the presence of ORL1 mRNA in mouse GI tract was determined by RT-PCR (Figure 2). Low abundant expression of an alternative ORL1 variant with an 81 bp insertion of the unspliced intron 3 was observed in mouse brain, in LMMP and mucosa of mouse ileum and in LMMP of mouse colon (Figure 1; PCR product of approximately 400 bp. This alternative splicing event generates an in-frame stop codon and therefore creates a C-terminally truncated ORL1 protein with unknown function. All investigated mouse tissues displayed expression of the short ORL1 mRNA variant lacking intron 3 with a PCR product size of approximately 320 bp (Figure 2).

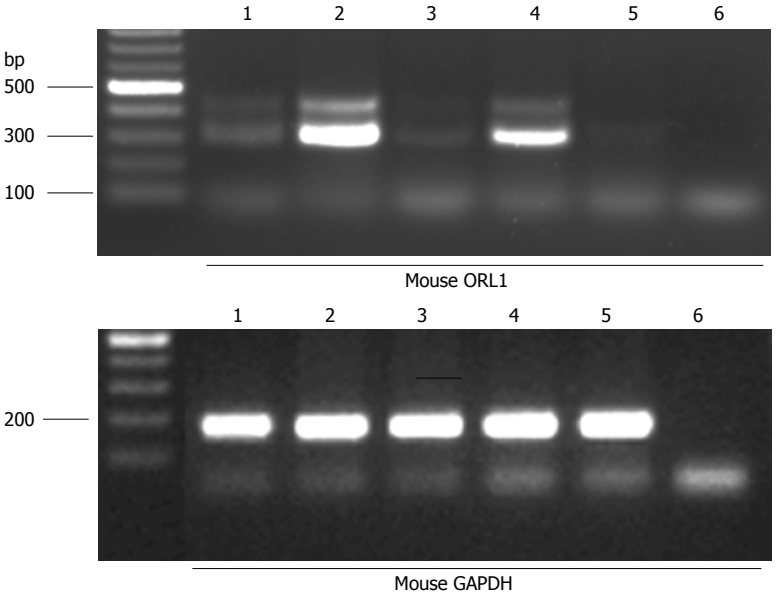

Figure 2 A representative gel for reverse transcriptase - polymerase chain reaction for opioid-receptor like-1 using total RNA. Two alternatively spliced mRNA variants of mouse opioid-receptor like-1 (ORL1) with a size of approximately 320 and 400 bp are expressed in mouse brain (lane 1), longitudinal muscle-myenteric plexus layer (LMMP) (lane 2) and mucosa (lane 3) of mouse ileum and in LMMP (lane 4) of mouse colon. In the mucosa of mouse colon, only the short ORL1 isoform is present (lane 5), whereas the negative control (lane 6) showed no specific polymerase chain reaction product. Lower panel shows results for GAPDH, which was used as internal control. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

ORL1 is co-expressed with NOS in mouse colon, but not ileum

In line with the single labeling, the RT-PCR data and the double labeling with the general neuronal marker PGP9.5 showed that a majority of myenteric neurons in mouse ileum and colon expressed IR for ORL1 (Figure 3). This ORL1 labeling was mainly restricted to the neuronal somata. Double staining with a nitrergic marker revealed high level of co-expression of ORL1 with NOS in mouse large intestine, but not in the small intestine. Corresponding single labeling studies, showed comparable staining, thus non-specific findings were ruled out (Figure 3).

Moreover, the presence of the endogenous peptide nociceptin was confirmed intracellularly in the majority of the myenteric neurons of the mouse colon and both NOS and PGP9.5 double-labeled with nociceptin (Figure 4A). A punctuate intracellular staining pattern suggested a localization in vesicles. Since granular staining was observed in 100% of the neurons, we performed additional control staining in cortex and hippocampus, *i.e.*, regions where distinct subpopulations of neurons are known to stain positive for nociceptin (Figure 4B).

Electrophysiological recordings show that nociceptin reduces excitatory and inhibitory junction potentials in the mouse colon

Under basal conditions, circular smooth muscle cells of mouse large bowel displayed stable resting membrane potentials (-53.9 ± 3.7 mV, n = 17). Stimulation of myenteric neurons with EFS gave rise to junction potentials sensitive to TTX.

In the colon (proximal), an EJP was followed by a biphasic IJP, with an initial fIJP followed by a sIJP. In the middle colon, EFS with the same stimulation parameter elicited a biphasic IJP without an EJP and in the distal colon only a monophasic IJP was seen.

The amplitudes of junction potentials were measured and compared to membrane potential prior to electrical stimulus application. The EJP represents excitatory cholinergic neurotransmission, the sIJP inhibitory nitrergic neurotransmission and the fIJP purinergic inhibitory neurotransmission as detailed earlier[33].

Increasing the nociceptin concentrations (1 nmol/L-1 μmol/L), led to a significant reduction in EJP in the mouse proximal colon and this effect was reversed by addition of [NPhe^1]NOC, (n = 6; Figure 5A). The fIJP was not affected by nociceptin, whereas the sIJP was significantly reduced. The effect of nociceptin on the sIJP was reversed by [NPhe^1]NOC (n = 6; Figure 5A). In the middle colon, nociceptin (1 nmol/L-1 μmol/L) reduced the sIJP, an effect that was reversed by [NPhe^1]NOC (n = 6; Figure 5B). The fIJP was not changed. Finally, in the distal colon nociceptin (10 nmol/L-1 μmol/L) significantly reduced the monophasic IJP, and this effect that was also reversed by [NPhe^1]NOC (n = 6; Figure 5C).

Influence of the ORL1 agonist nociceptin on the spatial distribution of EJP responses

The ORL1 agonist nociceptin was added in increasing doses (100 nmol/L-10 μmol/L) to the organ bath and the spatial distribution of the ascending EJP was reported. EJPs could be recorded up to a distance of 18-20 mm. Incubation of the tissues with nociceptin caused a reduction in EJP responses to electrical stimulation

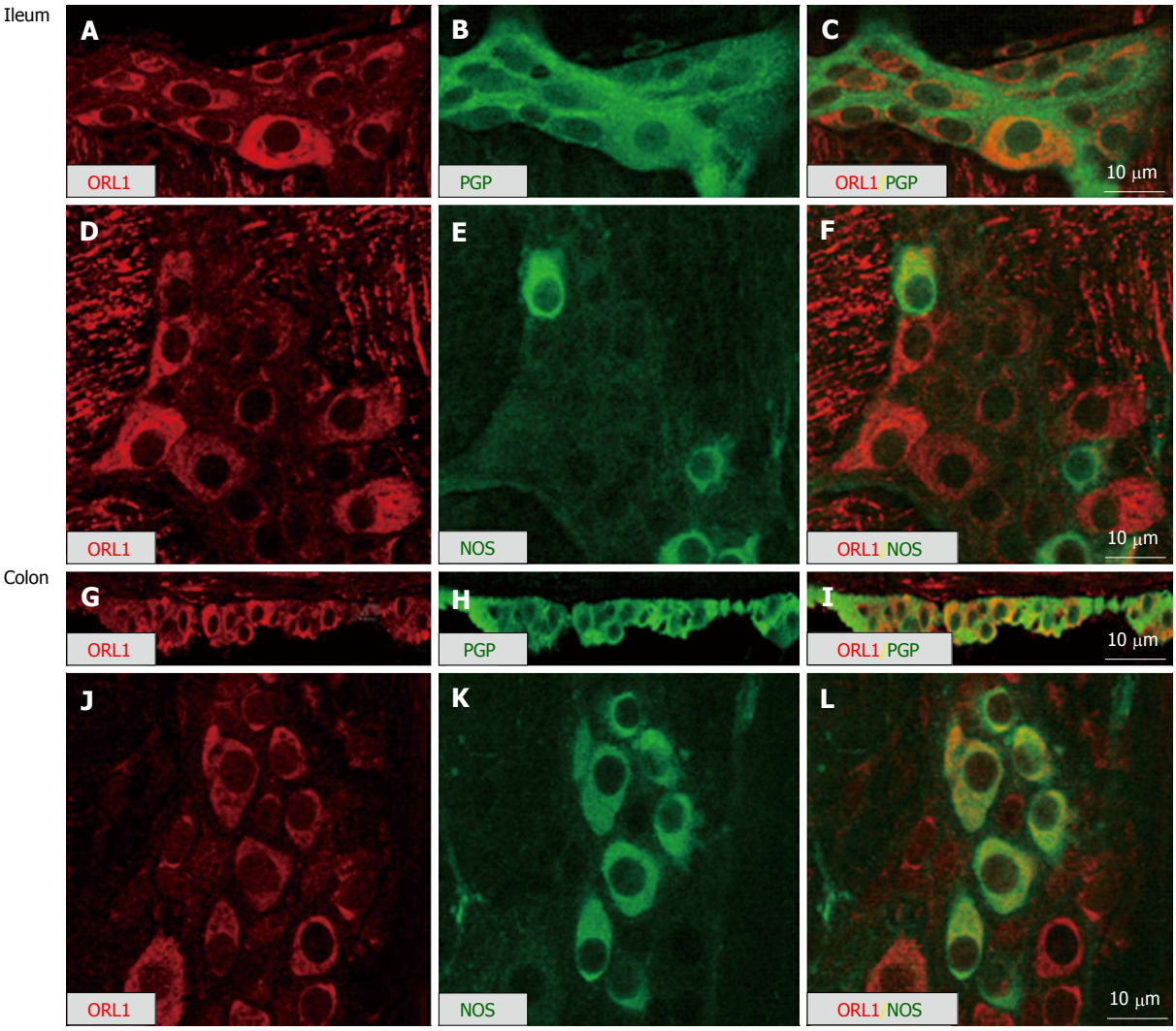

Figure 3 Double-labeling of myenteric plexus whole-mount preparations (A-F; J-L) and cryosections (G-I) of mouse ileum (A-F) and mouse colon (G-L) demonstrating co-labeling in myenteric neurons of opioid-receptor like-1 immunoreactivity with the neuron-endocrine marker protein gene product 9.5 or nitric oxide synthase. ORL1: Opioid-receptor like-1; NOS: Nitric oxide synthase; PGP: Protein gene product.

(Figures 6 and 7). For SE 1-6 this decrease represented an ORL1 activity on the motor neurons, whereas for SE > 7 the effects represented influences on interneurones, as characterized previously[34]. Furthermore, the distance over which the EJP was recordable, was concentration dependently shortened. This suggests that ORL1 agonism reduces the excitatory responses not only on amplitude, but also on the spatial fast propagation in the myenteric reflex (ascending part) (Figure 7A). The ORL1 antagonist [NPhe^1]NOC (1 μmol/L), when given alone, did not change the spatial distribution of EJPs (Figure 7B).

Influence of TTX, hexamethonium, atropine, and nociceptin in the partitioned electrophysiological chamber

In additional experiments with two separately perfused electrophysiological chambers, TTX (3 mmol/L), when applied to the stimulatory chamber, reduced the EJP in the chamber where recording was performed (data not shown). Atropine (1 μmol/L) when added to the stimulation chamber did not alter the EJP in the recording chamber (data not shown). Hexamethonium (100 mmol/L) when given to the stimulatory chamber reduced the EJP significantly in the chamber where recording was performed [control 29.6 ± 4.3 mV, hexamethonium (100 mmol/L) 24.5 ± 4.7 mV; $n = 5$, $P < 0.05$]. In separate experiments nociceptin was given to the stimulatory chamber and significantly reduced the EJP in the chamber where the anal recording was performed [control 27.9 ± 5.3 mV, nociceptin (1 μmol/L) 21.9 ± 4.5 mV; $n = 7$, $P < 0.05$].

DISCUSSION

Due to powerful actions on GI motility opioids are clinically used to slow increased GI transit. However, opioids are associated with side effects like addiction, respiratory depression and sedation, limiting their use. The ORL1 receptor and its endogenous ligand, nociceptin, have been known for approximately 15 years, but their effects on GI motility are still not

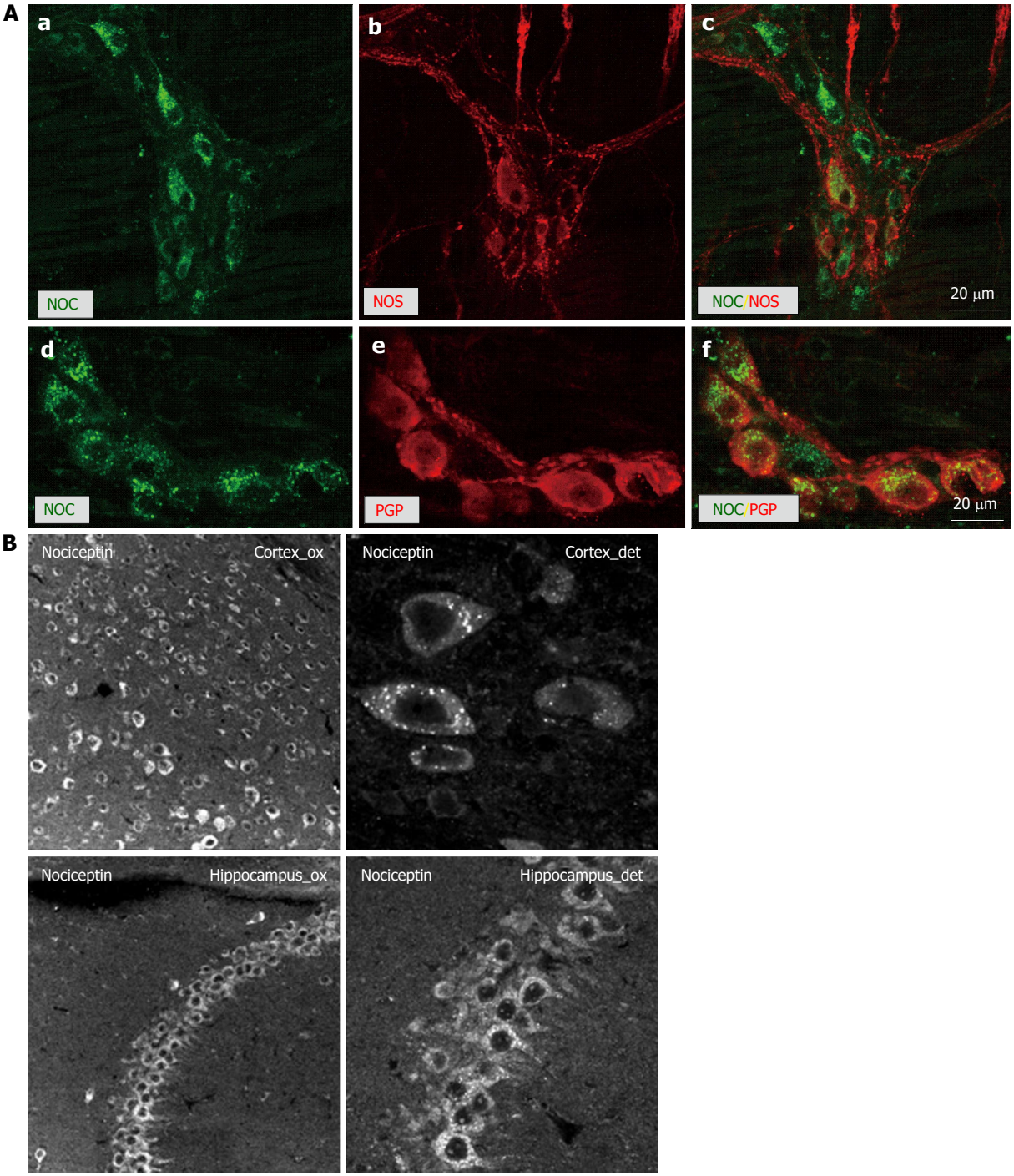

Figure 4 Double-labeling of myenteric plexus whole-mount preparations with nociception, nitric oxide synthase and neuron-endocrine marker protein gene product 9.5 immunoreactivity. A: Double-labeling of myenteric plexus whole-mount preparations of mouse colon demonstrating co-labeling in myenteric neurons of nociceptin (NOC) immunoreactivity with nitric oxide synthase (NOS) (A-C) and the neuron-endocrine marker protein gene product 9.5 (PGP9.5) (D-F); B: A granular appearance of the nociceptin staining in the myenteric neurons was also observed in brain sections, which served as additional controls. Individual immunopositive neurons are recognizable along the entire cortex, be it with slight region-dependent differences in staining intensity. In the hippocampal region also neuronal processes stain for nociceptin. The diffuse distribution of nociceptin corresponds with literature data (see also datasheet Abcam). No other structures apart from neurons appear to be stained.

fully understood. Since ORL1 activation, unlike the classical opioids, appears to be devoid of central side effects, drugs targeting the ORL1 receptor may be of future use in humans. The present study showed that nociceptin and ORL1 are present in the mouse colon and both are involved in the regulation of motility and neurotransmission. Most importantly, this study for the first time proves that the co-expression of ORL1 with NOS is crucial for nociceptin effects on GI motility *in vivo*. It also demonstrates that not only motor neurons, but also interneurons are modulated by nociceptin in an ORL1-dependent manner.

The effect of nociceptin on the GI motility has already been investigated by several groups and the

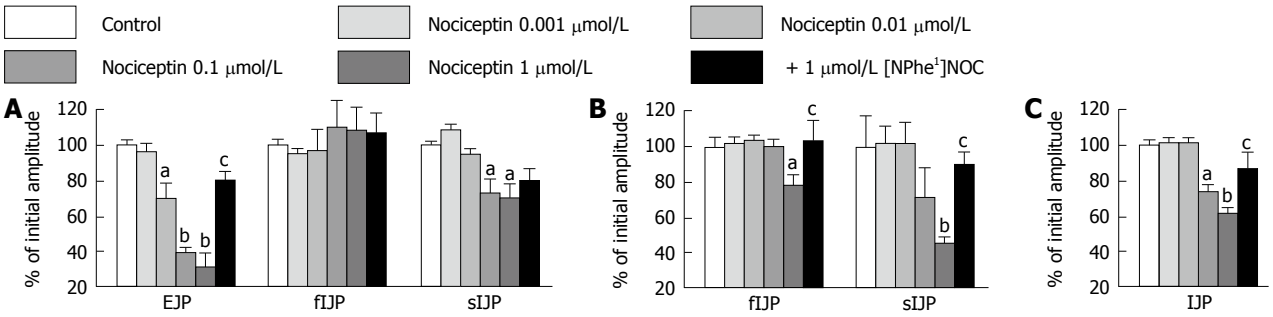

Figure 5 Effect of nociceptin (1 nmol/L-10 μmol/L) on electrically induced junction potentials. A: EJP, fIJP and sIJP for proximal colon; B: fIJP and sIJP for middle colon; C: IJP for distal colon. The last bar of each set shows the antagonizing effect of [NPhe^1]NOC (1 μmol/L). Data are mean ± SEM of n = 5-8. $^aP < 0.05$; $^bP < 0.01$ *vs* control; $^cP < 0.05$ *vs* nociceptin alone. EJP: Excitatory junction potentials; IJP: Inhibitory junction potentials; fIJP: Fast IJP; sIJP: Slow IJP.

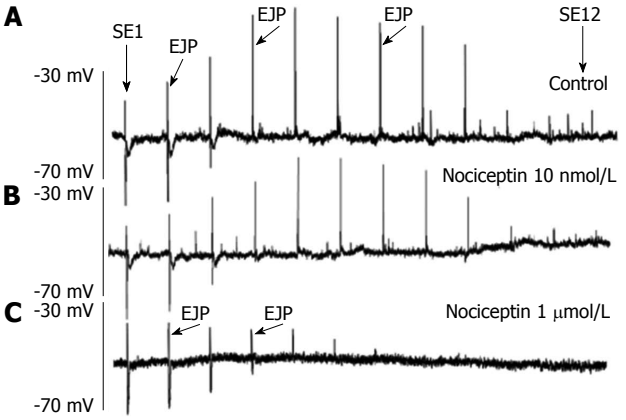

Figure 6 Effect of nociceptin (1 nmol/L, 1 μmol/L) on the spatial distribution of the excitatory junction potentials. Tracing A shows the spatial distribution of the excitatory junction potentials (EJP) recorded in smooth muscle cells. Stimulation site (SE)1 corresponds to stimulation nearby the recording site, whereas SE12 represents stimulation located 20 mm in anal direction. Thus the original recording shows the spatial distribution of ascending electrophysiological responses following anal stimulation recorded in 1.67 mm intervals. Tracings B and C show the effect of increasing concentrations of nociceptin (10 nmol/L and 1 μm) on the spatial distribution of junction potentials. Note the marked reduction of EJP in a concentration-dependent fashion and the shift of the maximal distance over which an EJP can be recorded.

results are often contradictory, depending on the site of administration, dose used and the animal model employed in the study[23,26,35,36]. Our observations *in vivo* clearly showed a differential, ORL1-dependent action of nociceptin in the mouse ileum and colon, where it accelerates and decreases the intestinal motility, respectively. In an attempt to investigate the possible influence of the ORL1 expression patterns on the effect of nociceptin, RT-PCR and immunohistochemistry studies were then executed. We found that ORL1 mRNA and immunoreactivity are present in the GI tract and there is practically no difference in expression levels between small and large intestine, excluding this factor from further investigations. Interestingly, we found the expression of an alternatively spliced ORL1 mRNA variant[37] in mice that has not been reported in other rodents like rats[25]. It remains unresolved whether the presence of ORL1 splice variants is associated with different receptor functionality; however, in view of our latter findings this seems rather unlikely. Immunohistochemistry localized ORL1 staining in the majority of myenteric neurons of the colon and co-localisation with NOS suggests that ORL1 is expressed on inhibitory neurons, what was previously suggested for rats[25]. Of note, there was no co-expression of ORL1 with NOS in the mouse ileum, what may indicate the lack of involvement of nitrergic pathways in the action of nociceptin in the small intestine and may explain in part the effect that we observed in mice.

Using specific antibodies, nociceptin has previously been localized mainly on fibers of myenteric neurons of rat colon and neuronal cell bodies of guinea pig colon[9,36]. Our study extends these observations to mouse colon and for the first time reports that nociceptin appears to be present in vesicles in the cell body of the majority of myenteric neurons, where it double labels with NOS and PGP9.5. In contrast to the guinea pig, where nociceptin can be found only on a limited number of neurons, in the mouse nociceptin is present in the majority of neurons.

We proceeded with our investigation by performing electrophysiological recordings and found that nociceptin significantly reduced electrically-induced contractions *in vitro*; moreover, these effects showed regional differences. The notion that the ORL1 receptor is involved modulation of intestinal motility was indicated by the finding that [Nphe^1]NOC antagonized the effects of nociceptin. Since the nociceptin effects were unchanged in naloxone presence no other opioid receptors are involved and this is in agreement with observations published by others[27,38,39]. Furthermore, we found in agreement with others no effect of nociceptin or [Nphe^1]NOC on pharmacologically stimulated smooth muscle or basal tone[22,24,25,40] and are strongly supported by our immunohistochemical studies, which localize ORL1 receptors on neuronal, but not on smooth muscle cells.

The most important findings in our study were illustrated by further intracellular recordings in the mouse colon. Nociceptin produced a significant inhibitory effect on EJPs, which, in addition, was the lowest in the middle colon compared to effects in proximal and distal colonic tissues. Furthermore, sIJP were reduced by nociceptin, suggesting ORL1 involvement in excitatory cholinergic and inhibitory neurotransmission. The sIJP is known

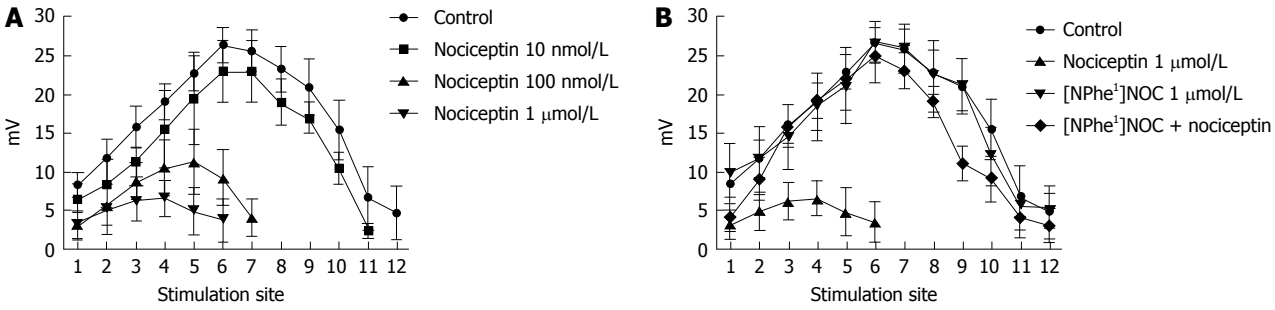

Figure 7 Effect of nociceptin (1 μmol/L) and [NPhe^1]NOC (1 μmol/L) on the spatial distribution of the excitatory junction potentials. A: A concentration-dependent reduction in excitatory junction potentials (EJP) amplitude at the different stimulation sites induced by nociceptin (10 nmol/L-1 μmol/L). Note that nociceptin caused, besides a reduction in the EJP amplitude, shortening of the distance over which the EJP can be recorded; B: [NPhe^1]NOC (1 μmol/L) had no effect on EJPs, but antagonized the effect of nociceptin (1 μmol/L).

to resemble the nitrergic inhibitory neurotransmission, which is further stressed by our immunohistochemical findings, showing that the ORL1 was co-localized with NOS, as well as our observations *in vivo*.

The present study shows am involvement of nociceptin and the ORL1 in ascending myenteric neuronal network circuits in the colon and increases our current understanding about ORL1 neurotransmission. EJP, which represents excitatory cholinergic neurotransmission was reduced by nociceptin[33], in a [Nphe^1]NOC-reversible manner at all involved sites of stimulation, but also shortened the transmission distance. This observation leads us to postulate that not only motor neurons, but also interneurons are modulated by nociceptin. The nociceptin-induced reduction of the EJP amplitude at the shorter distances (SE1-6) points to an action at ORL1 on motor-neurons. However, the shortened distance of transmission of the EJP was caused by ORL1 actions on inter-neurons. Here ORL1 activation reduced spreading of the electrical signal and excitatory neurotransmission. The shortened transmission distances reported here may be explained by different neuronal mechanisms, *e.g.*, reduced pre- or postsynaptic neurotransmitter release or neuronal excitability as underlying mechanisms[41,42].

The experiments with hexamethonium show that transmission involving ganglions is involved in EJP transmission, since EJPs were abolished by hexamethonium beyond 10 mm. In our study EJPs at the closer stimulation sites were reduced and this reduction effect was found to be higher than the remainder amplitude in presence of hexametonium, which suggests that the ORL1 influenced neurotransmission on motor neurons.

[Nphe^1]NOC alone had no influence on the electrophysiological parameter observed, but did antagonize the effect of nociceptin, lending clear evidence to the hypothesis that the receptors on motor neurons and interneurons are ORL1 receptors. Our experiments in the separatable electrophysiological chamber clearly indicated that ORL1 activation minimizes interneuron neurotransmission. This happens to an extent comparable to that seen with the ganglionic blocker hexamethonium. Therefore, our study shows for the first time that ORL1 receptors are located on myenteric plexus interneurons and additionally on neuromuscular junctions of motor neurons.

In summary, our study shows for the first time that nociceptin activates ORL1 on motorneurons and interneurons, which leads to a decrease in cholinergic excitatory and inhibitory nitrergic neurotransmission. In the cholinergic excitatory pathways nociceptin reduces not only the local transmission of EJP, but also the spatial spreading of ascending excitatory responses at distances up to 20 mm. Since ORL1 activation, unlike other opioid receptor-active drugs, is not associated with central side effects, compounds acting at the ORL1 may be good candidates for the future treatment of disorders associated with increased colonic transit, such as diarrhea or diarrhea-predominant irritable bowel syndrome.

COMMENTS

Background

The heptadecapeptide nociceptin, also known as orphanin FQ, is the only known endogenous agonist at the opioid-receptor like-1 (ORL1) receptor. From a structural perspective nociceptin is closely related to the dynorphin family, but differs from other endogenous opioids in that it does not possess the N-terminal tyrosine residue. Over the last decade, organ bath experiments revealed that nociceptin induces contractions by reducing inhibitory neurotransmission and causes relaxation by reducing cholinergic neurotransmission. More complex preparations disclosed that nociceptin reduces the excitatory components of the peristaltic reflex and thus slows gastrointestinal (GI) motility.

Research frontiers

The aim of the study was to investigate the endogenous nociceptin system in attempt to elucidate the role and function of ORL1 receptors in the GI tract. Based on the effects of nociceptin on the GI motility observed *in vivo*, localization and co-localization of the peptide and its receptor were examined in the mouse ileum and colon. Electrophysiology was employed to investigate whether cholinergic excitatory junction potentials, purinergic fast inhibitory junction potentials (IJPs) and nitrergic slow IJPs are changed by nociceptin, if nociceptin has effects on long-distance neurotransmission and motorneurons and interneurons are involved. The authors' results shed new light on the endogenous nociceptin system physiology, what may have important clinical implications in future.

Innovations and breakthroughs

The present study shows that nociceptin and ORL1 are present in the mouse colon and both are involved in the regulation of motility and neurotransmission. Most importantly, this study for the first time proves that the co-expression of ORL1 with nitric oxide synthase is crucial for nociceptin effects on GI motility *in*

vivo. It also demonstrates that not only motor neurons, but also interneurons are modulated by nociceptin in an ORL1-dependent manner.

Applications

Since ORL1 activation, unlike other opioid receptor-active drugs, is not associated with central side effects, compounds acting at the ORL1 may be good candidates for the future treatment of disorders associated with increased colonic transit, such as diarrhea or diarrhea-predominant irritable bowel syndrome.

Terminology

The authors' observations *in vivo* clearly showed a differential, ORL1-dependent action of nociceptin in the mouse ileum and colon, where it accelerates and decreases the intestinal motility, respectively. The authors proceeded with our investigation by performing electrophysiological recordings and found that nociceptin significantly reduces electrically-induced contractions *in vitro*; moreover, these effects showed regional differences.

Peer-review

Overall this manuscript is well conducted and written. The hypothesis is also interest and successful.

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ORIGINAL ARTICLE

Observational Study

Beverage intake preference and bowel preparation laxative taste preference for colonoscopy

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Data sharing statement: No.

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Abstract

AIM: To examine whether non-alcoholic beverage intake preferences can guide polyethylene glycol (PEG)-based bowel laxative preparation selection for patients.

METHODS: We conducted eight public taste test sessions using commercially procured (A) unflavored PEG, (B) citrus flavored PEG and (C) PEG with ascorbate (Moviprep). We collected characteristics of volunteers including their beverage intake preferences. The volunteers tasted the laxatives in randomly assigned orders and ranked the laxatives as 1st, 2nd, and 3rd based on their taste preferences. Our primary outcome is the number of 1st place rankings for each preparation.

RESULTS: A total of 777 volunteers completed the study. Unflavored PEG was ranked as 1st by 70 (9.0%), flavored PEG by 534 (68.7%) and PEG with ascorbate by

173 (22.3%) volunteers. Demographic, lifestyle characteristics and beverage intake patterns for coffee, tea, and carbonated drinks did not predict PEG-based laxative preference.

CONCLUSION: Beverage intake pattern was not a useful guide for PEG-based laxative preference. It is important to develop more tolerable and affordable bowel preparation laxatives for colonoscopy. Also, patients should taste their PEG solution with and without flavoring before flavoring the entire gallon as this may give them more opportunity to pick a pattern that may be more tolerable.

Key words: Bowel preparation; Laxatives; Colonoscopy; Taste tests; Colon cancer; Screening

Core tip: There is a need to improve patients' experience with bowel preparation process in order to optimize both colonoscopy uptake. Polyethelene glycol (PEG) is the most widely used laxative but many patients do not readily tolerate it because of its taste. We evaluated whether beverage intake preference pattern can be a useful guide for predicting tolerability of bowel preparation laxative in multiple public taste tests. Our study suggested that no demographic or lifestyle factors predicted bowel preparation taste preference for PEG-based preparations. We recommend that patients should taste PEG formulation before flavoring it to assist them in choosing a more tolerable pattern of ingestion.

Laiyemo AO, Burnside C, Laiyemo MA, Kwagyan J, Williams CD, Idowu KA, Ashktorab H, Kibreab A, Scott VF, Sanderson AK. Beverage intake preference and bowel preparation laxative taste preference for colonoscopy. *World J Gastrointest Pharmacol Ther* 2015; 6(3): 84-88 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i3.84.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i3.84

INTRODUCTION

Colorectal cancer (CRC) remains a leading cause of cancer-related deaths[1] despite evidence from case-control[2-4], cohort[5] and randomized control studies[6-8] that screening reduces the risk of death from the disease because a large proportion of age-eligible adults in the United States have not been screened[9,10]. This is a major public health problem. Although there are multiple acceptable options for CRC screening[11], colonoscopy is the only modality for surveillance, diagnostic and therapeutic purposes.

Colonoscopy requires a full bowel preparation using oral laxative agents. However, a substantial percentage of patients do not readily tolerate their bowel laxatives for colonoscopy. Inadequate bowel preparation wastes limited endoscopic resources in addition to patients' and providers' time and reduces the enthusiasm for repeat screening among patients[12]. Furthermore, inadequate endoscopy has been associated with subsequent colorectal cancer[13] underscoring the importance of achieving a high quality preparation for colonoscopy.

Given that the palatability of substances varies among people, we postulated that beverage intake preferences may be a useful guide to personalize bowel preparation recommendations for patients. We tested this hypothesis by conducting public taste tests among volunteers to correlate their beverage intake patterns to their preferred bowel preparations' taste.

MATERIALS AND METHODS

Public taste tests

The study was approved by the Institutional Review Board of Howard University (IRB-12-MED-17). We conducted eight public taste test sessions at Howard University Hospital lobby from July 2012 to March 2013. The objective of the taste test was to determine participants' preferences for 3 commercially procured preparations of polyethylene glycol-(PEG) 3350 used for bowel preparation for colonoscopy. These were: (1) Unflavored PEG; (2) citrus flavored PEG; and (3) PEG with sodium ascorbate and ascorbic acid (Moviprep®, Raleigh, NC).

The study was open to any volunteer (visitors, ambulatory care patients, students, and staff) if they were at least 18 years old. The study was explained to each participant and written informed consent was obtained. Each participant completed an intake form providing information on their demographic characteristics (age, sex, self identified race, highest education attained, income, marital status and self reported weight and height), lifestyle choices (smoking status and alcohol intake) and their non-alcoholic beverage intake preferences and patterns for coffee, tea and carbonated drinks (soda). Subsequently, volunteers tasted and drank 10 cc (equivalent to 2 teaspoons) of each laxative in randomly selected order (ABC, BCA, CAB) and rated the laxatives based on their preferences in an ordinal fashion with the preferred laxative rated as 1st, then 2nd for the next preferred and 3rd for the least preferred laxative. We offered confectioneries to each volunteer on completion of their participation in the study.

Exposure and outcome assessment

Information on how each volunteer prefers to drink coffee was collected with five options: (1) I do not drink coffee; (2) Without milk/cream; without sugar/sweetener; (3) Without milk/cream; with sugar/sweetener; (4) With milk/cream; without sugar/sweetener; and (5) With milk/cream; with sugar/sweetener. Similar information was obtained for tea intake. They were also asked if they drink regular carbonated drinks (soda), diet soda, and whether they prefer the taste of diet soda to regular soda. Our primary outcome was the number of 1st place ranking for each laxative preparation.

Table 1 Association of demographic and lifestyle characteristics with bowel preparation taste preference

Characteristics	*n*	Preferred unflavored PEG (*n* = 70)		Preferred flavored PEG (*n* = 534)		Preferred moviprep (*n* = 173)	
		n (%)	OR (95%CI)	*n* (%)	OR (95%CI)	*n* (%)	OR (95%CI)
Age ⩾ 50 yr							
No	421	32 (7.6)	Reference	283 (67.2)	Reference	106 (25.2)	Reference
Yes	356	38 (10.6)	1.45 (0.89-2.38)	251 (70.5)	1.17 (0.86-1.58)	67 (18.8)	0.69 (0.49-0.97)
Sex							
Male	340	21 (6.2)	Reference	242 (71.2)	Reference	77 (22.7)	Reference
Female	432	48 (11.1)	1.90 (1.11-3.24)	288 (66.7)	0.81 (0.60-1.10)	96 (22.2)	0.98 (0.69-1.37)
Race							
Non-blacks	120	12 (10.0)	Reference	83 (69.2)	Reference	25 (20.8)	Reference
Blacks	657	58 (8.8)	0.87 (0.45-1.68)	451 (68.7)	0.98 (0.64-1.49)	148 (22.5)	1.10 (0.69-1.78)
Marital status							
Unmarried	455	38 (8.4)	Reference	314 (69.0)	Reference	103 (22.6)	Reference
Married	169	13 (7.7)	0.91 (0.47-1.76)	110 (65.1)	0.84 (0.57-1.22)	46 (27.2)	1.28 (0.85-1.91)
College education							
No	418	41 (9.8)	Reference	299 (71.5)	Reference	78 (18.7)	Reference
Yes	343	27 (7.9)	0.79 (0.47-1.31)	228 (66.5)	0.79 (0.58-1.07)	88 (25.7)	1.50 (1.06-2.12)
Yearly income < $25000							
No	437	33 (7.6)	Reference	302 (69.1)	Reference	102 (23.3)	Reference
Yes	268	29 (10.8)	1.49 (0.88-2.51)	181 (67.5)	0.93 (0.67-1.29)	58 (21.6)	0.91 (0.63-1.31)
BMI							
< 25 kg/m^2	223	18 (8.1)	Reference	150 (67.3)	Reference	55 (24.7)	Reference
25-29 kg/m^2	258	25 (9.7)	1.22 (0.65-2.30)	172 (66.7)	0.97 (0.66-1.42)	61 (23.6)	0.95 (0.62-1.44)
⩾ 30 kg/m^2	275	26 (9.5)	1.19 (0.63-2.23)	199 (72.4)	1.27 (0.87-1.87)	50 (18.7)	0.68 (0.44-1.05)
History of smoking							
No	485	38 (7.8)	Reference	334 (68.9)	Reference	113 (23.3)	Reference
Yes	286	32 (11.2)	1.48 (0.90-2.43)	195 (68.2)	0.97 (0.71-1.33)	59 (20.6)	0.86 (0.60-1.22)
Alcohol							
No	311	26 (8.4)	Reference	216 (69.5)	Reference	69 (22.2)	Reference
Yes	457	42 (9.2)	1.11 (0.66-1.85)	312 (68.3)	0.95 (0.69-1.29)	103 (22.5)	1.02 (0.72-1.44)
Health history							
Diabetes							
No	661	51 (8.8)	Reference	453 (68.5)	Reference	150 (22.7)	Reference
Yes	107	11 (10.3)	1.19 (0.60-2.35)	76 (71.0)	1.13 (0.72-1.76)	20 (18.7)	0.78 (0.47-1.32)
Hypertension							
No	521	48 (9.2)	Reference	341 (65.5)	Reference	132 (25.3)	Reference
Yes	248	22 (8.9)	0.96 (0.57-1.63)	188 (75.8)	1.65 (1.17-2.33)	138 (15.3)	0.53 (0.36-0.79)

Missing data: sex = 5; marital status = 153; education = 16; income = 72; BMI = 21; smoking status = 6; alcohol = 9; diabetes = 9; hypertension = 8. PEG: Polyethylene glycol; BMI: Body mass index.

Statistical analysis

We calculated the percentages of volunteers who preferred each laxative's taste and compared the characteristics of participants who selected each laxative as 1st *vs* those who did not. Missing variables were set to missing without the use of dummy variables. We used logistic regression models to compare the characteristics of volunteers who chose each laxative as 1st to the rest of the volunteers and calculated odds ratios (OR) and 95%CI. We performed similar analyses and compared the pattern of beverage intake for coffee, tea, and soda with those who did not drink these beverages. We evaluated the predictive accuracy of the models by calculating the area under the receiver operating characteristics curve (AUC). We used Stata® statistical software version 11.2 (College Station, Texas) for our analyses.

RESULTS

A total of 777 volunteers completed the taste test. The mean age of volunteers was 45.1 years (range 18-83 years), 432 (56.0%) women and 657 (84.6%) blacks. Seventy (9.0%) participants preferred unflavored PEG as first choice, 534 (68.7%) preferred flavored PEG while 173 (22.3%) preferred PEG with ascorbate.

Overall, no demographic or lifestyle characteristics adequately predicted the preference for any bowel laxative. Volunteers who were older than 50 years (OR = 0.69; 95%CI: 0.49-0.97) and those with hypertension (OR = 0.53; 95%CI: 0.36-0.79) were less likely to prefer PEG with ascorbate as first choice. Although those with hypertension were more likely to prefer flavored PEG (OR = 1.65; 95%CI: 1.17-2.23) but the predictive accuracy was low (AUC = 0.55). Volunteers with college education were more likely to prefer PEG with ascorbate (OR = 1.50; 95%CI: 1.06-2.12), but the predictive accuracy was also low (AUC = 0.55). Similarly, women were more likely to prefer unflavored PEG (OR = 1.90; 95%CI: 1.11-3.24), albeit with low predictive accuracy (AUC = 0.57) (Table 1). The coffee, tea and carbonated drinks intake pattern of volunteers were not associated with laxative taste preferences (Table 2).

Table 2 Association of beverage intake preferences with bowel preparation taste preference

Beverage intake	*n*	Preferred unflavored PEG (*n* = 70)		Preferred flavored PEG (*n* = 534)		Preferred Moviprep (*n* = 173)	
		n (%)	OR (95%CI)	*n* (%)	OR (95%CI)	*n* (%)	OR (95%CI)
Coffee intake pattern							
Don't drink coffee	265	22 (8.3)	Reference	183 (69.1)	Reference	60 (22.6)	Reference
No milk, no sugar	41	4 (9.8)	1.19 (0.39-3.66)	27 (65.9)	0.86 (0.43-1.73)	10 (24.4)	1.10 (0.51-2.38)
With sugar, no milk	42	2 (4.8)	0.55 (0.13-2.44)	34 (81.0)	1.90 (0.84-4.29)	6 (14.3)	0.57 (0.23-1.42)
With milk, no sugar	65	9 (13.9)	1.78 (0.78-4.06)	42 (64.6)	0.82 (0.46-1.45)	14 (21.5)	0.94 (0.49-1.81)
With milk, with sugar	336	31 (9.2)	1.12 (0.63-1.99)	230 (68.5)	0.97 (0.69-1.38)	75 (22.3)	0.98 (0.67-1.44)
Tea intake pattern							
Don't drink tea	138	14 (10.1)	Reference	96 (69.6)	Reference	28 (20.3)	Reference
No milk, no sugar	89	6 (6.7)	0.64 (0.24-1.73)	63 (70.8)	1.06 (0.59-1.90)	20 (22.5)	1.14 (0.60-2.18)
With sugar, no milk	336	30 (8.9)	0.87 (0.44-1.69)	240 (71.4)	1.09 (0.71-1.69)	66 (19.6)	0.96 (0.59-1.57)
With milk, no sugar	25	2 (8.0)	0.77 (0.16-3.62)	16 (64.0)	0.78 (0.32-1.90)	7 (28.0)	1.53 (0.58-4.02)
With milk, with sugar	153	14 (9.2)	0.89 (0.41-1.94)	96 (62.8)	0.74 (0.45-1.20)	43 (28.1)	1.54 (0.89-2.65)
Carbonated drinks							
Regular soda intake							
No	269	27 (10.0)	Reference	180 (66.9)	Reference	62 (23.1)	Reference
Yes	482	41 (8.5)	0.83 (0.50-1.39)	336 (69.7)	1.14 (0.83-1.57)	105 (21.8)	0.93 (0.65-1.33)
Diet soda intake							
No	493	43 (8.7)	Reference	336 (68.2)	Reference	114 (23.1)	Reference
Yes	218	19 (8.7)	1.00 (0.57-1.76)	155 (71.1)	1.15 (0.81-1.63)	44 (20.2)	0.84 (0.57-1.24)
Prefers the taste of diet soda to regular soda							
No	582	47 (8.1)	Reference	402 (69.1)	Reference	133 (22.9)	Reference
Yes	111	13 (11.7)	1.51 (0.79-2.89)	76 (68.5)	0.97 (0.63-1.51)	22 (19.8)	0.83 (0.50-1.38)

PEG: Polyethylene glycol.

DISCUSSION

In this large study of volunteers in public taste tests, demographic, lifestyle and beverage intake patterns of volunteers did not predict their taste preferences for the studied bowel laxatives commonly used in the preparation process for colonoscopy. This suggests that these characteristics are not clinically useful to guide the selection of laxatives for colonoscopy. It is unclear why beverage intake patterns of our participants did not predict their preferences for bowel laxatives examined in this study. However, we speculate that beverage intake patterns are probably more unique to the individuals and can be varied in composition more readily than the limited taste range of the bowel laxatives. It will be important to develop better tasting and more acceptable bowel preparation laxatives and make them available and affordable to all patients.

Improving bowel preparation experience of patients is an important step to enhance uptake of CRC screening using colonoscopy. Previous interventions have involved reduction in the salt content and flavoring of the solutions by manufacturers. For those with low socio-economic status, these newer products are often not accessible because they are generally not considered to be "preferred brands" and are either not covered by their third party payers or covered with substantially higher co-pays. Bowel preparations containing PEG is the predominant laxative used in the preparation process for colonoscopy but salty taste and large volume of these solutions limit their tolerability. PEG is generally covered by health insurance and is relatively inexpensive. The effect of flavoring of PEG on patients' tolerability is uncertain. In a taste test involving 5 PEG preparations tasted by 100 subjects, Diab *et al*[14] reported that the majority of subjects preferred the flavored products while 22% rated unflavored PEG as their first choice. Furthermore, Hayes *et al*[15] reported that flavoring PEG (Colyte®) solution did not improve bowel preparation as compared to unflavored PEG.

An approach to ameliorate this challenge will be for manufacturers to provide free samples of their laxatives for patients to try at their endoscopists' offices. However, this may not be a viable option particularly as the relationship of pharmaceutical industries with care providers is under close scrutiny in many institutions and provision of free "test" samples medications has been abolished in many institutions. Therefore, it is imperative to develop palatable bowel preparation laxatives and make them affordable.

A notable strength of our study is that we studied the taste preference of a large number of volunteers. However, a limitation of our study is that we drew our inference from preferences that were based on tasting a small volume of laxatives by participants. However, if a small volume of a solution tastes really bad, it is highly unlikely that a large volume of it will be tolerable. Nonetheless, we acknowledge that it is conceivable that the sheer volume of solution to actually consume for colonoscopy preparation may further influence the overall experience of patients. Although our study was open to the general public, it was conducted at a single institution. Furthermore, the majority of our participants were black and the experience of other race-ethnicities may be different since beverage intake patterns and preferences may vary based on social characteristics.

In conclusion, the demographic characteristics, lifestyle choices and beverage intake preferences

of volunteers in this large taste test did not predict preferences for PEG-based bowel preparation laxatives to be a clinically useful guide to improve the experience of patients undergoing CRC screening. There is a need to develop palatable and affordable bowel preparation laxatives.

ACKNOWLEDGMENTS

Abstracts from this study were presented at the Digestive Diseases Week in May 2013 in Orlando, Florida and at the American College of Gastroenterology meeting in San Diego in October 2013.

COMMENTS

Background

There is a great need to improve the bowel preparation process in order to increase colon cancer screening uptake. The current study evaluated whether the beverage intake pattern for coffee, tea and carbonated drinks can guide the preference of volunteers for polyethylene glycol (PEG)-based bowel preparation for colonoscopy.

Research frontiers

Adequacy of bowel preparation for screening colonoscopy is a quality measure. This underscores the need to improve bowel preparation quality during colonoscopy, and overall bowel preparation experience of the population when undergoing colonoscopy.

Innovations and breakthroughs

The current study examined whether personalized uniqueness of beverage intake of coffee, tea and carbonated drinks can be useful to guide the selection of PEG-based bowel preparation laxative for patients. This has not been investigated previously.

Applications

To summarize the practical applications of their research findings, so that readers may understand the perspectives by which this study will affect the field and future research. Beverage intake preferences for coffee, tea and carbonated drinks did not predict the preferences for PEG-based bowel preparation laxative among volunteers. This suggests that taste preference is probably too unique and individuals should probably taste the unflavored PEG-based laxative prior to flavoring during the bowel preparation process.

Terminology

Bowel preparation is the process of ensuring that the colon is free of stool during colonoscopy and involves the consumption of laxatives. It is important to tolerate the laxatives, which of often consumed in large volumes, to achieve optimal bowel cleansing.

Peer-review

Better tolerable bowel preparation would increase the rates of screening colonoscopy and therefore benefit the public.

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CASE REPORT

Gastrointestinal Kaposi's sarcoma: Case report and review of the literature

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Author contributions: Lee AJ and Mourad B collected the patient's clinical data; Lee AJ, Brenner L and Munoz JC performed the endoscopy and obtained biopsies for diagnosis; Monteiro C analyzed the histopathology; Lee AJ, Munoz JC and Vega KJ performed the literature review and wrote the paper.

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Abstract

Kaposi's sarcoma (KS) of the gastrointestinal tract is not an uncommon disease among individuals with acquired immunodeficiency syndrome (AIDS). The majority is asymptomatic, and for this reason, gastrointestinal KS (GI-KS) remains undiagnosed. With continued tumor growth, considerable variation in clinical presentation occurs including abdominal pain, nausea, vomiting, iron deficiency anemia (either chronic or frank gastrointestinal bleeding), and rarely mechanical obstruction alone or combined with bowel perforation. Endoscopy with biopsy allows for histological and immunohistochemical testing to confirm the diagnosis of GI-KS among those with clinical symptoms. In previous studies, dual treatment with highly active antiretroviral therapy and systemic chemotherapy have been associated with improved morbidity and mortality in individuals with visceral KS. Therefore, investigators have suggested performing screening endoscopies in select patients for early detection and treatment to improve outcome. In this review, we describe a 44 years old man with AIDS and cutaneous KS who presented for evaluation of postprandial abdominal pain, vomiting, and weight loss. On upper endoscopy, an extensive, infiltrative,

circumferential, reddish mass involving the entire body and antrum of the stomach was seen. Histologic examination later revealed spindle cell proliferation, and confirmatory immunohistochemical testing revealed human herpes virus 8 latent nuclear antigen expression consistent with a diagnosis of gastric KS. Following this, we present a comprehensive review of literature on KS with emphasis on gastrointestinal tract involvement and management.

Key words: Kaposi sarcoma; Acquired immunodeficiency syndrome; Gastrointestinal endoscopy; Epidemiology; Gastrointestinal tumor symptoms

Core tip: Gastrointestinal Kaposi's sarcoma (GI-KS) is more common than originally thought and the majority of patients are asymptomatic and remain undiagnosed. Therefore, clinicians should maintain a high suspicion, especially among acquired immunodeficiency syndrome patients. Screening endoscopies are frequently performed and a recent retrospective study suggested using certain clinical factors (low CD4 cell count of < 100 cell/μL, men who have sex with men, and presence of cutaneous Kaposi's) which may predict occurrence of GI-KS endoscopically. Endoscopy to detect GI-KS prior to clinical symptom development is desired as initiation of combined highly active antiretroviral therapy and liposomal doxorubicin therapy has shown to improve outcome.

Lee AJ, Brenner L, Mourad B, Monteiro C, Vega KJ, Munoz JC. Gastrointestinal Kaposi's sarcoma: Case report and review of the literature. *World J Gastrointest Pharmacol Ther* 2015; 6(3): 89-95 Available from: URL: http://www.wjgnet.com/2150-5349/full/v6/i3.89.htm DOI: http://dx.doi.org/10.4292/wjgpt.v6.i3.89

INTRODUCTION

Kaposi's sarcoma (KS) is a low grade tumor of the vascular endothelium[1]. In the United States, the majority of affected individuals have human immunodeficiency virus (HIV) infection[2]. Although KS can develop at any HIV infection stage, it occurs more commonly in the setting of advanced immune suppression[2,3]. In the Western population, the prevalence of acquired immunodeficiency syndrome (AIDS) associated KS is 6%-30%, and affected individuals usually manifest with cutaneous disease and/or visceral involvement, of which the gastrointestinal (GI) tract is the most common extracutaneous site[4]. In untreated AIDS, the GI tract is involved in approximately 40%-51%, but only one in five of affected individuals have GI symptoms[5]. The majority are clinically silent (79%), and therefore, most visceral KS remain unidentified[4]. In recent studies, treatment using combined highly active antiretroviral therapy (HAART) and systemic chemotherapy was associated with a significant improvement in morbidity and mortality[6]. Therefore some experts have suggested screening endoscopy in select patients for early detection and initiation of treatment to improve survival outcome[7].

CASE REPORT

A 44 years old man presented to the emergency department for evaluation of worsening abdominal pain following oral intake. Abdominal pain was described as cramping and associated with severe nausea and vomiting of ingested food. Clinical symptoms were unrelated to food type (acidic, dairy, or gluten-containing groups), and occurred after either solid or liquid intake. At evaluation, he was unable to tolerate anything by mouth and reported an associated 10 pound weight loss over a 1 mo period. Past medical history was significant for AIDS and cutaneous Kaposi sarcoma diagnosed by skin biopsy 4 years previously. Home medications included topical Imiquimod therapy for cutaneous KS, and recent initiation of HAART (2 mo prior to presentation) for low CD4 cell count which was poorly tolerated due to GI discomfort. On physical examination, multiple, plaque-like, violaceous, skin lesions were found in both lower extremities with associated edema in the right leg and foot. In addition, similar lesions were also found in the neck and trunk; the patient reported these lesions to be increasing in size. Laboratory studies revealed an absolute CD4 cell count of 101 cells/μL, HIV-RNA level of 168 copies/mL, and a mild anemia with hemoglobin of 11 g/mL. Other biochemical markers were normal including amylase, lipase, and liver function panel. Further evaluation with computed tomography imaging of the abdomen/pelvis with and without contrast was remarkable for a prominent, concentric gastric wall thickening involving the majority of the stomach. An upper endoscopy revealed an extensive, circumferential, infiltrative, reddish mass throughout the entire gastric body which extended into the fundus and antrum (Figures 1-3). Endoscopic biopsy specimens were obtained from the gastric lesions, and hematoxylin and eosin examination revealed spindle cell proliferation with extensive lymphoplasmocytic cell infiltrates (Figure 4). A confirmatory immunohistochemical test showed human herpes virus 8 latent nuclear antigen (HHV8 LNA) expression suggestive of HHV8, supporting the diagnosis of gastric KS (Figure 5).

DISCUSSION

Evolution of an old disease

KS was described in 1872 by a Hungarian dermatologist, Moritz Kohn Kaposi, who was the first to identify five cases of "idiopathic multiple pigmented sarcomas of the skin"[8]. In one case, the patient expired from gastrointestinal bleeding with a postmortem report that revealed unspecified visceral lesions in the lung and GI

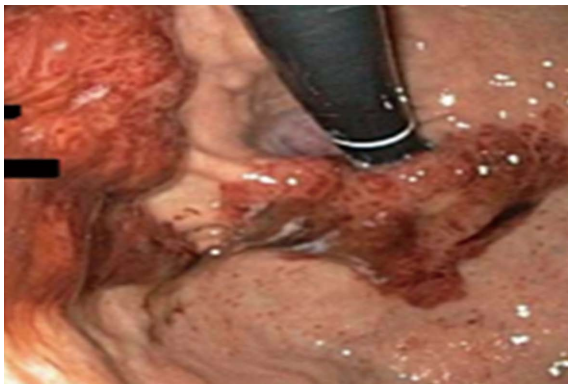

Figure 1 Endoscopic image of the gastric cardia demonstrating Kaposi's sarcoma lesion extension from the gastric body.

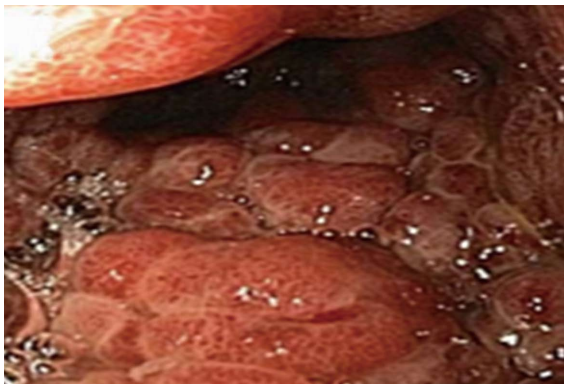

Figure 2 Endoscopic image of the gastric body showing an extensive, infiltrative, and circumferential Kaposi's sarcoma mass involving the entire body.

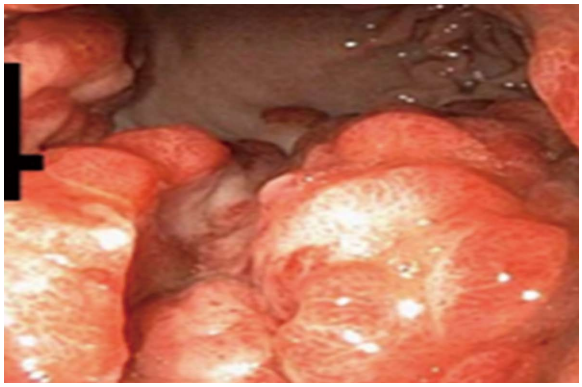

Figure 3 Endoscopic image of the gastric antrum with Kaposi's sarcoma lesion infiltration from the body.

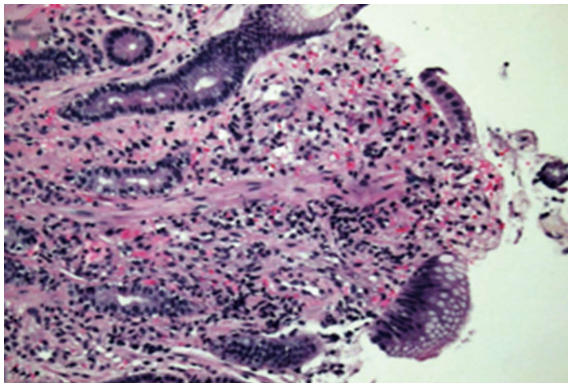

Figure 4 Hematoxylin and eosin stain of slit-like spaces with spindle cell proliferation and associated red blood cell extravasation seen in the lamina propria.

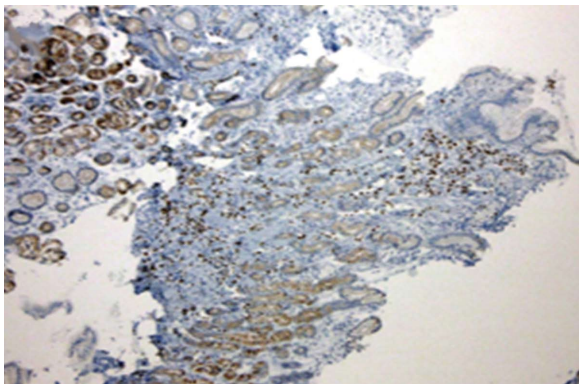

Figure 5 Immunohistochemical stain for human herpes virus 8 showing a strongly positive latent nuclear antigen staining of the spindle cells along the slit-like spaces.

tract. In 1981, with the onset of the HIV epidemic, the first case of AIDS-associated KS was described[9]. Since, an increasing number of KS among AIDS patients prompted speculations of a possible infectious agent involved in tumor pathogenesis[10]. By 1994, Chang *et al*[11] isolated a unique DNA sequence in KS tissue which was later classified as a new herpesvirus species now known as KS-associated herpesvirus or HHV8[11]. Since its discovery, HHV8 has been detected in over 95% of all AIDS and non-AIDS associated KS lesions, suggesting it as the causative agent in tumor development[12].

Epidemiology and prevalence

Numerous serological studies have demonstrated a direct correlation between HHV8 infection and KS incidence. This data also indicated significant geographic variation in HHV8 seroprevalence existed. Overall, low seroprevalence is reported in North America, Northern Europe and Asia, intermediate seroprevalence in Eastern Europe, Mediterranean, and Caribbean countries, and high seroprevalence in Central (Uganda, Zambia, and South Africa) and Southern Africa[12]. Although a cause and effect relationship between HHV8 and KS disease is clear, some exceptions to this pattern have been observed. For example in Africa, cases of endemic (or African) variant of KS were reported in Uganda and Zambia, but not in Gambia or the Ivory Coast although all four countries have similar seroprevalence rates[13]. Similar circumstances have been reported in Italy, Egypt, and Chinese subpopulations where despite high seroprevalence of HHV8, KS incidence is low[14-16]. These discrepancies suggest that other, at present, unidentified factors may be required for clinical expression.

Classification of KS

KS is classified into four clinical variants which have coevolved in certain human populations: classic KS, endemic (or African) KS, Iatrogenic (or immunosuppression related) KS, and epidemic (or AIDS related) KS[12]. In the United States, AIDS and immunosuppression-related causes are the two most common forms of KS tumor, and also the more likely variants causing visceral organ involvement. Despite efforts to classify KS, all forms are associated with HHV8 infection, and differences are observed in primary tumor location and rate of disease progression. Therefore, these epidemiological variants of KS merely represent different clinical manifestations of the same pathologic process.

Classic KS: The classic variant occurs predominantly in elderly men of Mediterranean, Eastern Europe, and Ashkenazi Jewish descent, and is uncommon in North America. It is a slow growing tumor which typically presents as a solitary or multifocal cutaneous lesion in the lower extremities. Systemic progression is rare and visceral disease is observed in less than 10% of cases[17].

Endemic or African KS: The African or Endemic KS variant consists of two disease subtypes, African cutaneous and African lymphadenopathic. It is more aggressive, and generally affects children of endemic African countries. These entities are not associated with HIV infection[18]. Cutaneous and nodal manifestations are common, with systemic disease less frequent.

Iatrogenic or immunosuppression-related KS: Iatrogenic or immunosuppression-related KS affect individuals with suppressed immunity as a result of organ transplantation or other medical conditions requiring long term immunosuppressant therapy. Cutaneous lesions are typically the presenting feature with systemic progression occurring in up to 50% of cases. In two case series of renal transplant recipients, KS developed in approximately 0.5%-5% of organ recipients which occurred in a median time of 29-31 mo after surgery[19-21]. In these patients, treatment with either reduction or discontinuation of the offending agent often led to tumor regression, and in some circumstances, complete resolution of disease.

Epidemic or AIDS related KS: Epidemic or AIDS-related KS is the most common form encountered in the United States. It can present at any stage of HIV infection, but more frequently in the setting of severe immune suppression[22]. Clinical features are similar to those with immunosuppression-related KS, presenting with cutaneous lesions and visceral KS disease arising as disease progresses[2].

Clinical features

The majority of patient with GI-KS will have cutaneous lesions at diagnosis. In most cases, skin lesions are not physically debilitating, but can cause severe disfigurement. Cutaneous KS are generally well-demarcated, erythematous to violaceous, as well as flat, plaque-like or nodular in appearance[2]. A solitary skin lesion is common in the early stage, but it can evolve into multicentric, coalescing lesions with progression. Large lesions are often associated with edema, but isolated lymphedema have been also reported in the absence of cutaneous disease. In most cases, these lesions present initially in the distal segment of the lower limbs, with proximal region involvement that parallel disease progression[23]. However, in AIDS-related KS, skin lesions more frequently affects the head, neck and upper torso regions[2].

Forty to fifty-one percent of patients with cutaneous lesions develop visceral KS which can involve the gastrointestinal tract, lungs, and less commonly the liver, spleen, kidney, and heart[5]. The GI tract is the most common extracutaneous site, and the majority of affected individuals are without clinical symptoms[4,5]. However, with tumor growth, considerable variations in symptoms can present including abdominal pain, nausea, vomiting, and iron deficiency anemia from chronic or frank GI bleeding. In rare cases, the tumor burden has also led to mechanical obstruction, intussusception, and perforation of the bowel wall[1].

Given such high incidence of GI-KS, endoscopic evaluation is required in AIDS patients that present with GI symptoms. However, in patients that are asymptomatic, the benefit of endoscopy remain a topic of debate. In Japan, screening endoscopies are frequently performed in HIV infected patients with the primary aim of early detection and initiation of treatment. A recent retrospective study looked at predictive factors for GI-KS and reported that patients with low CD4 cell count of < 100 cells/μL, men who have sex with men (MSM), and presence of cutaneous KS were more likely to have GI-KS, suggesting that clinicians consider endoscopy in these select patients even in the absence of clinical symptoms[7].

Endoscopy

Gastrointestinal KS can involve any part of the GI tract from the oropharynx to the rectum, but it occurs most frequently in the stomach and small intestine. In two case series of 83 AIDS patients, the incidence of GI-KS was 40%-51%, of which 12%-24% were in the upper tract, 8%-12% were in the lower tract, and 15%-20% had multifocal lesions involving both the upper and lower GI tract[5,24]. GI-KS is endoscopically evident by appearance which ranges from a red maculopapular lesion to a darker, nodular or polypoid lesion. In more severe disease, patients may present with a volcano-like mass with a central umbilication or ulceration which can bleed on contact[1].

Although the majority of GI-KS disease can be identified easily on endoscopy, in some situations it may resemble common benign lesions (peptic ulcer and/or granulation tissue) as well as malignant neoplasms

(gastrointestinal stromal tumor, spindle cell melanoma, angiosarcoma)[25,26]. For these reasons, biopsy specimens should be obtained to allow confirmatory testing with histopathology and immunohistochemistry testing. However, an endoscopic biopsy diagnosis is possible in only 15%-23% of cases, mainly owing to the submucosal nature of tumor growth[24,27,28]. In the absence of mucosal invasion, an endoscopically obtained biopsy may be too superficial and key features may be difficult to appreciate on histopathology. In such cases, an endoscopic ultrasound guided biopsy increases the diagnostic yield and is recommended[7].

Histology and immunohistochemistry

On histopathology, KS is classically characterized as spindle cell proliferation that forms irregular vascular channels or slits in the submucosal bowel layer. It is associated with extensive red blood cell extravasation and hemosiderin-laden macrophage deposits which gives it a characteristic red to dark, bruise-like appearance. Additionally, significant lymphoplasmocytic infiltration occur which can lead to tumor swelling and cause pain response[25].

The presence of spindle shaped cells can exclude many benign and malignant lesions. However, in some circumstances, subtle vascularity and certain histological features of KS may overlap with other GI spindle cell tumors (gastrointestinal stromal tumor-GIST, spindle cell melanoma, and angiosarcoma) causing diagnostic uncertainty. To make a diagnosis of KS, the presence of HHV8 is necessary and immunohistochemical testing is recommended for all specimens with spindle cell morphology. HHV8 LNA is an immunomarker for this viral agent, and expression in spindle cell nuclei is considered to be 99% sensitive and 100% specific for KS[29].

Other biomarkers that are expressed include CD34 and CD117 (or c-KIT), which overlap with other stromal tumors making it a less reliable marker for KS diagnosis. Similar to GI-KS, gastrointestinal stromal tumors also expresses CD34 and CD117, but it is distinguished by the absence of HHV8 LNA, and positive DOG 1 expression[29]. Furthermore, spindle cell melanomas also share similar endoscopic and histologic features with GI-KS, but these tumors typically lack the CD34 marker and express S100 immunopositivity[29].

Management

The treatment approach varies depending on the epidemiological variant of KS, and therapy is individualized based on the disease profile. In this segment, we will be focusing on the current treatment options for AIDS-associated KS with gastrointestinal tract manifestations.

AIDS-related KS is currently not a curable malignancy and the rate of disease progression is variable. Treatment with HAART is recommended in affected individuals if not already on therapy at diagnosis. Aside from the obvious improvement in immune status, the use of HAART inhibits the production of HIV tat protein which is responsible for HIV viral replication and KS cell growth, invasion and angiogenesis[28]. Since the introduction of HAART in 1995, the incidence of KS has substantially declined (25.6 cases per 100 person-years in the early 1990's to 7.5 per 100 person-years in 1996-1997), and patients stable on HAART with good virologic response had a slower rate of disease progression and prolonged survival[29,30].

Among patients with localized cutaneous KS, HAART with either topical gel therapy or local radiation may suffice. However, in patients with extensive mucocutaneous disease or visceral organ involvement, combined HAART and systemic chemotherapy is indicated. Liposomal doxorubicin is the chemotherapeutic agent of choice in which the pegylated liposome component allows for the drug to accumulate preferentially in KS tissue, reducing the dose requirement and drug toxicity. In one study, a combined HAART and liposomal doxorubicin therapy was superior to stand alone HAART therapy with an overall response rate of 72% compared to 20% in the HAART alone group[6]. Chemotherapy with vinca alkaloids, bleomycin, and doxorubicin has fallen out of favor due to variable response rates and substantial drug toxicity[31-34]. Moreover, in randomized control trials, liposomal doxorubicin was also superior to combined chemotherapy regimens, further supporting liposomal doxorubicin as the drug of choice[35,36].

Despite favorable outcomes with dual HAART and liposomal doxorubicin treatment, the incidence of disease relapse is reported to be 13% per year with the majority in the first year after completing chemotherapy[37]. Among patients that require retreatment with liposomal doxorubicin, one-third will have treatment failure. In clinical trials, paclitaxel has demonstrated a comparable response rate of 59% and is currently the second-line agent approved by the Food and Drug Administration[38]. A third option is interferon-alpha which was the initial medication approved for AIDS-related KS. It has a similar clinical response rate but requires several months for tumor response, limiting usefulness in clinical practice[2].

Other drugs have been studied including anti-angiogenic compounds thalidomide and IM-862, and immunomodulators such as retinoid compounds[2]. All have shown suboptimal response rates. Antiviral agents have also been investigated in small trials, and in a single *in-vitro* drug sensitivity evaluation, HHV8 was very sensitive to cidofovir, moderately sensitive to ganciclovir, and weakly sensitive to foscarnet and acyclovir suggesting it as a possible alternative therapy[39,40]. However, currently no treatment recommendations have been drawn based on this study data for these compounds.

Conclusion

KS of the GI tract is far more common than originally thought, but the majority of patients are asymptomatic and remain undiagnosed. Therefore, clinicians should always maintain a high index of suspicion for GI-KS

especially in patients with AIDS. In Japan, screening endoscopies are frequently performed, and a recent retrospective study has suggested using certain clinical factors (low CD4 cell count of < 100 cells/μL, MSM, and presence of cutaneous KS) may predict the occurrence of GI-KS endoscopically. Performance of endoscopy to detect GI-KS prior to development of clinical symptoms is desired as initiation of combined HAART and liposomal doxorubicin therapy has shown to improve outcome.

COMMENTS

Case characteristics

A 44 years old man with a history of acquired immune deficiency syndrome presented with postprandial abdominal pain and nausea with vomiting.

Clinical diagnosis

Multiple plaque-like, violaceous skin lesions found in the lower extremities, trunk and neck suggestive of Kaposi's lesions.

Differential diagnosis

Peptic ulcer disease, gastric mass (adenocarcinoma, stromal tumor, Kaposi's sarcoma), and infectious esophagitis secondary to candida, herpes simplex virus, cytomegalovirus.

Laboratory diagnosis

Absolute CD4 cell count 101 cells/μL; human immunodeficiency virus-RNA 168 copies/mL; hemoglobin 11 g/mL; amylase, lipase, and liver function test were within normal limits.

Imaging diagnosis

Computed tomography scan showed a prominent concentric gastric wall thickening, and follow up upper endoscopy revealed a circumferential mass in the gastric body extending into antrum and fundus.

Pathological diagnosis

Hematoxylin and eosin examination revealed spindle cell proliferation with extensive lymphoplasmocytic cell infiltrates, and immunohistochemical testing confirmed human herpes virus 8 latent nuclear antigen (HHV8 LNA) expression supporting the diagnosis of gastrointestinal Kaposi's sarcoma (GI-KS).

Treatment

Patient was treated with highly active antiretroviral therapy and medical oncology referral was made to initiate systemic chemotherapy.

Related reports

Early diagnosis and treatment of GI-KS has shown to improve patient outcome. Since the majority of patients are asymptomatic, screening endoscopies are being performed in Japan. One retrospective study suggested using specific clinical factors to predict the occurrence of GI-KS which should be confirmed in a larger trial.

Term explanation

The HHV8 LNA stain is a confirmatory immunohistochemical test which is used to distinguish GI-KS from mimickers such as gastrointestinal stromal tumor, spindle cell melanoma, and angiosarcoma.

Experiences and lessons

This case suggests clinicians should maintain a high index of suspicion for GI-KS and a more judicious use of endoscopy may be helpful in improving patient outcome. Specific clinical factors have been shown to help predict the occurrence of GI-KS, which allows clinicians to identify at-risk patients for endoscopic evaluation. However, this should be confirmed in a larger trial.

Peer-review

It is a good case report of a gastrointestinal KS. Moreover, It is performed a comprehensive review and updating of literature about this.

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