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EDITORIAL

Drug-induced autoimmune liver disease: A diagnostic dilemma of an increasingly reported disease

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Abstract

The aetiology of autoimmune hepatitis (AIH) is uncertain but the disease can be triggered in susceptible patients by external factors such as viruses or drugs. AIH usually develops in individuals with a genetic background mainly consisting of some risk alleles of the major histocompatibility complex (HLA). Many drugs have been linked to AIH phenotypes, which sometimes persist after drug discontinuation, suggesting that they awaken latent autoimmunity. At least three clinical scenarios have been proposed that refers to drug-induced autoimmune liver disease (DIAILD): AIH with drug-induced liver injury (DILI); drug induced-AIH (DI-AIH); and immune mediated DILI (IM-DILI). In addition, there are instances showing mixed features of DI-AIH and IM-DILI, as well as DILI cases with positive autoantibodies. Histologically distinguishing DILI from AIH remains a challenge. Even more challenging is the differentiation of AIH from DI-AIH mainly relying in histological features; however, a detailed standardised histologic evaluation of large cohorts of AIH and DI-AIH patients would probably render more subtle features that could be of help in the differential diagnosis between both entities. Growing information on the relationship of drugs and AIH is being available, being drugs like statins and biologic agents more frequently involved in cases of DIAILD. In addition, there is some evidence on the fact that patients diagnosed with DIAILD may have had a previous episode of hepatotoxicity. Further collaborative studies in DIAILD will strengthen the knowledge and understanding of this intriguing and complex disorder which might represent different phenotypes across the spectrum of disease

Key words: Drug-induced liver injury; Autoimmune hepatitis; Drugs; Drug-induced autoimmune hepatitis; Drug-induced autoimmune liver disease

Core tip: Drug-induced autoimmune liver disease (DIAILD) is a poorly defined and under-reported liver disorder, and, probably, a underestimated liver disease. A small number of drug-induced liver injury (DILI) cases exhibit features typical of autoimmune hepatitis (AIH). To differentiate between true AIH triggered by drugs (DI-AIH) and immune mediated DILI still remains a challenge. Patients diagnosed with DIAILD have frequently had a previous episode of hepatotoxicity. We consider that some basic requirements are needed to be considered before supporting a drug as a trigger of AIH and they should be taken into account by authors, reviewers and editors when cases are published and made available to the scientific community.

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INTRODUCTION

The cause and pathogenesis of autoimmune hepatitis (AIH) is unknown[1]. AIH is characterised by the following clinical features: (1) presence of raised aminotransferases with normal or minimal elevations of alkaline phosphatase; (2) association with hypergammalobulinemia and raised immunoglobulin G; (3) female gender preponderance; (4) high titres of a variety of autoantibodies; (5) immunogenetic background; (6) good response to immunosuppressive treatment; and (7) the presence of extrahepatic autoimmune manifestations[2,3]. In liver biopsy specimens, the presence of interface hepatitis is characteristic[3]. In 1999, an international group developed a diagnostic score system[4]; which was later on simplified in 2008[5].

The aetiology of AIH is uncertain, but the disease can be triggered in susceptible persons by an external factor, such as viruses, drugs or herbal remedies[1,2].

Many clinical observations suggest that drugs are potential triggers in some patients[6,7]. Several drugs have been identified to cause AIH that may persist after discontinuation, suggesting that they triggered true autoimmunity. These include oxyphenisatin, nitrofurantoin, minocycline, chlometacin and alpha-methyl dopa[3,8]. Other drugs have been infrequently reported to lead to AIH, making the association less probable. Recently, a growing number of drug-induced autoimmune liver disease (DIAILD) reports meeting the established international criteria[4,5] and showing clinical data (hypergammaglobulinemia, ANA/ASMA), typical liver biopsy findings and HLA-DR status, which allow to establish a causal relationship[6,9-11].

For instance, Björnsson *et al*[12], in a series of 261 patients with AIH, reported 9.2% (24 patients) of patients with drug-induced AIH. A French group[10] searched for a potential causative drug in a consecutive series of 65 patients with AIH, and identified that 12% of the cases were drug-induced, highlighting the frequency of this disorder. In this line, 5 out of 29 (17%) consecutive patients with AIH from Spain were diagnosed with drug-induced AIH[13].

Very recently, Licata *et al*[14] reported 12 patients from a series of 136 drug-induced liver injury (DILI) subjects that were diagnosed as drug-induced AIH (8.8%). All were treated with corticosteroids and remission was achieved after six months in 10 of the cases (83%).

Although no particular drug has been definitively identified as a true aetiological trigger agent for AIH, it is interesting to note that drug-metabolizing enzymes of phase I and phase II are common targets of autoimmunity in idiopathic AIH and viral hepatitis[2].

It is important to distinguish drugs as triggers of a self-perpetuating autoimmune liver disease from immune-mediated drug-induced liver injury (IM-DILI). Immune-mediated DILI nearly always resolves[2,12] or becomes quiescent when drugs are withdrawn. Another possibility is that AIH was quiescent and remains undiagnosed until a drug triggered a new autoimmune process. Thus, to attempt to make a proper diagnosis of the type of immune process affecting the liver is challenging. Björnsson *et al*[12] reported 24 cases of drug-induced AIH (22 minocycline-nitrofurantoin cases, 11/11). Immunosuppressive therapy withdrawal was successful in all the 14 cases in which it was attempted. On the contrary of the AIH cases in this series 65% relapsed upon corticosteroid withdrawal. Heurgué *et al*[10], observed one spontaneous remission, three relapses and four remissions without relapse in 8 patients out of 65 with drug-induced AIH.

In the Spanish DILI Registry, out of 742 DILI cases, 16 were diagnosed as DIAILD[9] and 25% of these cases had another autoimmune associated disease that may require persistent immunosuppressive treatment. Such cases may preclude the therapy to be discontinued and, therefore, one cannot ascertain the course of AIH upon immunosuppressive drug withdrawal. Therefore, to state that relapse after corticosteroid discontinuation might distinguish DIAILD from classical AIH is a very attractive conclusion, but far away from the complex reality of this disorder since in many cases the coexistence of other autoimmune disorders does not allow for stopping immunosuppressive therapy[15,16]. To further complicate the differentiation between AIH and DILI, we must underline the fact that there does not seem to be any specific histological features for either of the processes and the pathological features may show only subtle differences, pointing toward an immune-mediated liver disease versus hepatic toxicity[17].

CLASSIFICATION

There are several possible combinations of DILI and AIH (see Table 1). In 2002 Liu *et al*[2] distinguished 2 types: drugs as a potential triggers of drug-induced AIH (DI-AIH), which supposes a self-perpetuating liver disease, and immune-mediated DILI (IM-DILI), which is an acute or chronic process depending of the duration of the exposure of the liver to the hepatic insult (viruses, herbal remedies, drugs) and disappears or becomes quiescent when the drug is withdrawn[2]. In 2011, Weiler-Normann and Schramm[18] established a classification of DILI and AIH proposing possible connections with suggested diagnoses and clinical characteristics. First, AIH with DILI: The reactivation of a known AIH upon introduction of a new drug is possible, but it is very difficult to demonstrate a causal relationship, as it might be coincidental (chance by association) Often, there is advance fibrosis on histology (see Table 1). Second, DI-AIH, reveals a patient that has not been diagnosed before, or even just the predisposition to AIH that is awakened by DILI. An immune reaction in a genetically predisposed individual may lead to a chronic process, perpetuating the AIH in these patients, with a permanent need for immunosuppression. Usually, they have typical HLA-DR associated with true AIH (Table 1). Third, IM-DILI: acute

Table 1 Classification of drug-induced autoimmune liver disease

AIH with DILI	Patients with known AIH
	AIH quiescent: the drug may be the trigger of a new bout
	AIH under IS or corticosteroids treatment: Reactivation of known AIH upon intro duction of a newdrug (very difficult to demonstrate a causal relationship as it might be coincidental
	Often advanced fibrosis on histology
DI-AIH	Patients with a low grade disease not diagnosed before or predisposition to AIH
	Drug produce an immune reaction that lead to a chronic process:
	Perpetuating the AIH
	Permanent need of IS
	Habitually typical HLA-DR associated
IM-DILI (Autoimmune hypersensitivity)	Fever, eosinophilia, lymphadenopathy, rush
	Indistinguishable from true AIH: Mandatory IS treatment
	Frequently spontaneous remission after drug cessation
	Usually complete response to treatment and sustained remission without relapse
	It is the most frequent drug-induced immune process in the liver attributable to drugs
Mixed autoimmune type	Patients with mixed clinical features of DI-AIH and IM-DILI
	Complete response to IS treatment but with chronic course after withdrawal
	Patients under IS treatment for another autoimmune disease. Withdraw IS drugs is not possible. Remission cannot be evaluated
DILI with positive autoantibodies	Patients with positive autoantibodies
	The probability of developing DIAILD increases in second DILI episodes indepe dently of the causal agent

AIH: Autoimmune hepatitis; DILI: Drug-induced liver injury; IS: Immunosuppressants; IM-DILI: Immunomediated DILI; DIAILD: Drug-induced autoimmune liver disease; HLA: Human leukocyte antigen.

or chronic liver injury (depending of the duration of the exposure to the drug), as has been explained by Liu and Kaplowicz[2], that resolves or becomes quiescent with drug withdrawal. This scenario can be caused by a number of drugs, such as minocycline and nitrofurantoin[2,12]. These individuals may have spontaneous remission of acute hepatitis after drug cessation, or have a hepatocellular or mixed type of liver damage that does not improve after drug-withdrawal. Fever, eosinophilia, lymphadenopathy and rash may be present, but not always. In these situations, which are indistinguishable from true AIH, the immunosuppressive (IS) treatment is mandatory and may be life-saving. Most of these patients do not need IS forever as they usually have a complete response to treatment and a sustained remission without relapse[12,16,18,19]. This type of adverse drug reaction, which can be viewed as autoimmune hypersensitivity, is the most frequent drug-induced immune process in the liver that is attributable to drugs (see Table 1)[2,12].

A second DILI episode (recurrent DILI) involving a different drug is a rare event, but if it happens, AIH or AIH-like DILI is frequent, and reported in up to 40% of cases in the series published from the Spanish DILI Registry[20]. Differentiating IM-DILI from DI-AIH in these cases is very difficult as the clinical presentation may be the same[20].

Nevertheless, we think that a fourth type must be added: mixed autoimmune type. These patients have mixed clinical features that belong to DI-AIH and IM-DILI types. Thus, there are published cases of IM-DILI induced by minocycline which have a complete response to IS treatment, but experience a chronic course after drug withdrawal[15,21,22]. Therefore, the same drug can be DI-AIH or IM-DILI, depending on the clinical characteristics and outcome of the episode. Another possibility is that the patient requires IS treatment for an autoimmune disease, different to AIH, in which case we cannot withdraw IS drugs. In these cases, we cannot differentiate DI-AIH or IM-DILI because the patient needs chronic IS treatment for another autoimmune disease. Therefore, one cannot ascertain the course of AIH upon IS drug withdrawal. For example, chronic uveitis has been associated with AIH[23,24], and may require permanent IS treatment.In the Spanish DILI Registry[25], 16 out of 742 DILI cases, were diagnosed as DIAILD[9]; 25% of these cases had another associated autoimmune disease, which may require persistent IS treatment. In a French series of eight patients[10], 25% of them had another AI associated disease.

Finally, there are patients that present DILI with positive autoantibodies. However, its significance is unknown and requires further studies. In 2002, Ohmoto and Yamamoto studied 64 patients admitted to their hospital with DILI and identified 6 with positive ANA[26]. They found a higher prevalence of associated autoimmune diseases in the ANA positive group such as AIH, rheumatoid arthritis, Hashimoto's disease. The authors suggested that patients with DILI and ANA might also have autoimmune disease and should be followed over the long-term, even if liver function has recovered. On the other hand, Hinrichsen *et al*[27] reported a case of phenprocoumon-induced hepatitis with positive autoantibodies. After the drug was stopped, no clinical or laboratory features of autoimmune liver disease were present.

Recently, the Spanish-Latin American DILI Network has published a series of 73 DILI cases, in which 29% presented positive autoantibodies, mainly ANA. Six cases were DIAILD (AIH DILI) (8%) and 5 cases (7%) had

experienced a second DILI episode[28].

Therefore, this analysis further support that the probability of presenting positive antibodies increases in second DILI episodes, as previously shown by Andrade *et al*[29].

HISTOLOGY

Liver histology shows apoptosis, necrosis and inflammatory infiltrates including mononuclear cells, neutrophils, eosinophils and lymphocytes, or cholestasis and paucity of bile ducts with moderate portal inflammation. Granulomas may also be seen[2]. Distinguishing DILI from AIH is challenging histologically. Recently, Suzuki *et al*[17] showed that while a histologic overlap exists for these pathologies, sufficient differences exist for pathologists and they can use the pattern of injury proposed by Suzuki *et al*[17] to suggest a correct diagnosis. Interface hepatitis, focal necrosis and portal inflammation were present in all of the evaluated cases but were more severe in AIH than in hepatocellular DILI. Portal and intra-acinar plasma cells, rosette formation, and emperipolesis were features that favoured AIH. All Ishak inflammation scores were more severe in AIH than in cholestasic DILI. They did not identify any histologic features differentiating AIH from DIAILD (DI-AIH) in a small subgroup analysis (7 cases), but it might still be possible that detailed standardised histologic evaluation using a larger cohort of DIAILD (DI-AIH) *vs* AIH can identify histologic features that can be helpful in the differential diagnosis. Björnsson *et al*[12], in a recent work, revealed that similar histological grades and stages were present in patients with IM-DILI (DI-AIH) *vs* AIH. However, none of the IM-DILI (DI-AIH) patients had cirrhosis at baseline and it was present in 20% of the AIH patients. Czaja[16], based on the referred work, confirmed that cirrhosis is a rare histologic feature in nitrofurantoin AIH cases at presentation, which may help to distinguish it from classical AIH (0 *vs* 13%). In a recent review of the histological patterns found in cases of DILI[30], it was pointed out that, contrary to minocycline, significant fibrosis and cirrhosis occurs with nitrofurantoin. The French group[10,31] biopsied all of their cases (Minocycline $n = 3$; nitrofurantoin $n = 2$; atorvastatin $n = 1$; fenofibrate $n = 1$; isotretinoin $n = 1$), showing a fibrosis score F3-F4 in 57% *vs* 48% in the DIAILD (AIH-DILI) and AIH groups, respectively. Necroinflammatory activity A3, with Metavir, was revealed in 75% *vs* 65%. They concluded that the 2 groups showed similar histological lesions of fibrosis/cirrhosis. Recently, Licata *et al*[14] studied a series of 12 patients (4 nimesulide, 1 ketoprofen, 3 amoxicillin-calvulanate, 1 ceftriaxone, 1 epigallocatechin gallate and 1 hypericum perforatum -herbal drugs-, and dimethoate-toxic agent). All DIAILD patients were treated with corticosteroids and none developed cirrhosis. Appleyard *et al*[32] published a series of 3 cases, one of which had pre-cirrhosis/cirrhosis at diagnosis. The Spanish Registry of Hepatotoxicity[9] presented a case report of DIAILD that showed cirrhosis in the liver biopsy at presentation. Therefore, the absence of histological cirrhosis at presentation would not help to discern between these two entities.

Ju *et al*[33] have studied the histological features of DILI patients in Korea, with special focus on relevancy of AIH. The study showed that characteristic histologic features of AIH, as interface hepatitis and lymphoplasmocytic infiltrates, were present in up to one third of DILI patients.

FREQUENCY

Recently Czaja[16] stated that the best estimate of the frequency of drug-induced autoimmune-like hepatitis among patients with classical features of AIH is 9%[12]. There are other reports that show higher figures[15]. Heurgué *et al*[10], in France, identified 8 cases (12%) with drug-induced AIH in a consecutive series of 65 patients with AIH, fulfilling all requirements recommended in the review to confirm the diagnosis (Table 2). In Gipuzkoa, Spain, from 1994 to 2009, 29 cases of AIH were diagnosed, 5 of which were considered AIH-DILI, which resulted in a 17% frequency[13]. We believe that these differences in frequencies may be explained by the fact that AIH-DILI is often misdiagnosed.

If we study patients diagnosed with DILI, Licata *et al*[14] reported 8.8% of the patients presenting features of DIAILD (DI-AIH). In the Spanish DILI Registry[9], out of 742 recruited cases, 16 presented DIAILD criteria (2.15%).

Why is there a growing number of case reports?

Indeed, the diagnosis of AIH is often made in the setting of a patient being treated with multiple drugs. If the diagnostic scale points out probable AIH, the possible role of the drug is generally underscored, and immunosuppressive treatment is started. On the other hand, if the AIH scale is not conclusive and/or histology findings are more consistent with DILI, the case is assumed to be a DILI case, particularly if the clinical symptoms resolve after discontinuation of the suspected drug (DIAILD may be a self-limiting process), and the possibility of unmasking AIH by the action of a drug is disregarded. To further complicate the differentiation between AIH and DILI we must underline the fact that there is no specific histological features of either process and the pathological features may show only subtle differences pointing to the immune-mediated liver disease versus hepatic toxicity[17]. These considerations lead us to suggest that DIAILD might be underreported nowadays.

When a patient is admitted to hospital because of acute hepatitis and the drug that is thought to be responsible is identified, treatment is stopped.

If transaminases decreased more than 50% in 1 week to 1 month, and the DILI case fulfils other Council for International Organizations of medical Science (CIOMS) criteria such as a favourable temporal sequence, alternative pharmacological and clinical conditions have been ruled out, dechallenge with the drug is followed by improvement, and rechallenge if present is positive, then the DILI case is probably related to the drug[34,35]. Sponta-

neous recovery supports this possibility and, generally, if autoantibodies have been solicited to the laboratory and are positive, we do not consider the possibility of a drug as a trigger of IM-DILI.

If aminotransferases remain permanently raised, suffer an increase during follow-up, and autoantibodies are positive, AIH is assumed; if the AIH-scale reveals a probable AIH, treatment with IS is begun as soon as possible. There is complete exclusion of the trigger paper of a drug treatment if it is present prior to acute hepatitis, or if the drug is retired but without giving its importance to it; if true, AIH may resolve spontaneously.

New drugs: the growing use of drugs such as statins and biologic agents, which have been related to DILI with autoimmune features, which has prompted an increase in the diagnosis of DIAILD[36-42].

The diagnosis DIAILD represents a challenge to the clinician as there are neither histological, nor clinical features that are pathognomonic of true AIH, and HLA haplotypes do not convincingly distinguish between either entity. Indeed, as previously commented many cases of drug-induced AIH tend to be misdiagnosed as classical AIH cases. The CIOMS scale is unable to distinguish between AIH and DIAILD, as the scale fails to accommodate all relevant information for diagnosing DIAILD. Causality may also be challenged by the lack of spontaneous improvement after drug withdrawal in this form of DILI.

PREDICTION OF AUTOIMMUNE HEPATITIS-RISK FACTORS

Susceptibility

Autoantibodies: Many autoimmune diseases are chronic conditions that progress over the years, and are characterised by the presence of autoantibodies that may precede the overt disease by months or years[43]. In AIH, the hallmark is the presence of circulating autoantibodies. If ANAs are detected in female patients with DILI, the coexistence of an autoimmune disease is possible[26].

***HLA* genes and haplotypes:** Susceptibility to AIH, via a genetic predisposition, has been clearly associated with class Ⅱ human leukocyte antigen (*HLA*) genes, more specifically to the DRB1 locus[44]. This is the major associated gene locus. Predisposition to AIH type I is associated with HLA-DRB1*0301, DRB3*0101, and DRB1*0401 alleles in European and North-American Caucasoid, encoding the HLA-DR3, DR52 and DR4 molecules, respectively. Czaja *et al*[45] determined in 1993 the following class Ⅱ HLA associations with AIH: 44% DR3, 32% DR4, 9% DR3-DR4, and 15% other antigens. The study of different geographical areas and ethnic groups revealed different results. In Japan and Argentinean Caucasoid adults, susceptibility was associated with HLA-DRB*1-0405 (DR4): Mexican adults presented DRB1*0404 (DR4), and Caucasoid children and adults from Argentina presented DRB1*1301 (DR13)[45-49]. In Brazil, the haplotype DRB1*1301 is the most frequently related to type 1-AIH[44]. A secondary association with HLA-DRB1*0301 has been detected in this country[50]. Another haplotype, DRB1*07, seems to produce susceptibility in German and Brazilian populations[51,52]. Type 2 AIH is associated with the haplotypes HLA-DRB1*0301 and HLA-DRB1*07[50]. Recently, Oliveira *et al*[50] searched for additional susceptibility factors in the extended MHC region. They have studied genes located in the MHC class Ⅲ region, in addition to class I HLA-B and MICA genes, to verify if the specific haplotypes DRB1*1301 or DRB1*0301 could be involved in the susceptibility of paediatric patients in Brazil[53]. The ancestral haplotype comprising *TNFA*-308A, *TNFA*-238G, *LTA*+252G, *LTA*+80C, *NFKBIL1*-63A, *BAT1*-348C, *BAT1*-22C, *HLA-B**08 and *MICA**08 was more common in DRB1*03 positive patients than in controls (40% *vs* 14%), showing a seven-fold increased risk of disease. Finally, a variety of class Ⅲ haplotypes was also present in HLA-DRB1*13 patients, without a predominant pattern. The most common of the 98 haplotypes present in patients were completely absent in controls. The extended haplotype analysis in this sample of AIH-1 patients highlighted not only the genetic diversity present in the Brazilian population, and was also in accordance with the previously documented microdiversity within the MHC region. The DRB1*1501 allele may protect from disease[46].

Triggers

Triggers may induce AIH. AIH may be induced by drugs[12], herbal remedies[11], different viruses and bacteria[54-58] and vaccines[59,60].

Exposure to all of these potential triggers may produce immune responses that especially target the liver, mainly in predisposed individuals, as we have seen previously; therefore, chronic hepatitis with autoimmune features might develop. If not recognised promptly and the responsible agent is not withdrawn, such responses can evolve to chronic hepatitis (resembling viral hepatitis) - alpha-metil dopa, halothane, hydralazyne, minocycline, nitrofurantoin, and oxyphenisatin - or to a chronic non-suppurative cholangitis (resembling PBC) - chlorpromazine[1].

Identification of these risk factors might help us to think about this disease and halt treatment with the offending trigger as soon as possible. Withdrawal of the offending agent may lead to a rapid resolution of the process.

Genetic polymorphisms: Several genetic polymorphisms of drug metabolising enzymes, particularly CYP, have been identified, which may produce reactive metabolites[2]. Differences in the metabolism of drugs genetically conditions may produce protein adducts and susceptibility to DI-AIH. The occurrence of more than one case within a family supports the theory that genetic factors are involved[61]. It has been shown that a familiar sensitivity to the toxic effect of the metabolites exists, which may produce an inherited defect in the defence of

Table 2 Elements to be reported when a case of drug-induced autoimmune liver disease is suspected

Previously obtained ANA
Evolution:
During the treatment with the suspicious drug
After drug withdrawal
Check for the presence of HLA-DR:
HLA-DRB1*0301,0401,07,1301
Drug type
Time to onset from the beginning of the treatment
AIH diagnosed:
During the course of treatment
After withdrawal of the drug
AIH scales for diagnosis
International autoimmune hepatitis group report (4)
Simplified score (5)
Previous DILI episodes
Response to corticosteroids
Autoimmune titres evolution
IgG values evolution

AIH: Autoimmune hepatitis; DILI: Drug-induced liver injury.

the liver from the injury produced by these toxic metabolites[2]. Anti-convulsants and sulphonamides have shown a familiar inheritance of *in vitro* lethal effects against the lymphocytes by their metabolites[62,63].

Sex

In AIH, there is a female sex predominance. Women are affected more frequently than men (sex ratio 3.6:1) and the disease is seen in all ethnic groups and across all age intervals[56]. Björnsson *et al*[12] published 78% (184/237) of females with AIH *vs* 92% in the DI-AIH (20/24); in the nitrofurantoin group the findings were 11/11, with numbers of 10/11 in the minocycline group. In the Spanish DILI Registry, sixteen cases out of the 742 cases (2.15%) of idiosyncratic DILI were identified[9]. There were 10/16 women. Heurgué *et al*[10] reported 8 patients with DI-AIH out of 65 AIH cases diagnosed consecutively. The female/male sex ratio was 87% *vs* 82%, revealing no statistically significant differences. Sugimoto *et al*[11] recently published a series of 7 patients with AIH that developed after a first DILI episode. Six out of the seven affected patients were women. In other smaller series, a female preponderance seems to be the general rule[32]. Czaja *et al*[16] reported that not only does DIAILD (DI-like AIH) occur almost exclusively in women, but that the drug injuries in the liver are more severe than in men[2,12,64,65]. Indeed, female sex has been found to be a risk factor for acute liver failure development after a DILI episode[25].

DILI

Multiple episodes of DILI in the same patient with drugs of similar structure or function as well as unrelated drugs may induce immune-related hepatotoxicity[20]. Between 1994 and 2009, Lucena *et al*[20] identified 9 patients out of 742 in the Spanish DILI Registry (1.21%), with evidence of two distint DILI episodes produced by different drugs. In each individual, the type of injury was the same in the two episodes, regardless of the causative drug. Second episodes were associated with features of AIH up to more than 40% (4/9) of cases, making it unclear whether this is drug-induced unmasking of true DI-AIH or IM-DILI (DILI with autoimmune features).

Age

Elderly individuals have an increased risk of drug toxicity[16]. The risk of DILI increases with age for certain drugs, such as those that are implicated in autoimmune-like hepatitis (nitrofurantoin, halothane, and isoniazid).

Association with other autoimmune diseases or induction by biologic agents

Biologic agents: Biologic agents are increasingly being used for rheumatological and systemic autoimmune diseases. The BIOGEAS project[39,40], created by the Spanish Society of Internal medicine, has retrieved more than 800 cases of AI diseases secondary to biological therapies. In this study, 19 cases of AIH have been reported. Statins[36,37] have been reported to induce AI diseases, typically in patients with other AI diseases.

Spontaneously: AI diseases frequently appeared to be associated between them[36,37]. Multiple examples are published in the literature[66].

Drugs

Nowadays, more than 900 drugs, toxins and herbal remedies have been reported to cause liver injury. Recently, it has been reported that at least 24 drugs, probably more, have been associated with AI chronic hepatitis mimicking AIH[67], but more and more new agents are being implicated[67-83]. With the appearance of new statins, biologic agents, and antibiotics, it will be quite normal to see more new agents being reported as being responsible for new drug-induced AIH cases (Table 3).

Elements to be reported when a case of DIAILD is suspected

Some basic requirements are needed to consider a drug as a trigger of AIH. These basic requirements must be debated and consensuated prior to the publication of a suspected new case. The elements that we think that must be included in a suspected DIAILD case before publication are outlined in Table 2.

CONCLUSION

DIAILD is still a poorly defined and under-reported liver disorder and is also a probably underestimated liver disorder. A small number of DILI cases exhibit features typical of AIH. To differentiate between true AIH triggered by drugs (DI-AIH) and IM-DILI still remains a challenge. Patients diagnosed with DIAILD have frequently had a previous episode of hepatotoxicity. The CIOMS scale has a limited value to ascertain causality in DIAILD. Hopefully, the collaborative efforts in DILI research will

Table 3 Drugs and drug-induced autoimmune liver disease

Well stablished drugs:
Minocycline
Nitrofurantoin
Oxyphenisatin, alpha-methyl-dopa, clometacin.
Emerging drugs:
Statins
Biologics agents:
Infliximab
Others: adalimumab, etanercept, efalizumab, ipilimumab
Other drugs:
Less compelling association (infrequent reports): atomoxetine, diclofenac, fenofibrate, pemoline, phenprocoumon, dihydralazine, tielinic acid, benzarone

enhance our knowledge of this intriguing hepatic disease. We consider that some basic requirements are needed to be considered before supporting a drug as a trigger of AIH and they should be taken into account by authors, reviewers and editors when cases are published and made available to the scientific community.

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REVIEW

Lipid lowering effects of iodothyronines: *In vivo* and *in vitro* studies on rat liver

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is emerging as one of the most common liver diseases, leading to the increasing interest for new therapeutic approaches for its treatment. NAFLD primarily depends on a hypercaloric and/or unbalanced diet leading to overweight and obesity. The liver, in fact, plays a central role in lipid metabolism by importing free fatty acids from the blood and synthesizing, storing, oxidizing and exporting lipids. Furthermore, the liver is the target for the thyroid hormones, thyroxine (T_4) and 3,3',5-triiodo-L-thyronine (T_3), that stimulate the basal metabolic rate and lead to body weight loss. In the last decade, other iodothyronines have been shown to possess biological relevance and play some thyromimetic activities; in particular, 3,5-diiodo-L-thyronine (T_2) gained large interest. The global effect of iodothyronines on liver lipid metabolism results from the balance between direct and indirect actions on the hepatocyte, leading to stimulation of lipid synthesis, oxidation and autophagy. In this review, the results so far obtained on both *in vivo* and *in vitro* models of hepatosteatosis are summarized in order to obtain an updated picture of the lipid-lowering effects of iodothyronines on mammalian liver.

Key words: Iodothyronines; Liver steatosis; Lipid metabolism; Non-alcoholic fatty liver disease; Hepatocytes

Core tip: This review summarizes the recent insights about the mechanisms underlying the lipid lowering action of iodothyronines. In the last decades, extensive studies investigated the possible use of iodothyronines in the treatment of obesity and dysmetabolic syndromes. Since the pharmacological use of thyroid hormones has found severe limitations because of their thyrotoxic effects, the identification of iodothyronines retaining anti-obesity and hypolipemic efficacies, while being devoid of thyrotoxicity, gained great interest. The review discusses the recent studies employing both *in vivo* and *in vitro* models of hepatosteatosis, with particular attention to the *in vitro* studies demonstrating the direct anti-steatotic effect of iodothyronines.

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IODOTHYRONINES AND METABOLISM

Thyroid hormones (THs) secreted by the thyroid gland comprise two main iodothyronines: 3,5,3',5'-tetraiodothyronine (thyroxine or T_4) and 3,5,3'-triiodo-L-thyronine (T_3) (Figure 1). T_4 is the major form secreted by the thyroid and the most abundant TH in circulation, while T_3, the active form, is mainly generated by peripheral deiodination of T_4. T_3 may be further deiodinated to yield different diiodothyronines such as 3,5-diiodo-L-thyronine (T_2) (Figure 1). In the past, T_3 was assumed to be the only active iodothyronine *in vivo*, but recent evidence suggested that other iodothyronines, such as 3',5',3-l-triiodothyronine (rT3) and T_2, may be of biological relevance[1,2].

THs influence a large number of physiological pro-

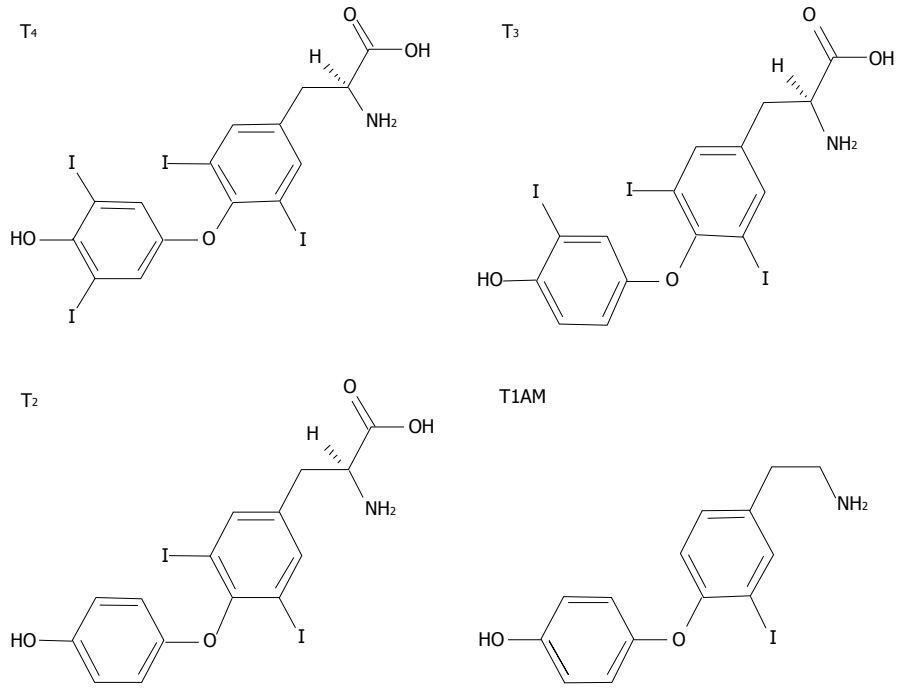

Figure 1 The chemical structures of three biologically active iodothyronines and one derivative: thyroxine, 3,3',5-triiodothyronine, 3,5-diiodothyronine and 3-iodothyronamine. T4: Thyroxine; T3: 3,3',5-triiodothyronine; T2: 3,5-diiodothyronine; T1AM: 3-iodothyronamine.

cesses in vertebrates, including growth, development and differentiation. THs have stimulatory effects on metabolic activity, thus inducing thermogenesis (the so-called calorigenic effect) that represents a major component of the energy expenditure in endotherms. Of particular interest is the effect of THs on lipid metabolism resulting from the balance between stimulation of lipid synthesis and lipid oxidation (Figure 2). The liver represents one of the main target tissues of THs. At the hepatic level, T_3 stimulates cholesterol synthesis and its metabolism into bile acids[3] and cholesterol uptake[4], and it induces lipogenic enzymes, including fatty acid synthase (FAS) and acetyl-CoA-carboxylase[5]. In addition to the lipogenic action, T_3 also leads to a general reduction in the hepatic triglyceride (TAG) content, likely through stimulation of lipolytic pathways[6]. Moreover, it has been suggested that TH-stimulated lipogenesis/lipolysis "futile" cycle may contribute to the calorigenic effect[7].

The metabolic effects of iodothyronines have long been investigated because of their potential use as drugs to treat obesity and lipid metabolism disorders[8]. However, due to the simultaneous undesirable side effects, such as the induction of a thyrotoxic state (tachycardia, muscle wasting, bone loss), the employment of T_3 or T_4 to stimulate body weight loss or treat metabolic syndrome has been limited. At the same time, the development of TH agonists/analogs retaining lipid-lowering and anti-obesity efficacies, while being devoid of thyrotoxic effects, has received great interest as a potential therapeutic advancement. The research of the last years has identified several iodothyronines other than T_3 and T_4 that display some thyromimetic activities. Among them, T_2 assumed a great interest as it mimics several effects of T_3 on energy metabolism[9-11] without inducing thyrotoxic effects[12]. A single dose of T_2 (25 μg/100 g body wt) stimulated the resting metabolic rate (RMR) of hypothyroid rats and increased the liver oxidative capacity to the same extent as the same dose of T_3[13]. Moreover, T_2 significantly reduced serum triglyceride and cholesterol levels and increased liver oxygen consumption[10].

With regards to the calorigenic effects of THs, several cellular targets have been proposed but none has received universal acceptance. By virtue of their central role in the energy-transduction pathway, mitochondria are natural candidates to mediate the calorigenic activity of iodothyronines[14]. Single injections of T_2 or T_3 into hypothyroid rats stimulated RMR[15], in association with an increase in oxygen consumption[16]. It is widely accepted that iodothyronines may exert two kinds of effects on mitochondria: (1) a rapid stimulation of respiration (within minutes/hours); and (2) a long term effect leading to mitochondrial biogenesis and mitochondrial mass increase. The calorigenic activity of THs has long been ascribed to uncoupling of mitochondrial oxidative phosphorylation, but the mode by which they promote mitochondrial proton leak is still unresolved. Harper *et al*[17] related the T_3-induced increase in mitochondrial proton leak to an increased permeability of the phospholipid bilayer due to a change in the lipid composition of the inner mitochondrial membrane. On the other hand, T_2, and to a lesser extent T_3, was shown to bind the Va subunit of the cytochrome oxidase complex, thus abolishing the allosteric inhibition due to ATP binding and stimulating enzyme activity[18]. Recently, Yehuda-Shnaidman *et al*[19] reported that mitochondrial uncoupling by T_3 was transduced both *in vivo* (in rats) and *in vitro* (Jurkat cells) by gating of the

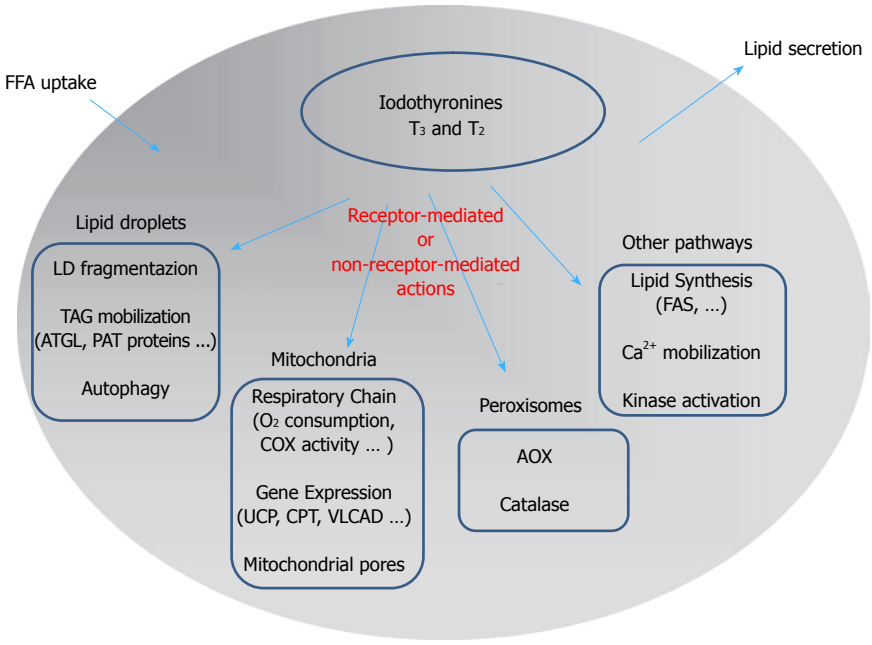

Figure 2 Schematic representation of the mechanisms underlying the control of lipid metabolism by iodothyronines in the hepatic cell: A summary of the possible signaling pathways involved in iodothyronine actions is presented. The classic "receptor-mediated" pathway describes the action of iodothyronines through the thyroid hormone receptors (TR). The "non receptor-mediated" pathway occurs through the interaction of iodothyronines with different cellular targets. FFA: Free fatty acids; LD: Lipid droplet; AOX: Acyl-CoA oxidase; ATGL: Adipose triglyceride lipase; CPT: Carnitine palmitoyl-transferase 1; FAS: fatty acid synthase.

mitochondrial permeability transition pore.

Interestingly, T_2 administration has been demonstrated to be able to stimulate RMR and to also reduce body weight in humans. In a pilot study, two euthyroid subjects were treated with increasing doses of T_2 (from 100 to 900 mcg/d, three times a day for 8 d) and for a further 3 weeks with 3000 mcg/d. A reduction in body weight of -4% was observed without effects at the cardiac level[20].

The pharmacological effects of some derivatives of thyronines called thyronamines have been also investigated. Scanlan *et al*[21] described the synthesis and biological properties of 3-iodothyronamine (T1AM), a novel thyronamine that was shown to be an endogenous component of biogenic amine extracts from rodents. T1AM has a carbon skeleton identical to that of T_4 and theoretically, it could be produced from T_4 by enzymatic decarboxylation and deiodination (Figure 1). T1AM treatment rapidly induced a hypometabolic state and hypothermia in rodents, with opposite effects compared with those typical of THs.

MECHANISMS OF ACTION OF IODOTHYRONINES

In the past, it was a common notion that TH actions were mediated by specific nuclear thyroid hormone receptors (TRs) acting as ligand-dependent transcription factors binding the "thyroid hormone response elements" (TREs) on the promoter region of thyroid hormone-responsive genes[22]. In the early 1960s, Tata and co-workers provided the first evidence for a "receptor-mediated" mechanism of T_3 action on energy metabolism[23]. In the 1980s, two distinct genes, *THRA* and *THRB,* were identified in humans and rodents, each encoding a different TR isoform (TRα and TRβ, respectively). The *THRA* gene was originally identified in chicken[24], while *THRB* was cloned from human and rat cDNA libraries[25]. Each isoform shows alternative splice variants (TRα1, TRα2, TRβ1 and TRβ2) with specific and distinct functions and tissue localization.

Although the "receptor-mediated" mechanism accounts for several actions of THs, other effects independent of TRs have been described, suggesting an alternative model for their action. Effects of iodothyronines that are not initiated by binding to TRs are termed 'non-receptor-mediated' mechanisms[26] and could involve a multiplicity of signaling pathways, such as phosphorylation of effector proteins[27], binding to surface receptors[28], Ca^{2+} mobilization[29], alteration of mRNA stability[30], modification of membrane fluidity and permeability[2]. The possibility that TH action was mediated by interactions with membrane surface receptors was confirmed by using cell impermeant agarose-conjugated T_3. The results clearly indicated that both free and conjugated hormones led to activation of extracellular signal-regulated kinase (ERK1/2s)[31] and affected Ca^{2+} homeostasis[32]. Moreover, THs were shown to interact with theαVβ3 integrin receptor triggering the ERK1/2 pathway[33]. Although the "non-receptor mediated" effects are sometimes called "non-genomic", this term is rather confusing as these pathways may also in turn affect gene transcription [34].

In conclusion, it is now widely accepted that TH effects may result from a synergism between "receptor-mediated" and "non-receptor mediated" mechanisms. Moreover, we can distinguish between early and late effects of THs (also called "short-term" and "long-term" effects), the first being evident within minutes or a few

hours, whereas the second occurs over several hours or days[34,35]. However, the latency of a response is not sufficient to discriminate between "receptor-mediated" and "non-receptor" mediated effects.

HEPATIC STEATOSIS: *IN VITRO* AND *IN VIVO* MODELS

With the rapidly growing prevalence of obesity throughout the Western countries, morbidity and mortality related to its complications are on the rise. Severe obesity is generally associated with TAG accumulation in non-adipose tissues like liver, muscle and pancreas and leads to a high risk of co-morbidities, including nonalcoholic fatty liver disease (NAFLD), cardiovascular disease and diabetes (for a review see[36]). NAFLD is a pathological condition associated with over-accumulation of TAGs in the liver and represents the most common of all hepatic disorders and the most frequent cause of chronic liver disease[37,38]. The earliest stage of NAFLD is hepatic steatosis characterized by the deposition of cytoplasmic TAGs as macro- and/or micro-vesicular lipid droplets in more than 5% of hepatocytes. Simple steatosis may progress to nonalcoholic steatohepatitis (NASH), cirrhosis and finally hepatocellular carcinoma[39]. NAFLD is now considered the hepatic manifestation of the metabolic syndrome and has insulin resistance as its hallmark. NAFLD is a syndrome with multifactorial etiology for which there is no effective treatment, although weight loss may halt disease progression and revert histological changes[36].

In hepatocytes, steatosis results from an imbalance between lipid availability (deriving from circulating lipid uptake or *de novo* lipid synthesis) and lipid disposal (through FFA oxidation or TAG secretion)[40]. Typically, the main cause of steatosis is an overflow of free fatty acids (FFAs) into the liver that may eventually trigger lipoperoxidative stress and hepatic injury[39,41]. In the liver, FFAs are stored as TAGs through their esterification with glycerol or, alternatively, catabolized by oxidation to generate adenosine triphosphate (ATP). Excess TAGs are accumulated inside lipid droplets (LDs) that regulate storage and traffic of lipids (for a review see[42]). Typically, LDs are composed of a core of neutral lipids surrounded by phospholipids and proteins of the PAT protein family (acronym referring to the first members identified)[43]. The main PAT proteins are the adipocyte differentiation-related protein (ADRP, also called PLIN2), the oxidative tissue-enriched PAT protein (OXPAT or PLIN5) and the tail-interacting protein (TIP47 or PLIN3)[44]. ADRP expression is increased in rat models of NAFLD and in isolated hepatocytes[45]. PAT proteins are under the control of peroxisome proliferator-activated receptors (PPARs), a subfamily of lipid-activated transcription factors[46] consisting of three members, PPARα, PPARγ and PPARδ, with distinct functional roles[47,48]. In the liver, PPARα enhances lipid catabolism and mobilization[49], PPARδ induces glycolysis/lipogenesis and PPARγ promotes lipid synthesis and LD formation[50]. In summary, PPARα and PPARδ mainly act in energy burning, whereas PPARγ regulates energy storage, although an overlapping in their function has been described[40-51]. Moreover, PAT proteins regulate action of hepatic lipases that mobilize TAGs stored in LDs towards oxidation or secretion[52], in particular, the adipose triglyceride lipase (ATGL) performs the first step in TAG hydrolysis.

In vivo models

Steatosis and steatohepatitis can be modeled in rodents by two main dietary protocols: a methionine and choline deficient (MCD) diet or a high-fat diet (HFD). Different dietary approaches produce different disease severities and work by specific mechanisms[53]. In rodents, a MCD diet quickly induces (2-4 wk) hepatic steatosis (mainly macrovesicular) that may progress to inflammation and fibrosis. MCD diet-induced NASH is reversible by switching to a diet with methionine and choline. Rodents fed MCD diets lose weight (due to the lower caloric intake) and do not show insulin resistance. By contrast, HFD increases body weight, body fat and induces insulin resistance in rodent models. In general, HFD feeding induces only mild steatosis (mainly microvesicular) and does not produce liver fibrosis. The term "HFD" encompasses a wide variety of diet formulas but in all of them about 30%-75% of total calories is derived from saturated fatty acids. This diet closely resembles the pathological and molecular alterations found in humans with NAFLD[53]. It can be emphasized that fatty liver is typically characterized by altered lipid metabolism, increased oxidative stress and abnormal pattern of cytokine production.

In vitro models

Hepatic steatosis in humans is typically associated with excess accumulation of oleic acid, a monounsaturated omega-9 fatty acid which represents the end product of *de novo* fatty acid synthesis. A number of studies using both primary cell cultures[54] and immortalized cell lines[55,56] proposed reliable cell models of hepatosteatosis in which the steatosis severity might be modulated and the TAG content was exactly quantifiable. These *in vitro* models represent a simple experimental system to investigate the mechanisms underlying the steatosis progression and the hepatocyte alterations by excluding the interference from the matrix and other non-hepatocytic cells. Over the past decade, several cellular models of hepatosteatosis have employed palmitate (C16:0) and oleate (C18:1) as exogenous fatty acids since these are common dietary long-chain FFAs and the most abundant FFAs in liver in both normal subjects and patients with NAFLD[57]. The human hepatoma cell line (HepG2) incubated with a mixture of oleate/palmitate (2:1 ratio) was used to study the cellular mechanisms involved in FFA-mediated lipotoxicity[55,58]. The same FFA mixture was used to induce steatosis in primary human hepatocytes[58]. In order to assess the different toxicity of saturated and unsaturated FFAs, primary mice hepatocytes and HepG2 cells were treated with various concentrations (0.05-0.5 mmol/L) of long

chain FFAs with different degrees of saturation; exposure to monounsaturated fatty acids resulted in lipid accumulation without changes in hepatocyte viability; in contrast, saturated fatty acids significantly decreased cell viability[59]. The effect of increasing concentrations of oleate alone (0.1-2.0 mmol/L) was also evaluated in order to clarify the pathophysiological changes associated with NAFLD[60].

LIPID-LOWERING EFFECTS OF IODOTHYRONINES ON *IN VIVO* MODELS OF HEPATOSTEATOSIS

In 1994, a first study reported that a daily intraperitoneal (*ip*) injection of T_3 (from 0 to 25 μg/100 g b.w.) to *ob/ob* mice decreased body weight and body fat and increased oxygen consumption and oxidative metabolism[61]. About ten years later, Goglia and coworkers described similar effects for T_2[10]. They showed that a daily ip injection of T_2 (25 μg/100 g b.w.) to rats simultaneously receiving HFD reduced both adiposity (about -50%) and body weight gain (about -13%) when compared with rats receiving HFD alone. Moreover, T_2 administration resulted in an almost complete disappearance of fat accumulation in the liver, a reduction in serum TAG and cholesterol levels (-52% and -18%, respectively), and a stimulation (about +42%) of FFA oxidation rate without inducing thyrotoxicity [10]. The effects of T_2 on liver metabolism seemed to involve mitochondria, even although peroxisomes are the main site for fat oxidation. In fact, long chain FFAs enter mitochondria through the activity of carnitine palmitoyltransferase 1 (CPT1) that was stimulated by HFD and further increased by T_2 [10].

Interestingly, dietary administration of T_3 was also able to both prevent and reverse hepatic steatosis in rats[62]. In fact, concurrent dietary administration of T_3 and MCD diet resulted in prevention of fatty liver and decrease in lipid peroxidation in rats fed a MCD diet for 10 weeks and then co-fed T_3 for 1 week. Similar effects were observed using the potent TR selective agonist GC-1 [62].

The hepatic effects of T_2 administration to HFD rats were investigated in more detail by Grasselli *et al*[63,64]. HFD feeding resulted in hepatic lipid accumulation under the form of numerous LDs, a condition resembling the microvesicular steatosis typical of NAFLD. Fat accumulation was associated with increased transcription of PPARα, a regulator for a number of genes involved in FFA catabolism, and of ATGL, a lipase mobilizing fat from LDs, together with a stimulation of anti-oxidant agents such as catalase and metallothioneins, in line with the increased production of reactive oxygen species (ROS) from mitochondria and peroxisomes as a consequence of fat accumulation[65]. In the liver of HFD rats, concomitant T_2 administration was able to prevent lipid accumulation, but also oxidative stress conditions associated with the diet[63]. Moreover, T_2 prevented the HFD-induced up-regulation of both PPARα and ATGL and stimulated - (AOX) expression, indicating a stimulation of peroxisomal FFA oxidation[64].

In addition to the above described reports demonstrating the ability of T_2 to prevent liver steatosis when administered simultaneously to HFD, other studies demonstrated that T_2 was also able to reverse hepatic steatosis after its induction through long term HFD and these effects were associated with a stimulation of mitochondrial uncoupling and a reduction in mitochondrial oxidative stress[66].

A recent paper investigated the changes in the rat liver proteome induced by T_2 treatment. The proteomic approach allowed identification of which proteins were differentially expressed in the liver of HFD rats as a function of T_2 treatment[67]. Upon T_2 administration, the rat liver proteome resembled that typical of a non-steatotic condition. In particular, high-fat feeding led to changes in the expressions of enzymes involved in a multiplicity of pathways (*i.e.*, lipid metabolism, antioxidant defense, respiratory chain, oxidative metabolism). Mitochondria, in particular, appeared as the major target for the metabolic/energy adaptations induced by lipid overload in the liver and showed the more marked changes in terms of proteome as a response to T_2 treatment. In mitochondria from HFD rats, enhanced activities of complexes I and V and reduced activities of complexes Ⅱ and Ⅳ were detected, even although the protein levels for all the complexes were increased. T_2-treatment stimulated complexes I and Ⅱ and normalized complex Ⅳ activity. On this basis, the authors suggest that the T_2-induced enhancement of oxidative capacity may actually be based on a stimulation of the individual respiratory chain complexes (I , Ⅱ and Ⅳ)[67].

LIPID LOWERING EFFECTS OF IODOTHYRONINES ON *IN VITRO* MODELS OF HEPATOSTEATOSIS

The above described *in vivo* studies could not distinguish between the direct antisteatosic effects of THs on the liver and their secondary effects due to upstream changes in endocrine or metabolic pathways. The employment of isolated hepatocytes allowed overcoming these problems.

Grasselli *et al*[51] assessed *in vitro* the direct effects of T_2 and T_3 (10^{-7}-10^{-5} mol/L doses for 24 h) using primary cultures of rat hepatocytes overloaded of lipids ("steatotic" hepatocytes) by exposure to the classical oleate/palmitate (2:1 ratio) mixture. The use of supraphysiological doses of iodothyronines depends on both their rapid metabolism *in vitro* and on their binding to the high concentration (1%) of albumin present in the culture medium. In accordance with reports showing altered expression of PPARs in murine models developing fatty livers[47], isolated "steatotic" hepatocytes exhibited increased expression of both PPAR-γ and PPAR-δ, as well as of ADRP, a PPAR-regulated PAT protein. As in liver of HFD rats[64], also in isolated "steatotic" hepatocytes an increased activity of AOX, the enzyme catalyzing peroxisomal β-oxidation, as well as of SOD and catalase, two antioxidant enzymes protecting cells from the higher ROS production associ-

ated with FFA catabolism, was described. A reduction in the number and average sizes of LDs was observed after treatment with T_2 or T_3, suggesting that iodothyronines lead to dispersion/fragmentation of LDs, thus making the stored TAGs more accessible to enzymes acting on catabolism/secretion of FFAs. Moreover, both T_2 and T_3 were able to reduce the FFA-induced up-regulation of PPARγ and PPARδ, the stimulation of AOX, SOD and catalase activities. These results clearly indicate the lipid-lowering effect of iodothyronines mainly depends on a direct action on the hepatic cell[51].

The use of primary rat hepatocytes allowed verification that the lipid-lowering effect of iodothyronines was a direct action on the hepatocyte but the involvement of thyroid hormone receptors in mediating this action remained to be elucidated. To this end, the same experiments were repeated using the FaO rat hepatoma cell line defective for functional TRs. FaO cells were exposed to the classical oleate/palmitate (2:1) mixture and then treated with T_2 or T_3 for 24 h (10^{-7}-10^{-5} mol/L doses). In FaO cells, TAG accumulation was associated with an increase in number and size of LDs and in PPARγ mRNA expression. The addition of T_2 or T_3 to "steatotic" cells reduced both the TAG content and the number and size of LDs and down-regulated expression of PPARα and PPARγ. Moreover, iodothyronines stimulated the fuel-induced O_2 consumption. Since iodothyronines prevented the ADP-induced transient stimulation of O_2 consumption, this indicated a mitochondrial uncoupling action. In conclusion, this study demonstrated that the lipid-lowering actions of both T_2 and T_3 on the hepatocyte occur *via* "non-receptor-mediated" mechanisms and involve a short-term action by stimulation of mitochondrial O_2 consumption[68].

IODOTHYRONINES AND AUTOPHAGY OF LIPID DROPLETS

Despite the advances in the understanding of the effects of THs on cellular metabolism, little is known about the mechanisms by which THs regulate energy consumption within the cell. This is particularly true for the events involved in the delivery of FFAs to mitochondria, a necessary step in converting stored intracellular triglyceride fuel into ATP.

Autophagy is a stress-induced catabolic process involving lysosome fusion that is conserved in almost all eukaryotes. Autophagy of lipid droplets, termed "lipophagy", has been shown to be a major pathway of lipid mobilization in hepatocytes[69] and its inhibition has been linked to development of fatty liver and insulin resistance[70]. The regulation of autophagy also appears to be important in the context of metabolic diseases, such as obesity. In a recent paper, Sinha *et al*[71] showed that T_3 induced both lipophagy in cultured liver cell lines and hepatic autophagy in the mouse liver. The authors observed that the T_3-stimulated autophagy of LDs depends on the presence of functional TRs and occurred before any stimulation of hepatic lipases or oxidation enzyme[71]. Moreover, in animals with impaired autophagy, the effect of THs on FFA oxidation was abolished. Therefore, they propose that T_3 may increase the delivery of FFAs to mitochondria for β-oxidation through induction of autophagy of LDs. In this light, T_3 or its analogs, through their proautophagic action, may be useful in the treatment or prevention of NAFLD and its associated complications.

CONCLUSION

In the last decades, extensive studies investigated the possible use of iodothyronines as pharmacological tools in the treatment of obesity, hyperlipidemia and dysmetabolic syndromes. The possible pharmacological use of the thyroid hormones T_3 or T_4 to stimulate body weight loss has found severe limitations because of the thyrotoxic effects associated with their long-term administration. For this reason, the identification of TH agonists/analogs retaining anti-obesity and hypolipemic efficacies, while being devoid of thyrotoxic effects, would represent a potential therapeutic advance.

Recent *in vivo* and *in vitro* studies have accumulated evidence on the lipid-lowering action of iodothyronines in the liver (Figure 2). The first studies showed that systemic administration of iodothyronines to rats receiving HFD resulted in a significant reduction in body weight gain and in the serum levels of triglycerides and cholesterol. At the organ level, the effects on the liver were very interesting, where iodothyronines could lower the excess lipid accumulation associated with HFD. These studies prosecuted by investigating the mechanisms of iodothyronine action. The development of *in vitro* models of hepatosteatosis using both primary cultures of rat hepatocytes and rat hepatoma cell lines allowed demonstration that the lipid lowering effects of iodothyronines depend on a direct interaction with the hepatic cell and is not mediated by thyroid hormone receptors. In conclusion, all the data summarized in this review clearly indicates that T_2 is able to reduce the lipid content of "steatotic hepatocytes", thus supporting the possible utilization of T_2 as a pharmacological tool in the treatment of dysmetabolic syndromes, such as NAFLD, and also in the light of its lack of thyrotoxic effects.

Although a preliminary study on humans has been published, clinical trials are needed to translate these effects to the treatment of human obesity. If reproduced in humans, these results may offer an interesting perspective on the possible pharmacological approaches to the above mentioned lifestyle-related dysfunctions.

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REVIEW

Update on inflammatory bowel disease in patients with primary sclerosing cholangitis

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Abstract

Patients with primary sclerosing cholangitis (PSC) complicated by inflammatory bowel disease (IBD) represent a distinct subset of patients with unique characteristics, which have serious clinical implications. The aim of this literature review was to shed light to the obscure clinical and molecular aspects of the two diseases combined utilizing current data available and putting issues of diagnosis and treatment into perspective. The prevalence of IBD, mainly ulcerative colitis in PSC patients is estimated to be 21%-80%, dependent on screening programs and nationality. PSC-associated colitis is likely to be extensive, characterized by rectal sparing, backwash ileitis, and generally mild symptoms. It is also more likely to progress to colorectal malignancy, making it imperative for clinicians to maintain a high level of suspicion when tackling PSC patients. There is no optimal surveillance strategy but current guidelines advocate that colonoscopy is necessary at the time of PSC diagnosis with annual endoscopic follow-up. Random biopsies have been criticized and a shift towards targeted biopsies using chromoendoscopy, laser endomicroscopy and narrow-band imaging has been noted. Techniques directed towards genetic mutations instead of histological abnormalities hold promise for easier, more accurate diagnosis of dysplastic lesions. Chemopreventive measures against colorectal cancer have been sought in these patients. Ursodeoxycholic acid seemed promising at first but subsequent studies yielded conflicting results showing anticarcinogenic effects in low doses (8-15 mg/kg per day) and carcinogenic properties in high doses (15-30 mg/kg per day).

Key words: Primary sclerosing cholangitis; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease

Core tip: Combination of primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD) has recently arisen as a challenging research field. Recent data highlight the specific clinical and genetic traits that differentiate PSC-IBD from the two diseases individually. We reviewed the literature on colorectal neoplastic susceptibility in this subset of patients and the underlying pathogenetic mechanisms. We also emphasize the technological advances that have provided novel diagnostic tools for more accurate detection of dysplastic lesions. Finally, we present current guidelines on follow-up as well as all evidence available as to whether ursodeoxycholic acid should be used prophylactically against colorectal cancer.

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic progressive disease characterized by inflammation and fibrosis of

medium size and large ducts in the intrahepatic and extrahepatic biliary tree[1,2]. This disorder results in multifocal intrahepatic and extrahepatic biliary strictures, leading to cholestasis, liver cirrhosis, portal hypertension, and ultimately, premature death from liver failure. It was first reported in the German literature in 1867 by Hoffman, but was described in more detail in the 1920s by two French surgeons, Delbet and Lafourcade. The term sclerosing cholangitis was first used in 1954 by Castleman and later by Schwartz and Dale in their review article[3]. Its etiology remains largely unknown, although it is strongly believed that autoimmunity is the main culprit. The differential diagnosis of PSC includes congenital diseases (*e.g.*, Caroli disease and choledochal cysts) and secondary cholangiopathy, as observed in patients with collagen vascular diseases (*e.g.*, systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis) and in those with infiltrative diseases (*e.g.*, mediastinal fibrosis, Riedel thyroiditis, eosinophilic cholangitis, and histiocytosis X). Parasitic, fungal, viral or bacterial infections or recurrent cholangitis itself, especially in patients who are immunocompromised, can cause multifocal liver abscesses that lead to a PSC-like appearance of the bile duct. This disease is associated with many cancers including cholangiocarcinoma, gallbladder cancer, hepatocellular carcinoma and colorectal cancer (CRC), thus establishing a link between chronic inflammation and carcinogenesis.

THE PSC-INFLAMMATORY BOWEL DISEASE INTERPLAY

The overwhelming majority of PSC cases have underlying inflammatory bowel disease (IBD). IBD is defined as a chronic condition characterized by immune-mediated inflammation of the gastrointestinal system. The prevalence ranges from 21% to 80%, with the higher rates seen in settings where screening programs are more intense and rectal and sigmoid biopsies are routinely obtained. A geographical variation also exists, with northern European and American societies exhibiting higher rates of PSC-IBD than southern regions and Asia. About 85%-90% of patients with PSC and IBD are comprised of ulcerative colitis (UC) patients and the remainder involves patients with Crohn's colitis or Crohn's ileocolitis[4]. The association of PSC and Crohn's disease (CD) was first described by Atkinson and Carroll in 1964[5]. A year later, Smith and Loe described an association between PSC and UC[6]. Conversely, it has been estimated that PSC occurs in about 5% of UC patients and 3% of CD patients[7].

IBD may be diagnosed at any time during the course of PSC. Until recently, the diagnosis of IBD more frequently preceded that of PSC, even by several years[8-11]. Nowadays, there has been a shift in the timing of diagnosis of IBD and PSC. PSC is most commonly diagnosed first, or at least there is a concomitant diagnosis of the two diseases. It is intriguing that *de novo* IBD may present after liver transplantation for PSC[12], and PSC may present several years after proctocolectomy for IBD. The altered trend in diagnostic timing can be attributed to two factors. First, the advent of noninvasive imaging techniques such as magnetic resonance cholangiography has enabled early diagnosis of pathological liver biochemistry. Second, the increasing awareness among physicians of the PSC-IBD association has led to early routine endoscopic screening in patients diagnosed with PSC, even in the absence of symptomatic colitis. A study comparing the interval from PSC to IBD diagnosis in 1993-1997 and 2003-2007 showed a decrease from 9 to 7 mo respectively[13].

The genetic factors for PSC development are still poorly understood. There is an obvious geographic clustering with high prevalence in northern countries compared to Southern Europe and Asia. It has been shown that first-degree relatives of PSC patients have a disease prevalence of 0.7%, representing a nearly 100-fold increased risk of developing PSC compared to that in the general population[14]. In siblings the prevalence even reaches 1.5%[15]. Taken together, these epidemiologic data and heritability studies have revealed a strong genetic background for PSC. Genome-wide association studies have shed some light on the subcellular maze of PSC and its overlap with IBD. Human leucocyte antigen (HLA) and non-HLA haplotypes have been identified. The HLA-A1 allele[16], HLA-C7[17], major histocompatibility complex class I chain-related A (MICA)*002 and 008/5.1 alleles[18,19], as well as the tumor necrosis factor (TNF)α promoter -308 A allele[20] were identified as risk loci for PSC susceptibility. Data from five different European countries (United Kingdom, Italy, Norway, Spain and Sweden) demonstrate that PSC is positively associated with three different HLA class II haplotypes: DRB1*03, DQA1*0501, DQB1*02 (which confers the highest relative risk for PSC development); DRB1*15, DQA1*0102, DQB1*0602; and the DRB1*13, DQA1*0103, DQB1*0603[21]. However, non-HLA associations have also been confirmed. Of note, MMEL1 and TNFRS14 on chromosome 1p36 encoding a membrane metalloendopeptidase-like protein of unknown function and a receptor for cytokines and membrane-bound ligands, respectively, have been identified as risk loci for PSC[22]. The risk of PSC has also been associated with the *FUT2* gene encoding fucosyltransferase[22], which is an enzyme that regulates expression of the ABO blood group antigens on the surface of epithelial cells. To date, there is scarcity of molecular evidence regarding the shared susceptibility loci between PSC and IBD. A Scandinavian study comparing PSC with UC patients showed distinct HLA associations[23]. No significant differences were noted between PSC patients with concurrent UC and PSC patients without IBD. This study provides genetic evidence that UC in PSC patients follows a distinct course and demonstrates phenotypic uniquity compared with UC in isolation. More recently, *REL*, *IL-2* and *CARD9* have been identified as genetic links between the two diseases[24].

Taking into consideration its associations with HLA haplotypes, autoimmune diseases and the presence of IBD in the majority of PSC patients, immunopathoge-

Table 1 Characteristics of inflammatory bowel disease associated with primary sclerosing cholangitis

Extensive colitis (with right-sided predominance)
Rectal sparing
Backwash ileitis
Mild or quiescent course
Increased risk of colorectal cancer
Increased risk of pouchitis in patients undergoing proctocolectomy with Ileal pouch anal anastomosis
Increased risk of peristomal varices in patients undergoing proctocolectomy with ileostomy

netic mechanisms have been sought in PSC pathogenesis. In this regard, two theories have been proposed: the leaky gut hypothesis and the gut lymphocyte homing hypothesis. According to the leaky gut hypothesis, bacteria or bacterial products enter the portal-venous system due to the increased intestinal permeability resulting from inflammation, and translocate to the liver. Bacteria trigger the release of cytokines by Kupffer cells and macrophages in the liver and lead to periductal fibrosis[8]. The gut lymphocyte homing hypothesis supports the notion that T lymphocytes primed in the inflamed gut may persist as long-lived memory cells, undergo enterohepatic circulation, and trigger portal inflammation in PSC *via* aberrantly expressed adhesion molecules in the liver and gut[25].

IBD IN PSC: A UNIQUE PHENOTYPIC EXPRESSION

There are many clinical and endoscopic features that differentiate patients with IBD and concomitant PSC and those with IBD in isolation (Table 1). Loftus *et al*[10] compared 71 patients with PSC who had IBD with a matched group of 142 patients with UC. Among the PSC patients, 86% had UC, 7% had CD, and 7% had indeterminate colitis. The PSC patients more frequently had pancolitis (87% *vs* 54%), rectal sparing (52% *vs* 6%), and backwash ileitis (51% *vs* 7%) than the control group. It is now commonly believed that the colitis associated with PSC is frequently extensive and characterized by rectal sparing and backwash ileitis[26,27]. These special traits impede the definitive classification of IBD. For instance, the presence of rectal sparing or ileitis may be misinterpreted for CD or indeterminate colitis, rather than UC. In addition, PSC-associated colitis runs a milder, quiescent course, sometimes with absent clinical manifestations, thus delaying diagnosis[28]. Another intriguing trait in PSC-IBD patients is the higher rate of colorectal neoplasia, which tends to be proximal, is diagnosed at a later stage and has a worse prognosis. The colorectal neoplastic potential in these patients will be discussed later.

Of note, PSC patients who have an ileal pouch anal anastomosis (IPAA) after colectomy have an increased risk of pouchitis compared to patients with UC without PSC[29,30]. The underlying mechanism for this complication remains obscure. There is also one report suggesting that patients with PSC and IPAA run an increased risk of development of dysplasia in the ileal pouch mucosa compared with UC patients without PSC, and that these patients consequently should be under intensive surveillance[31]. However, more studies are required to substantiate these claims. Interestingly, it has been suggested that PSC-IBD patients undergoing proctocolectomy with ileostomy develop peristomal varices more frequently than IBD patients without evidence of hepatobiliary disease[32]. Bleeding from these often is recurrent and is challenging to treat. This complication can be controlled with a portosystemic shunt or transjugular intrahepatic portosystemic shunt, but liver transplantation may be considered.

PSC in patients with IBD does not seem to run a different course when compared to patients without IBD. Nevertheless, one study has demonstrated that PSC in patients with concomitant IBD has a predilection for men, is more likely to manifest itself for the first time with abnormal liver biochemistry, and has intrahepatic and extrahepatic biliary tree strictures[33]. Proctocolectomy, as a surgical treatment for UC, has no effect on liver function tests, histology or survival of patients with PSC[34].

COLORECTAL NEOPLASTIC POTENTIAL IN PSC-IBD PATIENTS

The increased occurrence of CRC in IBD patients has been well documented since 1925, when it was first described by Crohn and Rosenberg[35]. The cancer risk involves both UC and CD[36-39], and has been linked with prolonged duration and extent of disease, associated PSC and active inflammation[40,41]. Data on the relative risk of CRC in IBD are not in agreement in different studies. The cumulative risk varies from 1.4% after 18 years[42] to 34% after 25 years from onset of disease[43]. Some studies even advocate that the risk is not increased at all[44].

Recent investigations have unveiled another relationship, that of PSC-IBD patients and CRC. The concept that PSC is associated with an increased risk of colorectal neoplasia in patients with UC was proposed by Broomé *et al*[45] in 1992. In a study of 17 patients with UC who were found to have dysplasia, carcinoma, and/or DNA aneuploidy, 28% had coexistent PSC. This led to the hypothesis that PSC is an independent risk factor for the development of colorectal neoplasia in patients with existing UC. There has been a wealth of studies since then reporting the connection of PSC-IBD and CRC, particularly highlighting the compounding neoplastic risk when the two disorders coexist as opposed to patients with IBD alone[46-53] (Table 2). Soetikno *et al*[54] performed a meta-analysis of 11 studies and described an odds ratio (OR) of 4.79 (95%CI: 2.89-5.76) when comparing patients with UC and PSC to UC patients without PSC. However, two studies (both from the Mayo Clinic but using different groups of patients) have yielded contradictory results rejecting the hy-

Table 2 Summary of studies evaluating primary sclerosing cholangitis as a risk factor for colorectal neoplasia in chronic ulcerative colitis

Ref.	UC case group (No)	Centre	End point (No)	Matched controls	Colectomy rate	Is PSC a risk factor?
Broomé *et al*[45]	Dys (17)	Hudding, Sweden	PSC (5)	Yes	0%	Yes
D'Haens *et al*[46]	Dys (29)	Chicago, United States	Cholestasis/PSC (10)	Yes	0%	Yes
Broomé *et al*[47]	PSC (40)	Hudding, Sweden	CRC/Dys (15)	Yes	30%	Yes
Brentnall *et al*[48]	PSC (20)	Seattle, United States	Dys (9)	No	0%	Yes
Leidenius *et al*[49]	PSC (45)	Helsinki, Finland	CRC/Dys (13)	Yes	29%	Yes
Marchesa *et al*[50]	PSC (27)	Clevelan, United States	CRC (4)/Dys (14)	Yes	All postop	Yes
Shetty *et al*[51]	PSC (132)	Clevelan, United States	CRC (17)/Dys (16)	No	0%	Yes
Loftus *et al*[52]	PSC (143)	Mayo Clinic, United States	CRC (8)	No	37%	No
Nuako *et al*[53]	CRC (171)	Mayo Clinic, United States	PSC (30)	Yes	14%	No

CRC: Colorectal cancer; CRN: Colorectal neoplasia; Dys: Dysplasia; PSC: Primary sclerosing cholangitis.

pothesis that there is an increased risk for CRC in PSC-IBD patients[52,53]. In general, the increased neoplastic potential in PSC-IBD patients could be ascribed to late diagnosis due to the subclinical course of colitis and the conservative treatment of mild flare-ups as opposed to colectomy, thereby increasing the duration and extent of colitis.

CRCs associated with PSC display a number of characteristics. They appear to have a more proximal localization with up to 76% right-sided distribution. A full colonoscopy is therefore mandatory for surveillance purposes. CRCs in this subset of patients are diagnosed at a more advanced stage and tend to be fatal. In a recent study, PSC patients with IBD and CRC were found to be younger at onset of IBD than patients who had IBD and CRC without PSC (19 *vs* 29 years; $P = 0.04$). The time interval from onset of colitis until diagnosis of CRC was, however, similar in the two groups (17 *vs* 20 years; $P = 0.02$)[55].

PATHOGENETIC MECHANISMS OF CRC IN PSC

The mechanisms underlying the pathogenesis of CRC in IBD patients have been rigorously investigated and many differences in comparison with sporadic CRC have been addressed[56,57]. Even though IBD-CRC usually follows a dysplasia-cancer pattern, as in sporadic cancer, molecular and genetic events seem to occur in an unconventional sequence. Alterations to the *p53* tumor suppressor gene occur earlier in colitis-related CRC[58], whereas adenomatous polyposis coli (*APC*) gene alteration is usually a later event[59]. The reverse applies to sporadic CRC. It is also noteworthy that p53 mutations can be present in nondysplastic mucosa in IBD-CRC, but only in dysplastic areas in sporadic cancer[60]. Another noteworthy difference is that low-grade dysplasia is often in flat lesions in CRC-IBD, which are difficult to detect endoscopically, whereas such dysplasia occurs within raised polyps in sporadic cancer. The role of microsatellite instability, hypermethylation, chromosomal instability, interleukin (IL)-23/IL-17 signaling and E-cadherin (CDH1) has been addressed in studies but is not yet fully understood[61-64]. Polymorphisms of the mismatch repair genes *MLH1* and *MSH2* have been incriminated for the pathogenesis of IBD and related malignancy[65,66].

The direct impact of PSC on colorectal carcinogenesis has not yet been delineated. Another theory highlights the significance of the cholestasis-associated secondary bile salt pool in the colon[67]. Bile acids such as deoxycholic acid and lithocholic acid (LCA) are thought to contribute to tumorigenesis through disruption of the balance between colorectal crypt cell proliferation, differentiation and apoptosis[68-71]. Mucosa in carcinoma displayed an increased frequency of bile acid receptors compared with normal tissue[72]. A higher fecal bile acid concentration was found in patients with UC who developed neoplasia compared with those without UC[73]. Folate deficiency has also been implicated in the pathogenesis of CRC in IBD patients. Folate deficiency arises from sulfasalazine use to treat UC, which is a competitive inhibitor of folate absorption. Folate supplementation was associated with a 62% reduction in the incidence of neoplasia in patients with pancolonic UC compared with placebo[74]. This theory, however, contradicts epidemiological reports according to which maintenance therapy in UC reduces risk of carcinogenesis[75].

SURVEILLANCE RECOMMENDATIONS

Periodic surveillance colonoscopy is the milestone of cancer prevention in IBD[76,77]. PSC in combination with IBD further enhances the risk for CRC as described above and necessitates increased alertness. That along with the fact that IBD in PSC patients usually follows an asymptomatic, subclinical course raises the need for routine colonoscopy at the time of PSC diagnosis, which should be repeated on an annual basis[78]. However, a recent study by Imam *et al*[79] showed a low risk of colonic neoplasia in young patients with a combined diagnosis of PSC and IBD, with an estimated prevalence of 1.3% and an incidence of 0.4% per year. This finding raises the question whether annual surveillance is unnecessary in this selected group of patients.

Three categories of dysplasia have been identified according to the IBD Dysplasia Morphology Study Group[80]: (1) negative for dysplasia; (2) indefinite for dysplasia; and (3) positive for dysplasia, which is further subdivided into low-grade dysplasia (LGD) and high-grade

dysplasia (HGD). Each of these categories necessitates a different approach. A finding of indefinite dysplasia dictates a repeat colonoscopy in 3-6 mo. The management of LGD is debatable with no clear evidence of optimal approach. St Mark's Hospital[81] demonstrated a 54% cumulative probability of LGD progressing to HGD or CRC. Mayo Clinic reported a 33% 5-year progression[82], while other studies described an even lower rate[83,84]. Thus, in the case of LGD different options should be discussed with patients and informed consent for conservative or operative management should be obtained. Patients with multifocal flat LGD in one screening or unifocal LGD in more than one screening should prompt prophylactic total proctocolectomy. HGD, however, needs unquestionable referral for total proctocolectomy due to the increased risk of concurrent or subsequent malignancy[85].

There has been increased skepticism in the medical community about the random biopsies used for surveillance purposes. New methods enable targeted biopsies to be obtained from identifiable lesions. Chromoendoscopy, confocal laser endomicroscopy and narrow-band imaging (NBI) are new promising techniques that are likely to replace old-fashioned random biopsies that have proven their inadequacy in many studies[86,87]. Chromoendoscopy uses the application of indigo carmine or methylene blue to stain dysplastic areas on the colonic mucosa. Hurlstone *et al*[88] examined 700 patients in a prospective case-control trial and diagnosed 69 dysplastic lesions with chromoendoscopy and only 24 with random biopsies ($P < 0.001$)[88]. Confocal laser endomicroscopy enables the histological visualization of the mucosa in real time. It is easily inferred that this technique requires specialized training for histological interpretation. A randomized controlled trial was conducted by Kiesslich *et al*[89] showing an increase of 4.75 times in yield of neoplasia when using endomicroscopy ($P = 0.005$). NBI uses optical fibers to enable a clear visualization of vessels, pit pattern and soft tissue structures. A study showed that NBI cannot be recommended as a chromoendoscopy substitute because it detected fewer lesions than chromoendoscopy in chronic colitis, although most were not dysplastic[90].

Newer techniques that target genetic alterations rather than histological abnormalities have been proposed to increase detection efficacy. Deletions and point mutations of tumor-suppressor genes such as *p53*, *Rb*, *APC*, *mcc* and the Sialosyl-Tn antigen have been found in dysplastic lesions and could be a useful tool for early diagnosis[91,92]. DNA evaluation by flow cytometry could reveal aneuploidy and predict suspicious areas likely to progress to CRC. The main drawback is that aneuploidy is not always a prerequisite for cancer occurrence and its presence does not always lead to malignancy.

CHEMOPREVENTION

Many studies have investigated the potential protective effects of different drug agents against malignancy in patients with UC. Pinczowski *et al*[93] was the first to report that CRC risk is diminished by therapy with 5-aminosalacylic acid (5-ASA) in 1994. Ever since, studies have failed to demonstrate a clear relationship between 5-ASA therapy and CRC, rendering its potential protective effect presumptive rather than definitive. Azathioprine and mercaptopurine have not been shown to have a beneficial effect with regards to CRC in IBD[85]. Likewise, research has yielded inconclusive results regarding the use of corticosteroids, nonsteroidal anti-inflammatory drugs or folates for chemopreventive purposes.

Ursodeoxycholic acid (UDCA) is a drug commonly used in PSC patients due to its safe profile and favorable effects on the biochemical parameters of the disease. *In vitro* and animal studies have revealed a chemoprophylactic effect of UDCA. UDCA appears to arrest proliferation of colon cancer cell lines *in vitro*[94]. CRC induced by N-methylnitrosourea[95] and azoxymethane[96,97] in rats appeared to respond to UDCA therapy with a decrease in size. UDCA also decreased fecal concentrations of deoxycholic acid in animals, suggesting a potential protective effect through control of bile acid concentration in colon[98]. Several molecular mechanisms have been proposed to explain the chemoprophylactic effect of UDCA, including downregulation of cyclo-oxygenase-2 expression[97], prevention of carcinogen-induced changes in protein kinase C isoforms[99], suppression of epidermal growth factor receptor[100], cell cycle modulation by inhibiting the expression of cyclin D1 and promoting that of E-cadherin[101], and stabilization of mitochondrial membranes against damaging free radicals[102]. These results triggered a number of investigations in humans in order to shed light on the real effect of UDCA. Two studies have confirmed the protective effect of the drug. Tung *et al*[103] conducted a retrospective review of 59 patients showing a reduced OR of 0.18 (95%CI: 0.05-0.61) of colonic dysplasia after ursodiol use. Pardi *et al*[104] also performed a randomized placebo controlled study evaluating the effect of UDCA in the subgroup of UC patients with concomitant PSC. Patients who received a low dose of UDCA (13-15 mg/kg per day) showed a relative risk of 0.26 for developing CRC or dysplasia. Wolf *et al*[105] showed in a retrospective study of 120 patients that there was no reduction of CRC or dysplasia in the UDCA group. A hallmark study by Eaton *et al*[106] has recently reversed the long-standing conviction that UDCA has a place in CRC prevention in PSC-IBD patients. Using high doses of UDCA (28-30 mg/kg per day), they showed an increased risk of CRC in the UDCA group. The majority of patients developed colorectal neoplasia after > 2 years of use. This association remained significant after adjusting for smoking history and UC duration. High-dose UDCA also resulted in an increased risk of liver transplantation and/or death[107]. The discrepancy between different studies can be attributed to their inherent limitations and their failure to adjust for confounding factors such as age at onset of colitis, extent of colitis, family history of CRC, cigarette smoking, use of other drugs such as 5-ASA and folate, and use of the same criteria for dysplasia classification.

There has been speculation with regard to mechanisms underlying the toxic and carcinogenic UDCA properties. The most prevalent theory implicates the alteration of colonic bile acid milieu when high doses are used. An increase in serum UDCA and LCA levels in the treatment group has been reported[108]. That combined with results from *in vitro* studies stating that bile acids stimulate cell invasion in a dose-dependent fashion and reduce apoptosis could possibly provide a plausible explanation of the differing effects when low and high UDCA doses are used[109-111]. It is therefore prudent to recommend UDCA chemoprevention only to a high-risk subset of patients, including those with a personal or family history of CRC, and those with long-standing extensive colitis. This rationale has been incorporated to recent European guidelines[112].

In conclusion, PSC-IBD patients represent an important public health concern. Significant steps have been made towards the elucidation of the pathogenetic mechanisms underlying this complex disease. HLA and non-HLA susceptibility genes have been thoroughly studied and proven their association with PSC-IBD. Further investigations are warranted to reveal PSC- and IBD-specific genes and clarify their real impact on the disease. Genome-wide association studies could be invaluable in this direction but are severely undermined by the rarity of the disease and therefore the limited number of PSC patients that can be recruited. In terms of diagnosis, biomarkers currently in use are liver function tests and histology. A couple of new methods have been introduced to facilitate the evaluation of PSC patients. Fibroscan and a breath test assessing the elasticity and metabolic capacity of the liver respectively have paved the way for rapid, non-invasive diagnosis. Their diagnostic accuracy in PSC, however, remains under scrutiny.

CRC is a well-established risk for PSC-IBD patients. Aggressive colonoscopic surveillance is therefore imperative, even in those who have undergone liver transplantation[113]. In an attempt to relieve the socioeconomic and medical burden that PSC-IBD poses, many studies have explored potential pharmaceutical agents that may retard disease progression and protect against colorectal neoplasia. Antibiotics, immunomodulators, UDCA and antifibrotic agents have attracted the attention of researchers but their full potential has not yet been unraveled. Recent meta-analyses have demonstrated that UDCA in low to medium doses seems to have a chemoprophylactic effect, whereas high doses are carcinogenic[114,115]. Further investigations are required to test the efficacy of existing drug agents and promote the development of new ones. Understanding and harnessing molecular events seems a pivotal step towards this direction.

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REVIEW

Effects of resveratrol in experimental and clinical non-alcoholic fatty liver disease

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Abstract

The prevalence of obesity and related conditions like non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and therapeutic options are limited. Alternative treatment options are therefore intensively sought after. An interesting candidate is the natural polyphenol resveratrol (RSV) that activates adenosinmonophosphate-activated protein kinase (AMPK) and silent information regulation-2 homolog 1 (SIRT1). In addition, RSV has known anti-oxidant and anti-inflammatory effects. Here, we review the current evidence for RSV-mediated effects on NAFLD and address the different aspects of NAFLD and non-alcoholic steatohepatitis (NASH) pathogenesis with respect to free fatty acid (FFA) flux from adipose tissue, hepatic *de novo* lipogenesis, inadequate FFA β-oxidation and additional intra- and extrahepatic inflammatory and oxidant hits. We review the *in vivo* evidence from animal studies and clinical trials. The abundance of animal studies reports a decrease in hepatic triglyceride accumulation, liver weight and a general improvement in histological fatty liver changes, along with a reduction in circulating insulin, glucose and lipid levels. Some studies document AMPK or SIRT1 activation, and modulation of relevant markers of hepatic lipogenesis, inflammation and oxidation status. However, AMPK/SIRT1-independent actions are also likely. Clinical trials are scarce and have primarily been performed with a focus on overweight/obese participants without a focus on NAFLD/NASH and histological liver changes. Future clinical studies with appropriate design are needed to clarify the true impact of RSV treatment in NAFLD/NASH patients.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Steatosis; Resveratrol; AMP-activated protein kinase; Silent information regulation-2 homolog 1; Anti-oxidants; Anti-inflammatory agents; Animal studies; Clinical trial

Core tip: The prevalence of obesity and related conditions like non-alcoholic fatty liver disease (NAFLD) is increasing. Therapeutic options are limited and alternative treatment options are sought after. An interesting candidate is resveratrol (RSV), a known AMP-activated protein kinase and silent information regulation-2 homolog 1 activator with anti-oxidant and anti-inflammatory properties. Here, we review the current evidence

for RSV-mediated effects and address the different aspects of NAFLD and non-alcoholic steatohepatitis pathogenesis. We review the *in vivo* evidence from animal studies and clinical trials. Uniformly, animal studies report a decrease in hepatic triglyceride accumulation and improvements in histological fatty liver changes, whereas results from the few clinical trials are equivocal.

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INTRODUCTION

The prevalence of obesity is increasing worldwide and consequently related conditions like non-alcoholic fatty liver disease (NAFLD) have increased. NAFLD now affects up to one-third of adults and a growing number of children in developed countries[1-3]. Early stages of NAFLD involve pathological accumulation of triglyceride (TG) in the liver, a fairly benign condition. However, some individuals elicit an inflammatory response that can progress to cirrhosis, cirrhosis complications and an increased risk of liver cancer[4]. NAFLD is now the third leading cause of liver transplantation in the United States[5]. Thus, NAFLD and especially the subtype non-alcoholic steatohepatitis (NASH) are thought to become a major health issue in the United States and throughout the world[6].

Therapeutic options are limited and include weight loss, which is hard to obtain and sustain[7], bariatric surgery, Vitamin E and glitazone treatment, especially the latter with a risk of significant side effects[8-11]. Alternative treatment options are therefore warranted and intensively sought after[12-15].

A potential new therapeutic option is the polyphenol resveratrol (RSV). RSV is found in a number of plants, although in low concentrations. It is known as an activator of AMP-activated protein kinase (AMPK) and silent information regulation 2 homolog 1 (SIRT1), thereby mimicking a condition of caloric restriction *in vivo*. In addition, RSV has anti-oxidant and anti-inflammatory properties. All of these effects could in theory be beneficial for the treatment of NAFLD and a number of experimental and clinical studies have been performed.

The aim of the present review is to provide a comprehensive description of the rationale for RSV treatment for NAFLD and to review the present evidence from RSV intervention in experimental and clinical NAFLD studies.

RSV ACTIONS

RSV is primarily recognized as an AMPK and SIRT1 activator[16-18]. AMPK and SIRT1 are both central in the metabolism of many different cell types, rendering RSV with pleiotropic effects in various tissues. To date, studies have been unable to determine if RSV activates AMPK, SIRT1 or both, directly or indirectly[19], a matter of ongoing debate[20]. Regardless, the effects of the enzymes are closely interdependent. Recently, Park *et al*[21] proposed a mechanism involving a direct RSV-mediated inhibition of cAMP-specific phosphodiesterases and identified the cAMP effector protein Epac1 as a key mediator, which may lead to activation of first AMPK[21] and then SIRT1 through the up-regulation of NAD^+[20]. Furthermore, RSV may also act independently of AMPK/SIRT1; however, the mechanisms are not clarified.

Through the activation of AMPK, SIRT1 and alternative routes including anti-inflammatory and anti-oxidant actions, RSV may inhibit the development or progression of steatosis and steatohepatitis. Former attempts to use the AMPK activator metformin in the treatment of NAFLD have largely been abandoned because clinical studies showed no effect on histological NASH changes, despite a general decrease in hepatic steatosis and transaminase levels[22-24].

AMPK

The AMPK pathway regulates energy homeostasis, both intracellularly and at the whole-body level. Through the action of upstream kinases, AMPK responds to changes in the AMP/ATP ratio and thus serves as an intracellular sensor of energy levels, *e.g.*, in the situation of fasting, calorie restriction or accelerated ATP consumption[25].

AMPK activation in the liver shuts down anabolic processes like cholesterol and TG biosynthesis by reducing the activities of, *e.g.*, sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS). AMPK activation also promotes catabolic processes, *e.g.*, fatty acid (FA) β-oxidation by inactivation of acetyl-CoA carboxylase (ACC) and promotion of carnitine palmitoyltransferase-1 (CPT-1) activity[26-28]. *In vivo*, it has been shown that chronic AMPK activation limits TG accumulation in both high-fat and control diet fed rats[29]. These AMPK-mediated effects have been shown in *in vitro* and *in vivo* studies, using RSV as an AMPK activator[28]. As an example, RSV treatment of HepG2 cells in high glucose media dose-dependently attenuated enhanced FAS expression, increased ACC activity and elevated TG accumulation[30].

In adipose tissue, the AMPK effects are similar, impairing lipolysis and promoting mitochondrial β-oxidation, thereby decreasing the level of circulating FFAs and the FFA load on the liver[26]. Both hepatic and peripheral insulin sensitivity is augmented.

SIRT1

SIRT1 is a member of the sirtuin family and a NAD^+-dependent deacetylase that acts as a master metabolic sensor of NAD^+. Thus, it adapts gene expression and metabolic activity in response to the intracellular energy state. SIRT1 is mainly found in the nucleus, where it functions

as a transcriptional repressor through histone, transcription factor, co-factor and enzyme deacetylation[31]. Following the SIRT1 metabolic effects, the molecule is thought to link calorie restriction and healthy aging and/or longevity. A number of studies have confirmed this *in vivo* and *in vitro*[32-34], while few have disputed it[35].

In the liver, SIRT1 is implicated in the control of energy metabolism through deacetylation and activation of especially peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and the lipid-sensing transcription factor peroxisome proliferator-activated receptor (PPARα), resulting in increased FA β-oxidation[36]. PGC-1α stimulates mitochondrial biogenesis, thereby increasing the mitochondrial content in hepatocytes[37]. In unison with AMPK, SIRT1 deacetylates and regulates SREBP-1c and liver X receptor (LXR), which govern lipid and cholesterol metabolism[38-41]. Adenovirus-mediated overexpression of SIRT1 specifically in mouse liver has been shown to reduce liver fat by down-regulation of SREBP-1c and FAS and up-regulation of expression of genes that control FA β-oxidation[42].

SIRT1 is an inhibitor of inflammation, repressing especially NF-κB transcription and activation as shown in liver and adipose tissue[31,36,43]. Anti-inflammatory effects of RSV have also been demonstrated in *in vitro* and *in vivo* studies, however, better documented in adipose tissue[44-48] than in hepatic cells or tissue[18,49-52].

Targeting SIRT1 activation for treatment of NAFLD has been suggested[53] as SIRT1 expression is decreased in dietary NAFLD models and NAFLD patients[54-56] and moderate SIRT1 overexpression protects mice from developing NAFLD[57].

NAFLD PATHOGENESIS

The pathogenesis of NAFLD and NASH is far from clarified and especially the factors that drive disease progression towards a more progressive, inflammatory phenotype are not fully characterized. Recently, a "multiple parallel hit hypothesis" has been proposed by Tilg and Moschen. Here, TG accumulation is viewed as an "innocent bystander", while a number of different parallel hits lead to NASH development[58]. Thus, it appears that there are 3 types of NAFLD patients: the "Good Fat Storer" (the NAFLD patient with a benign course); "the Bad Fat Storer" (the patient who develops immediate NASH); and the "Unfortunate Good Fat Storer" (the NAFLD patient that experiences additional hits and becomes a NASH patient)[59]. Only the latter two may require pharmacological treatment however, bearing in mind that NAFLD may be an independent risk factor for type 2 diabetes[60,61].

STEATOSIS

Hepatic steatosis occurs most often in the setting of obesity and metabolic syndrome and is the result of lipid overload, primarily with increased free fatty acid (FFA) flux and TG accumulation. Several mechanisms are involved[58,62] and some may be targeted directly or indirectly by RSV treatment. An illustration of the proposed RVS effects on NAFLD pathogenesis is shown in Figure 1.

Increased FFA supply due to increased lipolysis from adipose tissue

Insulin resistance (IR) results in increased lipolysis of TG in adipose tissue, resulting in elevated levels of circulating FFAs. Hepatic uptake of both diet- and lipolysis-derived FFAs is unregulated with limitless hepatocyte uptake *via* fatty acid transporters (*e.g.*, CD36). The bulk of hepatic TG (two-thirds) is derived from circulating FFA from lipolysis[62]. RSV may decrease lipolysis in adipose tissue through improvement of peripheral insulin sensitivity, as documented in several studies[63,64]. Furthermore, RSV may favorably modulate the expression of fatty acid transporters[65-67].

Overnutrition

Dietary fat contributes approximately 15% to the overall FFA load on the liver[62]. Increased fat intake increases circulating FFA, whereas elevated carbohydrate intake, especially in the form of fructose, increases *de novo* lipogenesis[68].

Increased de novo hepatic lipogenesis from dietary carbohydrates and amino acids

Normally, *de novo* synthesis accounts for 5% of hepatic fat content. However, in subjects with NASH, up to 25% of the hepatic fat content may be caused by *de novo* lipogenesis[62]. The process is regulated independently by insulin and glucose. In the postprandial and in the IR state, insulin stimulates the transcription factor SREBP-1c that promotes transcription of all genes involved in lipogenesis, among them ACC, FAS and peroxisome proliferator-activated receptor (PPARγ)[69,70]. Glucose stimulates lipogenesis through stimulation of the transcription factor carbohydrate response element binding protein (ChREBP)[71]. An upstream activator of both SREBP-1c and ChREBP is LXR, which is a transcription factor governing lipid and cholesterol metabolism[72,73].

Results from *in vitro* and *in vivo* studies show that RSV inhibits hepatic lipogenesis by AMPK/SIRT1-mediated inhibition of SREBP-1c, ACC and FAS activity[27,28,37,44,74].

Inadequate fatty acid oxidation

Under normal physiological conditions, the mitochondria handle FFA by β-oxidation. Inadequate β-oxidation may be involved in NAFLD development due to the increased FFA flux through the liver[62,68] and both de- and increased β-oxidation rates are reported[75]. Regardless, SREBP-1c inhibits FA oxidation by indirect inhibition of CPT-1.

RSV may increase FA β-oxidation by stimulating mitochondrial biogenesis through PGC-1α activation[37], increasing mitochondrial number[37,76], increasing uncoupling protein 2 expression[76] and by increasing CPT-1 expression and activity[27,77].

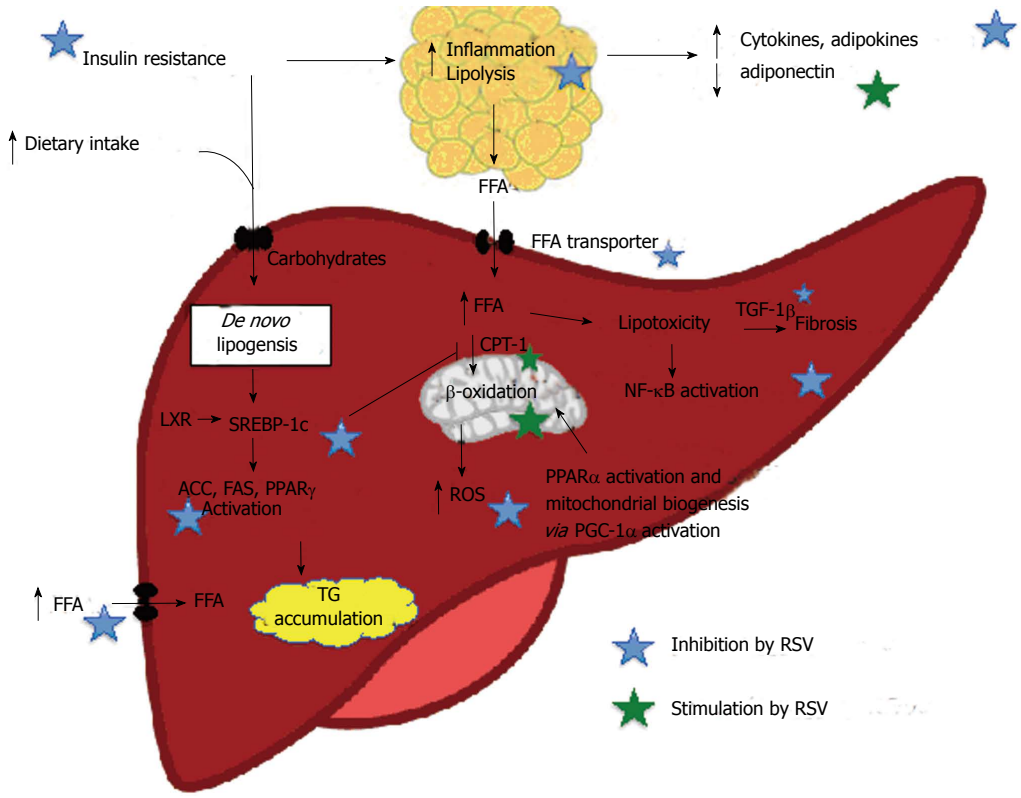

Figure 1 Proposed resveratrol effects on nonalcoholic fatty liver disease pathogenesis, AMP-activated protein kinase and silent information regulation-2 homolog 1 dependent and non-dependent mechanisms. Evidence of *in vivo* effect demonstrated especially a RSV-mediated inhibition of adipose tissue lipolysis, inhibition of hepatic de novo lipogenesis and an increase in FA β-oxidation. ACC: Acetyl-CoA carboxylase; CPT-1: Carnitine palmitoyltransferase-1; FAS: Fatty acid synthase; FFA: Free fatty acids; NAFLD: Non-alcoholic fatty liver disease; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1 α; PPARγ/α: Peroxisome proliferator-activated receptor γ/α; ROS: Reactive oxygen species; SREBP-1c: Sterol regulatory element-binding protein-1c; TG: Triglyceride; TGF-1β: Tumor growth factor 1β.

STEATOHEPATITIS

Hepatic inflammation is the hallmark of NASH and the inflammation is driven by several inflammatory hits that may include both intra- and extrahepatic factors[58].

Among the intrahepatic factors are the excess cholesterol, FFA and lipotoxic intermediates, which elicit a number of damaging effects, collectively named "lipotoxicity"[78]. This is recently reviewed in comprehensive reviews[78,79]. Converging the harmful factors is the NF-κB pathway in immune cells, hepatocytes and hepatic stellate cells (HSC), resulting in an inflammatory, pro-fibrogenetic and pro-apoptotic hepatic environment. NF-κB activation enhances transcription of pro-inflammatory cytokines[80,81], with increased hepatic transcription of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and the TNFα receptor, as shown in NASH patients[82]. Kupffer cells and damaged hepatocytes release, *e.g.*, transforming growth factor 1β (TGF-1β) that acts on quiescent HSC, inducing an activated state and thereby fibrosis. In addition, extracellular molecules, endotoxins, such as lipopolysaccharide (LPS) (*via* toll-like receptor 4), TNFα, IL-1, IL-6 and reactive oxygen species, can induce NF-κB-mediated activation of the HSCs.

The anti-inflammatory effects of RSV are also documented in the liver. Animal studies have shown reduced hepatic macrophage infiltration[51] and TNFα levels[49,52,83], as well as inhibition of the NF-κB pathway[50,52,83]. Only one study has focused on NASH-like fibrosis[52], probably due to the lack of appropriate animal models. However, other hepatic fibrosis models have shown RSV-mediated mitigating effects on markers of hepatic fibrosis[84-87] and HSC activation[86,88,89].

A number of studies report endoplasmatic reticulum stress, lipid peroxidation and oxidative stress as causative or early events in NASH pathogenesis[90-93]. RSV is a known anti-oxidant compound and has been shown to lower hepatic oxidative stress in rodent diabetes and NAFLD models[49,51,94-98]. One potential mechanism is interference in the Keap1/Nrf2 pathway, leading to up-regulation of anti-oxidant enzymes[99,100].

The extrahepatic inflammatory factors include dietary factors (*e.g.*, trans-fatty acids and fructose), gut-derived factors (*e.g.*, microbiota composition and bacterial byproducts) and adipose tissue-derived factors (*e.g.*, hypoadiponectinemia, adipo and cytokines, namely leptin, resistin, IL-6, TNF-α and monocyte chemotactic protein-1 (MCP-1)), that induce a state of whole-body low-grade inflammation in obesity[101]. Several lines of evidence document the anti-inflammatory effects of RSV in adipose tissue, especially by inhibiting NF-κB activation[46,102,103], lowering IL-6 levels[47,104] and macrophage infiltration[103], and modulating circulating cytokines and adipokines[45,50,103,105]. RSV-mediated reduction of LPS-

induced liver pathology and oxidative stress has also been reported[106,107].

In summary, RSV has AMPK/SIRT1 activating, anti-inflammatory and anti-oxidant effects that may act in unison, combating the different hits in the pathogenesis of NAFLD and NASH development.

RESVERATROL *IN VIVO*

Animal studies of RSV effects on NAFLD are numerous and span a wide variety of models, intervention periods and RSV doses. The studies can be divided into low-dose [RSV doses 7-45 mg/kg bodyweight (BW) daily] and high-dose studies (RSV 45-300 mg/kg BW daily). Some of the studies have hepatic steatosis as the primary endpoint, others as secondary. The RSV treatment is generally started from the beginning of the study and therefore most of the studies concentrate on the preventive effect of RSV and not the therapeutic effect. Almost uniformly, the studies report beneficial effects of RSV treatment on NAFLD pathology. In addition, RSV treatment in experimental models generally reduce circulating insulin and glucose levels[18,37,45,46,50-52,63,105,108-110] and, in some instances, weight[50,52,110-113], circulating transaminases[44,52,77,110] and lipids[45,50,65,96,108,111,112].

In Table 1 we present a list of published RSV animal studies with hepatic histological NAFLD/NASH data.

MOUSE STUDIES

In 2006, Baur *et al*[37] published a much-cited study on the effect of RSV on the health and survival of mice on a high-fat diet (HFD). HFD and low-dose RSV (10 mg/d) were fed to the mice from senescence to death. Besides increased survival and a number of beneficial metabolic effects, the study showed RSV-mediated hepatic AMPK activation, ACC inhibition, decreased FAS transcription and increased mitochondrial number. RSV also decreased liver weight and the degree of steatosis. This study triggered a number of other mouse studies, often using C57BL/6J diet-induced obese (DIO) mice.

Regarding NAFLD data, the studies report a decrease in hepatic TG [45,50,65,96,111,114] and/or cholesterol accumulation[45,108,111-113] and liver weight[37,65,112], along with improvement in histological fatty liver changes[37,46,51,52,65,96,108,111,113-115]. Only a few studies report NASH changes in the histological specimens. Ahn *et al*[65] and Li *et al*[52] find that RSV supplementation represses development of histological steatosis and steatohepatitis and also fibrosis in the latter study. Tauriainen *et al*[115] find that high-dose RSV represses steatosis and hepatocyte ballooning in a model with minimal hepatic inflammation.

Although tested in a few studies, only two mouse studies are able to verify RSV-mediated AMPK activation in liver tissue[105,114]. Also, no subsequent mouse studies have investigated hepatic PGC-1α deacetylation as a marker of SIRT1 activation. However, other markers of AMPK/SIRT1 activation have been documented in several mouse studies, among them inhibition of FAS expression and activation[65,109,111], inhibition of ACC activation[97,114], augmentation of FA β-oxidation[111], inhibition of PPARγ and SREBP-1c expression and stimulation of PPARα expression[52,65]. In mouse models, RSV treatment inhibits NF-κB activation[50,52] and lowers hepatic expression of inflammation markers[52]. Furthermore, oxidative stress is alleviated by RSV treatment in a number of mouse models[51,52,97].

In a long-term study of high-dose RSV treatment alone and in combination with another polyphenolic compound, namely quercetin, transcriptomic and metabonomic data demonstrated that combination therapy results in a significant restoration of gene sets in functional pathways of glucose and lipid metabolism (glycolysis and FA β-oxidation), inflammation/immunity, liver function and the cardiovascular system, which were altered by HFD feeding[108].

Also, in mutant Werner syndrome mice (showing premature signs of aging, *e.g.*, fatty liver), RSV treatment reversed liver steatosis and lipid peroxidation[109]. Microarray and biological enrichment analyses on liver tissues suggested that RSV mainly decreases lipogenesis and increases genes involved in the insulin signaling pathway and glutathione metabolism. The authors also observed a lower prevalence of hepatocellular carcinoma, however, an increase in lymphomas and other solid tumors was observed.

RAT AND HAMSTER STUDIES

Numerous different rat models of NAFLD report on RSV effects on NAFLD relevant endpoints, along with a single hamster study. Similar to the mouse studies, the conclusions are positive overall. The studies show a decrease in liver weight[67,74,77], hepatic TG[27,44,66,67,74,76,77] and/or cholesterol accumulation[66,67], and histological fatty liver[49,66,67,74,76,77].

The first to describe RSV effects on NAFLD in rats was Shang *et al*[74], using a HFD rat model in which the HFD was started 6 wk prior to the high-dose RSV treatment (100 mg/kg BW daily). This study therefore focused on the therapeutic effects of RSV. Besides alleviating NAFLD changes, it demonstrated that high-dose RSV treatment promotes phosphorylation and activation of AMPK and suppresses expression of FAS and SREBP-1c. This is backed by a recent study in which the HFD was added to a high amount of sucrose. Alberdi *et al*[27] found that low-dose RSV treatment for 6 wk activates AMPK and PGC-1α, increases CPT-1 and decreases ACC activities with no change in the mRNA expression of SREBP-1c, PPARα, SIRT1 and PGC-1α. Yet, not all rat studies find AMPK activation either. At variance, our group found no increase in AMPK phosphorylation or expression of related genes in spite of improvement in fatty liver changes, along with an increase in the hepatic mitochondrial content[76].

Obese Zucker rats have been used in low-dose RSV studies[44,77], with a reduction in hepatic lipid content and alanine aminotransferase levels, along with activation of

Table 1 Rodent resveratrol studies with histological liver data

Ref.	Focus	Model	RSV dose	RSV exposure (wk)	Histology/ LW	TG/CH content	Liver AMPK activation	Suggested RSV actions/mechanisms
Baur *et al*[37] (2006)	Longevity	Middle-aged DIO mice	22.4 mg/kg BW = 0.04% in diet	27	+/+	ND	+	Elevated PGC-1α deacetylation as marker of enzymatic SIRT1 activity. Phosphorylation of ACC
Ahn *et al*[65] (2008)	NAFLD/NASH	DIO mice (1% CH in diet)	1.25 g/kg diet	8	+/+ Decrease in NASH	+/-	ND	Reduced hepatic expression of FAS, PPARγ, CD36. Increased PPARα expression
Kang *et al*[46] (2010)	Insulin signaling	DIO mice	30 mg/kg BW	2	+/ND	ND	-	Increased Akt phosphorylation, improving insulin signaling
Labbé *et al*[109] (2011)	NAFLD, metabolic profile, longevity	Werner syndrome mice	0.04% RSV in diet	Life-long (≤ 22.5 mo)	+/ND	ND	-	Decreased FAS expression Decreased HCC prevalence
Tauriainen *et al*[115] (2011)	Obesity, NAFLD	DIO mice	2 or 4 g/kg diet	15	+/ND	ND	ND	Increased SIRT1 expression in liver tissue. High-dose most effective
Cho *et al*[111] (2012)	NAFLD	DIO mice (1% CH in diet)	7 mg/kg BW or 30 mg/kg BW	10	+/ND	+/+	ND	Suppressed FAS activity. Activation of FA β-oxidation in liver. The lower dose is more efficient than the higher dose
Zhou *et al*[108] (2012)	Overall transcriptomic and metabolic profiling	DIO mice	0.04% or 0.02% RSV + quercetin 0.02%	26	+/ND	-/+	ND	Modulation of inflammation and FA β-oxidation. Combination with quercetin was more effective than RSV alone
Jeon *et al*[51] (2012)	Cognitive deficit	DIO mice	200 mg/kg BW	20	+/ND	ND	ND	Attenuation of hepatic lipid peroxidation and macrophage infiltration
Shiozaki *et al*[97] (2012)	Longevity	SAMP10 mice	0.04% RSV	20	+/ND	ND	ND	Inhibition of ACC. Improved mitochondrial number, redox status and activity
Gao *et al*[114] (2013)	NAFLD	T0901317-treated mice	200 mg/kg BW	< 1	+/+	+/ND	+	Inhibition of ACC. Unchanged expression SREBP-1c and related genes
Li *et al*[52] (2013)	NAFLD	HFS mice	50 mg/kg BW	3	+/ND Decrease in NASH	ND	ND	Inhibition of NF-κB-induced inflammation and MDA-induced oxidative stress. Protection against NASH fibrosis
Bujanda *et al*[49] (2008)	NAFLD	Fasting/ feeding special diet, rats	10 mg daily	4	+/-	ND	ND	Hepatic TNF-α decrease. Improved oxidant/antioxidant markers (MDA, NOS, SOD, *e.g.*, catalase)
Shang *et al*[74] (2008)	NAFLD	HFD rats	100 mg/kg BW	10	+/+	+/-	+	Suppressed *SREBP-1c* and *FAS* gene expression
Poulsen *et al*[76] (2012)	NAFLD	HFD rats	100 mg daily	8	+/-	+/ND	-	Increased UCP2 expression Increased mitochondrial number
Gomez-Zorita *et al*[103] (2012)	NAFLD	Obese Zucker rats	15 mg/kg BW or 45 mg/kg BW	6	+/+	+/?	ND	Increased CPT-1α and ACO No effect on activity of lipogenic enzymes
Bagul *et al*[94] (2012)	Oxidative stress	High fructose fed rats	10 mg/kg BW	8	+/ND	ND	ND	Attenuation of hepatic oxidative stress, *e.g.*, with increased level of NRF2
Franco *et al*[96] (2013)	Oxidative stress and NAFLD	Obese 'early weaned' rats	30 mg/kg BW	4	+/ND	+/ND	ND	Decreased markers of oxidative stress
Xin *et al*[66] (2013)	NAFLD	HFS rats	50 or 100 mg/kg BW	13	+/ND	+/+	ND	Decreased hepatic LDLr and SRB1 mRNA and protein express
Cho *et al*[67] (2008)	Hyperlipidemia	HFD hamsters	0.25 g/kg diet	8	+/+	+/+	ND	Decreased HMG-CoA expression and modulated lipoprotein expression
Burgess *et al*[110] (2011)	Metabolic syndrome	HFD mini-swine	100 mg/kg daily	11	(+)/ND	ND	ND	Improved insulin sensitivity

+ positive finding; - negative finding; ND: Not determined. LW: Liver weight; BW: Body weight; TG: Triglyceride; CH: Cholesterol; AMPK: AMP-activated protein kinase; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1 α; SIRT1: Silent information regulation 2 homolog 1; ACC: Acetyl-CoA carboxylase; NAFLD/NASH: Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis; DIO: Diet induced obesity; FAS: Fatty acid synthase; PPARγ/α: Peroxisome proliferator-activated receptor γ/α; SAMP10: Senescence-accelerated mouse P10; HCC: Hepatocellular carcinoma; FA: Fatty acids; SREBP-1c: Sterol regulatory element-binding protein-1c; TNF-α: Tumor necrosis factor-α; MDA: Malondialdehyde; NOS: Nitric oxide synthase; SOD: Superoxide dismutase; HFD: High fat diet; UCP2: Uncoupling protein 2; CPT-1α: Carnitine palmitoyl transferase-1α; ACO: Acyl-coenzyme A oxidase; NRF2: Nuclear factor-like 2; HFS: High fat/sucrose diet; LDLr: Low-density lipoprotein receptor; SRB1: Scavenger receptor class B member 1; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A.

AMPK and increased CPT-1 activity, which is important for the rate of FA β-oxidation. Resveratrol treatment also improved the inflammatory status of visceral adipose tissue[44] and reduced liver oxidative stress[77]. Here, the ef-

fect on lipogenetic enzyme activity was equivocal.

Similar results on inflammatory and oxidative status was found by Bujanda *et al*[49] in a model in which cycles of fasting and feeding with a high carbohydrate-fat free diet induced steatosis. Low dose RSV for 4 wk lowered hepatic TNFα levels and reduced markers of lipid peroxidation and hepatic oxidative stress.

To our knowledge, only one study reports no RSV effect on hepatic lipid levels. Andersen *et al*[116] used a dietary rat model and high-dose RSV treatment for 8 wk, yet found no decrease in liver TG, FFA or cholesterol content. Also, they found no effect on liver SIRT1 protein expression.

Taken together, the current evidence shows that RSV prevents NAFLD-like hepatic steatosis in rodent NAFLD models. This may be caused by inhibition of adipose tissue lipolysis, inhibition of hepatic *de novo* lipogenesis and an increase in FA β-oxidation. A graphic illustration of the proposed RVS effects on NAFLD pathology is shown in Figure 1. Development of steatohepatitis may be attenuated by an inhibition of adipose tissue and hepatic inflammation and reduction of oxidative stress. However, few studies have used appropriate animal NASH models and there is only one study documenting an alleviating effect on NASH fibrosis. RSV could exert some of these effects through AMPK activation but AMPK activation is not found in all studies. Also, hepatic SIRT1 activation has not been verified in an experimental NAFLD model. Studies focusing on the therapeutic effect as opposed to the preventive effect of RSV on NAFLD and especially NASH are few but warranted.

OTHER ANIMAL MODELS

In a porcine model of metabolic syndrome, Burgess *et al*[110] found that high-dose RSV treatment had mitigating effects on insulin resistance and transaminase levels. Oil red O staining showed a decrease in hepatic lipid accumulation. However, a HE stain found no difference in histology between the control, HFD and HFD with RSV groups, signifying that steatosis was not sufficiently induced in this model.

CLINICAL TRIALS

So far, only a few clinical RSV trials on efficacy outcomes have been concluded and none of these studies have focused on fatty liver disease *per se*. Two studies on obese but otherwise healthy male participants report liver data. In the study by Timmers *et al*[117], 11 participants received a daily dose of 150 mg RSV or placebo for 30 days in a double-blind, cross-over design. Results suggest a number of beneficial metabolic effects, among these a reduction of liver transaminases and liver fat by magnetic resonance (MR) spectroscopy. In another study, 24 participants received a dose of 1.5 g RSV or placebo daily for 4 wk[118] and there was no effect on liver fat content (MR spectroscopy) or transaminase levels. This is consistent with another study in 45 non-obese postmenopausal women receiving a dose of 75 mg for 12 wk where no effect on liver fat (MR spectroscopy) or any other physiological parameter could be demonstrated[119].

Future clinical studies should focus on patients with biopsy verified NAFLD and NASH to determine any efficacy of RSV treatment in this setting.

CONCLUSION

So far, clinical studies of RSV effects on steatosis are scarce and the overall positive effects seen in rodent studies are still missing. None of the studies have included verified NAFLD patients or histological data and the studies differ significantly in the RSV dose used. New clinical studies should focus on the RSV effects in patients rather than healthy or near-healthy individuals[120], in this case, histologically verified NAFLD/NASH patients. In this setting, the focus should be on the therapeutic effects of RSV and not its preventive effects, as reported in the majority of animal studies.

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REVIEW

Insulin sensitizers for the treatment of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in the Western world and is closely associated with metabolic syndrome, which includes hypertension, central obesity, dyslipidemia and insulin resistance. NAFLD includes a wide spectrum of liver alterations, ranging from simple hepatic steatosis to variable degrees of fibrosis, cirrhosis and even hepatocellular carcinoma. Although the etiology and progression of the disorder remain poorly understood, insulin resistance is considered to play a pivotal role in the pathogenesis. Insulin sensitizers such as biguanides, thiazolidinediones (TZDs), glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase 4 inhibitors have been studied as therapeutic approaches for NAFLD in recent years. Metformin improves insulin sensitivity and serum alanine transaminase and aspartate transaminase (ALT/AST) levels in the majority of subjects; however, it has no significant effect on liver histology. TZDs improve insulin sensitivity, serum ALT/AST levels and histology in some cases, but there are some concerns about the safety of long-term therapy. Selection of appropriate patients for avoiding side effects and the treatment of underlying disease are the main points. These drugs are the best choice for the treatment of NAFLD in patients with type 2 DM who are also candidates for treatment with an insulin sensitizer. The present review provides an overview of insulin sensitizers in the treatment of NAFLD.

Key words: Insulin sensitizers; Metformin; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Thiazolidinediones

Core tip: Non-alcoholic fatty liver disease (NAFLD) is increasing significantly due to the obesity epidemic. Insulin resistance, mainly caused by obesity, plays a primary role in NAFLD pathogenesis. Medications that improve insulin sensitivity are theorized to be useful in the treatment of NAFLD. Therefore, recent studies have explored the role of insulin sensitizers to improve biochemical and histological features of NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) was first described by Ludwig *et al*[1] as a liver disease that mimicked alcoholic hepatitis in histopathological features, but without a history of excessive drinking. A recent definition of NAFLD consists of evidence of hepatic steatosis either by imaging or by histology and no secondary hepatic fat accumulation from causes such as alcohol consumption, hereditary disorders and steatogenic medication[2]. The

Figure 1 The relationship between obesity, insulin resistance and hepatic steatosis. VLDL: Very low-density lipoprotein; TG: Triglyceride.

spectrum of the disease reaches from simple hepatic steatosis to lobular inflammation (non-alcoholic steatohepatitis or NASH), fibrosis, cirrhosis and hepatocellular carcinoma[3,4]. It is the most common cause of chronic liver disease in all industrialized regions of the world[5-7].

BACKGROUND

The estimated prevalence of NAFLD varies in a wide range depending on the population studied and methods used for diagnosis. The disease has been reported in up to 10%-15% of normal weight individuals and 90% of obese persons[8-10]. The prevalence of NAFLD in patients with type 2 diabetes mellitus and hyperlipidemia is approximately 70% and 50%, respectively[11]. Ageing, male gender and ethnicity, such as being Hispanic, are associated factors that increase the prevalence of NAFLD. Elevated liver enzymes, histopathology of liver biopsy and imaging techniques such as ultrasound and magnetic resonance spectroscopy are different methods used for definition.

Previous studies have shown that 40% of patients with NAFLD may go on to develop NASH. The most common cause of cryptogenic cirrhosis is NASH and it progresses to advanced fibrosis in 32% to 37% of patients[12]. The patients with advanced fibrosis and cirrhosis also have higher risk of hepatocellular carcinoma[13-15]. Patients with NAFLD and NASH have increased cardiovascular mortality as well as liver-related mortality[16-18]. This is due to increased pericardial fat, increased carotid intima thickness and abnormal electrocardiogram changes[19-21]. Ramilli *et al*[22] showed that the prevalence of carotid plaques was close to 60% in patients with NAFLD, while it was 38% in patients without NAFLD.

Type 2 DM, obesity and the associated insulin resistance have been shown as independent factors for fibrosis progression[23]. Although a lot of risk factors have been defined, the major underlying mechanisms of the disease progression have not been clearly understood. The pathogenesis of NAFLD is closely related to obesity that leads to insulin resistance and significant metabolic alterations in liver occur in the setting of insulin resistance[24] (Figure 1). The prevailing hypothesis for explaining the pathway and involved mechanisms is the so-called two hit model[25,26]. At the first hit, hepatic steatosis develops due to insulin resistance and liver fat accumulation induced by excessive free fatty acid production, increased fatty acid oxidation and decreased hepatic triglyceride export. Following this step, the second hit includes increased oxidative stress which is characterized by excessive reactive oxygen species (ROS) in the liver. Progression from NAFLD to NASH is promoted by ROS through lipid peroxidation, cytochrome P450 activation and pro-inflammatory cytokine production[27]. Considering the complexity and unexpected progression of the disease, environmental and genetic factors are approved as contributors of a third hit.

Insulin resistance is the most specific metabolic risk and pathophysiological feature of NAFLD, with most patients having insulin resistance. In diabetic patients, a correlation between the severity of insulin resistance and grade of hepatic steatosis has also been shown[28]. On the basis of these data, studies of NAFLD treatment are mostly focused on improving insulin resistance and a pharmacological approach targeting improving insulin resistance are the more promising therapeutic candidates among categories that include antioxidants, lipid-lowering agents and anti-obesity drugs.

INSULIN-SENSITIZING MEDICATIONS

Metformin

Metformin was first used in medical practice in the 1950s and has been considered the first-line treatment of type 2 diabetes after receiving approval by the United States Food and Drug Administration (FDA) in 1994. Metformin belongs to a class of insulin-sensitizer drugs and acts through reducing hepatic glucose output, increasing insulin-stimulated glucose uptake in peripheral tissue and stimulating fatty acid oxidation in adipose tissue[29]. Adenosine monophosphate-activated protein kinase is the main player in mediating metformin effects.

Animal studies demonstrated that metformin reverses aminotransferase abnormalities, steatosis and inflammation in mouse models of NAFLD and NASH[30,31]. During last decade, many clinical trials have evaluated the useful effects of metformin on patients with NAFLD and NASH[32-41] (Table 1). Only a few of these studies were randomized and the results are conflicting.

The first nonrandomized study was carried out by Marchesini in 2001 and it included 20 biopsy proven NASH patients. For 4 mo the patients were treated with 1.5 g/d metformin and they observed a decrease in ami-

Table 1 Summary of metformin trials in adult patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis

Ref.	Study type	Subject number	Therapy	Compared with	Duration	NAFLD *vs* NASH	Liver enzymes	Histology
Marchesini *et al*[32]	Open label, single arm	20	Metformin	Baseline	4 mo	NASH	Improved	Not assessed
Nair *et al*[33]	Open label, Single arm	15	Metformin	Baseline	48 wk	NAFLD	Transiently improved	Mildly improved
Uygun *et al*[34]	Open label, RCT	36	Metformin	Diet/Exercise	6 mo	NASH	Improved	Not improved
Bugianesi *et al*[35]	Open label, RCT	110	Metformin	Vitamin E/Diet	12 mo	NAFLD	Improved	Improved
Duseja *et al*[36]	Open label, RCT	50	Metformin	Diet	6 mo	NAFLD	Improved	Not assessed
de Oliveira *et al*[37]	Open label, Single arm	20	Metformin and NAC	Baseline	12 mo	NASH	Improved	Improved
Loomba *et al*[38]	Open label, Single arm	28	Metformin	Baseline	48 wk	NASH	Improved	Improved
Haukeland *et al*[39]	Open label, RCT	48	Metformin	Diet/Exercise	6 mo	NAFLD	Improved	Not improved
Garinis *et al*[40]	Open label, RCT	50	Metformin	Diet	6 mo	NAFLD	Improved	Not assessed
Shargorodsky *et al*[41]	Open label, RCT	63	Metformin	Placebo	12 mo	NAFLD	Not improved	Not assessed

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; RCT: Randomized controlled trials.

notransferase levels[32]. The limitation of the study was the lack of histological evaluation and a control group. In 2004, Uygun *et al*[34] conducted the first randomized control trial comparing dietary modification to dietary modification plus metformin for six months. Aminotransferase levels and insulin sensitivity improved in the metformin treated group but there was no significant differences in necroinflammatory activity or fibrosis between groups. Bugianesi *et al*[35] presented an open label trial consisting of 110 patients who were randomized to receive either metformin 2 g/d (55 patients), vitamin E 800 IU/d (28 patients) or dietary-induced weight loss (27) patients for 12 mo. Liver transaminase levels were significantly decreased in the metformin group and there was also a histological improvement in hepatic steatosis, inflammation and fibrosis in the subset of 17 patients taking metformin. Haukeland *et al*[39] demonstrated that treatment with metformin for 6 mo was no better than placebo in terms of improvement in liver histology in patients with NAFLD. Another recent randomized control trial showed that metformin only transiently improved aminotransferase levels in patients with NASH[41]. A meta-analysis including five randomized controlled trials (RCT) concluded that metformin did not improve steatosis, lobular inflammation, hepatocellular ballooning and fibrosis in patients with NASH[42]. These results were independent of drug dose, treatment duration or diabetic state. Therewithal, a recent guideline indicates that metformin has no significant effect on liver histology and therefore it is not recommended as a specific treatment for liver disease in adults with NASH[2]. The largest RCT, "The Treatment of NAFLD in Children" (TONIC), investigated the effects of metformin in a pediatric population. The results of this study demonstrated that metformin was not associated with improvement in histology and reduction in serum alanine transaminase (ALT) levels[43]. On the basis of all these data, the AASLD guideline for the diagnosis and treatment of NAFLD concluded that metformin has no significant effect on liver histology and is not recommended as a specific treatment of NASH. A position statement on NAFLD/NASH based on an EASL special conference has not recommended metformin for specific liver-directed therapy of NASH[44].

Some studies demonstrated that high insulin and IGF levels have important roles in hepatic fibrosis and hepatocellular carcinoma[45,46]. An inverse association between cancer risk and long term metformin therapy has been found in previous studies. A recent meta-analysis showed that metformin was associated with an estimated 62% reduction in the risk of liver cancer among patients with type 2 diabetes[47]. Chen *et al*[48] demonstrated that each incremental year increase in metformin use results in 7% reduction in the risk of hepatocellular cancer. While the main anti-tumor effect of metformin is not clear in reducing lipogenesis and lipogenic expression, inhibition of hepatocyte proliferation and induction cell cycle arrest at G_0/G_1 phase *via* adenosine monophosphate (AMP)-activated protein kinase, reduction endogenous reactive oxygen species are possible estimated mechanisms.

In summary, metformin improves insulin sensitivity and serum ALT and aspartate transaminase levels in the majority of subjects; however, it has no significant effect on liver histology. The precise dose and duration of treatment is unknown and the beneficial effects on serum ALT only continued during treatment. Metformin has no apparent increase in the risk of lactic acidosis[49] and unlike the thiazolidinediones, it is not encumbered by weight gain or potential hepatotoxicity. According to current data, it cannot be suggested for the specific treatment of NAFLD or NASH but can be given in patients with both NAFLD/NASH and type 2 DM.

Thiazolidinediones

Thiazolidinediones (TZDs) are a class of oral antidiabetic drugs that induce a nuclear transcription factor, peroxisome proliferator activated receptor-γ (PPAR-γ), by binding selective ligands[50]. PPAR-γ is predominantly expressed in adipose tissue and leads to decreased hepatic fat content and improves glycemic control with insulin sensitivity. TZDs also increase plasma adiponectin levels,

Table 2 Summary of thiazolidinedione trials in adult patients with non-alcoholic fatty liver disease/non-alcoholic steatohepatitis

Ref.	Study type	Subject number	Therapy	Compared with	Duration	NAFLD *vs* NASH	Liver enzymes	Histology
Caldwell *et al*[53]	Open label, single arm	10	Troglitazone	Baseline	< 6 mo	NASH	Improved	Mildly improved
Neuschwander-Tetri *et al*[54]	Open label, single arm	30	Rosiglitazone	Baseline	48 wk	NASH	Improved	Improved
Promrat *et al*[55]	Open label, single arm	18	Pioglitazone	Baseline	48 wk	NASH	Improved	Improved
Sanyal *et al*[56]	Open label, RCT	20	Pioglitazone + vitamin E	Vitamin E	6 mo	NASH	Improved	Improved
Belfort *et al*[57]	Blinded, RCT	55	Pioglitazone	Placebo	6 mo	NASH	Improved	Improved
Idilman *et al*[58]	Open label, RCT	74	Rosiglitazone	Metformin/life style modification	48 wk	NASH	Improved	Improved
Ratziu *et al*[59]	Blinded, RCT	63	Rosiglitazone	Placebo	12 mo	NASH	Improved	Improved
Omer *et al*[60]	Open label, RCT	64	Rosiglitazone	Metformin	12 mo	NAFLD	Improved	Improved
Sanyal *et al*[61]	Blinded, RCT	274	Pioglitazone	Placebo and vitamin E	24 mo	NASH	Improved	Improved

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; RCT: Randomized controlled trials.

activate AMP-activated protein kinase and induce fatty acid stimulation[51].

A lot of human and animal studies have investigated the effect of TZDs on liver enzymes and histology to date. In rat models, pioglitazone and rosiglitazone prevented activation of hepatic stellate cells *in vitro* and improved hepatic steatosis and fibrosis *in vivo*[52].

The first human study was conducted by Caldwell in 2001[53]. Troglitazone was studied in 10 patients who had biopsy-proven NASH and the results were associated with improved aminotransferase levels. There was no change in histology and the drug was withdrawn from clinical use due to severe idiosyncratic hepatotoxicity. Neuschwander-Tetri *et al*[54] showed that rosiglitazone (4 mg twice daily for 48 wk) significantly decreased liver enzymes and improved steatosis, ballooning and inflammation scores in 30 patients who had biopsy-proven NASH. In addition to no change in fibrosis, the liver enzymes levels reverted to pretreatment values 6 mo after withdrawal of the drug. Clinical trials evaluating the effect of thiazolidinediones on patients with NAFLD and NASH are summarized in Table 2[53-61]. Omer *et al*[60] conducted an open label RCT including biopsy-proven NAFLD individuals to compare rosiglitazone with metformin. After 12 mo treatment, study results reported that rosiglitazone was more effective in metabolic control and histological improvement but fibrosis did not change significantly.

An early study with pioglitazone in 18 patients who had biopsy-proven NASH resulted in a decrease in aminotransferase levels with histological improvement[55]. The first double-blind, placebo-controlled trial using pioglitazone compared with placebo included 55 patients with NASH for 6 mo[57]. Insulin sensitivity, serum ALT levels, steatosis and necroinflammation, except fibrosis, were significantly ameliorated in the pioglitazone group.

In the largest trial completed to date for evaluation of the role of pioglitazone, 247 subjects with biopsy proven NASH were randomized to vitamin E, pioglitazone or placebo for 96 wk[61]. Compared with placebo, both agents, pioglitazone and vitamin E, were associated with reductions in liver steatosis, lobular inflammation, hepatocellular ballooning and improvement in insulin resistance and serum aminotransferase levels. However, there was no improvement in fibrosis scores in the pioglitazone treated group. The "Fatty Liver Improvement with Rosiglitazone Therapy" (FLIRT) trial compared rosiglitazone with placebo in 63 patients[59]. Rosiglitazone improved serum aminotransferase levels, insulin sensitivity and hepatic steatosis. The two year extended trial (FLIRT2) demonstrated that improvement in liver enzyme levels continued but there was no further improvement in liver histology[62].

A meta-analysis including six trials demonstrated reduction in steatosis and hepatocyte ballooning but no improvement in inflammation or fibrosis compared with control[63]. In contrast to this study, Mahady *et al*[64] found improvement in inflammation and fibrosis in addition to reduction in steatosis and hepatocyte ballooning in a meta-analysis including seven randomized trials.

The largest meta-analysis that included 11 RCTs (862 participants, 38% diabetic) showed that TZDs improve steatosis, hepatocellular ballooning and necroinflammation, delay fibrosis progression and ameliorate hepatic, muscle and adipose tissue insulin resistance with more consistent cardiovascular benefits with pioglitazone[42].

Although the results of studies suggest some benefits from TZDs, a major problem also emerges: safety of long-term therapy and adverse effects. The use of rosiglitazone has been highly restricted in the United States and prohibited in Europe due to the increased risk of coronary events. On the other hand, pioglitazone is associated with adverse events such as bladder cancer, bone loss, weight gain, painful swollen legs and congestive heart failure. After evaluation of the overall results, it would be a good choice to use TZDs for the treatment of NAFLD only in patients with type 2 DM who are also candidates

for treatment with a TZD. The AASLD guideline recommended that pioglitazone can be used to treat only patients with biopsy-proven NASH; however, it also raised the concern about its long term safety and efficacy in patients with NASH[2]. The guideline also stressed that most of the clinical studies had been done in non-diabetic patients and thus the effect of TZDs on NASH of diabetic patients was not established. The position statement of a special EASL conference has recommended that pharmacological therapy of NASH could be a 1-2 year course of therapy with glitazone[44].

Dipeptidyl peptidase 4 inhibitors

Dipeptidyl peptidase 4 (DPP4) inhibitors are a new class of drugs and include sitagliptin, vildagliptin and saxagliptin. DPP4 is a membrane associated peptidase with a widespread organ distribution and deactivates a variety of bioactive peptides such as glucagon like peptide-1 (GLP-1). Inactivation of GLP-1 causes glucose intolerance, diabetes mellitus and hepatic steatosis. In a study including 31 NASH patients, Balaban *et al*[65] reported that serum DPP-4 levels were higher in patients with NASH compared to controls. Furthermore, the serum DPP-4 activity and staining intensity in liver were correlated with histopathological grade of NASH and hepatosteatosis.

In rat models, DPP-4 inhibitors improve hepatic steatosis by increasing insulin sensitivity and decreasing hepatic triglyceride levels[66,67]. To date, there is no published controlled trial with these agents in humans.

GLP-1, a hormone excreted by intestinal L cells, regulates blood glucose by stimulation of glucose-dependent insulin release. GLP-1 has a direct effect on hepatocytes by inducing genes responsible for fatty acid oxidation and insulin sensitivity[68]. GLP-1 analogs (exenatide, liraglutide) have been approved by the FDA for treatment of patients with type 2 diabetes mellitus. Ding *et al*[69] demonstrated that exenatide improves insulin sensitivity and reduces hepatosteatosis in rats with fatty liver. In another animal study liraglutide treatment reduced hepatic steatosis[70]. A case series including 8 patients with type 2 diabetes and biopsy-proven NAFLD showed that exenatide improves serum liver enzyme levels but has no effect on histopathology[71]. A recent meta-analysis including 4442 patients indicated that liraglutide decreased aminotransferase levels and that this effect was dose-dependent[72]. However, controlled studies are needed to show the efficacy of GLP-1 analogs in NAFLD and NASH treatment.

CONCLUSION

NAFLD is a complex, multifactorial and major public health problem with an increasing prevalence worldwide. Insulin resistance is very common in this disease and the goal of the therapy should include improving insulin sensitivity. Insulin sensitizing agents could be convenient drugs to reach this target. Metformin has been accepted to have no significant effect on liver histology and is not recommended as a specific treatment for liver disease in adults with NAFLD. TZDs have been most extensively evaluated in published trials to date and they have modest effects on liver histology. The long term safety and efficacy of TZDs in patients with NAFLD is lacking. Selection of appropriate patients to avoid side effects and treatment of the underlying disease causing insulin resistance, such as obesity, are crucial main points. NAFLD patients with metabolic syndrome and obesity are likely to be the best candidates to be treated with TZDs. According to current data, unfortunately insulin sensitizers do not satisfy expectations for the treatment of NAFLD. Future RCTs with adequate size and duration are still needed to assess the clinical outcomes in patients with NAFLD.

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REVIEW

Mechanisms of fibrogenesis in liver cirrhosis: The molecular aspects of epithelial-mesenchymal transition

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Abstract

Liver injuries are repaired by fibrosis and regeneration. The cause of fibrosis and diminished regeneration, especially in liver cirrhosis, is still unknown. Epithelial-mesenchymal transition (EMT) has been found to be associated with liver fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis reinforced the concept that activated hepatic stellate cells are not the only key players in the hepatic fibrogenic process and that other cell types, either hepatic or bone marrow-derived cells could contribute to this process. Following an initial enthusiasm for the discovery of this novel pathway in fibrogenesis, more recent research has started to cast serious doubts upon the real relevance of this phenomenon in human fibrogenetic disorders. The debate on the authenticity of EMT or on its contribution to the fibrogenic process has become very animated. The overall result is a general confusion on the meaning and on the definition of several key aspects. The aim of this article is to describe how EMT participates to hepatic fibrosis and discuss the evidence of supporting this possibility in order to reach reasonable and useful conclusions.

Key words: Epithelial-mesenchymal transition; Liver; Fibrosis; Transforming growth factor-beta1; Biological markers

Core tip: The cause of fibrosis and diminished regeneration, especially in liver cirrhosis, is still unknown. Epithelial-mesenchymal transition (EMT) has been found to be associated with liver fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis reinforced the concept that activated hepatic stellate cells are not the only key players in the hepatic fibrogenic process. The aim of this article is to describe how EMT participates to hepatic fibrosis and discuss the evidence of supporting this possibility in order to reach reasonable and useful conclusions.

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INTRODUCTION

Chronic liver damage can be triggered by different mechanisms (*e.g.*, viral hepatitis, metabolic liver diseases, or chronic alcohol consumption)[1] and are accompanied by changes in several key biochemical pathways involved in hepatic tissue homeostasis. One of the most important alterations is hepatic fibrosis, which is characterized by deposition of extracellular matrix (ECM) components around the sinusoidal layer in the space of Disse, together with molecular reorganization of the matrix components resulting in an altered composition[2].

Fibrosis is comparable with a wound-healing response

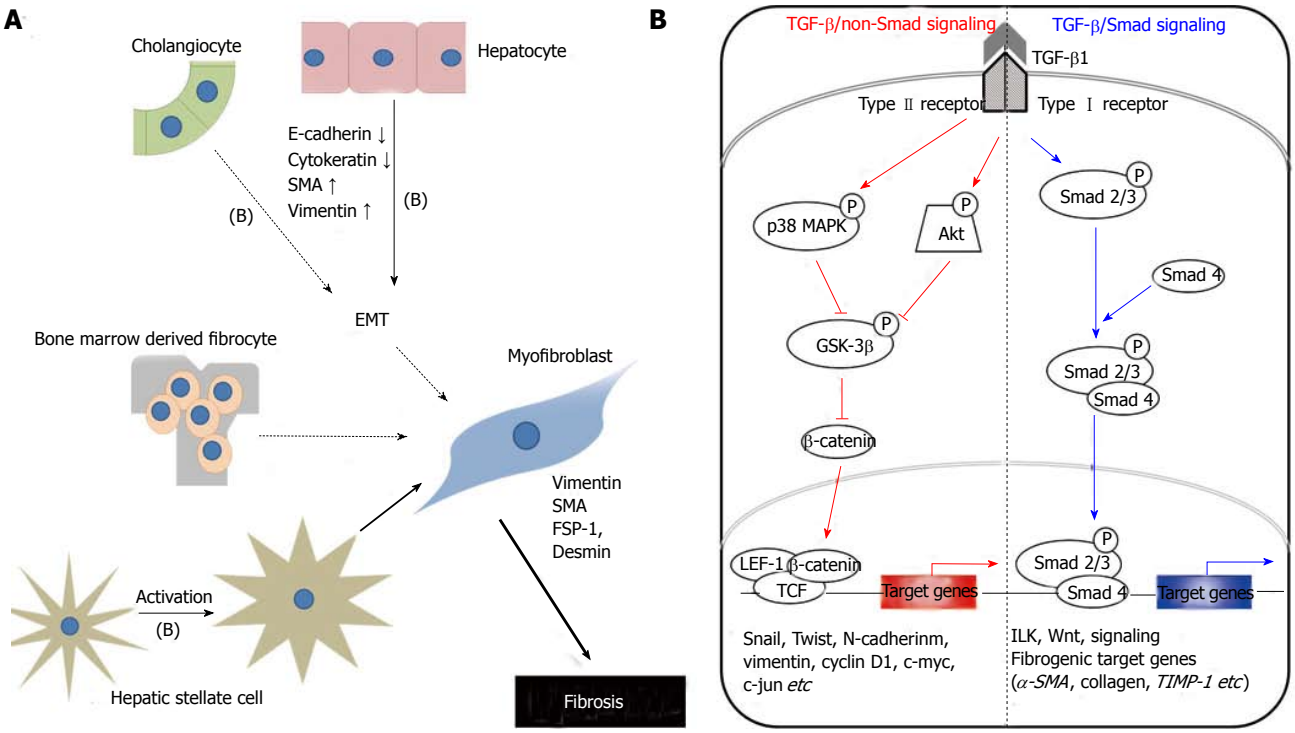

Figure 1 Mechanisms of hepatic fibrogenesis. A: The proposed sources of hepatic myofibroblasts: Resident cells (hepatic stellate cells and portal fibroblasts); bone marrow-derived mesenchymal cells, and EMT from hepatocytes and cholangiocytes. Different insults initiate inflammation and then cause hepatocyte stellate cells activation and hepatocyte and biliary cell damage, necrosis and EMT. Continuous insults will shift those EMT-like cells to complete EMT cells and finally myofibroblasts, the main producer of extracellular matrix, which may be one of the main causes of an early loss of regenerative capacity. A similar process also occurs in biliary cells. Some cytokines play an important role to affect the adjacent cells and promote EMTs, such as TGF-β1 (B); B: Schematic presentation of the major intracellular signal transduction pathways of TGF-β1 in liver fibrosis. TGF-β1 is a chief inducer of the EMT process, and p-Smad2/3, p38 MAPK and ILK function as mediators of the intracellular signaling pathway. TGF-β1 signals via heteromeric transmembrane complexes of type I and type II receptors that are endowed with intrinsic serine/threonine kinase activity (ALK activin receptor-like kinase). Upon type-II-mediated phosphorylation of the type I receptor, the activated type I receptor initiates intracellular signalling by phosphorylating receptor regulated-Smad2 and Smad3. Activated Smads form heteromeric complexes with Smad4 and these complexes accumulate in the nucleus where they mediate transcriptional responses. p-Smad2/3: Phosphorylated-Smad2/3; MAPK: Mitogen-activated protein kinase; GSK-3β: Glycogen synthase kinase-3β; ILK: Integrin-linked kinase; TCF/LEF-1 complex: T cell factor/lymphoid enhancer-binding factor-1 complex; EMT: Epithelial-mesenchymal transition; TGF: Transforming growth factor.

being out of control. Repair mechanisms aim at the replacement of injured cells. However, contrary to the pure regeneration of tissue in fibroplasia, connective tissue substitutes normal parenchyma[3]. The ultimate result is organ failure. In the liver, the final common pathway is cirrhosis, characterized by accumulation of ECM. In human beings liver fibrosis is associated with dysregulated growth of hepatocytes and results in the formation of regenerative nodules, dysplastic nodules, and hepatocellular carcinomas[4]. At present, no therapeutic concepts have been developed to treat and reverse fibrosis[5].

Striking increases in our understanding of the pathogenesis of liver fibrosis include the identification of the main cellular effectors, key cytokines regulating the EMT process, and determinants of ECM turnover[3].

FIBROGENESIS OF HEPATIC STELLATE CELLS, MYOFIBROBLASTS AND HEPATOCYTES IN LIVER CIRRHOSIS

Recent work regarding liver fibrosis centers on the myofibroblast as a pivotal cell type due to its contractile nature and synthesis repertoire[6]. The sources of myofibroblasts are still matters of discussion. Undisputedly, a "myofibroblast" phenotype is observed with hepatic stellate cell (HSC) after exposure to profibrogenic cytokines[3].

Liver myofibroblasts stand for a wide repertoire of functions that emphasize the dynamic nature of the wound-healing response, including synthesis of fibrillar collagens, contractile and migratory activities, secretion of chemotactic and vasoactive factors, and the secretion of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs)[3]. The origin of myofibroblasts in the injured liver is now under scrutiny, although evidence hints at HSCs as a major source[3]. Myofibroblasts can be derived from local mesenchymal cells recruited from the bone marrow or could derive from other cellular sources by EMT, a physiologic process in embryogenesis and of relevance for cancerous cell transformation[7] (Figure 1A).

Studies with animals and human tissue indicate that bone marrow stem cells infiltrate the liver and contribute to the myofibroblast population after damage. This may occur directly or through an intermediary cell, such as quiescent HSCs or $CD45^+$ fibrocytes[8,9]. Several studies have indicated that bone marrow-derived mesenchymal stem cells could be a source of multi-lineage cells for

various organs. They have the capacity to differentiate into hepatocytes, biliary epithelial cells, sinusoidal endothelial cells and even Kupffer cells in the presence of a suitable hepatic microenvironment[10,11]. There is growing evidence to suggest that bone marrow-derived stem cells are recruited during both progression and regression of liver fibrosis[8,12-16].

Most studies in the past decade have focused on HSCs and analyzed characteristic features like plasticity and transdifferentiation to myofibroblasts, a phenotype that can be readily recapitulated in tissue culture[3]. Current concepts envision activated HSCs as a crucial profibrogenic source, while the majority of hepatocytes are believed to undergo necrosis or apoptosis, thereby providing space for proliferating cells. Besides the resident hepatic cells, infiltrating neutrophils, macrophages, T and B cells, and eosinophils participate in the inflammatory response and may perpetuate the damage, whereby activated macrophages and neutrophils clean up tissue debris, dead cells, and invading organisms[17].

HSCs are located in the space of Disse of hepatic sinusoids between hepatocytes and sinusoidal endothelial cells[1] as liver-specific pericytes interposed by sparse connective tissue and closely adhering to sinusoidal endothelial cells[18]. They also directly face hepatocytes and maintain a quiescent phenotype with the main function to store vitamin A[19]. During liver injury, HSCs lose their vitamin A content[20] and undergo activation triggered by exposure to cytokines and growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β1, which derive from activated Kupffer cells and damaged hepatocytes. During activation, HSCs transform into a myofibroblast-like phenotype[21-23] losing their typical star-shape[20], characterized by the expression of α-smooth muscle actin (SMA)[24], by the production of ECM and matrix degrading enzymes, such as MMPs[21-23] and TIMPs[25,26]. Activated HSCs are thought to migrate from the sinusoids into necrotic areas and produce a variety of ECM[19].

Some authors[27] have proposed that HSC could be transitional cells derived from epithelial cells that have undergone partial EMT[28] or even a particular type of oval cell/hepatocyte precursor[29]. This hypothesis was based, at least in part, on the finding of an adult subpopulation of primary rat HSC expressing the progenitor cell marker CD133 and differentiating into either myofibroblasts or hepatocytes when cultured under different *in vitro* conditions[30].

Another source of myofibroblast may be portal fibroblasts that are described in fibrotic diseases with a portal component (*e.g.*, viral hepatitis and autoimmune conditions)[31]. In coherence with experimental data on hepatocytes, TGF-β1 is required for myofibroblast transdifferentiation of this cell type[32]. The portal connective tissue in healthy liver is surrounded by quiescent portal fibroblasts, which constitute a second population of liver cells implicated in portal fibrosis[33]. Derived from small portal vessels, they express markers distinct from HSC (*e.g.*, elastin)[32]. Proliferation of biliary cells is often accompanied by proliferation of portal fibroblasts, which form onion-like configurations around biliary structures and acquire a myofibroblast phenotype, and are thus implied in the early deposition of ECM in portal zones[34]. It is generally believed that substantial signaling from biliary epithelial cells leads to portal fibroblast activation, although the key factors remain to be identified[20]. In complex studies employing the "lineage tracing" methodological approach have documented, in different animal models of liver fibrogenesis, that some hepatocytes or cholangiocytes acquire "mesenchymal markers" implicated in cell motility and survival, but are not involved in active fibrillar ECM deposition and, therefore, cannot be considered profibrogenic cells[35].

Dooley *et al*[3] have provided *in vitro* and *in vivo* evidence that profibrogenic TGF-β1 functions during liver damage are directed toward hepatocytes. A TGF-β1-induced gene expression profiling of hepatocytes indicates a minor role of apoptosis and induction of fibrogenesis- and EMT-related genes. While the definite occurrence of EMT in this cell type *in vivo* needs further investigation (*e.g.*, by double-transgenic animals expressing fluorescent proteins under the control of hepatocyte- and myofibroblast-specific promoters), their results suggest that hepatocytes and TGF-β1 signaling in this cell type play a prominent role for fibrogenesis. In patients with chronic hepatitis B virus (HBV) infection, the activation of the TGF-β1 pathway was also shown by the accumulation of phosphorylated Smad2 in hepatocyte nuclei. Furthermore, the induction of Snail, a transcription factor known to repress E-cadherin expression, and the co-expression of type I collagen and transferrin in HBV livers, indicated that hepatocyte EMT was a feature of human liver fibrosis[36]. Therefore, the hepatocytes may be a contributor to hepatic fibrosis, especially when they are chronically injured[4].

TGF-β/SMAD AND NON-SMAD SIGNALLING PATHWAY IN LIVER FIBROSIS

TGF-β1 is recognized as a major profibrogenic cytokine and is a potent inducer of HSC proliferation and collagen production[37]. Furthermore, TGF-β1 expression is also associated with morphologic alterations like EMT in fetal[38] and adult hepatocytes[39], and changes in survival signaling pathways[40].

TGF-β1 binds to TGF-β1 receptor type Ⅱ (TbR-Ⅱ), and it recruits the TGF-β1 receptor type Ⅰ (TbR-Ⅰ)[41]. TbR-Ⅰ subsequently phosphorylates Smad2 and Smad3, which form hetero-oligomers with Smad4. They translocate from the cytoplasm to the nucleus, where they regulate transcription of target genes[42] (Figure 1B). R-Smad signaling is limited by the inhibitory effects of inhibitory Smad6 and 7[3].

Dysregulated TGF-β1 signaling is implicated in multiple developmental disorders and various human diseases, including cancer and autoimmune illnesses[43]. Its

over-expression is linked to liver fibrosis in diverse animal models[44] and in human patients with chronic liver diseases[45]. TGF-β1 crucially regulates ECM deposition by controlling the expression of ECM network components such as fibrillar collagens and fibronectin, ECM-degrading protease inhibitors, such as plasminogen activator inhibitor (PAI)-1 and TIMPs. Its activity is strongly induced during chronic liver damage with links between TGF-β1 and connective tissue growth factor in the HSC activation process[46], which in turn acquire myofibroblastic features and produce ECM proteins.

Epithelial cell transdifferentiation comprises alterations in cellular morphology characterized by changes in cell polarity and loss of adhesion protein expression[3]. TGF-β1 can initiate and maintain this process in a variety of biological systems and pathophysiological contexts by activating major signaling pathways and transcriptional regulators integrated in extensive cellular networks[47]. In MDCKII cells, claudin-1, claudin-2, occludin and E-cadherin disappear within 72 h of exposure to TGF-β1. It is suggested that this expression loss occurs through a Smad-independent mechanism, involving mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase pathways with expression of Snail. On the other hand, a complete loss of E-cadherin and transition to the mesenchymal phenotype additionally requires Smad signaling, which results in formation of β-catenin/lymphoid enhancer factor-1 complexes that induce EMT[48]. Participation of TGF-β1 in the regulation of Notch signaling has been reported previously at the onset of EMT in epithelial cells from mammary gland, kidney tubules, and epidermis[49]. A set of the previously mentioned genes and others described to be involved in EMT, including Snail and Notch2, were identified as TGF-β1 target genes in hepatocytes. Some studies[39,50] have suggested on hepatocyte plasticity showing up-regulation of α1(Ⅰ) collagen mRNA expression and type Ⅰ collagen deposition in mouse hepatocytes and α (alpha) mouse liver 12 cells as a result of Smad2/3/4-dependent induction of Snail-1.

EMT IN HEPATIC FIBROGENESIS

EMT is a process that is normally evident in embryonic stages of development and recently has been investigated as a mechanism of cancer cell migration and metastasis[51,52]. A classification of EMT has been recently proposed to distinguish between these different types of EMT[53]. It is characterized by the loss of epithelial characteristics (E-cadherin) and the acquisition of a mesenchymal phenotype (vimentin and fibronectin)[54,55]. According to the functional consequences and biological context, EMT is divided into three subtypes[53,55,56]: Type Ⅰ EMT occurs during embryogenesis, in which it produces motile cells but does not lead to ECM deposition or intravascular invasion. Type Ⅱ EMT induces a morphogenetic change during organ fibrosis or wound healing, which is associated with ECM production and muscle-like characteristics. Type Ⅲ EMT is involved in carcinoma-metastatic transition.

Evidence of EMT in fibrosis was first demonstrated in the kidney. *In vitro*, adult renal tubular epithelial cells were shown to undergo EMT[57,58]. Thereafter, induction of renal fibrosis in mice by unilateral ureteral obstruction (UUO) showed that epithelial marker expression (*i.e.*, E-cadherin) was lost in tubular epithelial cells, while the mesenchymal marker α-SMA expression was increased[59]. A cell tracing method later established that mice submitted to UUO display EMT-derived fibroblasts that contribute to the fibroblastic population[60].

Because the liver is an organ prone to fibrosis and because the origin of fibroblastic cells in fibrotic liver is still debated, the possibility that liver epithelial cells participate to fibrosis by EMT is appealing. Such hypothesis was strengthened by the observation that HSC lines express E-cadherin, while hepatic epithelial progenitor cells are positive for α-SMA[61]. Kaimori *et al*[39] then demonstrated that freshly isolated hepatocytes were able to convert to mesenchymal cells *in vitro*. Hepatocyte EMT, characterized by a decrease in E-cadherin expression and concomitant acquisition of mesenchymal markers (vimentin and type I collagen), was observed when cells were incubated with the profibrogenic cytokine, TGF-β1[39]. Zeisberg and co-workers were the first to report *in vivo* evidence for hepatocyte EMT[50]. They demonstrated that hepatocyte EMT was observed in CCl_4 induced liver fibrosis by developing transgenic mice specifically expressing a molecular tag in hepatocytes (*i.e.*, β-galactosidase). In these mice challenged with CCl_4, 45% of the cells expressing the mesenchymal marker fibroblast-specific protein-1 (FSP-1) were also positive for β-galactosidase expression. Furthermore, the inhibition of the TGF-β1 pathway limited the extent of liver fibrosis in the CCl_4-injected mice[50]. *In vitro* TGF-β1 treatment induced higher vimentin expression in cirrhotic liver-derived hepatocytes than in normal liver-derived hepatocytes[4]. Taken together, these results suggest that hepatocyte EMT is triggered by TGF-β1 and contributes to liver fibrosis. Some studies have proposed that EMT leads to myofibroblast accumulation through a two-stage process. In the first stage, epithelial cells adopt a mesenchymal phenotype, whereas in the second stage these mesenchymal cells further transition to myofibroblasts as part of what has been termed an epithelial-to-myofibroblast transition (EMyT)[62-64].

Substantial experimental evidence supports the occurrence of EMT in embryonic development and tumor metastasis, processes in which the motility phenotype of the transitioned cells is essential[56,65,66]. For tissue fibrosis, however, there are conflicting data on whether or not EMT occurs[67]. Many studies of EMT in fibrosis have failed to define EMT rigorously or to differentiate between the transition to a mesenchymal (EMT) *vs* a myofibroblast (EMyT) phenotype. Type I collagen expression is the most direct measure of fibrogenesis, and the literature suggests that α-SMA-positive cells are the primary effectors of fibrogenesis[55,62,68,69]. Nevertheless, surrogate fibroblast markers have often been used to identify EMT, most notably FSP-1, despite some data suggesting that it is nonspecific[68,70,71].

Mendez *et al*[72] examined the expression of four different mesenchymal markers, including FSP-1, vimentin, α-SMA, and procollagen I . Their lack of colocalization with yellow fluorescent protein (YFP) in the setting of fibrosis supports the conclusion that in these models EMT does not contribute to fibrosis. The complete absence of its colocalization with YFP in their study suggests that liver epithelial cells do not transition to either mesenchymal cells or myofibroblasts in the mouse models examined.

The hypothesis of hepatocyte EMT contributing to liver fibrosis has been also challenged by a cell lineage strategy in mice[67]. A lineage tracing study in which β-galactosidase was expressed under the control of the hepatocyte marker albumin in transgenic mice expressing a collagen marker provided strong evidence against hepatocyte EMT in the CCl_4 model of fibrosis[73]. Triple transgenic mice with permanent cell labeling were produced to track hepatocyte-derived cells and type I collagen-expressing cells. Hepatocytes isolated from these triple transgenic mice were able to undergo EMT in culture when incubated with TGF-β1. However, in mice challenged by CCl_4, no cells exhibited a double labeling specific for both hepatocytes and collagen expressing cells[73]. These observations also suggest that hepatocytes may not undergo EMT *in vivo*, while the observed transition *in vitro* might be an experimental artifact.

EMT IN CHOLANGIOCYTES

The assumption that liver epithelial cells undergo EMT in liver fibrosis cannot however be ruled out for biliary epithelial cells. Indeed, biliary epithelial cell EMT could represent a cellular mechanism supporting histological observations[70]. For instance, primary biliary cirrhosis (PBC), a prototypical biliary-type liver disease, is characterized by both the loss of biliary epithelial cells and the concomitant development of periportal fibrosis.

The bile duct basement membranes undergo degradation in fibrogenic liver diseases and that cholangiocytes, the other major hepatic epithelial cell type, assume fibroblast-like, non-cuboidal shapes. Therefore, it became obvious that the next step was to investigate whether or not biliary cells could undergo EMT in chronic liver disease[27]. It is well established that proliferating cholangiocytes within the so-called "ductular reaction" (*i.e.*, "reactive cholangiocytes"), detectable in all types of chronic liver disease, express a variety of pro-fibrogenic growth factors and cytokines and are likely to contribute to fibrosis and inflammation by promoting activation, proliferation, and collagen synthesis in the surrounding pro-fibrogenic cells[74-80]. Nevertheless, the possibility of a direct contribution of cholangiocytes to fibrosis *via* EMT was suggested by Omenetti and his colleagues[81] showing *in vitro* a complete EMT in an immature cholangiocyte cell line treated with activated HSC conditioned medium.

Biliary epithelial cell EMT was confirmed by another study analyzing liver of patients with PBC, primary sclerosing cholangitis or alcoholic liver disease. Irrespective of the underlying etiology, biliary epithelial cells from ducts associated with the ductular reaction were positive for FSP-1 and vimentin[82]. In biliary atresia, a disease defined by a destructive inflammatory obliterative cholangiopathy with portal tract fibrosis and ductular proliferation[83], biliary epithelial cells were shown to express FSP-1 and vimentin, while hepatocytes were not. Moreover, the authors of this study show that the expression of mesenchymal markers in biliary epithelial cells is observed in all liver disease with a ductular proliferation component[84]. The common bile duct ligation (BDL) is an experimental liver fibrosis model that induces strong ductular reaction. In mice submitted to BDL, biliary epithelial cells undergo EMT as shown by α-SMA and type I collagen expression[85].

Evidence of cholangiocyte EMT was recently challenged with the lineage-tracing methodology previously used for the investigation of hepatocyte EMT[27]. Along these lines, Scholten and the colleagues[86] employed the Cre-Lox technology for lineage tracing and studied several mouse strains expressing Cre under cholangiocyte-, HSC-, or FSP-1-specific promoters in two established models of liver fibrosis, *i.e.*, chronic CCl_4 intoxication and common BDL. In this case the fundamental experiment was tracing the fate of cells expressing K19, a bile ductular cell-specific marker, after permanent genetic Cre-mediated labeling of cholangiocytes. The key result of this study was that, although myofibroblast markers were often found in the close proximity of the K19+ progeny of cholangiocytes, the two signals never overlapped in either CCl_4 or BDL fibrosis. Based on these and other observations reported in the paper, the authors concluded that cholangiocyte EMT does not occur in their experimental models.

It may be that in human livers EMT occurs in cirrhosis, a state not well modeled in rodents, and may require a florid ductular reaction, which is also poorly mimicked by rodent models. Alternatively, this discrepancy may reflect the limitations of immunohistochemistry-based lineage-tracing methodology[67]. Future work should focus on better understanding the direct contribution of dysfunctional epithelial cells to liver fibrosis, as well as determining the mechanistic relationships between fibrogenesis and the progenitor cell activation characteristic of the ductular reaction. This will ultimately require the development of animal models of biliary fibrosis that better reflect human disease[67].

SPECIFIC CELLULAR MARKERS IN EMT

The most widely used marker identifying myofibroblasts is the cytoskeletal protein α-SMA that is a part of the contractile machinery and is involved in cell motility[27]. In adult normal tissue, α-SMA expression is mostly restricted to vascular smooth muscle cells, but in most chronic inflammatory and fibrogenic disease states, it is often found in myofibroblasts of different derivation, and this expression is interpreted as an active involvement of these cells in fibrogenesis (*i.e.*, "activated myofibroblast").

Table 1 Useful biomarkers for identifying epithelial-mesenchymal transition

Biomarkers	Myofibroblast	Hepatic stellate cell	Hepatocyte	Cholangiocyte
α-SMA[1]	+	+	-	-
Vimentin[1]	+	+	-	-
Desmin[1]	+	+	-	-
ICAM-1	+	+	-	-
Collagen type Ⅳ	+	+	-	-
Fibronectin	+	+	-	-
Fibulin-2	+	-	-	-
IL-6 mRNA	+	-	-	-
NCAM	+	-	-	-
Synaptophysin	+	-	-	-
Neurotrophin	+	-	-	-
Neural growth factor	+	-	-	-
αB-crystalline	+	-	-	-
Tyrosine kinase	+	-	-	-
FSP-1[1]	+		-	-
HSP47[1]	+		-	-
CD95L	-	+	-	-
α2-macroglobulin	-	+	-	-
P100	-	+	-	-
Reelin	-	+	-	-
Fascin	-	+	-	-
E-cadherin	-	-	+	+
Cytokeratin	-	-	+	+
K19	-	-	-	+
Albumin	-	-	+	-
Slug[1]	+	?	-	-
Twist[1]	+	?	-	-
Snail[1]	+	?	-	-

[1]Previously proven markers associated with epithelial-mesenchymal transition. α-SMA: α-smooth muscle actin; ICAM-1: Intercellular adhesion molecule-1; IL-6: Interleukin-6; NCAM: Neural cell adhesion molecule; FSP-1: Fibroblast-specific protein-1.

Accordingly, α-SMA cannot be a good lineage marker since its expression is activated by disease states and, in addition, does not denote function. Regardless of this, there is supportive evidence that epithelial cells express intermediate filaments such as α-SMA and vimentin following tissue injury[4,87].

A multitude of studies have shown that epithelial cells, including hepatocytes, when cultured *in vitro* retain epithelial features including polarity and specific protein expression (*i.e.*, albumin for hepatocytes), but when chronically stimulated with TGF-β1 or serum factors acquire a pattern of gene expression that is somehow typical of myofibroblasts *in vivo* and in the mesenchyme during development[39,88-91]. These genes are often represented by Slug, Twist, Snail, α-SMA, vimentin, desmin, FSP-1, and discoidin domain receptor tyrosine kinase 2. Some of these markers have been used to identify epithelial cells that are in the midst of undergoing an EMT associated with chronic inflammation. These cells continue to exhibit epithelial-specific morphology and molecular markers, such as cytokeratin and E-cadherin, but often show the concomitant expression of the FSP-1 and α-SMA. These aspects have been proposed to represent the intermediate stages of EMT, when epithelial markers continue to be expressed but new mesenchymal markers have already been acquired, and, overall, these observations have led to the notion of the so-called "partial EMT"[53].

Previous work has demonstrated in a model of fetal hepatocytes that TGF-β1 treatment induces EMT-like morphologic changes in 50%-60% of the hepatocyte population, whereas the remaining hepatocytes undergo apoptosis[38,40]. This means that EMT can be elicited by several oncogenic pathways (Src, Ras, integrin, Wnt/β-catenin and Notch)[27,92]. In particular, Ras-MAPK has been shown to activate two related transcription factors known as Snail and Slug[93]. Both of these proteins are transcriptional repressors of E-cadherin and their expression induces EMT.

Chronic liver damage leads to fibrotic degeneration of parenchyma, characterized by the formation of fibrotic septa. Except for HSCs, portal myofibroblasts can produce collagen in the liver[94]. Although both cell types show similar expression patterns of intercellular adhesion molecule-1, desmin, vimentin, collagen type IV, fibronectin, and α-SMA, several differences between them have also been observed[94]. For instance, cultured portal myofibroblasts are positive for fibulin-2 and interleukin-6 mRNA, whereas CD95L, α2-macroglobulin, P100, and reelin are exclusively expressed by activated HSCs[94-100]. In addition, neural cell adhesion molecule, synaptophysin, neurotrophin, neural growth factor, αB-crystallin, and tyrosine kinases are markers that distinguish HSCs from portal myofibroblasts[99,101]. Experiments using these markers have shown that myofibroblastic cells in fibrotic septa strongly resemble portal myofibroblasts, that they may originate and migrate from the portal tract, and that they are different from sinusoidal HSCs[19].

Uyama *et al*[19] found that fascin is present in intralobular sinusoidal areas, but not in the periportal areas or fibrotic septa of the human liver. In addition, the localization of fascin in the sinusoidal area is similar to that of vimentin. They concluded that fascin was localized in human HSCs, but not in (myo)fibroblasts of the periportal area or fibrotic septa.

Evidence favoring biliary EMT comes largely from immunohistochemical studies of fibrotic human and rodent livers that identified cholangiocytes coexpressing epithelial markers (especially the cholangiocyte marker K19) and mesenchymal markers (*i.e.*, FSP-1, vimentin, and HSP47)[64]. Table 1 shows the summary of useful biomarkers for identifying EMT.

CONCLUSION

The pathogenesis of liver fibrosis is now better understood than ever before. It is increasingly recognized that the fibrogenic cells in the liver are heterogenous in both their formation and their behaviour.

EMT is an established process in embryo development and plays an important role in liver fibrosis. Discussions now arise on the involvement of EMT in organ

fibrosis. The possibility that EMT could contribute to hepatic fibrogenesis in chronic liver diseases reinforced the concept that activated HSCs are not the only key players in the hepatic fibrogenic process and that other cell types, either hepatic or extrahepatic (bone marrow-derived cells and circulating fibrocytes) could contribute to this process. The presence of cells expressing both epithelial and mesenchymal markers suggests that EMT is a feature of liver fibrosis, however the ability of these cells to produce ECM *in vivo* has not yet been documented.

Therefore, the knowledge relative to the interpretation of what is defined as EMT in chronic fibrogenic disorders of the liver represents a scientific treasure that has prompted discussion, animated debates and has ultimately provided further maturity in this field of research. Definitely, there is now need for a more insightful analysis of the real pathophysiological meaning of these observations beyond their morphologic and biological features. Nevertheless, the future holds great promise for EMT as a viable therapeutic target.

EMT research in the next few years promises to be exciting, as new mouse models and molecular probes are identified to address the identities of the EMT-inducing microenvironmental signals, the nature of the cellular response of such signals and signaling machinery within epithelial cells.

Future research will surely be required on uncovering the origin of all fibrogenic cells within the liver and the molecular similarities and differences among the EMT programs.

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MINIREVIEWS

Pediatric non-alcoholic fatty liver disease: New insights and future directions

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Abstract

One of the most common complications of childhood obesity is the non-alcoholic fatty liver disease (NAFLD), which is the most common form of liver disease in children. NAFLD is defined by hepatic fat infiltration > 5% hepatocytes, as assessed by liver biopsy, in the absence of excessive alcohol intake, viral, autoimmune and drug-induced liver disease. It encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis, which, in turn, can evolve into cirrhosis and end stage liver disease. Obesity and insulin resistance are the main risk factors for pediatric NAFLD. In fact, NAFLD is strongly associated with the clinical features of insulin resistance especially the metabolic syndrome, prediabetes and type 2 diabetes mellitus (T2D). In particular, it has been clearly shown in obese youth that the prevalence of metabolic syndrome, pre-diabetes and type 2 diabetes increases with NAFLD severity progression. Evidence that not all of the obese patients develop NAFLD suggests that the disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition. Recently, a non-synonymous SNP (rs738409), characterized by a C to G substitution encoding an isoleucine to methionine substitution at the amino acid position 148 in the patatin like phospholipase containing domain 3 gene (*PNPLA3*), has been associated with hepatic steatosis in a multiethnic cohort of adults as well as in children. Another important polymorphisms that acts with *PNPLA3* to convey susceptibility to fatty liver in obese youths is the rs1260326 polymorphism in the glucokinase regulatory protein. The pharmacological approach in NAFLD children poorly adherent to or being unresponsive/partially responsive to lifestyle changes, is aimed at acting upon specific targets involved in the pathogenesis. There are some therapeutic approaches that are being studied in children. This article reviews the current knowledge regarding the pediatric fatty liver disease, the new insights and the future directions.

Key words: Non alcoholic fatty liver disease; *PNPLA3*; Obesity; Insulin resistance; Glucokinase regulatory protein; Fructose

Core tip: The prevalence of hepatic steatosis is increased in the last three decades concomitantly with the increased prevalence of pediatric obesity. Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease in children. The *PNPLA3* rs738409 and the glucokinase regulatory protein rs1260326 are the strongest variants associated with fatty liver in paediatrics. Important risk factors are obesity, insulin resistence, gender, ethnicity and excessive dietetic intake of n-6 polyunsatured fatty acids and fructose. New pharmacological approaches are object of study, in NAFLD children poorly adherent to or being unrespon-

sive/partially responsive to lifestyle changes.

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INTRODUCTION

In the last three decades with the increased prevalence of childhood obesity, there has been an increase also of the obesity complications in paediatrics. One of the most common complications of childhood obesity is the non-alcoholic fatty liver disease (NAFLD), which is the most common form of liver disease in children[1].

NAFLD is defined by hepatic fat infiltration > 5% hepatocytes, as assessed by liver biopsy, in the absence of excessive alcohol intake, viral, autoimmune and drug-induced liver disease[2,3]. It encompasses a wide spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which, in turn, can evolve into cirrhosis and end stage liver disease[3,4].

The prevalence of NAFLD has more than doubled over the past 20 years. According to a landmark study by Schwimmer *et al*[1] based on autoptic data obtained in 1138 children and adolescents of the San Diego county (CA), its prevalence in the general pediatric population is estimated to be nearly 13%, while among obese and overweight children and, particularly, adolescents it rises up to 46%[1]. Nevertheless, other studies report quite a wide range of steatosis prevalence, likely due to the different diagnostic methods used. In fact, although liver histology is important for NAFLD evaluation, performing biopsies is not always indispensable from a clinical point of view; therefore, surrogate markers are often used in epidemiological and clinical studies. One of the marker most commonly used is liver aminotransferase [aspartate aminotransferase, and alanine aminotransferase (ALT)] evaluation. Children with NAFLD typically have elevated liver enzymes values[5], which is why elevated serum levels of liver enzymes, even though may misrepresent the entity of intrahepatic damage, are used as a non-invasive test to screen for pediatric NAFLD[6].

RISK FACTORS FOR THE DEVELOPMENT OF PEDIATRIC NAFLD

Obesity and insulin resistance are the main risk factors for pediatric NAFLD[1,7,8]. In fact, NAFLD is strongly associated with the clinical features of insulin resistance especially the metabolic syndrome (MS), prediabetes and type 2 diabetes mellitus (T2D)[9-11]. In particular, it has been clearly shown in obese youth that the prevalence of metabolic syndrome, pre-diabetes and type 2 diabetes increases with progression of NAFLD severity[12].

This picture is strongly contributed by pubertal insulin resistance, a physiologic state characterized by an increased insulin resistance during the adolescence and resolving at the end of the pubertal development and probably consequent to the increase in growth hormone action during this stage of life[8,13]. In fact, although obesity is the most important cause of NAFLD among obese and adolescents, it is important to note that a transient insulin resistant state occurs during puberty[14], and that this state worsens the insulin resistance present in obese children in turn accelerating the progression to MS and type 2 diabetes. In healthy individuals this phenomenon is balanced by an increased insulin secretion by the beta cell, but in obese individuals the co-occurrence of obesity and puberty represents the perfect storm causing such a high degree of insulin resistance that the beta cell is not always able to produce enough insulin to maintain the glycemic control[15-17].

Two other critical risk factors for NAFLD development are represented by the gender and the ethnic background. In fact, NAFLD is more common in boys than in girls[15] with a male to female ratio of 2:1. This has been explained by the liver-protective role of estrogens, as well as by the potentially negative role of androgens in aggravating NASH[18,19]. The beneficial effects of estrogens on liver could be mediated by the beneficial effect on insulin action. Studies showed that insulin sensitivity is greater in premenopausal women compared with age-matched men, and metabolic-related cardiovascular diseases and type 2 diabetes are less frequent in premenopausal women[20,21]. Also, estrogens deficiency leads to increased fat mass and body weight in postmenopausal women, which has been associated with increased intraabdominal fat[22]. Moreover, Camporez *et al*[23] showed that, in mice, endogenous estrogens are important to protect against high-fat diet induced skeletal muscle insulin resistance, whereas E2 treatment in estrogen-deprived mice increased insulin sensitivity in both liver and skeletal muscle. Also, the estrogens effect is important in turn preventing diet-induced ectopic lipid deposition and hepatic and muscle insulin resistance.

The risk linked to the ethnic background has been investigated in large multiethnic populations. A cornerstone article by Browning *et al*[15] described for the first time that the prevalence of NAFLD is the highest in the American Hispanic population (45%) and the lowest among African Americans (24%), with the Caucasians showing an intermediate prevalence (33%). Ethnic differences could possibly be due to different degree of insulin resistance, and of visceral adiposity at equivalent body mass index, but may also be a result of genetics as well as socio-economic factors, including type of diet, exercise choice and living location[24].

The accumulation of fat, as triacylglycerol (TAG), in the hepatocyte is the fingerprint of fatty liver. The TAG accumulated in the liver mostly derive from adipose tissue lipolysis (60%) and hepatic de novo lipogenesis (26%) whereas only a small amount directly derives from the diet as chylomicron remnants (14%)[25]. A large body of

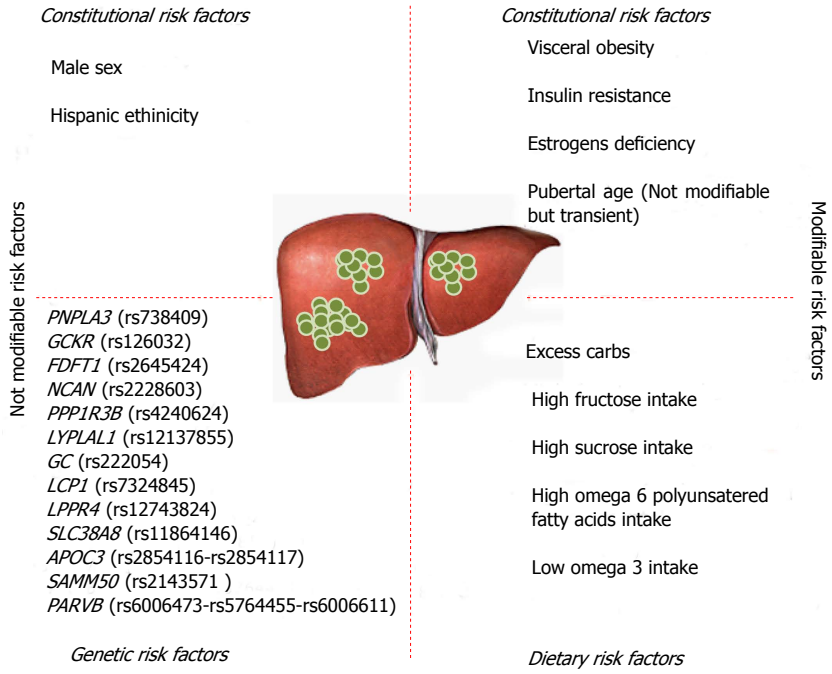

Figure 1 Risk factors for non-alcoholic fatty liver disease development. In this figure, all risk factors for non-alcoholic fatty liver disease (NAFLD) are summarized. We divided the risk factors in modifiable and not modifiable. Among not modifiable risk factors we listed *PNPLA3* rs738409, but as underlined in the text, the weight loss can modify the capacity of *PNPLA3* polymorphism to lead to hepatic steatosis. *PNPLA3*: Patatin like phospholipase 3 gene; *GCKR*: Glucokinase regulatory protein; *FDFT1*: Farnesyl-diphosphate farnesyltransferase 1; *NCAN*: Neurocan; *PPP1R3B*: Protein phosphatase 1 regulatory subunit 3B; *LYPLAL1*: Lysophospholipase-like 1; *GC*: Group-specific component; *LCP1*: Lymphocyte cytosolic protein-1; *LPPR4*: Lipid phosphate phosphatise-related protein type 4; *SLC38A8*: Solute carrier family 38 member 8; *APOC3*: Apolipoprotein C3 gene; *SAMM50*: Sorting and assembly machinery component; *PARVB*: Parvin beta.

evidence suggests that not only the amount, but also the quality of dietary fat plays a role in NAFLD development[26]. In particular, recently published literature provides clues that the dietary imbalance between omega-6 (n-6) and omega-3 (n-3) polyunsatured fatty acids (PUFAs) leads to development of an adverse cardiovascular and metabolic profile, thus contributing to the pathogenesis of NAFLD[27]. N-6 and n-3 are essential fatty acids; this means that they are not synthesized by human body. N-6 species are mainly represented by linoleic acid while n-3 are represented by alpha-linolenic acid, mainly found in plants and limited sets of seeds and nuts[28]. N-6 is readily converted by the body into other species such as omega-9 and so incorporated into triglycerides, or converted into arachidonic acid, which is the parent molecule of the main regulators of the inflammatory response including prostaglandins (ciclooxigenase pathway), leukotrienes (lipoxygenase pathway) and tromboxane[28]. It has been demonstrated that individuals with NAFLD have a lower dietary intake of n-3 PUFAs than healthy controls[29] and an increased n-6/n-3 PUFA ratio consumed in the diet[29,30]. Consistent with these data, lipidomic studies have shown that the intrahepatic fat in subjects with steatohepatitis is composed by an excess n-6 PUFA[31]. In particular, studying the three groups of subjects-NAFLD, NASH and healthy controls- it has been observed a progressive increase in the n-6/n-3 ratio from controls to NASH subjects[31].

Another dietary risk factor contributing to the development of NAFLD is the fructose. Nowadays, the majority of fructose consumption comes from the added sugars in the beverages more than from the fruit[32]. Strong evidence exists that high in fructose intake results in increased de novo lipogenesis (DNL), dyslipidemia, insulin resistance, and obesity in humans[33]. Stanhope *et al*[33], studying the effect of consumption of glucose- or fructose-sweetened beverages providing 25% of energy requirements for 10 wk in overweight and obese subjects, provided the evidence that the consumption of fructose, instead of glucose, specifically increases DNL, promotes dyslipidemia, decreases insulin sensitivity, and increases visceral adiposity in overweight/obese adults.

Progression from NAFLD to NASH

A recent study demonstrated that NAFLD in children is a progressive disease[34]. In that study the authors showed that 6% of subjects with early onset NAFLD develop cirrhosis and end-stage liver disease with the consequent need of liver transplantation.

The oxidative stress seems to explain the progression to NASH and liver fibrosis. Reactive oxygen species (ROS) can induce hepatocellular injury by the inhibition of the mitochondrial respiratory chain enzymes, the inactivation of glyceraldehyde-3-phosphate dehydrogenase and the inactivation of membrane sodium channels. ROS further cause lipid peroxidation, cytokine production, and induce Fas ligand, contributing to hepatocellular injury and fibrosis[12]. The risk of progression varies by ethnicity, in fact, as recently demonstrated, the African American obese children and adolescents show a lower degree of liver damage than Caucasians and Hispanics, independent of the degree of hepatic fat accumulation and insulin resistance. These data suggest that African Americans are protected from hepatic damage even in presence of high degree of hepatic fat accumulation and insulin resistance[35].

GENETIC PREDISPOSITION

Evidence that not all of the obese patients develop NAFLD suggests that disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition (Figure 1).

Recently, a non-synonymous SNP (rs738409), char-

Table 1 Current and future non-alcoholic fatty liver disease treatment strategies

Present	Under development	
Weight loss	Vitamin E	Pentoxifylline
Physical activity	Metformin	Farnesoid X receptor agonists
Reduced dietary sucrose intake	Probiotics	Toll-like receptors modifiers
Reduced dietary fructose intake	Oral treatment with omega 3	Glucagon-like peptid-1 receptor agonists resistant to DPP-4 mediated degradation
Reduced dietary omega 6 intake		DPP-4 inhibitors
Increased dietary omega 3 intake		

DPP-4: Dipeptidyl peptidase-4.

acterized by a C to G substitution encoding an isoleucine to methionine substitution at the amino acid position 148 in the patatin like phospholipase 3 gene (*PNPLA3*), has been associated with hepatic steatosis in a multiethnic cohort of adults[36] as well as in children[37,38]. *PNPLA3* encodes for a triglyceride hydrolase expressed in the liver and adipose tissue[39]. Metabolic studies in transgenic mice revealed that high level expression of PNPLA3^{I148M} in the liver, but not in adipose tissue, affected both hepatic triacylglycerol (TAG) synthesis and catabolism. A surprising finding was that the PNPLA3^{I148M} transgenic mice have significantly increased fatty acid synthesis and an altered spectrum of TAG-fatty acids in the liver, with no evidence of insulin resistance[40]. It is interesting that PNPLA3^{I148M} transgenic mice develop steatosis on a sucrose diet but not on a high-fat diet. Ingestion of sucrose stimulates de novo synthesis of fatty acids[41], whereas most of the hepatic fatty acids in livers of fat-fed mice are derived from circulating non esterified fatty acids (NEFAs). Perhaps PNPLA3 in hepatocytes is exposed preferentially to newly synthesized TAG and is shielded from fatty acids that enter the liver in lipoproteins or are synthesized from circulating NEFAs[40]. Alternatively, PNPLA3 may function specifically under conditions of insulin-stimulated lipid anabolism. The finding that PNPLA3 is virtually absent from livers of fasting animals and is strongly upregulated both transcriptionally[42-45] and post-translationally[39] by carbohydrate refeeding is consistent with the latter hypothesis.

Moreover, purified recombinant PNPLA3 has been found to have 5 enzymatic activities: triacylglycerol, diacylglycerol, and monoacylglycerol hydrolysis[42,46] as well as acyl-CoA thioesterase[42] and lysophosphatidic acid acyltransferase activity[47]. These different activities are not equally affected by the I148M substitution. In vitro assays, the I148M substitution results in a substantial loss of triacylglycerol, monoacylglycerols, and diacylglycerols hydrolytic activity[33]; a modest reduction in acyl-CoA thioesterase activity[33]; and an increase in lysophosphatidic acid acyltransferase activity[47]. None of these activities alone can explain all of the changes in TAG metabolism observed in the *PNPLA3* I148M transgenic mice. Therefore, some of the metabolic changes observed in these animals are likely to be secondary rather than direct consequences of altered *PNPLA3* activity.

The effect of this polymorphism on liver damage seems to be driven by the size of abdominal fat, expressed as waist to height ratio (W/Hr)[38]. More recently it has been demonstrated that weight loss reduce the effect of this polymorphism in obese children[48].

Other findings suggest also that the influence of *PNPLA3* on hepatic fat in obese children and adolescents might be modulated by dietary factors such as n-6/n-3 polynsatured fatty acids (PUFA) intake[49]. Finally, the rs738409 *PNPLA3* polymorphysm is considered, regardless of metabolic profile, a risk factor for liver disease. In fact, in a recent study, in a hepatitis C-infected population, the *PNPLA3* polymorphism influenced the development of liver steatosis[50]. Moreover, it was also demonstrated as a novel genetic marker associated with progressive ALD (alcoholic liver disease)[51].

Another polymorphism that acts along with the *PNPLA3* gene variant to convey susceptibility to fatty liver in obese youths is the rs1260326 polymorphism in the glucokinase regulatory protein (*GCKR*). This polymorphism is associated with hepatic fat accumulation along with large VLDL and triglyceride levels[52].

Speliotes *et al*[53], in addition to *GCKR,* identified variants in novel loci *NCAN* and *LYPLAL1* associated with both increasing computer tomography (CT) hepatic steatosis and histological NAFLD and identified variants in another locus, protein phosphatase 1 regulatory subunit 3B (*PPP1R3B*), associated with CT steatosis but not histologic NAFLD[53]. Recently Kitamoto *et al*[54] found that *PNPLA3, SAMM50* sorting and assembly machinery component (*SAMM50*), parvin beta (*PARVB*) genetic regions was significantly associated with NAFLD in the Japanese population. Adams *et al*[55] showed that SNPs in two genes expressed in liver were associated with NAFLD in adolescents: group-specific component (*GC*) and lymphocyte cytosolic protein-1 (*LCP1*). SNPs in two genes expressed in neurons were also associated with NAFLD: lipid phosphate phosphatise-related protein type 4 (*LPPR4*) and solute carrier family 38 member 8 (*SLC38A8*)[55].

TREATMENTS

Diet and lifestyle changes

The goal of lifestyle interventions is a gradual and controlled weight loss achieved by diet and physical exercise (Table 1). This aim is difficult to achieve and only a small percentage of individuals is able to steadily lose weight and exercise regularly[56]. Weight loss in NAFLD patients improves hepatic insulin sensitivity by reducing hepatic NEFAs supply, improves extra-hepatic insulin sensitivity through better glucose utilization and reduces ROS generation and adipose tissue inflammation[56].

Currently, there are no evidence-based guidelines establishing the optimal intervention. The only effective interventions are physical activity and dietary changes. In fact, reduction in sugar/sucrose and in soft drinks

rich in fructose, most probably not only acts through a reduction in IR and lipogenesis, but also counteracts the recently evidenced hepatic pro-inflammatory/fibrogenetic role of fructose[57]. It should be taken in mind that diet in childhood must be balanced to allow a healthy and harmonic growth, including wellness of bone structures. To intervene on dietary changes does not only mean to reduce the caloric intake, but also the single components of the diet (Table 1). In fact, also diet composition plays an important role in the development of NAFLD, as an increased dietary intake of monounsaturated and polyunsaturated fatty acids (mainly omega 3 PUFA) has been associated with a reduction of hepatic fat content, representing a reasonable intervention especially in the pediatric population[58].

Pharmacological interventions for NAFLD

The pharmacological approach, in NAFLD children poorly adherent to or being unresponsive/partially responsive to lifestyle changes, is aimed at acting upon specific targets involved in etiopathogenesis (Table 1).

Antioxidants, by reducing oxidative stress, protect susceptible components of biological membranes from lipid peroxidation, and may, therefore, prevent the progression of simple steatosis to NASH. The most studied antioxidant in children with NAFLD is alpha tocopherol (vitamin E) and warrants consideration in obesity-related liver dysfunction for children unable to adhere to low-calorie diets[59]. Sanyal *et al*[60] showed that vitamin E therapy, as compared with placebo, was associated with a significantly higher rate of improvement in NASH (43% *vs* 19%, $P = 0.001$) in adults without diabetes. There was no benefit of pioglitazone over placebo for improvement of NASH but serum alanine and aspartate aminotransferase levels were reduced as well as with vitamin E.

For its pathogenic role, insulin resistance appears as an adequate therapeutic target. Metformin is the only insulin-sensitizing agent evaluated in children.

Lavine *et al*[61] in a more recent large, multicenter, randomised double-bind placebo-controlled trial (TONIC study), evaluated the effect of daily dosing of 800 IU of vitamin E (58 patients), 1000 mg of metformin (57 patients) or placebo (58 patients) for 96 wk of NAFLD course. The patients (aged 8-17 years) with biopsy-confirmed NAFLD and persistently elevated levels of ALT, without diabetes or cirrhosis, were randomly assigned to 1 of 3 groups. At 96 wk neither vitamin E nor metformin was superior to placebo in attaining the primary outcome of sustained reduction in ALT level in pediatric NAFLD; vitamin E and metformin groups, however, showed an improvement in histological hepatocellular ballooning in NAFLD and NASH.

Another, single-arm, open-label, small pilot study on metfotmin (500 mg twice daily for 24 wk), conducted in 10 non-diabetic children with biopsy proven NASH and elevated ALT levels showed reduction of hepatic steatosis, as evaluated with Magnetic Resonance Spectroscopy (MRS) and low serum ALT levels[62].

FUTURE DIRECTIONS

A growing body of evidence[63] shows that the gut microbiota controls obesity and visceral fat storage. Specific variations in gut microbiota in early life may determine a major risk factor of obesity and its complications later in life[64]. Small intestinal bacterial overgrowth (SIBO) (a frequent condition in obese individuals, mainly prompted by slowing of the oro-coecal transit time) may promote NAFLD progression to non-alcoholic steatohepatitis by enhancing intestinal permeability and by favouring absorption of endotoxins with pro-inflammatory and pro-fibrogenetic effects on the liver[65].

Probiotics are live microorganisms which when consumed in adequate amounts, confer an healthy benefit to the host[66]. Gut microbiota manipulation with probiotics in rodents with fatty liver reduces intestinal inflammation and improves the epithelial barrier function[67,68]. Therefore, probiotics could represent a new effective treatment also in NAFLD human patients (Table 1). Loguercio and colleagues have shown that probiotics may reduce NAFLD liver injury and may improve liver function tests[69].

Moreover, recent pharmacological studies in NAFLD animal models and in adult humans focusing on the effect of oral treatment with n-3 fatty acids, demonstrate that they have both anti-inflammatory and insulin sensitizing properties, suggesting a potential role in treatment of NAFLD[70]. In NAFLD children n-3-docosahexaenoic acid (DHA) treatment for 6 months improved ultrasonographic fatty liver and insulin sensitivity[71]. Because this treatment is well tolerated in pediatric population, DHA deserve further studies in the management of children with NAFLD.

A series of other interesting approaches, hitherto explored only in NAFLD animal models or in few pilot studies in adults will possibly become in future the object of study in pediatric population (Table 1), as well: (1) tumor necrosis factor-α (TNF-α) and other adipocytokines produced by adipose tissue are involved in NAFLD progression. Pentoxifylline, a phosphodiesterase inhibitor, exerts immunomodulatory functions by antagonizing the TNF-α pathway. In adults with NASH, pentoxifylline treatment showed good tolerability and could decrease serum ALT levels and improve histological features[72]; (2) the nuclear bile acid receptor, Farnesoid X receptor (FXR), strongly expressed in bowel and liver, is probably involved in NAFLD pathogenesis, by mediating control of lipids and glucose homeostasis, and controlling bacterial flora growth. Altogether, these effects may induce reduction of hepatic inflammation and fibrogenesis, through different mechanisms. Therefore, recently developed FXR agonists have a potential role in the pharmacological therapy of NAFLD/NASH[73]; (3) toll-like receptors (TLRs) are receptors sensing microbial components of gut microbiota. A number of recent evidences suggests the role of SIBO and increased intestinal permeability in NAFLD, by exposing *via* portal vein the liver to an high load of intestinal noxae including lipo-

polysaccharide and other pathogen-associated molecular patterns[74]. Furthermore, TLRs stimulation causes downstream activation of the inflammatory response. Pro-inflammatory patterns result in production of cytokines and chemokines implicated in progression from simple steatosis to steatohepatitis and fibro-cirrhosis; so therapeutic manipulation of innate immune system through TLRs modifiers, formerly evaluated for autoimmune diseases[75], might be a new potential therapeutic target for pediatric NAFLD, but further studies are necessary; and (4) glucagon-like peptid-1 (GLP-1) is an incretin secreted in response to food intake, allotted to multiple functions, including, the stimulation of glucose-dependent insulin secretion and inhibition of glucagon release. The enzyme dipeptidyl peptidase-4 (DPP-4) rapidly degrades circulating GLP-1 (half-life: 1-2 min). Recent animal model and NAFLD adults studies showed an effective role of GLP-1 receptor agonists resistant to DPP-4 (such as exenatide and liraglutide) or DPP-4 inhibitors (*e.g.*, some gliptins) as a promising new therapy in NAFLD for their ability in modulating fatty acid oxidation, decreasing lipogenesis, and improving hepatic glucose metabolism[76].

CONCLUSION

Non-alcoholic fatty liver disease, because of the rise in the prevalence of childhood obesity, is becoming one of the most important chronic liver disease among children. Evidence that only a sub-group of obese patients develop NAFLD suggests that disease progression is likely to depend on complex interplay between environmental factors and genetic predisposition (Figure 1). Recent researches led us to understand the genetic basis predisposing to NAFLD. Many genes have been identified and many other will be identified and, actually, the most important it appears to be *PNPLA3* gene. Probably, all the genetic polymorphisms implicated in NAFLD development could have a summarizing effect. In fact, if more predisposing NAFLD polymorphisms coexist in the same subject, the risk to develop NAFLD and to develop it more severely could increase. Other important findings are related to the diet. For example, strong evidence exists that high in fructose intake, usually present in beverages, results in increased de novo lipogenesis and then in increased risk of NAFLD. Moreover, also high n-6/n-3 PUFA ratio consumed in the diet could predispose the NAFLD development. All these findings must drive the clinical practice: the diet is the first and important approach for the NAFLD prevention and treatment. In fact, there is the striking evidence that the weight loss can reduce the effect of I148M polymorphisms on determining hepatic steatosis. In addition to weight loss, to reduce the fructose intake trough the beverages and increasing the n-3 fatty acids dietary intake could be also useful in contrasting the NAFLD.

In conclusion, waiting the new approaches, the dear and old diet is always a fundamental and irreplaceable NAFLD therapy.

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To treat or not to treat the "immunotolerant phase" of hepatitis B infection: A tunnel of controversy

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A thorough review of the updated published reports was carried out and a merge of the various management options, with a special point of view of the author, is stated.

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem, with an estimated 350 million people worldwide chronically infected and approximately 500000 who die annually from HBV-related liver diseases. Management of chronic HBV is challenging and waves of guidelines emerge every year. One of the hottest topics and a matter of debate is the management of patients in their early immunotolerant phase of infection. With the lack of evidence, dealing with this particular subset of patients creates a great conflict with opposing views. In this review, the author highlights the pros and cons of these views and proposes a reasonable solution to resolve this dilemma.

Key words: Liver biopsy; Hepatitis B Virus; Immunotolerant phase; Polymerase chain reaction; Nucleotide analogue

Core tip: In this mini review, the author discusses the management dilemma of this peculiar subset of patients suffering from chronic hepatitis B in the immunotolerant phase. As already known, the immunotolerant phase of hepatitis B virus may last for a long period and hence there may be a potential for subtle liver damage.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem, with an estimated 350 million people chronically infected worldwide. Fifteen to forty percent of these individuals will develop serious sequelae during their lifetime, with greater evolution to cirrhosis or hepatocellular carcinoma (HCC). The estimated 5-year rate of progression from chronic hepatitis B (CHB) to cirrhosis was estimated to be 12%-20% and the 5-year cumulative risk of developing HCC was also estimated to be between 10%-17% in patients with cirrhosis. These figures vary from country to country according to the disease endemicity and prevalence[1-3]. The natural history of CHB is complex and described to run through different immunological phases that may overlap. In its early phases, HBV infection is characterized by minimal liver damage on liver biopsy, a high level of HBV replication and positivity for HBe-antigen (HBeAg). These patients are asymptomatic and have normal levels of serum alanine aminotransferase (ALT). This phase is described as the "immunotolerant phase"[3,4].

Managing these patients creates a great conflict with two opposing views. One view is optimistic, conservative and relies upon the long-term course of benignity of the disease. They adopt the view of "leave the patient alone on close follow up". On the other hand, the other view is

pessimistic and relies upon the great risk of cancer development, even without cirrhosis. This latter view adopts the view of "to treat the patient and why wait". Between these two views, there are no real evidence based guidelines.

In this review, an extensive online research for English reviews and articles that tackle this subject by using the key words "immunotolerant", "HBV" and "management" was carried out. The author highlights the pros and cons of all views regarding the management strategies of this subject and makes a reasonable proposed solution for this dilemma.

IMMUNOTOLERANT PHASE: CHARACTERISTICS AND IMMUNOLOGICAL INSIGHT

The natural history of HBV infection is perplexing and its net result is an interplay between the viral replication and the host immune response. After primary infection, an immunotolerant phase characterized by a very high rate of viral replication but without liver injury takes place. The mechanism of this tolerance is not yet fully understood[5]. These patients are infected early in life through vertical or early horizontal infection. Such infection most often occurs in areas with high rates of endemic infection, low rates of maternal screening, and lack of widely available neonatal prophylaxis with HBV vaccine and hepatitis B immunoglobulin[6,7].

It is believed that before birth, HBeAg acts as a "tolerogen viral protein" in the fetus, and thus virus specific T-cells undergo deletion. This phase lasts from weeks to years, depending on the age at acquisition. After years/decades, this tolerance is somehow ruptured and the immune attack against infected hepatocytes to clear them begins, causing liver damage. During this "immune clearance phase", ALT levels increase and HBV DNA levels begin to decrease. Immune attacks of infected hepatocytes result in HBeAg seroconversion and this seroconversion is usually associated with sustained remission of liver disease. Selection pressures for the virus come from either competition between viral variants, which are different in their replicative efficiency, and the host immune activity[8-10].

It was found that the majority of young children who presented in the immunotolerant phase have either minimal chronic hepatitis or, more commonly, non-specific reactive hepatitis, in spite of persistently normal ALT activity[11-13].

Wang *et al*[14] studied seven patients (age range between 7-25 years) by follow up for at least 17 years with serial sampling for ALT activity and viral load. They concluded that the interplay between viral replication and host immunity explains the pattern of HBV dynamics within the host during the early stages of infection. That is, without immune selection, competition between peers increases the viral load and decreases the nucleotide diversity; in contrast, host immunity accelerates viral evolution and decreases copy numbers but increases diversity. The fully infected liver can yield between 10^9 to 10^{10} viruses per milliliter of serum, a level of production that would be expected to persist if infection were benign and the host were truly immunotolerant. Virus titers in adolescent and young adult carriers in the immunotolerant phase of infection tend to be lower, ranging from 10^7 to 10^9 copies per milliliter[15,16]. Some studies explain the declining of virus titers during the time in the immunotolerant phase by a low but persistent immune destruction of infected cells by the cytotoxic T-cell, leading to an adaptive immune response over time[14].

IMMUNOTOLERANT PHASE: MANAGEMENT OPTIONS AND DEBATES

Of particular concern is the fact that until now there is no drug therapy that is actually effective in achieving a sustained response against HBV in the immunotolerant phase[17].

The currently approved treatment options for chronic HBV infection are interferon and nucleoside analogues (NA). Interferon acts primarily as an immunomodulatory agent, while NAs have essentially antiviral effects. According to current consensus and guideline statements, treatment candidates are patients with active liver disease characterized by persistently elevated ALT levels and detectable HBV DNA (10^5 copy/mL) by most commercial assays, irrespective of their HBeAg/Ab status. These statements also concluded that HBeAg-negative inactive carriers do not need any treatment because of the absence of viral replication and liver injury. Also, patients in the immunotolerant phase should be followed up without treatment[18,19].

However, and in the light of the Risk Evaluation of Viremia Elevation and Associated Liver Disease study, a baseline high HBV DNA level was associated with a significant risk of hepatocellular carcinoma[20]. These results led to the debate on whether a HBV infected person with normal liver enzymes, unremarkable liver histology, but with a detectable level of HBV DNA (high or low regardless the cutoff), should be treated with antiviral drugs or not[20,21].

As a rule, most of the current guidelines recommend that patients with moderate/severe inflammation or bridging fibrosis/cirrhosis must be treated. Also, they recommend liver biopsy for the grey zone of patients who do not meet the typical criteria, have a detectable level of HBV DNA and/or fluctuating or persistently elevated ALT. The presence of significant inflammation or bridging fibrosis/cirrhosis is an indication for treatment[22,23].

Hence, and in the light of the previous statements, we can assume that there are two options regarding the management of the immunotolerant phase; the "why wait" view and the "close follow up" view.

The "why wait" view adopts the option to treat all patients with a persistently high level of viral replication regardless of the phase of infection and relying only on the presence of detectable DNA levels. They rely on the

high risk of cancer/cirrhosis development, considering the infection as not totally benign[24]. Therefore, earlier treatment intervention may be beneficial in preventing disease progression. A recently published study aimed to break this tolerance in children by treating a group of HBV-infected children in the immunotolerant phase with lamivudine and interferon and comparing them to an untreated group. They reported a cure rate in more than one-fifth of the studied cohort, a figure that is still primitive and not high[25].

On the opposing side, another strong option exists and adopts the view of "wait and observe". This view relies on some evidence. The first is the evidence of the benign long term course of the immunotolerant phase[26]. The second is the pooled results of poor response to antiviral therapy in this unique phase, which hardly reaches 19%[27]. The third is the proved emerging resistance on long term therapy[28]. The last is a heavy cost burden of treatment.

Wong *et al*[29] studied the risk of liver fibrosis progression in HBeAg-positive patients at different phases by recruiting two hundred and forty-seven HBeAg-positive patients without advanced fibrosis at baseline. They found that liver fibrosis progression is uncommon in HBeAg-positive patients and hence their results enforce the follow-up strategy.

As is known, the degree of fibrosis or inflammation on liver biopsy cannot be predicted by the level of HBV-DNA and ALT is also considered an imperfect surrogate marker for liver disease[30]. Therefore, without evidence of normal liver histology, the definition of immunotolerant disease depends mainly on the persistence of a normal ALT level as a major determinant. Nevertheless and unfortunately, the definition of a "normal" ALT level has been redefined several times and was subjected to a strong debate. The study of Prati *et al*[31] modified the normal upper limit for ALT to be 30 IU/mL for men and 19 IU/mL for women. Re-introducing these relatively low figures will endorse many more patients under the umbrella of raised ALT levels.

The most appropriate way to make this miss clear cut is to perform a liver biopsy. However, there are still some unanswered questions; *e.g.*, what is the optimal timing of liver biopsy during the natural history of this phase, how many times and at what intervals should it be done, which drug is the best to start with, *etc.*

The use of therapeutic vaccines may also help to break the tolerance. In spite of its preliminary application, the published results of the study of Buchmann *et al*[32] carry a great hope for a wide future applicability. They evaluated the potential use of a novel vaccine formulation, comprising particulate hepatitis B surface and core antigen and the saponin-based adjuvant, for its ability to stimulate T and B cell responses in C57BL/6 mice. Their results were promising and future intense research in this subject is deemed to be mandatory.

CONCLUSION

The immunotolerant phase of chronic HBV is a challenging problem, with an increasing awareness of its occurrence, especially in endemic areas. More intense studies are required for a better delineation of the pathogenesis and whether it is better is to break the tolerance or to wait for the natural clearance. Until then, the most suitable solution is to perform liver biopsy to stand on solid ground in choosing the best option, to wait or to interfere.

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ORIGINAL ARTICLE

Melatonin attenuates cisplatin-induced HepG2 cell death *via* the regulation of mTOR and ERCC1 expressions

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Abstract

AIM: To elucidate the effects of melatonin on cisplatin-induced hepatocellular carcinoma (HepG2) cell death and to identify potential cross-talk pathways.

METHODS: Hepatocellular carcinoma HepG2 cells were treated with melatonin and/or cisplatin for 24 to 48 h. Cell viability and the 50% cytotoxic concentration (CC_{50}) were calculated by MTT assays. The effects and intracellular events induced by the selected concentrations of melatonin (1 mmol/L) and cisplatin (20 μmol/L) were investigated. Cell death and survival detection were primarily evaluated using a fluorescence microscope to assess 4',6 diamideno-2-phenylindol DNA staining and acridine orange lysosome staining and then further analyzed with immunocytochemistry using an anti-LC3 antibody. The potential molecular responses mediated by melatonin against cisplatin after the combined treatment were investigated by reverse transcription-polymerase chains reaction and Western blot analyses of the genes and proteins associated with cell survival and death. A cell cycle analysis was performed using a flow cytometry assay.

RESULTS: Melatonin had a concentration-dependent effect on HepG2 cell viability. At 1 mmol/L, melatonin significantly increased the cell viability percentage and decreased reactive oxygen species production due to cisplatin. Melatonin reduced cisplatin-induced cell death, decreasing phosphorylated p53 apoptotic protein, cleaved caspase 3 and Bax levels but increasing anti-apoptotic *Bcl-2* gene and protein expression. When combined with cisplatin, melatonin induced S phase (DNA synthesis) cell cycle arrest and promoted autophagic events in HepG2 cells. Melatonin also had a concentration-dependent effect on Beclin-1 and its autophagic regulator mammalian target of rapamycin (mTOR) as well as the DNA excision repair cross complementary 1 (ERCC1) protein. The expression levels of these proteins were altered in HepG2 cells during cisplatin or melatonin treatment alone. In the combination treatment, melatonin reversed the effects of cisplatin by suppressing the over-expression of mTOR and ERCC 1 and enhancing the expression levels of Beclin-1 and microtubule-associated protein-light chain3-Ⅱ, leading to intracellular autophagosome progression.

CONCLUSION: Melatonin attenuated cisplatin-induced cell death in HepG2 cells *via* a counter-balance between the roles of apoptotic- and autophagy-related proteins.

Key words: Melatonin; Cisplatin; Hepatocellular carcinoma; Excision repair cross complementary 1; Mammalian target of rapamycin; Autophagy

Core tip: Melatonin has anti-oxidative stress and anti-

proliferative effects on cisplatin-treated hepatocellular carcinoma cells through a counter-balance between the roles of apoptosis and autophagy proteins. Melatonin also reduced cisplatin-induced DNA damage by decreasing the activation of excision repair cross complementary 1 in the DNA repair system. Thus, co-treatment with melatonin to ameliorate cisplatin adverse effects might be beneficial for Hepatocellular carcinoma therapy.

Bennukul K, Numkliang S, Leardkamolkarn V. Melatonin attenuates cisplatin-induced HepG2 cell death *via* the regulation of mTOR and ERCC1 expressions. *World J Hepatol* 2014; 6(4): 230-242 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i4/230.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i4.230

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of human death worldwide. Aberrant gene expression and mutations induced by any genotoxic agents that can cause DNA damage contribute to its development. Effective treatment for HCC is not yet available because all anticancer strategies have limitations associated with the tumor grade and stage of the disease. For example, chemotherapy is limited by the side effects of cytotoxic drugs that also kill normal cells and by the long-term side effects of these drugs, which are carcinogenic and can cause secondary cancers[1]. Moreover, the mechanisms controlling the responses of pro-oncogenes and tumor suppressor genes in the effected cells are not well defined. In cancer research, the human hepatocellular carcinoma (HepG2) cell line has been used as a model system for studies of hepatocarcinogenesis because it is a permanent cell line derived from a well-differentiated hepatocellular carcinoma patient. The HepG2 cell line has also been used as an in vitro model for studying liver metabolism and in drug targeting assays[2].

To date, several anticancer drugs have been used clinically. Cisplatin, a platinum-based drug that has a known molecular mechanism of action, is a drug of choice that has been widely chosen for the treatment of solid cancers[3,4]. Cisplatin platinum can form complexes with DNA, inducing DNA adducts and damage[5]. Once produced, the complex mediates a series of intracellular responses that lead to apoptosis and also activates the DNA repair system, which is capable of inducing recovery of the damaged DNA and participates in the restoration of normal cell systems. However, if the damage is too extensive, the repair mechanism fails, and the cell undergoing apoptosis dies[6]. Through this pathway, cisplatin has no selective effects on cancers or normal cells. When receiving chemotherapy with cisplatin, some individuals respond by showing excessive side effects, and some cancers develop resistance to cisplatin by mechanisms that are only partially known. To improve chemotherapeutic efficiency, investigators have emphasized the need to search for new drugs. An example would be to study the potency of cisplatin at low concentrations, which can protect against hepatotoxicity, and to study drug resistance mechanisms to identify a new drug to replace cisplatin.

Melatonin is a hormone that is produced in the pineal gland and has several normal physiological functions in the human body. Its known properties include circadian rhythm regulation[7], sleep induction[8], immunemodulation[9], neuroprotection[10], bone differentiation[11], and anti-microbial[12] and anti-oxidative effects[13]. Previous investigations have demonstrated that melatonin also possesses anti-proliferative effects, especially in cell lines derived from various malignancies such as lymphoma[14], prostate cancer[15], melanoma[16] and hepatocellular carcinoma[17]. Although most of the biological effects of melatonin are produced through the activation of melatonin receptors, some are due to its status as a powerful antioxidant that plays roles in the protection of nuclear and mitochondrial DNA[18]. The anti-proliferative effect of the human osteosarcoma cell line MG-63 was shown to be activated by melatonin if the concentration of melatonin reached an optimal value, which was a high concentration of 4-10 mol/L[19]. Further data have suggested that melatonin could be used as an adjuvant to increase responses to anticancer drugs and to ameliorate their side effects[20-22]. However, the effectiveness of melatonin as an adjuvant of chemotherapy most likely differs among cancer types, and the mechanisms of the action of melatonin are still unclear. Due to the selective effects of melatonin, the current oncology research aiming to enhance the apoptosis effect of cisplatin combined with melatonin has extended to alternative pathways in treated cells.

It is well accepted that cells under oxidative stress or exposed to DNA damage, hypoxia, nutrient deprivation, and intracellular pathogens can survive through an autophagy pathway[23]. This pathway involves the lysosomal degradation of cytoplasmic organelles or cytosolic components, allowing cells to eliminate damaged or harmful components and also to recycle the released amino acids and energy to maintain cellular homeostasis[24]. In mammalian cells, the pathway is regulated through a serine/threonine protein kinase called mammalian target of rapamycin (mTOR)[25]. Normally, mTOR is activated under nutrient-rich conditions and inhibits autophagy[26]. However, under stress conditions, mTOR plays a role in autophagy activation *via* an effector Beclin-1 that initiates core nucleation for autophagy formation[27]. Under the normal condition, Beclin-1 is inhibited by an interaction with the Bcl-2/Bcl-xL complex, but when p53 binds to Bcl-2, it frees Beclin-1, leading to autophagy[28]. In addition to losing collaborative proto-oncogenes and tumor-suppressor genes during DNA damage, the cells expressed several efficient DNA repair systems, such as the nucleotide excision repair (NER) pathway, to prevent cancer formation[29]. NER can eradicate a broad spectrum

of DNA damage lesions through the action of a specific endonuclease enzyme named excision repair cross complementary 1 (ERCC1), which functions at the incision step of the NER pathway. ERCC1 cleaves damaged DNA at upstream sites, leading to DNA re-synthesis and ligation to return the damaged DNA to its native state and configuration[30]. Therefore, increased or decreased levels of ERCC1 expression should indicate efficiency of the DNA repair system.

In this study, we hypothesized that autophagy is an important pathway that plays roles in the outcome of cell death or survival in HepG2 cell during cisplatin and melatonin chemotherapy. We explored the expression of genes and proteins that regulate autophagy processes and lead to cell death and also identified the possible mechanism or cross-talk pathways mediated by melatonin and cisplatin.

MATERIALS AND METHODS

Cell culture

A HepG2 cell line was purchased from the American Type Culture Collection (Rockville, MD, United States). The cells were cultured at 37 ℃ in a humidified 5% CO_2 incubator and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and 100 Units/mL penicillin-streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, United States).

Cell viability assay

HepG2 cells were seeded onto 96-well plates (2×10^4 cells/well) for 24 h and then treated with 0.5-5.0 mmol/L melatonin (Merck, Frankfurter , Germany), 2.5-80.0 μmol/L cisplatin (Sigma Aldrich, St. Louis, MO, United States), or the combination of both for 24 and 48 h. In the combination treatment, the selected concentration was based on the minimal concentration that induced the anti-proliferative effect of melatonin and the most tolerable concentration that induced the cytotoxic effect of cisplatin. Cell viability was measured using an MTT colorimetric assay; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Aldrich (St. Louis, MO, United States), a working solution was added to each well and incubated at 37 ℃ for 2 h. The optical density of each well was measured using a microplate reader at 570 nm and the reference wavelength of 690 nm. Cell viability was calculated as the percentage of viable cells in the drug-treated group versus the untreated control group. The concentration of the compound that decreased cell viability by 50% cytotoxic concentrations (CC50) was calculated. Each experiment was performed in triplicate, and each result was presented as the mean ± SE.

Measurement of intracellular reactive oxygen species production

HepG2 cells were seeded onto 96-well black, flat, clear-bottom plates (2×10^4 cells/well). After treatment, the intracellular levels of ROS were measured by staining the treated and untreated cells with 50 μmol/L 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) that was purchased from Sigma Aldrich (St. Louis, MO, United States) after incubation in the dark at 37 ℃ for 30 min. The fluorescence intensity was quantified by the relative percentage of untreated cells using a fluorescence microplate reader and used 500 μmol/L H_2O_2-treated cells as the positive control. Each experiment was performed in triplicate, and each result was presented as the mean ± SE.

4',6 diamideno-2-phenylindol DNA stain and acridine orange lysosome stain

HepG2 cells were seeded onto 24-well plates (2×10^4 cells/well) for 24 h and then treated with 1 mmol/L melatonin, 20 μmol/L cisplatin, or both for 24 and 48 h.

DNA stain: Treated cells were fixed with cold methanol, air dried, and stained with 4'-6-diamidino-2-phenylindole, (DAPI) (Boehringer, Ingelheim, Germany) at 37 ℃ for 30 min. The primary signs of apoptosis induction, namely, chromatin condensation, fragmented DNA, and/or apoptotic body formation, were evaluated under an inverted fluorescent microscope.

Acridine orange stain: Treated cells were stained with 5 μg/mL acridine orange (Sigma Aldrich, St. Louis, MO, United States) in serum-free medium at 37 ℃ for 15 min. The cells were examined under an inverted fluorescence microscope. Positive acidic vacuoles or stained lysosomes were observed as orange or red foci in the cytoplasm, and DNA bound-acridine orange was detected as a green signal.

Immunocytochemistry

To determine an autophagic event, treated cells grown on coverslips were fixed in 4% paraformaldehyde for 5 min, permeabilized with methanol, and blocked with 3% bovine serum albumin in phosphate buffer saline (PBS) for 1 h at room temperature. The cells were then incubated with an indicator of autophagy, an anti-LC3 antibody was purchased from Cell Signaling Technology (Danvers, MA, United States) and was diluted 1:400 in PBS, overnight at 4 ℃. After incubation, the cells were washed with PBS and incubated with an Alexa Fluor 555-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, United States) for 2 h at room temperature, washed with PBS, and counter-stained with DAPI. The immunofluorescently labeled cells were visualized under a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Semi-quantitative analysis (RT-PCR) of genes controlling apoptosis and autophagy

HepG2 cells were seeded onto 6-well plates (4×10^5 cells/well). After treatment with 1 mM melatonin, 20 μmol/L cisplatin, or both for 24 h, total RNA was extracted with TRI Reagent (Molecular Research Center Inc; Cincinnati, OH, United States). cDNAs were synthesized by RevertAid™ M-MuLV Reverse Transcriptase and amplified by polymerase chain reaction (PCR) with

Table 1 Genes of interests and the primer pairs for polymerase chain reaction

Genes	Function	Primers sequence (5'-3')	Size (bp)
Bax	Pro-apoptosis	F- AAAGCTAGCGAGTGTCTCAAGCGC R-TCCCGCCACAAAGATGGTCACG	366
Bcl-2	Anti-apoptosis	F-TTGTGGCCTTCTTTGAGTTCG R-TACTGCTTTAGTGAACCTTTT	332
mTOR	Autophagic inhibition	F-TCTCATGGGCTTCGGAACAA R-GTGAAGGCAGAAGGTCGGAA	318
ERCC1	DNA repair	F-CCCTGGGAATTTGGCGACGTAA R-CTCCAGGTACCGCCCAGCTTCC	273
GAPDH	House keeping	F-CATCACCATCTTCCAGGAGC R-CATGAGTCCTTCCACGATACC	307

mTOR: Mammalian target of rapamycin; ERCC1: Excision repair cross complementary 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

specific primer pairs, as indicated in Table 1, using a High Fidelity PCR kit (Thermo Fisher Scientific, Waltham, MA, United States). The PCR cycles were adjusted accordingly to primer annealing. The PCR products were analyzed by 1.5% agarose gel electrophoresis and gel staining with ethidium bromide for 30 min. The gels were photographed using gel documentation (UVP Bioimaging System, United States). The experiments were performed in triplicate, and the relative band densities of cDNA from the treated cells were compared to the untreated cells.

Western blot analysis of proteins associated with apoptosis and autophagy

The treated cells were lysed in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail were purchased from Merck (Frankfurter, Germany) on ice for 30 min. The samples were then centrifuged at 24000 *g* at 4 ℃ for 15 min. The cell lysates were collected, and the total protein concentrations were determined by a BCA assay (Merck, Frankfurter, Germany). Equal amounts of protein (30 μg) from each sample were separated by 8%-15% SDS-polyacrylamide gel electrophoresis at 100 V and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, United Kingdom). The membranes were blocked with 3% w/v bovine serum albumin (Sigma Aldrich, St. Louis, MO, United States) in Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature. Then, the membranes were incubated with a 1:1000 dilution of primary antibodies against Bax (Santa Cruz, CA, United States), p53, phospho-p53, Bcl-2, procaspase3, cleaved-caspase3, p-mTOR, ERCC1, Beclin-1 or LC3 (Cell Signaling Technology, Danvers, MA, United States) overnight at 4 ℃, followed by two extensive washings with TBST. The membranes were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h. The specific bands corresponding to the investigated proteins were visualized using enhanced chemiluminescence (ECL reagents, GE Healthcare, Buckinghamshire, United Kingdom). The signal intensities were determined by densitometry using Image-J software (National Institutes of Health, Bethesda, MD, United States). After stripping off the first probe, each membrane was re-probed with a β-actin antibody (Santa Cruz, CA, United States) to confirm the equal loading of protein in each experiment. The levels of protein expression were presented in relation to β-actin.

Flow cytometry analysis of DNA synthesis and cell cycle activity

HepG2 cells were seeded onto 6-well plates (4×10^5 cells/well). After treatment with 1 mmol/L melatonin, 20 μmol/L cisplatin, or a combination of both for 24 h, the cells were trypsinized, centrifuged, washed twice with cold PBS, and fixed overnight with 70% ethanol at -20 ℃. The cells were resuspended in staining buffer containing 50 μg/mL propidium iodide (Merck, Frankfurter, Germany), 3.8 mmol/L sodium citrate, and 50 μg/mL RNase A (Sigma Aldrich, St. Louis, MO, United States) and incubated in the dark at 37 ℃ for 30 min. The stained cell suspensions were processed for flow cytometry analysis to determine the amount of DNA at different phases of the cell cycle using a FACScanto apparatus (BD Pharmingen, San Diego, CA, United States) with the loaded software. A total of 30000 cells from each sample were collected for evaluation of each data file.

Statistical analysis

All experiments were performed in triplicate ($n = 3$). Data are presented as the mean ± SE for each group, and these were compared for significant differences using a one-way analysis of variance test, followed by a post-hoc analysis (Tukey's multiple comparison test) using Prism 5 (GraphPad Software Inc; San Diego, CA, United States).

RESULTS

Effect of melatonin and cisplatin on HepG2 cell viability

As shown in Figure 1, melatonin (concentration 0.5-5 mmol/L) and cisplatin (concentration 2.5-80 μmol/L) reduced the viability of HepG2 cells in a time-concentration dependent manner. The 50% CC_{50} of melatonin were 6.25 mmol/L and 3.74 mmol/L and of cisplatin were 38.53 μmol/L and 17.53 μmol/L, at 24 h and 48 h, respectively. The concentrations of melatonin and cisplatin used in the combination treatment were selected from the minimal anti-proliferative effect of melatonin, which significantly decreased percent cell viability, and the most tolerable cytotoxic effect of cisplatin, which induced cell death at a rate below 50%. Therefore, 1 mmol/L melatonin was used in combination with 20 and 30 μmol/L cisplatin for 48 h. Melatonin increased the viability of HepG2 cells compared with cisplatin treatment alone. The combined treatment significantly reduced cell viability compared with the control and significantly increased cell viability compared with the cisplatin treatment alone

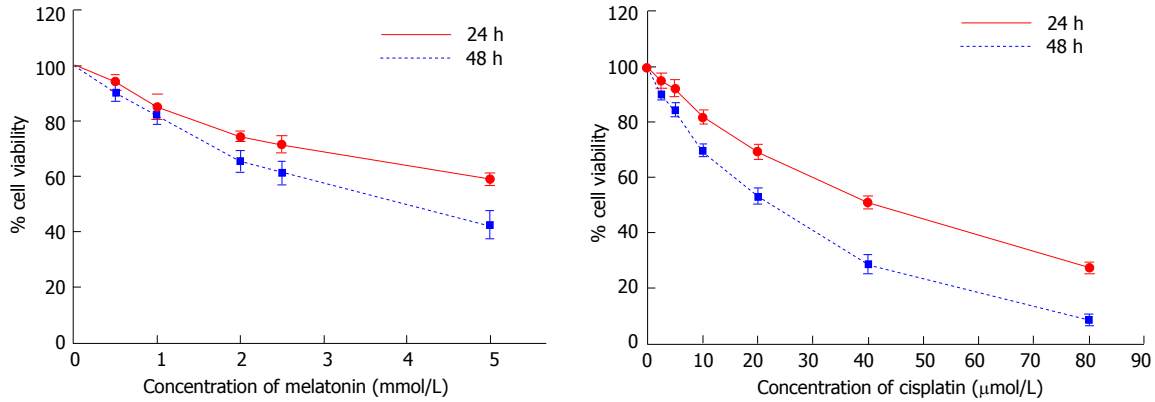

Figure 1 Effect of melatonin and cisplatin on cell viability analyzed using an MTT assay. Hepatocellular carcinoma (HepG2) cells were treated with various concentrations of melatonin and cisplatin for 24 h and 48 h. Both melatonin and cisplatin reduced the percent viability of HepG2 cells in a time and concentration-dependent manner ($P < 0.001$). The experiments were performed in triplicate, and the results are presented as the means ± SE.

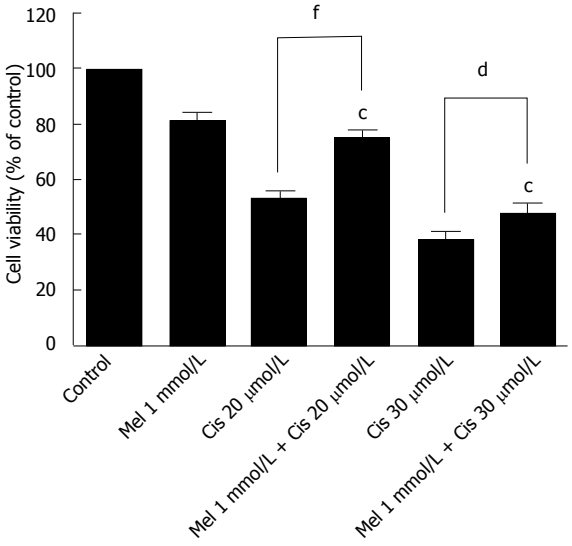

Figure 2 Effect of the combined treatment of melatonin and cisplatin on cell viability. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 and 30 μmol/L) for 48 h and then analyzed by an MTT assay. Melatonin reduced the cisplatin cytotoxic effect at 20 μmol/L cisplatin. The results are presented as the mean ± SE. ($^cP < 0.001$ compared with the control group, $^dP < 0.05$, $^fP < 0.001$ compared with cisplatin-treated group).

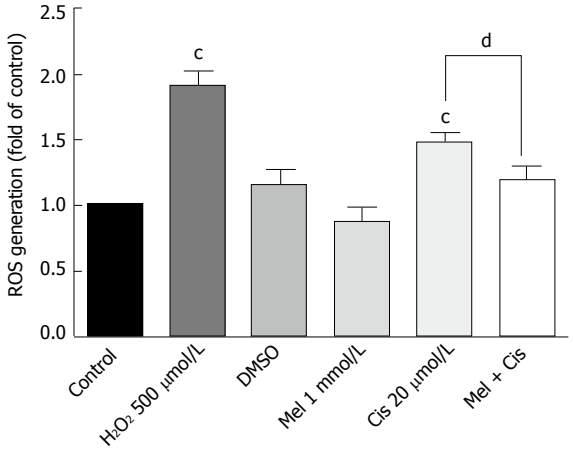

Figure 3 Melatonin reduced cisplatin-induced intracellular reactive oxygen species levels. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 24 h. The results are presented as the means ± SE. ($^cP < 0.001$ compared with the control group, $^dP < 0.05$ compared with the cisplatin-treated group).

(Figure 2).

Potential mechanisms and pathways of the melatonin-mediated attenuation of cisplatin-induced cell death

Anti-ROS production: To determine whether melatonin reduced the ROS production due to cisplatin, the biochemical basis of intracellular ROS was explored using a fluorescein-labeled dye, DCFH-DA. The results showed significantly increased intracellular ROS in cisplatin-treated cells. However, the combined treatment with melatonin (1 mmol/L) reduced the intracellular ROS level (Figure 3).

Reduction of cellular damage and apoptosis formation: The combined effect of melatonin and cisplatin was evaluated in unstained in HepG2 cells under an inverted light microscope (Figure 4A) and in HepG2 cells stained with DAPI under a fluorescence microscope (Figure 4B). Melatonin treatment resulted in no morphological changes and the absence of apoptotic nuclei. Cisplatin treatment induced intense morphological changes, with cell shrinkage and typical apoptotic nuclear features, such as nuclear condensation and apoptotic bodies. The combined melatonin and cisplatin treatment revealed unchanged cell morphology and a reduction of apoptotic bodies.

Regulation of apoptosis *vs* anti-apoptosis genes and protein expression: To determine apoptotic events in cells, the expression of the genes and proteins involved in apoptosis were analyzed. As shown in Figure 5, Western blotting results indicated that melatonin (1 mmol/L) had no significant effect on proteins associated with apoptosis (p53, p-p53, pro-caspase3 and cleaved-caspase3), whereas cisplatin significantly increased p53 and p-p53 levels.

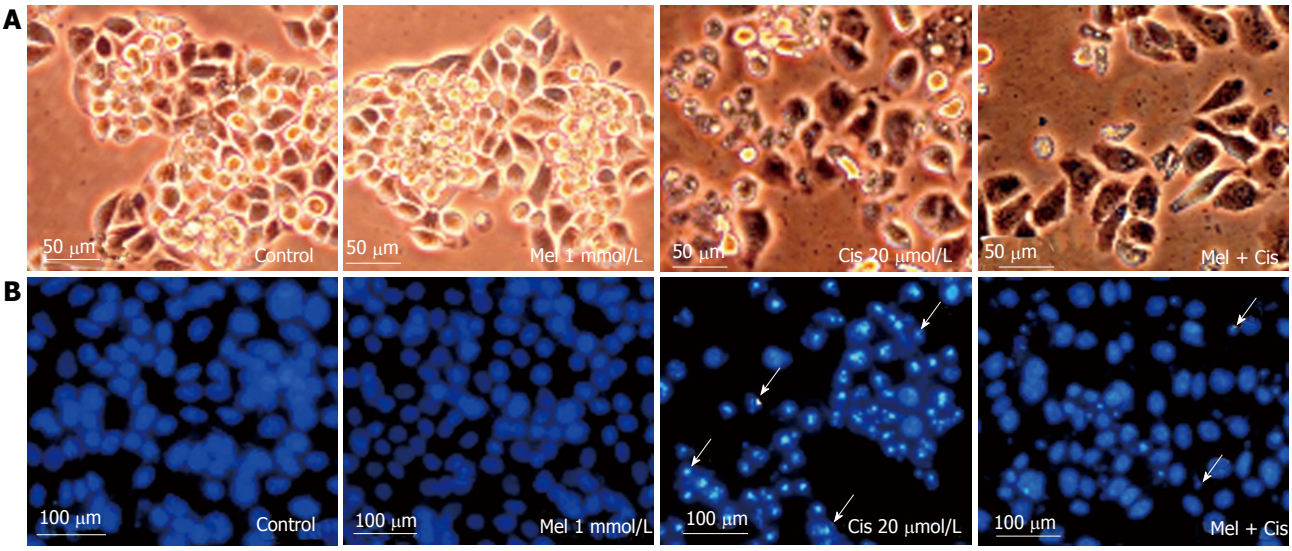

Figure 4 Histological changes. Hepatocellular carcinoma cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 48 h. The cells were stained with DAPI for fragmented DNA (apoptotic bodies) and observed under an inverted microscope. A: Morphological changes showing vacuoles in the cytoplasm of cisplatin-treated cells (scale bar = 50 μm); B: DAPI staining showing nuclear condensation and apoptotic bodies (white arrows) in cisplatin-treated cells. The combined treatment showed less apoptotic effects (scale bar = 100 μm).

Gene and protein expression analyses of pro-apoptosis Bax and anti-apoptosis Bcl-2 levels revealed slight decreases in the Bax/Bcl-2 ratio with melatonin treatment while resulting in a significantly increased Bax/Bcl-2 ratio with cisplatin treatment. In the combined treatment, melatonin significantly ameliorated the pro-apoptotic effects of cisplatin.

Cell cycle regulation: To determine the effects of the treatments on HepG2 cell cycle control, DNA accumulation was measured by flow cytometry at different phases of the cell cycle. The phases of cell cycle arrest were determined at G_0/G_1 by melatonin and at subG_0, G_0/G_1, and S by cisplatin. In the combined treatment, melatonin reduced cisplatin cell cycle arrest at the subG_0 through G_0/G_1 phases but significantly increased cell cycle arrest at the S phase (Figure 6).

Autophagy regulation: The combined effect of melatonin and cisplatin in HepG2-treated cells was evaluated for lysosomal staining with acridine orange dye and observation under an inverted microscope to measure fluorescence. Both melatonin and cisplatin caused slight increases in the acidic lysosomal compartments; an increase in acridine orange intensity was highly apparent in the combined treatment (Figure 7A). In the immunofluorescence assay with the anti-LC3 antibody, melatonin and cisplatin treatment alone induced minor autophagy events in HepG2 cells compared with the control. However, the combination of melatonin and cisplatin treatment significantly enhanced autophagy, as indicated by the fluorescence intensity of LC3 scattered throughout the cytoplasm (Figure 7B).

Alteration of autophagy regulators and the DNA repair system: To explore the potential pathways of melatonin and cisplatin effects on autophagy regulation and DNA repair process in HepG2-treated cells, the relationship between the proteins Beclin-1, mTOR and ERCC1 were analyzed. Melatonin (0.5 and 1 mmol/L) increased Beclin-1, p-mTOR, and ERCC1 in a concentration-dependent manner (Figure 8). Conversely, cisplatin (10 and 20 μmol/L) decreased Beclin-1, p-mTOR, and ERCC1 in a concentration-dependent manner. At the selected concentrations (1 mmol/L melatonin and 20 μmol/L cisplatin), melatonin significantly increased p-mTOR and ERCC1 from the basal levels, whereas cisplatin decreased Beclin-1 and increased p-mTOR and ERCC1 from the basal levels.

Evaluation of the autophagy process in melatonin- and cisplatin-treated HepG2 cells showed changes in Beclin-1 (an initiator of autophagy) and LC3-Ⅱ (a mature marker of autophagy). Melatonin (1 mmol/L) treatment alone slightly increased both Beclin-1 and LC3-Ⅱ, but cisplatin (20 μmol/L) treatment alone decreased Beclin-1 and slightly increased LC3-Ⅱ (Figure 9). The combined treatment showed that melatonin prevented cisplatin from affecting the level of Beclin-1 but significantly increased the effect of cisplatin on the LC3-Ⅱ level.

The analysis of mTOR and ERCC1 mRNA and protein expression in the treated HepG2 cells revealed that melatonin (1 mmol/L) or cisplatin (20 μmol/L) treatment alone significantly increased p-mTOR and ERCC1 from the basal levels. However, the combined treatment resulted in the suppression of both p-mTOR and ERCC1 compared with the basal cellular levels (Figure 10). (See a schematic overview of the results in Figure 11)

DISCUSSION

Cisplatin chemotherapy has been used to kill several solid cancer cells with satisfactory results. However, due to

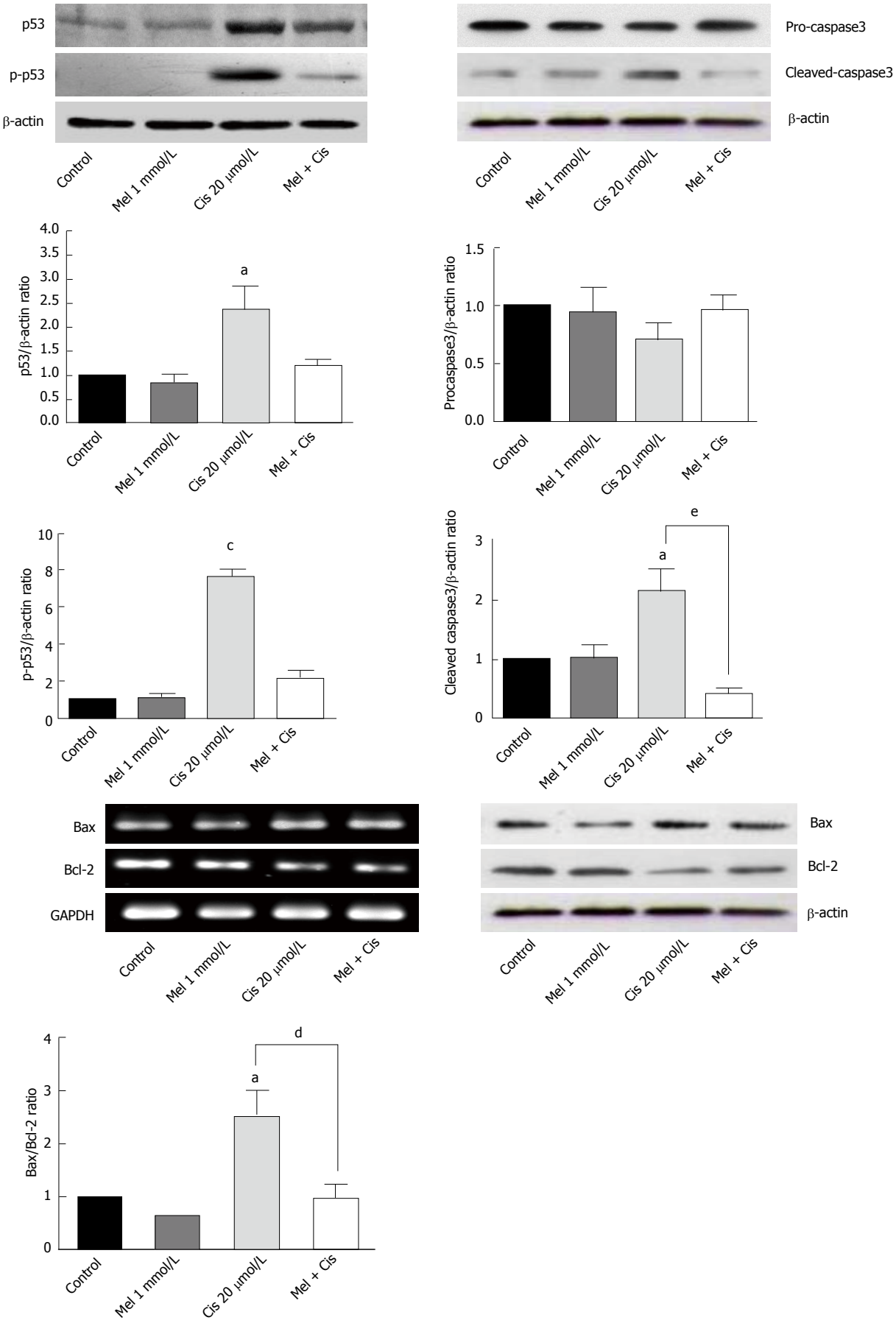

Figure 5 Reverse transcription-polymerase chain reaction and Western blot analyses of apoptosis regulators in treated hepatocellular carcinoma cells. Melatonin had minor effects on all apoptosis markers, whereas cisplatin significantly increased apoptosis via the activation of p53 and caspase3 and the Bax/Bcl-2 ratio. Melatonin reduced cisplatin effects in the combined treatment. ($^{a}P < 0.05$, $^{c}P < 0.001$ compared with the control group, $^{d}P < 0.05$, $^{e}P < 0.01$ compared with the cisplatin-treated group).

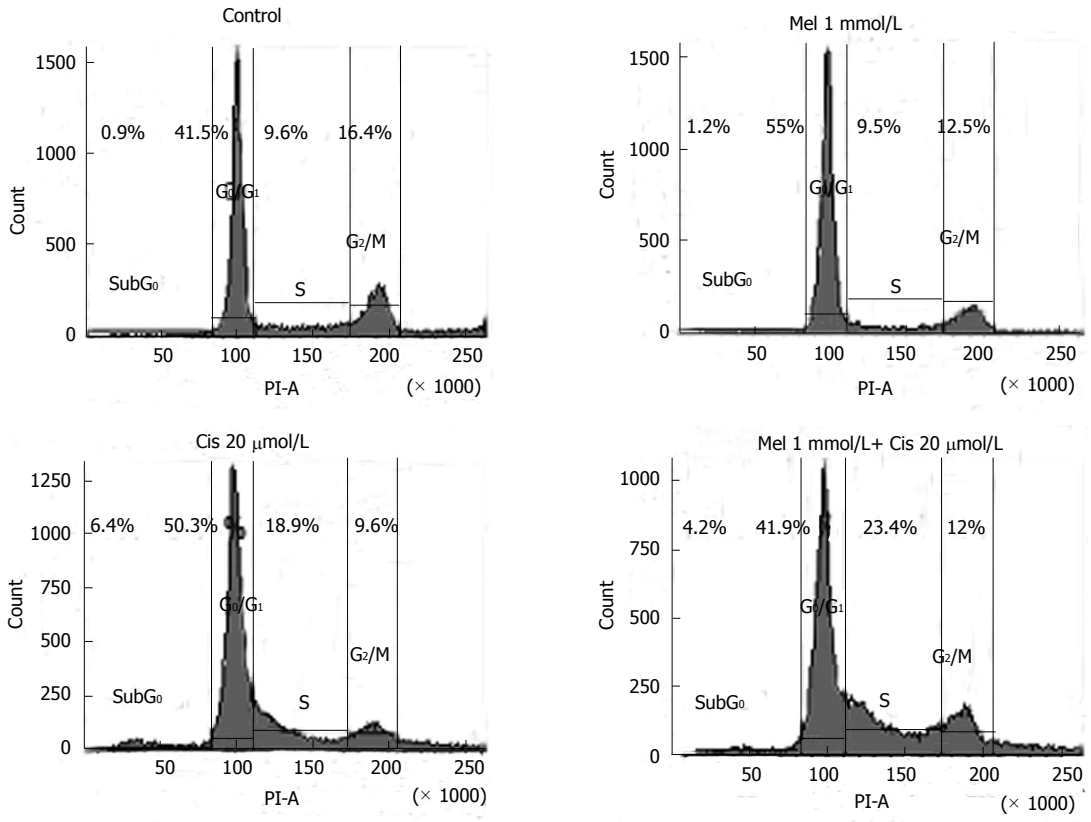

Figure 6 Effects of melatonin and cisplatin on the cell cycle, as evaluated using flow cytometric analysis. Hepatocellular carcinoma (HepG2) cells were treated with melatonin (1 mmol/L) and/or cisplatin (20 μmol/L) for 24 h. Melatonin induced DNA accumulation in the HepG2 cells at the G_0/G_1 phase, whereas cisplatin affected cell cycle arrest at the sub-G_0, G_0/G_1, and S phases. In the combined treatment, melatonin decreased the cisplatin effect on cell cycle arrest at the sub-G_0 through G_0/G_1 phases but significantly increased S phase cell cycle arrest.

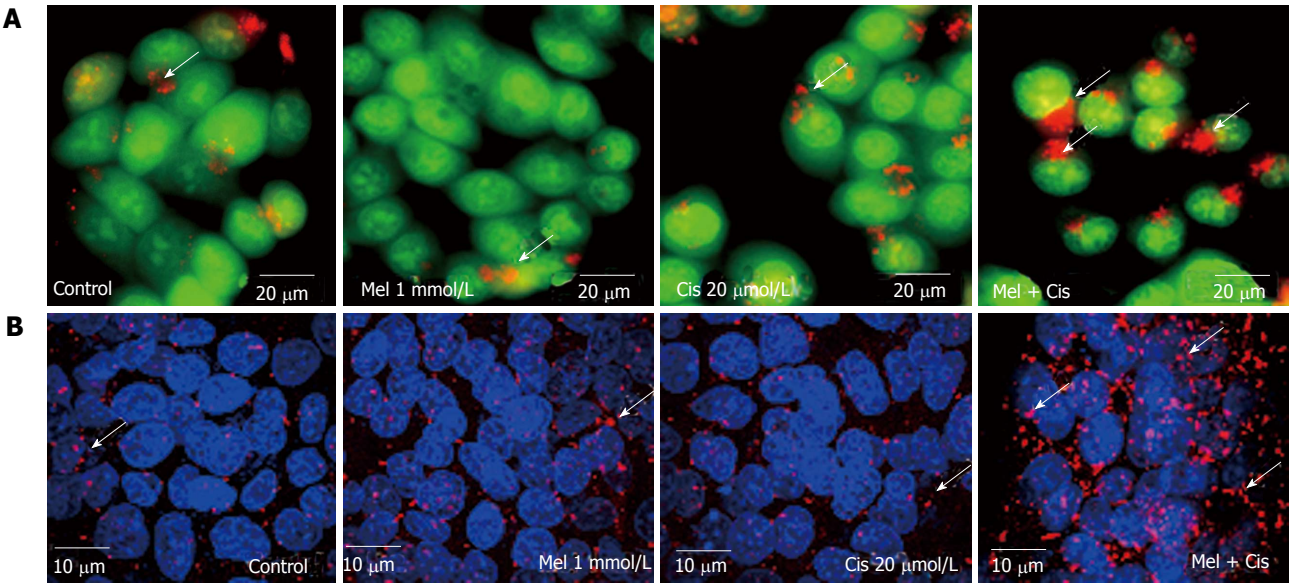

Figure 7 Autophagic detection by acridine orange staining and immunofluorescence. Hepatocellular carcinoma (HepG2) cells were treated with 1 mmol/L melatonin and/or 20 μmol/L cisplatin for 24 h and evaluated for autophagy formation. The experiments were performed in triplicate, and representative micrographs are shown. A: Acridine orange staining showed lysosomal (red or orange) staining in the cells of all treatments. The increased acidic lysosomes in the combination treatment suggests potential lysosomal activation (scale bar = 20 μm); B: Confocal immunofluorescence micrographs of representative treated-HepG2 cells immunolabeled with the anti-LC3 antibody (scale bar = 10 μm). Melatonin and cisplatin treatment alone induced some fluorescent immunoreactivity in the cytoplasm, but the combined treatment induced intense immunoreactivity.

its efficient cytotoxic effects, cisplatin produces adverse drug effects and chemo-resistance. Therefore, adjuvant therapy with melatonin has been proposed, and extensive studies were performed, with variable outcomes[22]. In the

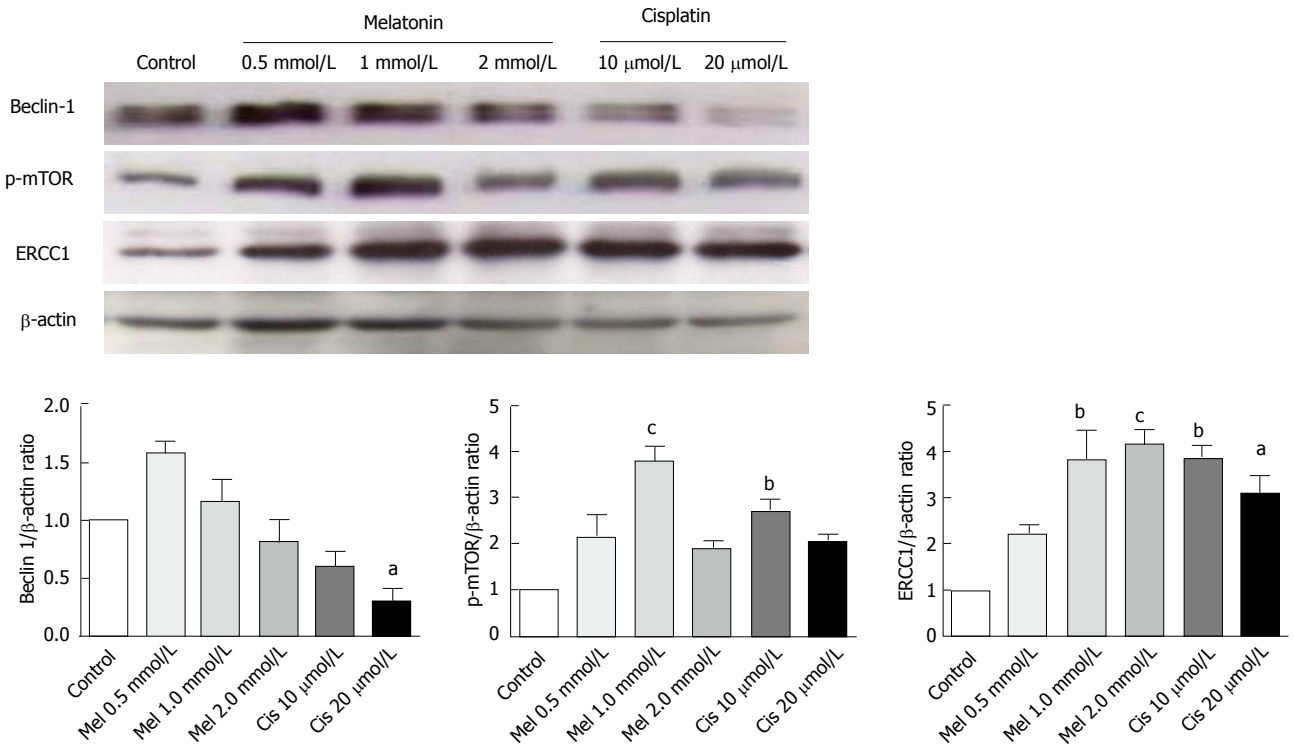

Figure 8 Analysis of autophagy and DNA repair-related proteins. Hepatocellular carcinoma cells were treated with various concentrations of melatonin and cisplatin. At the selected concentrations, 1 mmol/L melatonin and 20 μmol/L cisplatin have a similar effect by significantly increasing p-mTOR and ERCC 1 from the basal levels. However, the effect on Beclin-1 was the opposite, with melatonin increasing Beclin-1 and cisplatin decreasing Beclin-1 from the basal level. The results are presented as the mean ± SE. ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ compared with the control group). ERCC1: Excision repair cross complementary-1.

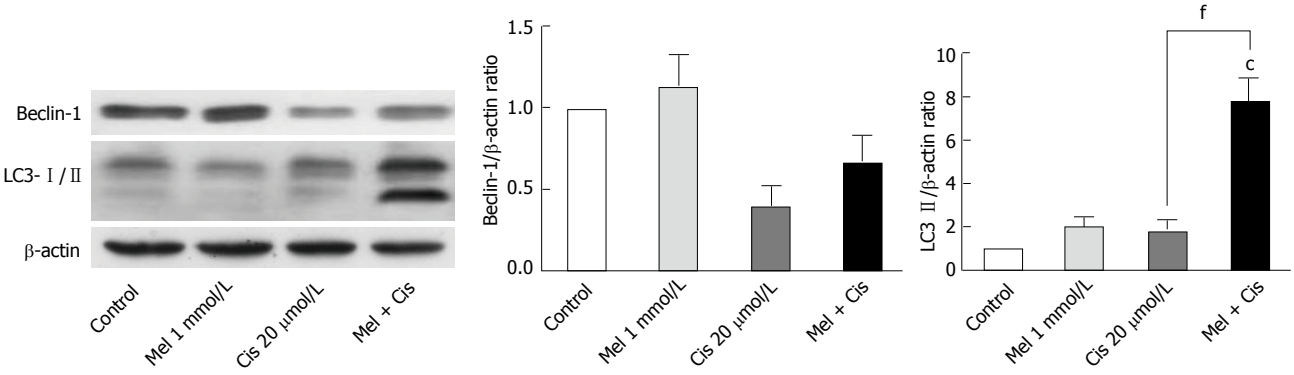

Figure 9 Alteration of autophagy-forming proteins. Hepatocellular carcinoma cells were treated with melatonin and/or cisplatin for 24 h. Melatonin slightly increased Beclin-1 and LC3-Ⅱ, whereas cisplatin significantly decreased Beclin-1 and slightly increased LC3-Ⅱ. Melatonin inhibited the cisplatin effect on Beclin-1 but increased LC3-Ⅱ in the combined treatment. ($^cP < 0.001$ compared with the control group, $^fP < 0.001$ compared with the cisplatin-treated group).

present study, we set out to determine whether melatonin could mediate a protective effect against cisplatin-induced apoptosis using HepG2 cells and attempted to identify the potential molecular pathways of melatonin action. The results demonstrated that the overall effect of this adjuvant in chemotherapy depends on a number of melatonin effects on cells. When melatonin was combined with cisplatin in the HepG2 cell line, the expression of the apoptosis mediators phosphorylated p53, cleaved caspase 3, and Bax decreased, whereas anti-apoptotic Bcl-2 increased. These biochemical results correlated well with decreases in the intracellular ROS level and the recovery of cell morphology. The results indicated the anti-oxidative stress property of melatonin, which directly scavenges reactive oxygen species induced by cisplatin anticancer drugs[31,32], thus reducing adverse cisplatin drug effects in hepatocytes.

In addition to the extended results from the alteration of the anti-apoptotic Bcl-2 level, we found that melatonin decreased the expression ratio of Bax/Bcl-2, which was affected by cisplatin. Thus, an apoptosis event in the combined treatment was switched to cell survival. The expression of p53 protein was also subjected to a switch by the melatonin and cisplatin combination, suggesting a direct effect of both on the cell cycle. The pro-death effect of cisplatin, which was mostly mediated by p53, indicated

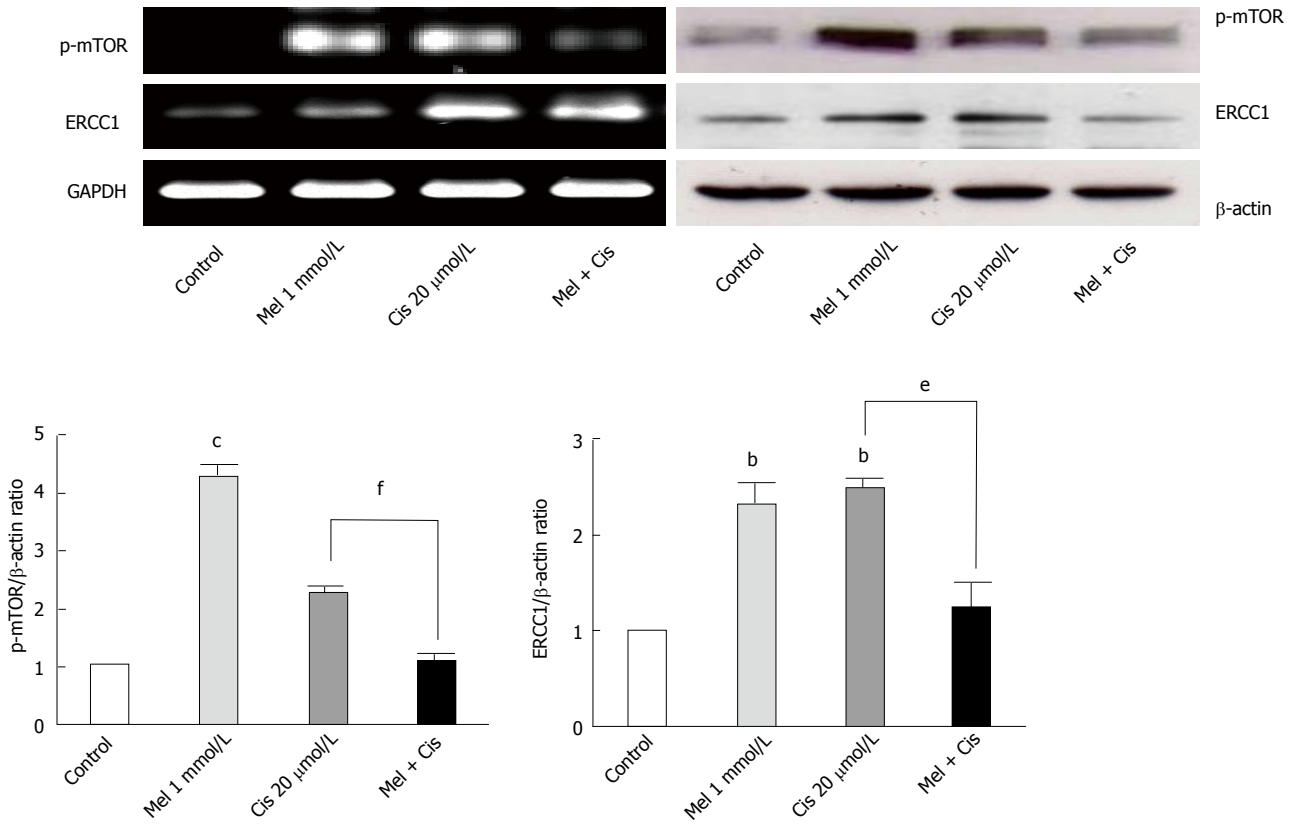

Figure 10 RT-PCR and Western blot analyses of autophagy regulators. Both melatonin and cisplatin increased p-mTOR and ERCC 1 levels in the HepG2 cells, but the combined treatment resulted in the suppression of both proteins. ($^{b}P < 0.01$, $^{c}P < 0.001$ compared with the control group, $^{e}P < 0.01$, $^{f}P < 0.001$ compared with the cisplatin-treated group). ERCC1: Excision repair cross complementary-1.

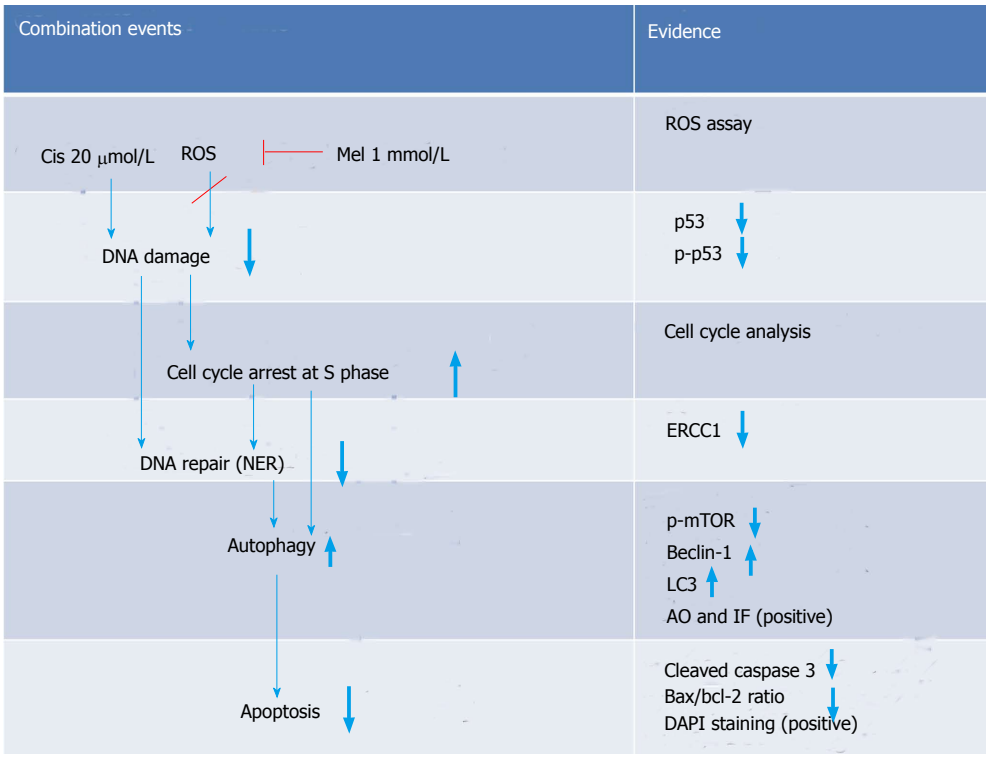

Figure 11 Schematic overview of the major findings in the combination group.

that the cell cycle specificity of cisplatin in HepG2 cells affected G_0/G_1 phase. In the combined treatment, the cell

cycle arrest was changed to the S and G_2/M phases. Therefore, melatonin must have increased the cisplatin effect on cell cycle arrest through inhibition at the S (DNA synthesis) and M (mitosis) phases. These are considered the most anti-proliferative (oncostatic) effects of melatonin. However, the cell cycle arrest by melatonin in the treated cells could also be mediated by other cell cycle control proteins, such as the calcium/calmodulin pathway[33] and autophagy regulation pathway. Both pathways are related to the anti-proliferation of cells and pro-survival.

In our study, DNA damage may have been induced in HepG2 cells by the cisplatin genotoxic chemical and ROS by-products of cell metabolism[34]. The subsequent response reactions include the activation of a DNA damage checkpoint, which arrests cell cycle progression and leads to apoptosis[35]. We demonstrated that there was a survival switch from cisplatin-induced apoptosis to autophagy in the combination treatment with melatonin. The indicators included acridine orange staining, an immunohistochemistry assay for autophagy, and the measurement of mRNA and proteins associated with autophagy [*i.e.*, mTOR, Beclin-1, and chain3-Ⅱ (LC3-Ⅱ)]. Compared with cisplatin treatment alone, the combination treatment showed a high intensity of positive fluorescence staining in the lysosomal compartment of the cell, suggesting increased autophagy activity. Cisplatin acted on p53 and Bcl-2 to induce apoptosis, but in the combined treatment, Bcl-2 was decreased to an extent lower than the basal level. This could occur through reduced binding between the Bcl-2/Bcl-xL complex and Beclin-1, followed by a release of Beclin-1 to initiate autophagy formation. Thus, the combined treatment could change apoptosis to autophagy.

The basal levels of Beclin-1 and mTOR, major regulators of autophagy, were evaluated in HepG2 cells. Cisplatin treatment alone up-regulated mTOR and down-regulated Beclin-1 expression compared with the basal levels, thus decreasing autophagy and promoting the apoptosis pathway. Melatonin treatment alone up-regulated both mTOR and Beclin-1 expression compared with the basal levels, and this up-regulation by melatonin played a significant role in the survival of HepG2 cells. In the combined treatment, melatonin caused autophagy through the final formation of the autophagosome and was shown by an increased microtubule-associated protein-light LC3-Ⅱ level via the up-regulation of p-mTOR and Beclin-1. This changed the apoptotic signal caused by cisplatin. Overall, melatonin attenuated cisplatin-induced HepG2 cell death by inducing an autophagy survival signal[36].

A novel autophagy regulation mediated by melatonin was identified in this study. Both melatonin and cisplatin exhibited concentration-dependent effects on mTOR, Beclin-1, and DNA ERCC1. ERCC1 is a marker of DNA repair, whereby ERCC1 is the rate-limiting enzyme in the NER system, a pathway known to remove cisplatin lesions from DNA[37]. The analysis of protein expression in this study revealed that increases in ERCC1 from the basal levels in HepG2 cells by each treatment were associated with an increase in mTOR expression, suggesting a tight relationship between DNA repair and autophagy. We also found that both mTOR and ERCC1 were significantly decreased by melatonin induction in the co-treatment group compared with the cisplatin-treated group. However, the only slight reduction in ERCC1 in our assay using rapamycin as an inhibitor of mTOR demonstrated that the melatonin effect on ERCC1 was not directly to the mTOR activation pathway (data not included). Therefore, melatonin reduced cisplatin-induced DNA damage, resulting in decreased activation of the DNA repair capacity and might be involved in transcriptional regulation or an epigenetic mechanism. As previously shown, p-p53 is a transcription factor for apoptotic gene expression and also plays a role in DNA repair via the subsequent up-regulation of genes in the NER pathway[38-40]. AP-1 is a transcription factor for autophagy and ERCC1 regulation in DNA damage pathways[41]. In the combined treatment, melatonin and cisplatin may encounter transcription inactivation. That is, after cisplatin treatment, DNA damage occurs, and ERCC1 is induced. An increased level and activation of mTOR induced by melatonin counter-balances the role of p-p53 in the DNA damage and repair process induced by cisplatin. This finding is the first report that melatonin can activate ERCC1 in a concentration-dependent manner.

Interestingly, significant decreases in both mTOR and ERCC1 compared with the basal levels were identified in the combined treatment, suggesting a positive response of the cells. The expression level of ERCC1 has been previously suggested to be a prognostic marker of cancers, and ERCC1 over-expression has been associated with to cisplatin resistance[42]. In our research, ERCC1 decreased compared with the cisplatin-treated group, indicating an improvement in cancer prognosis.

In conclusion, melatonin exerted an oncostatic effect on cisplatin-treated hepatocellular carcinoma cells via a counter-balance between the roles of apoptosis-related p53 and Bcl-2 and autophagy proteins. The alteration of Beclin-1, mTOR, and ERCC1 levels determined the pro-death and pro-survival effects of treatment in this cancer cell line. Therefore, during cisplatin treatment of hepatocellular carcinoma, the administration of a high dose of melatonin as an adjuvant in cancer therapy should ameliorate the adverse effects of cisplatin.

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COMMENTS

Background

Liver cancer is one of the major causes of death in humans worldwide due to

the limitation of drugs for treatment. Frequently, chemotherapy with platinum-based cisplatin is provided during the late stage of the disease. This drug causes excessive side effects and sometimes induces cancer drug resistance.

Research frontiers

Melatonin is a hormone produced in our body to maintain several normal physiological functions, which may be altered during the course of cancer. To reduce cisplatin side effects and still maintain the drug's efficacy, we treated human hepatocellular carcinoma (HepG2) cells with cisplatin and a high concentration of melatonin. The results showed that melatonin could reduce the oxidative stress effects of cisplatin by altering the stages of cell cycle arrest and apoptosis mediators such as p53. Melatonin switched cisplatin-induced apoptosis in HepG2 cell to autophagy-induced survival via Bcl-2, Beclin-1 and mTOR. In addition, melatonin reduced cisplatin-induced DNA damage by decreasing the activation of excision repair cross complementary-1 (ERCC1) in the DNA repair system.

Innovations and breakthroughs

Melatonin can activate DNA ERCC1 in a concentration-dependent manner. An analysis of protein expression in this study revealed that increases in ERCC1 from the basal levels in HepG2 cells by each treatment were associated with an increase in mTOR expression, suggesting a tight relationship between DNA repair and autophagy. In the combined treatment, melatonin and cisplatin may result in transcriptional inactivation between mTOR and ERCC1.

Applications

During cisplatin treatment of hepatocellular carcinoma, the administration of melatonin as an adjuvant in cancer therapy should ameliorate the adverse cisplatin effects while maintaining the drug's efficacy.

Terminology

ERCC1 is the rate-limiting endonuclease in the nucleotide excision repair (NER) pathway, which is the major pathway for removing cisplatin from the DNA strand. ERCC1 is a good predictive biomarker for cancer treatment with cisplatin. ERCC1-negative tumor patients were associated with a higher survival rate than ERCC1-positive tumor patients treated with adjuvant platinum-based regimens.

Peer review

This study has a good structure and is easy to read despite many abbreviations, even for inexperienced readers in this field. It is a very important and up-to-date subject. The work is interesting, technically correct, and well written and describes an important role for melatonin as an adjuvant to cisplatin, a therapy known to cause severe toxicity in the body. The studies were well-designed, clearly presented, highly mechanistic, and demonstrated novel pathways regulated by melatonin to improve health outcomes associated with hepatic cancer and cisplatin-induced hepatotoxicity. This study will open up new research areas in this field. The authors used myriad techniques and approaches to tackle this deadly disease.

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BRIEF ARTICLE

Murine model to study brain, behavior and immunity during hepatic encephalopathy

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Abstract

AIM: To propose an alternative model of hepatic encephalopathy (HE) in mice, resembling the human features of the disease.

METHODS: Mice received two consecutive intraperitoneal injections of thioacetamide (TAA) at low dosage (300 mg/kg). Liver injury was assessed by serum transaminase levels (ALT) and liver histology (hematoxylin and eosin). Neutrophil infiltration was estimated by confocal liver intravital microscopy. Coagulopathy was evaluated using prolonged prothrombin and partial thromboplastin time. Hemodynamic parameters were measured through tail cuff. Ammonia levels were quantified in serum and brain samples. Electroencephalography (EEG) and psychomotor activity score were performed to show brain function. Brain edema was evaluated using magnetic resonance imaging.

RESULTS: Mice submitted to the TAA regime developed massive liver injury, as shown by elevation of serum ALT levels and a high degree of liver necrosis. An intense hepatic neutrophil accumulation occurred in response to TAA-induced liver injury. This led to mice mortality and weight loss, which was associated with severe coagulopathy. Furthermore, TAA-treated mice presented with increased serum and cerebral levels of ammonia, in parallel with alterations in EEG spectrum and discrete brain edema, as shown by magnetic resonance imaging. In agreement with this, neuropsychomotor abnormalities ensued 36 h after TAA, fulfilling several HE features observed in humans. In this context of liver injury and neurological dysfunction, we observed lung inflammation and alterations in blood pressure and heart rate that were indicative of multiple organ dysfunction syndrome.

CONCLUSION: In summary, we describe a new murine model of hepatic encephalopathy comprising multiple features of the disease in humans, which may provide new insights for treatment.

Key words: Hepatic encephalopathy; Liver injury; Thioacetamide; Neurological dysfunction; Neuropsychomotor abnormalities; Intracranial hypertension; Cerebral herniation

Core tip: The study of hepatic encephalopathy is crucial for development of new therapies but has been dampened by the absence of murine models resembling the disease in patients. We showed that sequential thioacetamide injections cause extensive liver injury in mice, leading to increased ammonia levels, electroencephalography alterations and brain edema. In line with this, mice presented with poor psychomotor activity and survival rate. Liver injury and brain function impairment by thioacetamide resulted in systemic alterations such as coagulopathy, hemodynamic instability and lung inflammation, consistent with multiple organ failure. Therefore, this alternative model may provide tools for new therapeutic insights for hepatic encephalopathy.

Gomides LF, Marques PE, Faleiros BE, Pereira RV, Amaral SS, Lage TR, Resende GHS, Guidine PAM, Foureaux G, Ribeiro FM, Martins FP, Fontes MAP, Ferreira AJ, Russo RC, Teixeira MM, Moraes MF, Teixeira AL, Menezes GB. Murine model to study brain, behavior and immunity during hepatic encephalopathy. *World J Hepatol* 2014; 6(4): 243-250 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i4/243.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i4.243

INTRODUCTION

Acute liver failure (ALF) is a rare but severe clinical syndrome. It is typically characterized by jaundice, coagulopathy and encephalopathy resulting from sudden hepatic dysfunction without preexisting liver disease[1]. Drug-induced liver injury (DILI) is the main cause of ALF in the United States and Europe[2], whereas in developing countries viral hepatitis is the most important etiology. Although liver injuries may result from a wide range of situations, drug overdose may become the most important etiology of ALF worldwide in a few years[2] as life expectancy increases and the medicalization of the older population tends to increase[3]. Moreover, the worldwide cause of ALF tends towards DILI due to increasing public health measures (*e.g.*, vaccination)[1], making it an emergent widespread problem. ALF has a high mortality rate of approximately 30% due to multiple organ dysfunction, hepatic encephalopathy and sepsis. Unfortunately, therapeutic options are very limited since liver transplantation is the only definitive therapy available. Besides that, patients are kept under clinical management and supportive care until spontaneous liver recovery[1,4].

Hepatic encephalopathy (HE) is one of the major complications in ALF because of rapid brain edema, intracranial hypertension and cerebral herniation[5]. Clinically, HE is a neuropsychiatric syndrome that comprises a wide range of signs and symptoms from subtle altered mental status to stupor and coma[6]. The severity of HE has prognostic implications since patient outcome worsens as HE progresses. There is a chance of 65%-70% of spontaneous liver recovery in mild HE and less than 20% in severe HE[7]. Moreover, patients with ALF who manifest increased intracranial pressure during the course of illness are more likely to develop sepsis[8]. Hence, understanding HE pathophysiology is crucial for development of new and specific therapies that would slow down its progression and increase chances of better outcomes.

In this sense, a murine model that reproduces features of HE in humans would be key to improve our understanding in HE pathophysiology and may also be useful for drug testing and development. Here, we propose an alternative murine model of HE using sequential systemic thioacetamide (TAA) injections in a lower dosage instead of a single higher dose administration.

MATERIALS AND METHODS

TAA induced liver failure and hepatic encephalopathy

Female C57BL/6 mice and Lysm-eGFP (eGFP-expressing neutrophils) had free access to food and water. TAA-induced liver injury protocol was based on two consecutive days of treatment with thioacetamide (300 mg/kg) administered intraperitoneally. Treated groups received the first dose in time zero and an additional dose after 24 h. Controls received vehicle following the same chronogram. Mice were sacrificed after 24 or 48 h and liver, lung, blood and brain samples were collected for further analysis. All mice received glucose replacement (12/12 h; 5%; *s.c.*) and glycemia was monitored (12/12 h) with commercially available reactive strips and a glucometer (Accu-Chek, Performa). Body temperature was maintained at 37 ℃ by a thermal pad. Psychomotor activity was graded following an adapted clinical score, in which 0 = normal behavior; 1 = mild lethargy; 2 = decreased motor activity, poor gesture control, diminished pain perception; 3 = Severe ataxia, no spontaneous righting reflex; 4 = no righting reflex, no reaction to pain stimuli; and 5 = death[9]. All procedures were approved by Animal Care and Use Committee in UFMG (CEBIO n°051/2011). The investigation conformed to the standards of Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, 1996 revision). In a separate set of experiments, the liver was imaged using confocal intravital microscopy as described previously[10]. Lysm-eGFP mice received propidium iodide (200 μL of a 100 μmol/L stock solution; *i.v*) prior to the surgical procedure in order to visualize necrotic cells.

Serum and cerebral ammonia determination

Immediately after decapitation, the brain was rapidly removed from the cranial cavity and fast-frozen in liquid nitrogen. Subsequently, brain samples were macerated using a pestle in perchloric acid (ice-cold; 1mol/L) and centrifuged at 10000 *g* for 10 min at 4 ℃. The supernatant was collected for immediate dosage. Also, blood

samples were centrifuged for 7 min (7000 *g*) and plasma was collected. Ammonia concentration was estimated using Ammonia Assay kit (Sigma, United States) following manufacturer instructions.

Alanine aminotransferase

The alanine aminotransferase enzyme is present in the cytoplasm of hepatocytes and is highly specific for the liver. The measurement of serum alanine aminotransferase (ALT) is a gold-standard marker of liver damage. To determine the activity of ALT, blood samples were centrifuged and the serum was collected and dosed using a kinetic kit (Bioclin, Brazil).

Hemodynamic measurements using tail cuff method

Mean arterial pressure (MAP) and heart rate (HR) were evaluated by a volume pressure recording sensor and an occlusion tail-cuff, which measures mice blood pressure and HR noninvasively (Kent Scientific Corporation, Torrington, CT, United States)[11]. Mice were acclimated to the restraint and tail cuff inflation for two days before the beginning of the experiments. The restraint platform was maintained at 36-38 ℃. In each session, mice were placed in an acrylic box restraint, and the tail was inserted into a compression cuff that measured the blood pressure 10 times. Following the measurement cycle, the average of these values was considered for each mouse. MAP and HR were evaluated at 0, 24 and 48 h after TAA administration.

Procedures for electroencephalography recording

Surgery procedures: Mice (n = 8) were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Prophylactic treatment with antibiotics (enrofloxacin; 10 mg/kg; *s.c.*) was done in order to prevent post-surgical infections. The animals were submitted to surgery for electroencephalography (EEG) electrode implantation in the right and left parietal cortices. The electrodes for superficial EEG recordings were made with surgical screws (Fine Science Tools; model 19010-00; Foster City, CA, United States) previously soldered to Teflon-coated stainless-steel wires (A&M Systems; model 7916; Carlsborg, WA, United States) and introduced bilaterally in the parietal bones. A reference electrode, also made with surgical screws, was inserted in the nasal bone. All the electrodes were soldered to a common pin connector and anchored to the cranium with dental acrylic. Following surgery, animals were allowed to recover for at least 4 days before EEG recordings.

Experimental protocol: Mice were injected with thioacetamide in the same regime (n = 5) or saline (n = 3). Each mouse was recorded in three consecutive days, namely: day 1, before drug injection; days 2 and 3, after first and second drug injections, respectively. Mice were recorded for a period of 10 min each day. Bioelectrical activity was recorded with a video-EEG recording system (8:00-11:00 am). The EEG signal from both parietal cortices was amplified (1000 × gain) and filtered (1 Hz High pass, 500 Hz Low pass) by a signal conditioner (Aisha4 - Kananda® Ltda). Data were sampled at 1 kHz (12 bit DI-148U A/D converter - DATAQ® Instruments) and recorded in a computer hard disk for offline analyses. Spectral analysis and 3D rendering of EEG spectrum were done using MatLab® scripts.

Magnetic resonance imaging: Acquisition and analysis

MR image experiments were acquired using 4.7T NMR system (Oxford Systems) controlled by a UNITY Inova-200 imaging console (Varian). The imaging protocol consisted of coronal T2-weighted (TR = 3000 ms, TE = 50 ms) spin echo multislice scans, 16 contiguous 1 mm thick slices. Mice (n= 12) were anesthetized with halothane (4% induction, 1.5% maintenance) and oxygen (1.5 l/min) delivered by a facemask in a head holder to minimize artifact movements. Animals anesthetized for the duration of an imaging experiment (50 min) recovered with no apparent difficulty and could be used for subsequent imaging studies. Each mouse was imaged in three consecutive days, namely: day 1, before drug injection; days 2 and 3, after first and second drug injections, respectively. Brain masks, based on the anatomical scans, were done using a tablet driver (Bamboo Tablet Driver, V5.2.5 WIN, WACOM Technology Corporation, United States) and MeVisLab software (MeVis Medical Solutions AG, Fraunhofer). Additionally, densitometry analysis was done using MatLab® scripts and repeated measures. ANOVA was used to compare densitometry values on different days.

Statistical analysis

Statistical analyzes were performed using one-way ANOVA (Tukey's post test) and Student's t test. P values less than 0.05 were considered statistically significant. All data are presented as mean ± SE.

RESULTS

Repeated TAA injections caused neuropsychomotor changes, brain edema and hyperammonemia, with EEG spectrum suggestive of metabolic encephalopathy

As shown in Figure 1A, around 30% of mice treated with a single dose of TAA died after 24 h and more than 40% succumbed due to a second TAA administration. Also, a progressive increase in neurological score (reflecting a decreased neuropsychomotor activity) was observed (Figure 1B) and, probably due to such reduced mobility, TAA-treated mice presented with a significant weight loss in comparison to controls throughout the experiment (Figure 1C). In addition, TAA treatment caused serum hyperammonemia after 24 h (Figure 1D), which was also detected in the brain in later timepoints (48 h; Figure 1E). Taking into account the edematogenic effects of cerebral ammonia accumulation, we imaged mice brains using magnetic resonance (MR) to investigate potential morphological alterations, including suggestive areas of fluid accumulation. MR revealed a discrete but diffuse brain edema in TAA-treated mice, which was not observed in

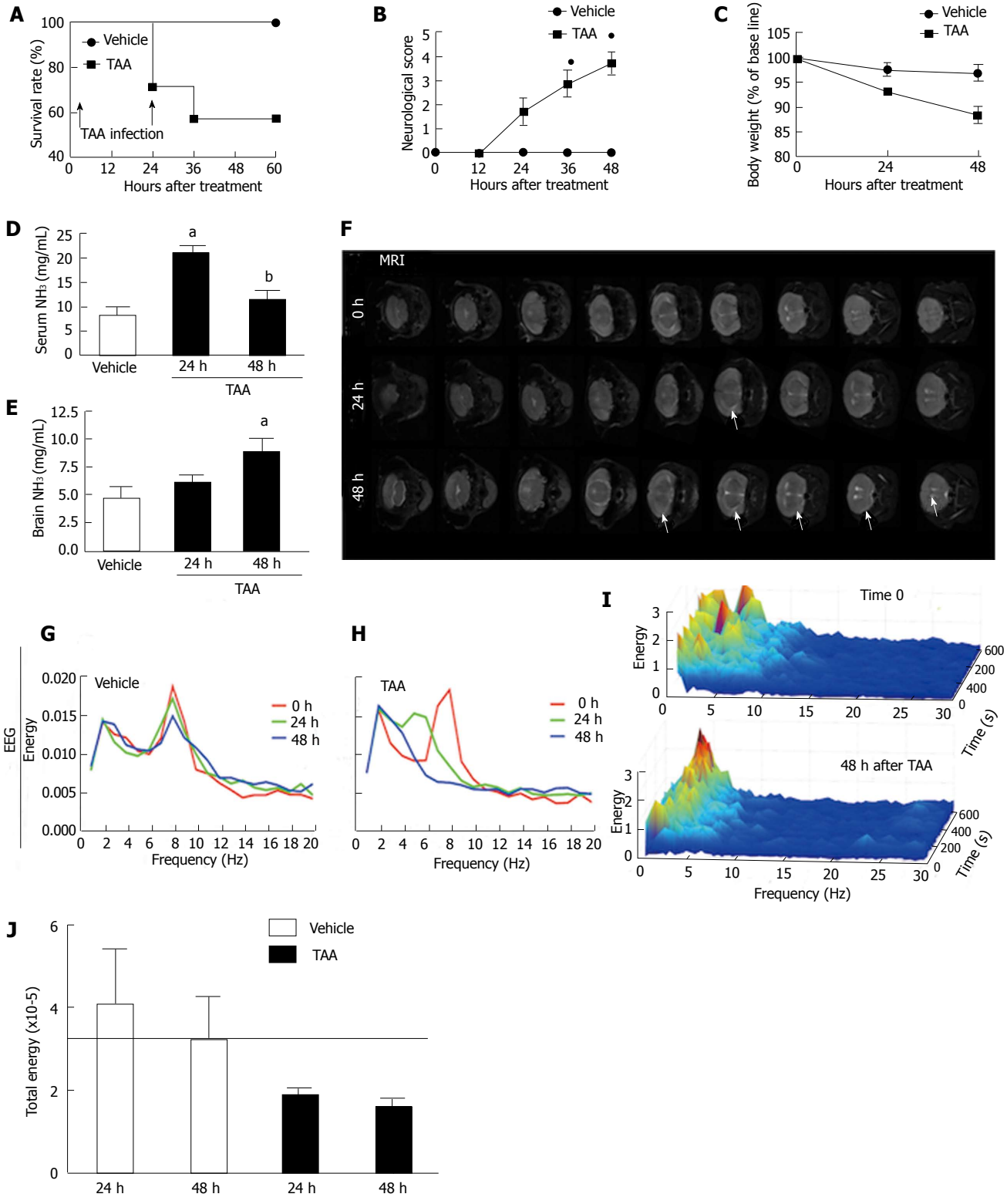

Figure 1 Thioacetamide treatment triggered neuropsychomotor changes, diffuse brain edema and hyperammonemia, with electroencephalography spectrum compatible with of encephalopathy. A: Survival rate following repeated TAA injections (arrows); B: Neurological score was assessed through all experimental procedure. Neuropsychomotor deficit increases as higher is the score average, with 1 = normal and 5 = death; C: Mice treated with TAA also presented with significant weight loss in comparison to controls; D, E: Serum ammonemia was detected following 24 h of TAA injection, while brain ammonia concentration increased gradually, reaching significant higher values after 48 h (E); F: In agreement, MRI from TAA-treated mice brains showed discrete, but diffuse edema as pointed by arrow heads. G, H: EEG analysis revealed that while controls had coincident EEG records during the experimental protocol (G), brain waves spectrum of TAA-treated mice (H) was compatible with metabolic encephalopathy; I: 3D rendering of 60 minutes EEG record (Z axis) plotted comparing energy (in Y axis) with frequency (X axis). Note the increase in higher frequencies waves (theta and alpha; 4-8 and 8-13 Hz, respectively) with a concomitant increase of lower frequency ones (mainly delta; up to 4 Hz). Best case results were depicted here; $N = 8$ for each group; J: Total EEG energy was also reduced in TAA-treated group. aIndicates statistical significance in comparison to controls and bin comparison to 24 h group. $P < 0.05$, analysis of variance (Tukey's post test). $N \geqslant 5$ for each group. TAA: Thioacetamide; EEG: Electroencephalography.

controls (Figure 1F). Increased fluid accumulation was more evident following 48 h of TAA treatment; however, such a feature was not prominent. In fact, no significant differences were observed in brain densitometry during analysis of MRI images (data not shown), suggesting that a mild brain edema was occurring in this model. To further dissect the neurological effects of TAA poisoning, we submitted mice to daily EEG registration throughout the experimental protocol. Analysis of the EEG spectrum revealed that while untreated mice had coincident EEG records during all experimental protocol (Figure 1G), TAA administration caused a progressive decrease in higher frequencies waves (*theta* and *alpha*; 4-8 and 8-13 Hz, respectively) with a concomitant increase of lower frequency ones (mainly *delta*; up to 4 Hz; Figure 1H-I). Such an EEG profile, with concentration of energy mainly in *delta* waves region and significant reduction in total energy (Figure 1J), confirmed that repeated TAA administration led to a diffuse brain lesion and metabolic encephalopathy.

Severe liver necrosis and inflammation triggered metabolic encephalopathy, coagulopathy and remote lung injury

It is well established that impaired liver function caused by extensive hepatocyte death leads to a general deficiency in metabolism, including ammonia depuration and synthesis of coagulation cascade factors[3]. Liver intravital microscopy revealed a marked increase in necrotic cells (stained by propidium iodide; in red) following TAA exposure (Figure 2A), which was also confirmed by histology analysis (Figure 2B). Also, we observed an exuberant neutrophil accumulation in liver necrotic areas, which increased throughout TAA intoxication process (Figure 2A; eGFP-expressing cells). Macroscopically, livers from TAA treated mice displayed extensive areas of necrosis (Figure 2C), which became more obvious after repeated TAA administration (48 h). Significantly higher serum levels of alanine aminotransferase (ALT) also indicated massive liver injury, which was sustained during the whole experimental period (Figure 2D). In fact, a complete lack of hemostatic function also confirmed hepatotoxicity and organ failure, suggesting that synthesis of liver-derived coagulation factors was seriously impaired. While controls had normal hemostatic parameters (MAP and HR), TAA-treated mice had a prolonged (undetermined) prothrombin and partial thromboplastin time (Figure 2E, F). Moreover, while control mice had normal hemodynamic parameters, the TAA-treated group had a significant drop in MAP (approximately 35%) with concomitant tachycardia, suggesting that mice might be evolving to a hemodynamic shock (Figure 2G, H). In this direction, we hypothesized that in our model TAA might also cause multiple organ failure and remote injury.

We have previously shown that necrosis-derived products may reach systemic circulation and trigger remote inflammatory responses in organs including the lungs[12]. Here, we evaluated the potential of TAA in induction of lung inflammation. Histopathological analysis showed that TAA-induced liver injury also caused pulmonary injury. After 24 h, lungs from mice had increased cellularity in lung parenchyma, alveolar edema and hemorrhage in comparison to controls (Figure 1I; arrows). Those observations were found to be more pronounced after 48 h, showing increased tissue damage, alveolar hemorrhage and lung architecture disruption, suggesting that lethality induced by TAA can also be due to exacerbation of lung inflammatory response in this model.

DISCUSSION

The hepatotoxic ability of TAA is well described in the literature and this drug has been extensively used in rat models of ALF and HE in sequential repeated dose administration[9,13-16]. Once in circulation, TAA is absorbed and bioactivated by hepatocytes. This metabolite will finally modify aminolipids and proteins, causing cell damage and death[17]. In mice, acute TAA poisoning causes centrilobular necrosis and an increase in plasma transaminases and bilirubin. We have shown that following two daily TAA injections, mice developed a massive and progressive liver damage and failure, with hyperammonemia in serum and brain, with discrete signals of cerebral edema.

Hyperammonemia is a hallmark of HE pathophysiology. In patients with ALF, the arterial ammonia level is directly related to severity of HE and development of intracranial hypertension[18]. Also, persistent hyperammonemia seems to be more important than a transient increase in the ammonia level regarding the occurrence of intracranial hypertension[19]. Most studies in mice used a single dose of a hepatotoxic drug which caused a briefer period of illness[20-22]. To supersede this, we developed a novel protocol using a lower TAA dose (300 mg/kg) and repeated administration (2 daily doses), trying to mimic a more realistic scenario closer to severe HE. We found that under these conditions, mice progressively presented with neuropsychomotor deficiencies (as assessed by neurological score)[23], which was accompanied by persistent liver injury and failure. Interestingly, serum ammonia concentration peaked after the first TAA administration, decreasing at 48 h. However, cerebral ammonia gradually increased throughout the experimental protocol, reaching significantly higher levels 48 h after TAA, suggesting that probably excessive serum levels of ammonia might be transferred to the brain due to deficient liver ammonia clearance. The mechanisms responsible for ammonia-mediated encephalopathy and brain edema are still under debate; however, it is accepted that increased blood-derived ammonia intensifies glutamine synthesis via amidation of glutamate, generating a hyperosmotic environment within brain cells (mainly astrocytes)[24]. Consequently, cerebral liquid accumulation leads to intracranial hypertension, herniation and coma. In agreement with this, magnetic resonance imaging from mice treated with TAA revealed a discrete, but crescent-shaped and diffuse

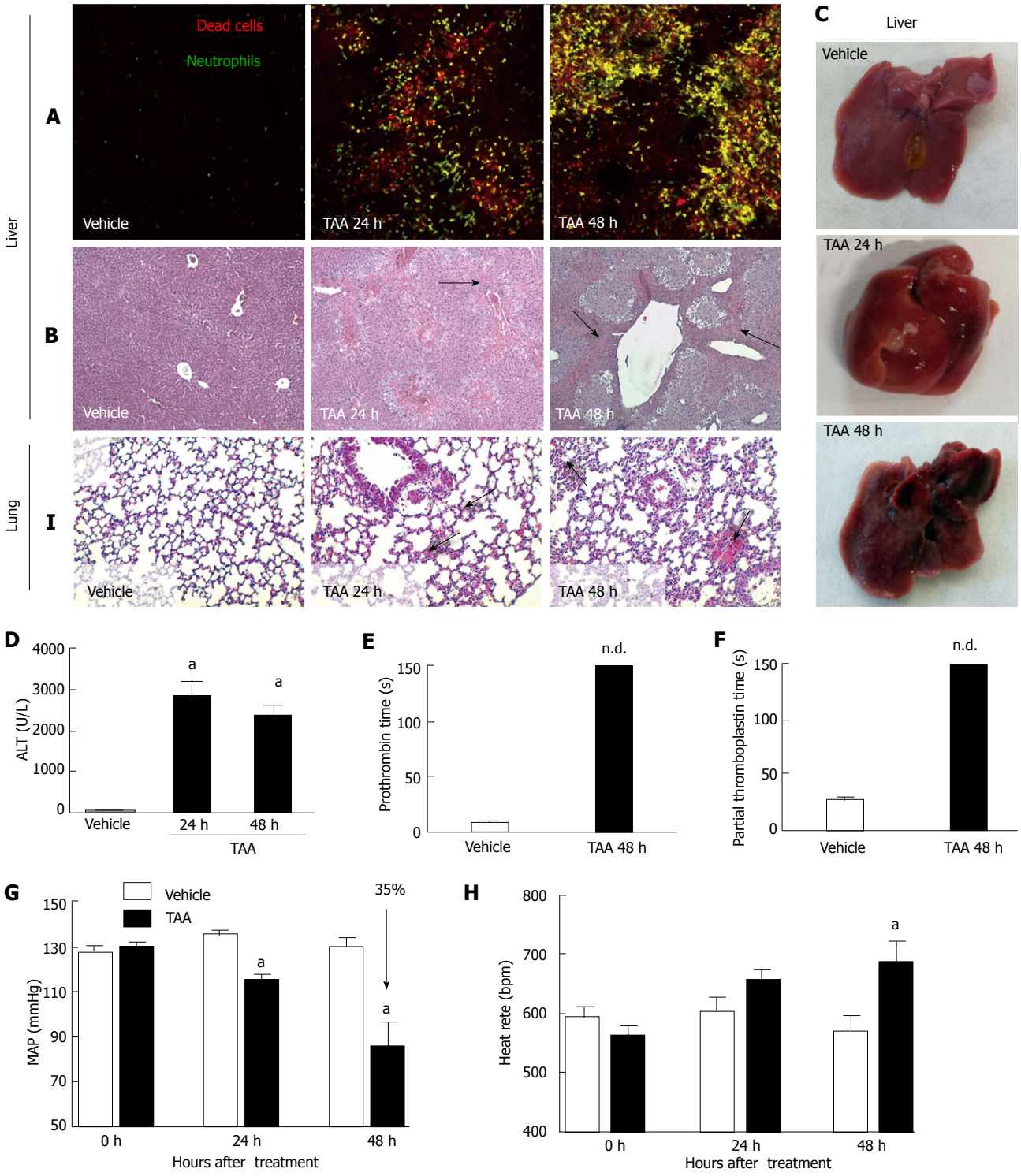

Figure 2 Thioacetamide caused severe liver necrosis and inflammation, which may explain mice metabolic encephalopathy, coagulopathy and remote lung injury. A: Liver intravital microscopy showing crescent number of necrotic cells (in red, propidium iodide) with concomitant neutrophil infiltration (in green, Lysm-eGFP mice); B: Liver sections stained by hematoxylin and eosin (4 × increase). Arrows indicate necrotic areas; C, D: Liver macroscopic analysis confirmed extensive and diffuse necrosis, which is also reflected by elevated serum transaminase activity (D); E, F: Liver failure was confirmed by prolonged prothrombin and partial thromboplastin times; G, H: Also, significant drop in mean arterial pressure (MAP; G) and increased heart rate (H), as assessed by tail cuff method, suggested that TAA-treated mice also evolved to hemodynamic shock; I: After TAA treatment hours, lungs from mice had increased cellularity in lung parenchyma, alveolar edema and hemorrhage in comparison to controls (arrows, 4 x increase) aIndicates statistical significance in comparison to controls. $^{a}P < 0.05$, ANOVA (Tukey's post test). N ⩾ 5 for each group. TAA: Thioacetamide.

brain edema[25]. In addition, through analysis of the EEG spectrum we found diffuse a brain lesion compatible with metabolic encephalopathy[26], suggesting that our DILI model reproduces some of the main clinical manifestations of HE[27]. In conjunct, our data suggest that this model may be suitable for further studies involving ALF,

hyperammonemia and brain edema.

We also investigated the mechanisms involved in liver damage triggered by TAA in mice. Intravital microscopy revealed that large areas of liver necrosis were infiltrated by neutrophils and such sterile inflammation has been described as a key factor for injury amplification[12]. Furthermore, we also observed repercussions in hemodynamic functions and inflammatory infiltration in the lungs. In this sense, the innate immune response triggered by necrosis-derived products may add to direct TAA-mediated hepatotoxicity to establish not only ALF, but also remote organ injury. Accordingly, TAA-treated mice displayed a severe impairment in hemostatic function compatible with an end-stage liver failure, with coagulopathy, hypovolemic shock and possible multiple organ failure. Thus, these factors should be also evaluated in future studies primarily dedicated to the liver or central nervous system.

In conclusion, we have shown that repeated lower doses of TAA might constitute as a novel murine model of HE and ALF, which reproduce several features of human disease. Also, we provided read-outs for disease grade and severity that comprise behavioral changes, brain edema and ammonemia, hemodynamic parameters and inflammatory response.

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COMMENTS

Background

Acute liver failure (ALF) is a rare but severe clinical syndrome. ALF is the result of massive liver injury, which can be caused mostly by drugs or viruses. During the disease, patients may suffer hepatic encephalopathy, multiple organ dysfunction and sepsis, leading to a high mortality rate. The incidence of ALF is increasing worldwide and liver transplantation and supportive medical care are the only therapies available for ALF.

Research frontiers

Hepatic encephalopathy (HE) is one of the major complications in ALF because of rapidly progressing brain edema and hypertension. Hence, understanding HE pathophysiology is crucial for development of new and specific therapies that would slow down its progression and increase chances of better outcomes. In this sense, a murine model that reproduces most features of HE in humans would be key to improve our understanding of HE pathophysiology and may also be useful for drug testing and development.

Innovations and breakthroughs

This study provided a new model to study hepatic encephalopathy and acute liver failure in mice. In this model, mice are treated twice with a low dose thioacetamide, causing extensive liver injury and characteristic complications of human HE, including high ammonia levels, altered electroencephalography (EEG), brain edema and poor neuropsychomotor function. In addition, mice presented with a systemic response similar to multiple organ failure syndrome, where lung inflammation, coagulopathy and hemodynamic alterations were observed.

Applications

This is a new model to study ALF and HE in mice, the most used animal model. Also, this is the first animal model to present such similar symptoms to the human disease, making it a more useful research tool for this biomedical area.

Terminology

The most important terms in this article are: ALF, HE and thioacetamide.

Peer review

The authors describe a murine model of acute hepatic failure to study hepatic encephalopathy by sequential administration of thioacetamide intraperitoneally. The hypothesis is convenient, the methodology for acute hepatic failure was suitable, the evaluation of hepatic encephalopathy was convenient and sophisticated, the results were interesting and the discussion appropriate.

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CLINICAL PRACTICE

Nuclear medicine dynamic investigations in the diagnosis of Budd-Chiari syndrome

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Author contributions: Dragoteanu M was the leader of the research team, coordinated the practical procedures, developed the method of using per-rectal portal scintigraphy and liver angioscintigraphy to investigate the liver hemodynamics, conducted the analysis of data and wrote the paper; Balea IA contributed to the data analysis and writing of the paper; Piglesan CD performed the practical procedures of the patients and acquisition of data.

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Abstract

AIM: To investigate the hepatic hemodynamics in the Budd-Chiari syndrome (BCS) using per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS).

METHODS: Fourteen consecutive patients with BCS were evaluated by PRPS between 2003 and 2012. Ten of them underwent LAS and liver scan (LS) with Tc-99m colloid. Eleven patients had clinical manifestations and three were asymptomatic, incidentally diagnosed at PRPS. The control group included 15 healthy subjects. We used new parameters at PRPS, the liver transit time of portal inflow and the blood circulation time between the right heart and liver. PRPS offered information on the hepatic areas missing venous outflow or portal inflow, length and extent of the lesions, open portosystemic shunts (PSS), involvement of the caudate lobe (CL) as an intrahepatic shunt and flow reversal in the splenic vein. LAS was useful in the differential diagnosis between the BCS and portal obstructions, highlighting the hepatic artery buffer response and reversed portal flow. LS offered complementary data, especially on the CL.

RESULTS: We described three hemodynamic categories of the BCS with several subtypes and stages, based on the finding that perfusion changes depend on the initial number and succession in time of the hepatic veins (HVs) obstructions. Obstruction of one hepatic vein (HV) did not cause opening of PSS. The BCS debuted by common obstruction of two HVs had different hemodynamic aspects in acute and chronic stages after subsequent obstruction of the third HV. In chronic stages, obstruction of two HVs resulted in opening of PSS. The BCS, determined by thrombosis of the terminal part of the inferior vena cava, presented in the acute stage with open PSS with low speed flow. At least several weeks are required in the obstructions of two or three HVs for the spontaneous opening of dynamically efficient PSS. The CL seems to have only a transient important role of intrahepatic shunt in several types of the BCS.

CONCLUSION: Dynamic nuclear medicine investigations assess the extent and length of hepatic venous obstructions, open collaterals, areas without portal inflow, hemodynamic function of the CL and reverse venous flow.

Key words: Budd-Chiari syndrome; Per-rectal portal scintigraphy; Liver angioscintigraphy; Caudate lobe; Hepatic veins

Core tip: Per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS) are reliable investigations of the liver hemodynamics in the Budd-Chiari syndrome (BCS). Diagnosis of the number, length and succession in time of hepatic vein obstructions allows identification

of hemodynamic varieties and stages of the BCS. Our new PRPS parameters, liver transit time and right heart to liver time, are used to diagnose obstructed hepatic veins, areas missing venous outflow or portal inflow, open collaterals, reverse splenic vein flow and hemodynamic role of the caudate lobe. LAS is useful in the differential diagnosis with portal occlusions, highlighting arterial-venous shunts and reverse portal flow.

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INTRODUCTION

The Budd-Chiari syndrome (BCS) is determined by hepatic venous obstruction localized from the small hepatic veins (HVs) to the terminal part of the inferior vena cava (IVC), resulting in increased sinusoidal pressure, hepatic congestion and portal hypertension (PHT)[1]. The natural outcome is poor in many cases, with a three year survival rate of about 10%[2,3]. Clinical manifestations are extremely varied and include ascites, jaundice, hepatomegaly, splenomegaly, collateral veins and upper right abdominal quadrant pain[4]. Ascites fluid has a characteristic high protein concentration (> 2.5 g/dL).

A widely accepted classification is based on etiology, site of obstruction, manifestations and duration of the disease[5]. Primary BCS is produced by thrombosis, its sequels or web obstruction[6]. Secondary forms are determined by malignant or parasitic obstruction of the lumen or by extrinsic compression, commonly produced by tumors[7]. The site involved by the obstruction and the affected HVs are commonly diagnosed through non-invasive imaging [ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT)] or by using venography[8].

Clinical approach accounts for the severity of disease (fulminant/non-fulminant) and its duration (acute, subacute or chronic). Subacute forms show signs or symptoms for less than six months and no evidence of liver cirrhosis. Chronic evolution is characterized by onset over six months, with evidence of PHT and cirrhosis[9]. However, the severity of liver damage may be inconsistent with apparent symptomatology. Recent clinical onset may be discordant with advanced liver fibrosis, suggesting a long course without clinical symptoms. Asymptomatic disease (10%-15% of cases) is usually associated with the obstruction of only one HV but also with spontaneous development of dynamically efficient intrahepatic collaterals and extrahepatic portosystemic shunts (PSS), incidentally diagnosed by imaging at surgery or necropsy[10]. For the patients with acute clinical onset but with advanced histological damage, a recent hepatic venous obstruction added to older obstructions of other HVs has to be suspected. In acute forms, the liver area without physiological outflow is hypertrophied and PHT occurs. Because of the higher pressure (35 mmHg) in the hepatic artery (HA) than in the portal vein (PV) branches (3-6 mmHg), the portal flow may be reversed.

Chronic alterations include parenchyma atrophy and extended fibrosis, gaining a cirrhotic appearance and considerably reducing both portal and arterial inflow. Splenomegaly is found in a third of patients[11].

Thrombosis is the usual cause for the occlusion of large HVs, while the obstructions of the IVC or of the small HVs are rarely thrombotic[12].

The anatomical varieties of the HVs have to be accounted for, both for the diagnosis and surgical treatment. A common opening into the IVC of the left hepatic vein (LHV) and middle hepatic vein (MHV) was reported in around 55%-60% of cases[13]. Other varieties, such as a common trunk of the MHV and right hepatic vein (RHV), separate opening of the three HVs into the IVC or the existence of an accessory RHV, were identified in various percentages[14-16]. The caudate lobe (CL) hemodynamic status is important as it may be an anastomosis between the obstructed HVs and the IVC. CL hypertrophy is described in 65%-75% of cases, with good sensitivity but not specificity[17]. A caliber of the CL vein higher than 3 mm is considered diagnostic for the BCS[18].

US usually demonstrates altered HVs and hypertrophy of the CL[19]. Duplex Doppler sonography is widely used, offering a good assessment of the blood flow through the HVs[20,21]. Color Doppler sonography allows a more reliable identification of abnormalities of the HVs than conventional sonography and detects collateral vessels not visible with other techniques. The lack of flow signals in the HVs, intrahepatic and extrahepatic collaterals, together with reverse, slow or turbulent portal flow, are characteristic findings. US techniques should be the first line investigations, due to a low cost and a diagnostic sensitivity of more than 75%. There are, however, cases where the occlusion of HVs is difficult to demonstrate by US, even by color Doppler imaging[22].

CT scans offer a good assessment of thrombosis of the HVs or IVC, global liver enlargement, abnormalities of liver structure, size and direction of the venous flow. The contrast-enhanced helical CT allows a good dynamic visualization of HVs[23]. MRI provides useful images of the hepatic venous outflow and thrombosis of HVs, as second line investigations together with CT[24,25]. It can be difficult to diagnose spontaneous intrahepatic anastomoses and prominent azygos and hemiazygos veins (especially in IVC thrombosis) on MRI[26]. The liver biopsy has limited value due to the inhomogeneous distribution of liver lesions in the BCS[27].

Per-rectal portal scintigraphy (PRPS) was used over the last decades to investigate PHT and PSS in chronic liver disease (CLD) by evaluating a per-rectal portal shunt index[28,29]. Detailed information may be acquired about liver hemodynamics by using the parameters introduced by us in the interpretation of PRPS dynamic curves - liver transit time (LTT) and right heart to liver

time (RHLT)[30].

Liver angioscintigraphy (LAS) evaluates the contribution of arterial inflow to the total liver perfusion. It is especially useful in the differential diagnosis between the BCS and obstructions of the portal branches. Liver areas missing portal inflow have compensatory increased arterial inflow due to the hepatic artery buffer response (HABR)[31] and a characteristic pattern at LAS, with abrupt arterial entry and a flattened portal segment.

Liver scan (LS) with Tc-99m labeled colloid performed after LAS may show changes in radiotracer capture by liver, spleen and spine. Increased radioactivity on the CL area is considered a characteristic finding in the BCS[32].

The aim of this study is to underline the diagnostic possibilities in the BCS of the PRPS and LAS, with auxiliary use of the LS. The goals are assessment of liver areas missing venous drainage, length and order of the appearance of lesions, arterial and portal perfusion changes, existence of dynamically efficient collaterals and of supplementary drainage through the CL.

MATERIALS AND METHODS

We evaluated 14 consecutive patients with the BCS between 2003 and 2012, 9 females and 5 males, between 20 and 63 years old. Eleven patients had clinically manifested BCS and 3 were asymptomatic, incidentally identified at PRPS among over 400 patients explored for chronic liver disease (CLD) staging. The control group included 15 healthy subjects from the laboratory casework, 7 men and 8 women, between 19 and 67 years old. All 14 patients with BCS underwent PRPS. LAS and LS were performed in 10 of them. Anonymity of the patients was respected. All persons gave their informed consent prior to the investigations, accepting inclusion in research studies. The study was realized as part of routine clinical practice.

All the investigations were performed by using a single photon emission computed tomography (SPECT) Orbiter Siemens gamma-camera with high resolution, low-energy, parallel collimator, connected to a Power Macintosh computer, using ICON dedicated software. We used Tc-99m sodium pertechnetate, eluted from Drygen generators (General Electric, Amersham, United Kindom) and Fyton colloid (Institute of Isotopes, Budapest, Hungary).

PRPS was performed using the method developed by Shiomi *et al*[33]. A solution containing 2 mL (296-370 MBq/8-10 mCi) of Tc-99m sodium pertechnetate was instilled at PRPS into the upper part of the rectum through a Nelaton tube, followed by 15 mL of air under pressure. Serial scintigrams were recorded every 2 s for 5 min. Radioactivity curves were built thereafter by computer on liver and heart areas to analyze the dynamics of the radiotracer absorbed from the rectum. The patients were told to fast from the evening preceding the test. Two enemas were performed in each patient, the first the previous evening and the the second one 2 h prior to the PRPS. The patients were placed in a supine position, with the camera detector in the anterior view, including in the field the liver and heart areas.

LTT and RHLT allow detailed assessment of liver hemodynamics in the BCS. These time parameters can be assessed for particular hepatic areas or for the whole liver. The portal inflow is missing in those areas where the tracer arrives (through the HA) with a delay equal to RHLT after entering into the right heart (RH). LTT increased between 25-40 s shows a higher resistance opposed to the portal inflow. Values over 50 s of the time interval between the entering of tracer into the liver and its arrival to the RH may occur in the acute stage of the BCS produced by obstruction of the terminal part of the IVC. This high delay is determined by the slow flow through the PSS open to the superior vena cava. LTT between 15-23 s reflects a decrease of the resistance opposed by the liver to the portal inflow due to the enlargement of intrahepatic small size shunts between terminal portal branches and small HVs. Open extrahepatic PSS of high flow are emphasized at PRPS by arrival of the portal tracer to the RH before entering into the liver. PRPS also detects alterations of portal and arterial perfusion in the liver areas which maintain their physiological venous outflow, highlighting the changes determined by the redistribution of flow from the affected areas.

The arterial upward inflexion (AUI) of the PRPS curve on a liver area is placed at a time interval equal to RHLT after the moment when the tracer entered into the RH. The beginning of the AUI segment on our figures is noted HA, marking the arrival of tracer to the liver through the HA. The moments when the portal tracer enters into the different areas of the liver are noted as Co, Lo, Mo and Ro. The entering of tracer into the RH is noted as Ho. The slope of the AUI gives information about the amplitude of arterial inflow. The portal segment of the PRPS curve (before the AUI) offers information about the portal inflow and the dynamic resistance encountered. Summed images at PRPS resulting from the overlapping of all the sequential images may highlight the normal aspect, increased accumulation or low quantity of tracer in a liver area. The presence of the spleen on the summed image highlights reverse flow in the splenic vein.

LAS was performed by rapid bolus injection of 8 to 15 mCi (300-450 MBq) of Tc-99m labeled colloid in a volume smaller than 0.5 mL, followed by computer dynamic recording of sequential images during 1 min at a 1 s rate. The detector's area included the heart, liver and kidneys. Six patients underwent LAS in the posterior-anterior view (P-A) and four in the anterior-posterior view (A-P). Patients were asked to fast 12 h before the LAS. Dynamic time-radioactivity curves for the liver, spleen and left or right kidney were built by computer. The moment of the peak of the kidney curve corresponds on the liver curve to the upward inflection point when the portal inflow of tracer adds to the arterial inflow. The interval of 8 s on the liver curve before the kidney curve's peak corresponds to the arterial segment, while the 8 s interval after the peak represents the portal segment[34].

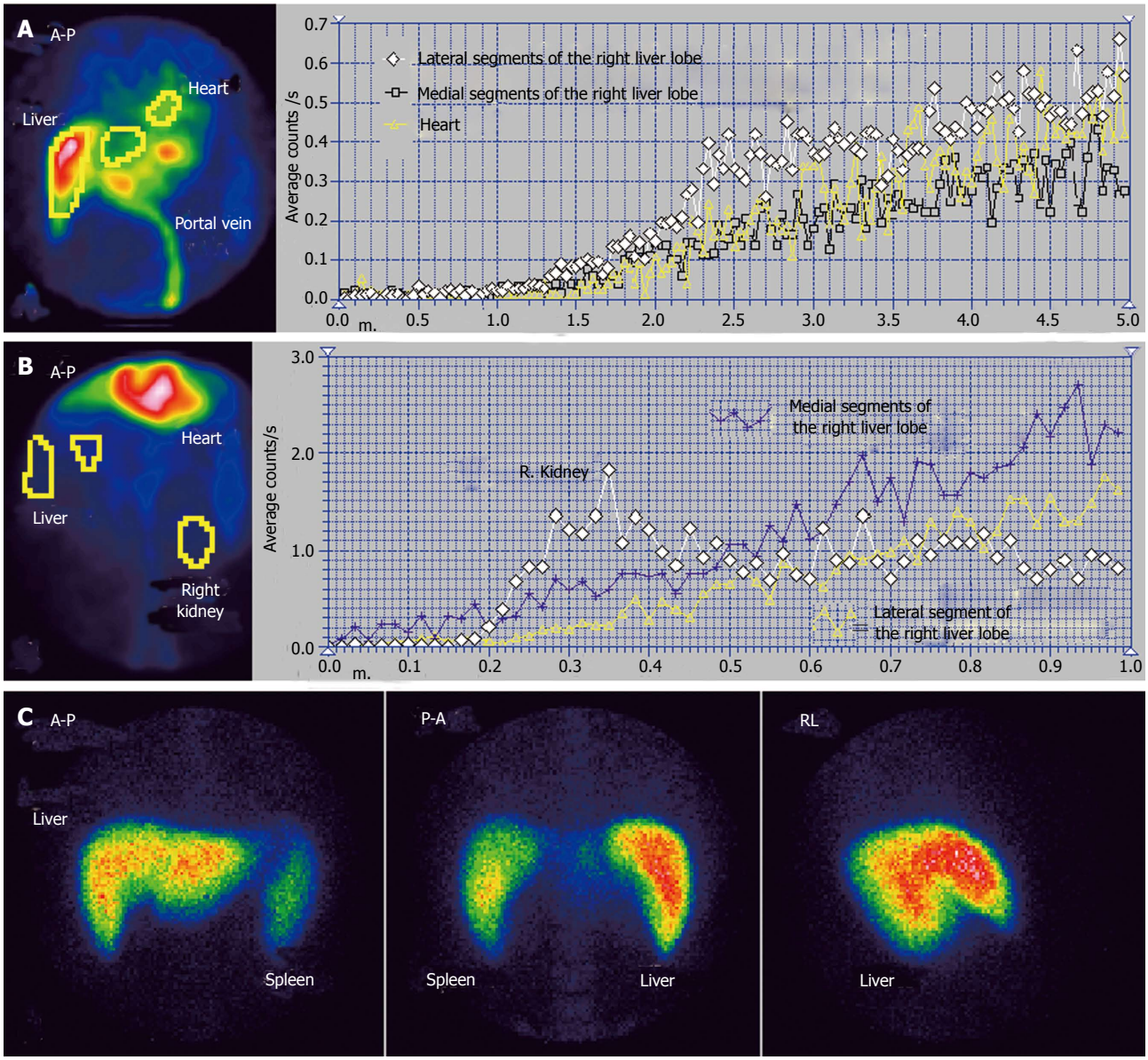

Figure 1 Budd-Chiari syndrome with obstruction of the middle hepatic vein. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

The shape of the LAS curve built on a liver area may highlight amplitude changes of the arterial flow, a higher resistance opposed to the blood flow or open arterial-venous shunts. Hepatic perfusion index (HPI) calculated at LAS is used to estimate the ratio between the arterial inflow and total liver perfusion[35]. HPI > 45% on an area of the right liver lobe (RLL) without tumors diagnoses a decrease in portal inflow, with reactive increase in the arterial flow determined by the HABR[36]. HPI > 100% emphasizes reverse flow in the portal vein. HPI can be accounted for the RLL only, as the left liver lobe (LLL) normally has an increased arterial inflow.

LS images were acquired beginning at least 15 min after the LAS, based on the same administration of Tc-99m labeled colloid. P-A, A-P and right lateral (RL) views were used. Time consuming SPECT was not considered to bring significantly more information on hemodynamic status than planar LS and was not performed.

RESULTS

We highlighted three hemodynamic categories of the BCS using PRPS and LAS: (1) BCS debuted by obstruction of one HV: asymptomatic, incidentally diagnosed and old obstruction of one HV followed by recent obstruction of the other two HVs; (2) BCS started by obstruction of two HVs: old obstruction of two HVs, followed by recent obstruction of the third HV, and old obstruction of two HVs, also followed by old obstruction of the third HV; and (3) BCS with acute onset due to simultaneous obstruction of all three HVs caused by obstruction of the terminal part of the IVC.

BCS with one obstructed HV, asymptomatic, incidentally diagnosed

We had three cases of asymptomatic BCS in our study, two with obstruction of the MHV and one with obstruc-

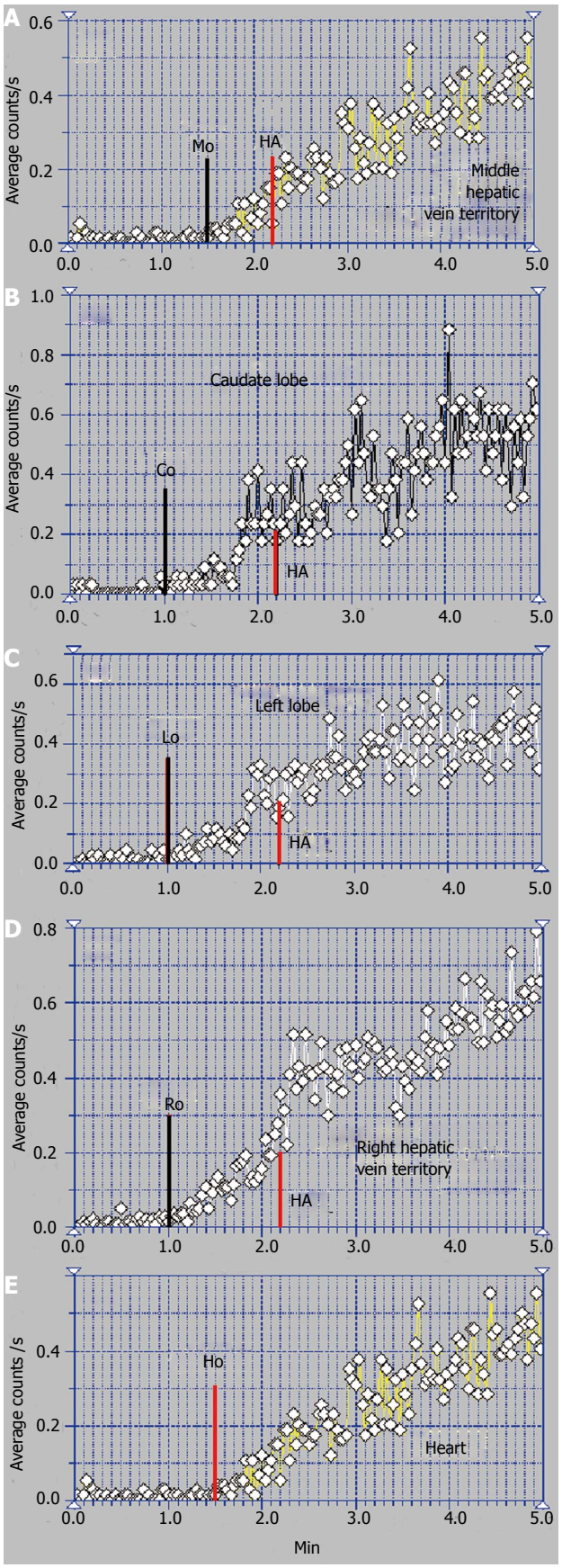

Figure 2 Per-rectal portal scintigraphy in Budd-Chiari syndrome with obstruction of the middle hepatic vein. A: Curve on the territory drained by the middle hepatic vein; B: Caudate lobe curve; C: Left lobe curve; D: Curve on the territory drained by the right hepatic vein; E: Heart curve.

tion of the RHV. All three were incidentally identified at PRPS.

The case presented in Figures 1 and 2 had MHV obstruction. The patient was a 36-year-old male, known to have chronic alcoholic hepatitis. US described inhomogeneous liver with regenerative nodules and dilated portal branches.

Summed image at PRPS (Figure 1A) reveals a decreased amount of radiotracer in the medial part of the RLL. The dynamic PRPS curves built on the RLL show highly different slopes on the medial and lateral segments. The arrival of portal tracer to the medial area was delayed about 30 s after the entrance into the lateral segments. Initial segment of the PRPS curve built on the medial area of the RLL has a slow slope, denoting moderately increased resistance opposed to the portal inflow (Figure 2A). The PRPS curve built on the lateral part of the RLL has an increased slope, showing a rise of its portal inflow (Figure 2D). The dynamic curve built on the CL has a low initial slope, indicating that the portal flow blocked due to the obstruction of the MHV was not drained through the CL. The LLL has a simultaneous entry of tracer as the CL and as the lateral area of the RLL, with a normal initial slope of the PRPS curve.

LTT is prolonged to 30 s, most likely due to the subjacent CLD, with increased resistance opposed to the portal inflow. The LS images confirm the diagnosis of alcoholic CLD, showing splenomegaly and hypertrophy of the LLL, with a normal aspect of the spine. Normal LS aspect of the medial area of the RLL suggests a subacute stage of the BCS, without cirrhotic changes of the affected parenchyma. PSS were not open, as the tracer arrived at the RH by passing through the liver. The LAS curve on the medial segments of the RLL is significant for arterial-venous shunts. The differential diagnosis accounted for the value of LTT and missing of the HABR, excluding a portal branch obstruction.

BCS with old obstruction of one HV and recent obstruction of the other two HVs

The patient presented in Figures 3 and 4 was a 32-year-old male, without a previous diagnosis of CLD. The patient complained of flatulence, vomiting and severe abdominal pain which had started several weeks earlier. US highlighted ascites and venous dilations. No blood flow in the HVs and no portal thrombosis were observed at the US, while numerous intrahepatic arterial-venous and venous-venous collaterals were described. Esophageal varices and gastric injuries were not seen on endoscopy.

The summed image at PRPS presents a sharply increased radioactivity on the CL area (Figure 3A). The LLL only has arterial inflow, receiving tracer after the RH with a delay equal to RHLT (Figure 4B). This suggests an old obstruction of the LHV, with chronic alteration of perfusion of the LLL. The clinical onset in this case resulted from a recent obstruction of the RHV and MHV, while the LHV had been obstructed for a long time. The PRPS curve on the RLL has low slopes on

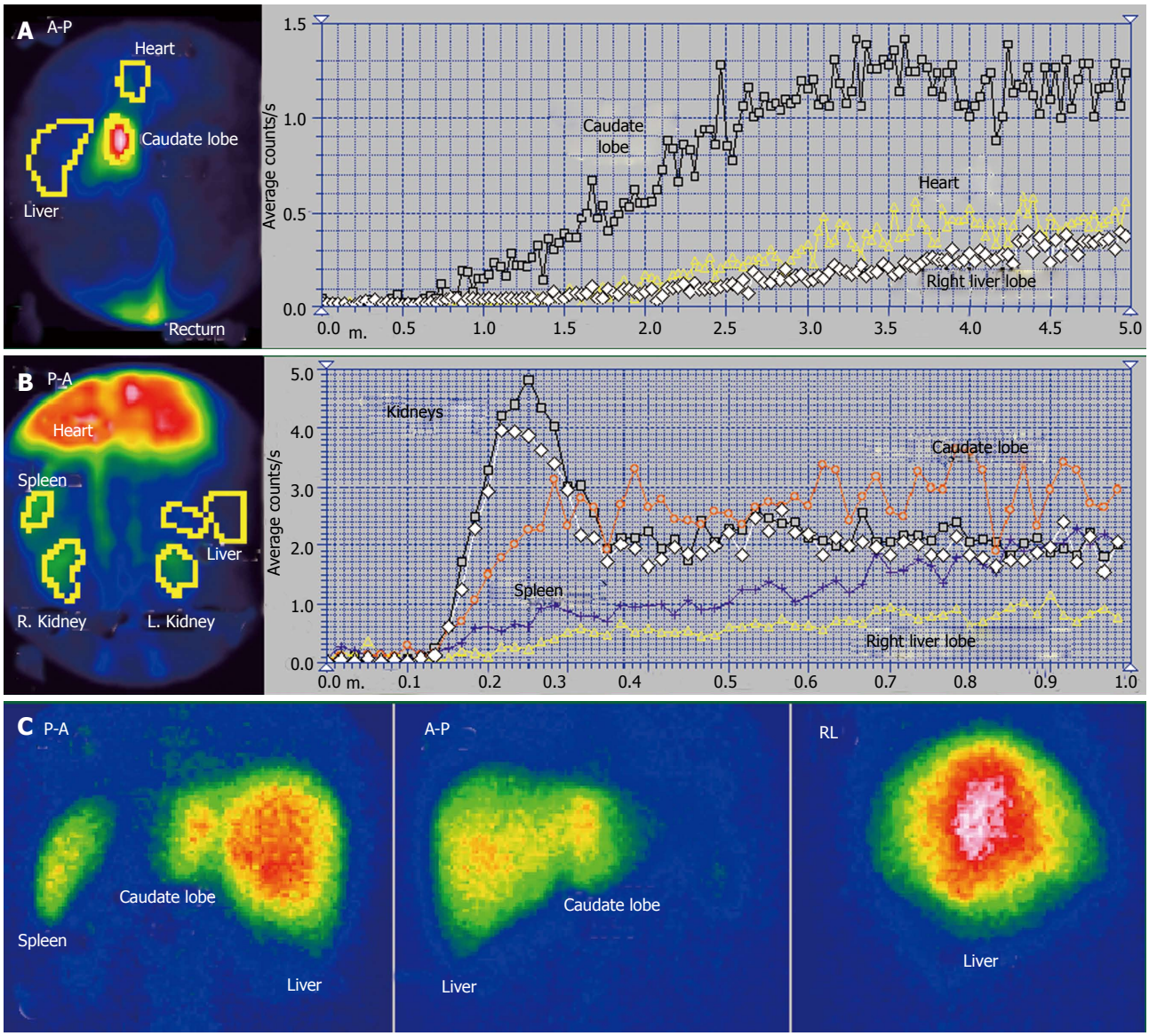

Figure 3 Budd-Chiari syndrome with old obstruction of the left hepatic vein and recent obstruction of the middle and right hepatic veins. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

the initial portal segment and on the AUI, showing that its portal and arterial inflows were decreased. The differential diagnosis excluded portal obstruction, accounting for small arterial inflow of the two liver lobes and missing the HABR.

Arrival of tracer to the RH at 30 s after entering into the CL shows that PSS were not open due to the earlier obstruction of one HV and the recent obstruction of the other two HVs (Figure 4). The tracer was detected in the RLL about 12 s after the CL, a slow portal inflow to the RLL being maintained.

Altered flow of the RLL was highlighted at the LAS (Figure 3B). After a short initial interval of about 1 s with normal arterial input, the LAS curve flattens on the rest of the arterial segment and has a low slope on the portal segment. The flattened curve on the RLL highlights the higher dynamic resistance encountered by the arterial inflow.

The ASH curve of the CL has an initial abrupt and high segment, due to the increased arterial inflow, followed by a flat portal segment and then by fluctuations of amplitude suggesting arterial-venous shunts (Figure 3B). The CL has a very high entering of portal tracer at the PRPS (Figure 4A). LTT on the CL is increased to 30 s due to the dynamic resistance opposed by the supplementary arterial and portal flows redirected from the RLL and LLL. The high amplitude of the PRPS curve on the CL was determined by the increased and slowed portal inflow. LS (Figure 3C) revealed increased radioactivity in the CL, inhomogeneous liver parenchyma and slightly increased radioactivity in the spleen.

The perfusion changes in the LLL and RLL suggest a subacute stage of the BCS. We underline that the old obstruction of the LLL together with more recent occlusion of the other two HVs did not open PSS. The arterial and portal blood flow of the whole liver was drained to the IVC through the CL.

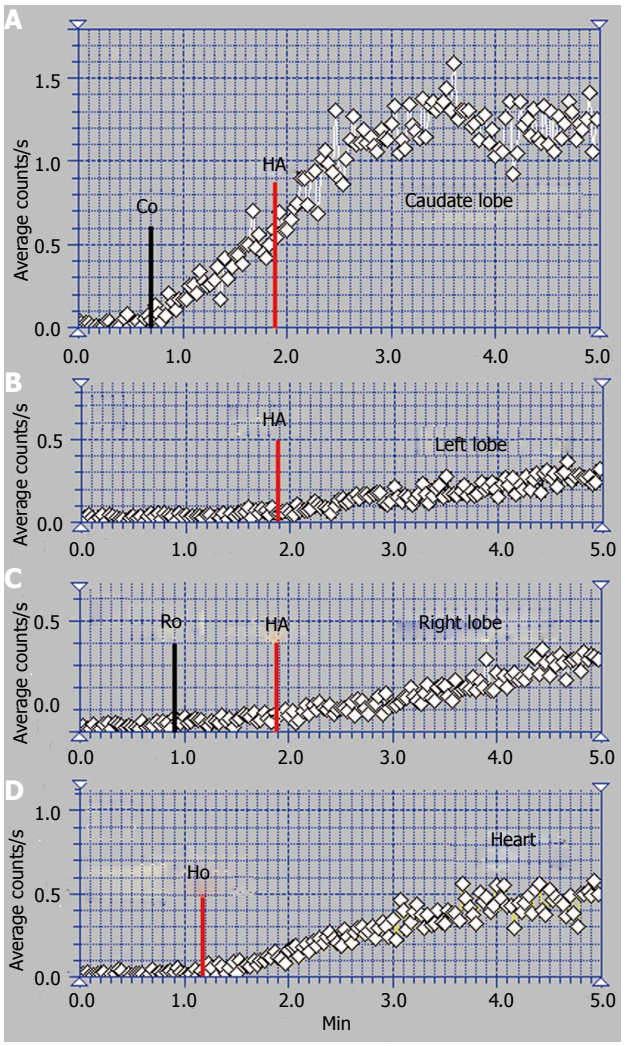

Figure 4 Per-rectal portal scintigraphy in Budd-Chiari syndrome with old obstruction of the left hepatic vein and recent obstruction of the middle and right hepatic veins. A: Caudate lobe curve; B: Left lobe curve; C: Right lobe curve; D: Heart curve.

BCS started by the common obstruction of two HVs, also followed by old obstruction of the third HV

The scintigraphy investigations in a patient with BCS with an old obstruction of the MHV and RHV and subsequent but also old obstruction of the LHV are presented in Figures 5 and 6. This 20 year old woman, a user of an oral contraceptive, was hospitalized with an impaired general condition and abdominal pain. The first symptoms appeared two and a half years earlier when the BCS was diagnosed. US at admission showed abundant ascites and PV dilation to 15 mm. Doppler US could not detect flow in the HVs.

The RLL received tracer at PRPS at a time interval equal to RHLT after the RH, emphasizing that the portal inflow was missing and the blood supply of the RLL came from the HA only (Figures 6C, D).

The tracer entered the LLL and the CL (Figure 6A, B) at about 25 s after reaching the RH, showing that their perfusion was mainly (but not completely) arterial. The initial low slopes of the CL and LLL curves were caused by increased resistance opposed to the portal inflow. The high slope of the AUI on the CL and LLL curves was determined by the large quantity of tracer that arrived through the HA. The slope of the AUI is smaller on the RLL than on the LLL and CL curves with about 50% (Figure 6C). Due to the older impairment of the RLL, its arterial perfusion per unit of area became lower than for the LLL and CL.

PSS were open, allowing the tracer absorbed from rectum to arrive faster to the RH than to the liver. The presence of the IVC on the summed PRPS image suggests the existence of open per-rectal PSS.

The ASH curve on the CL has a steep and biphasic entrance during the arterial segment. The portal phase has a drop in amplitude and subsequent fluctuations (Figure 5B). Increased arterial perfusion and existence of arterial-venous shunts are highlighted in the CL. The high arterial inflow suggests that the CL maintained part of its role of intrahepatic shunt to the IVC for the arterial inflow redirected from the rest of the liver. The ASH curve on the RLL shows increased arterial perfusion and reverse portal flow, with HPI = 115%.

The CL is visible on the LS, contrasting to the low colloid capture in the RLL (Figure 5C). The normal LS aspect of the spine and spleen argues against alcoholic or viral cirrhosis.

BCS with old obstruction of two HVs followed by recent obstruction of the third HV

We encountered one case of the BCS syndrome with old RHV and MHV obstruction and very recent occlusion of the LHV. One of these patients was a 32-year-old woman, an oral contraceptive user, with ascites in high quantity, increasing abdominal girth and without viral or alcoholic cirrhosis.

The time interval at PRPS (Figure 7) between the dynamic curves built on the heart and on the whole liver is equal to RHLT, showing that both the LLL and RLL missed portal inflow and the extrahepatic PSS of high flow were open. The high initial slope of the PRPS curve on the heart confirms that the PSS were hemodynamically efficient.

The highly increased radioactivity in the congested LLL on the summed PRPS image suggests that its deprivation of physiological venous drainage was very recent, while the obstruction of the MHV and RHV was old. The CL cannot be specifically distinguished on the PRPS summed image or by building dynamic curves on its area, therefore not playing a specific hemodynamic role. The image of the spleen on the summed PRPS image suggests inversion of flow in the splenic vein, while presence of the IVC suggests open per-rectal shunts.

BCS with acute onset by simultaneous obstruction of all three HVs (occlusion of terminal portion of the IVC)

Two of the BCS patients in our study had fulminant symptoms. US and CT scans argued for obstruction of

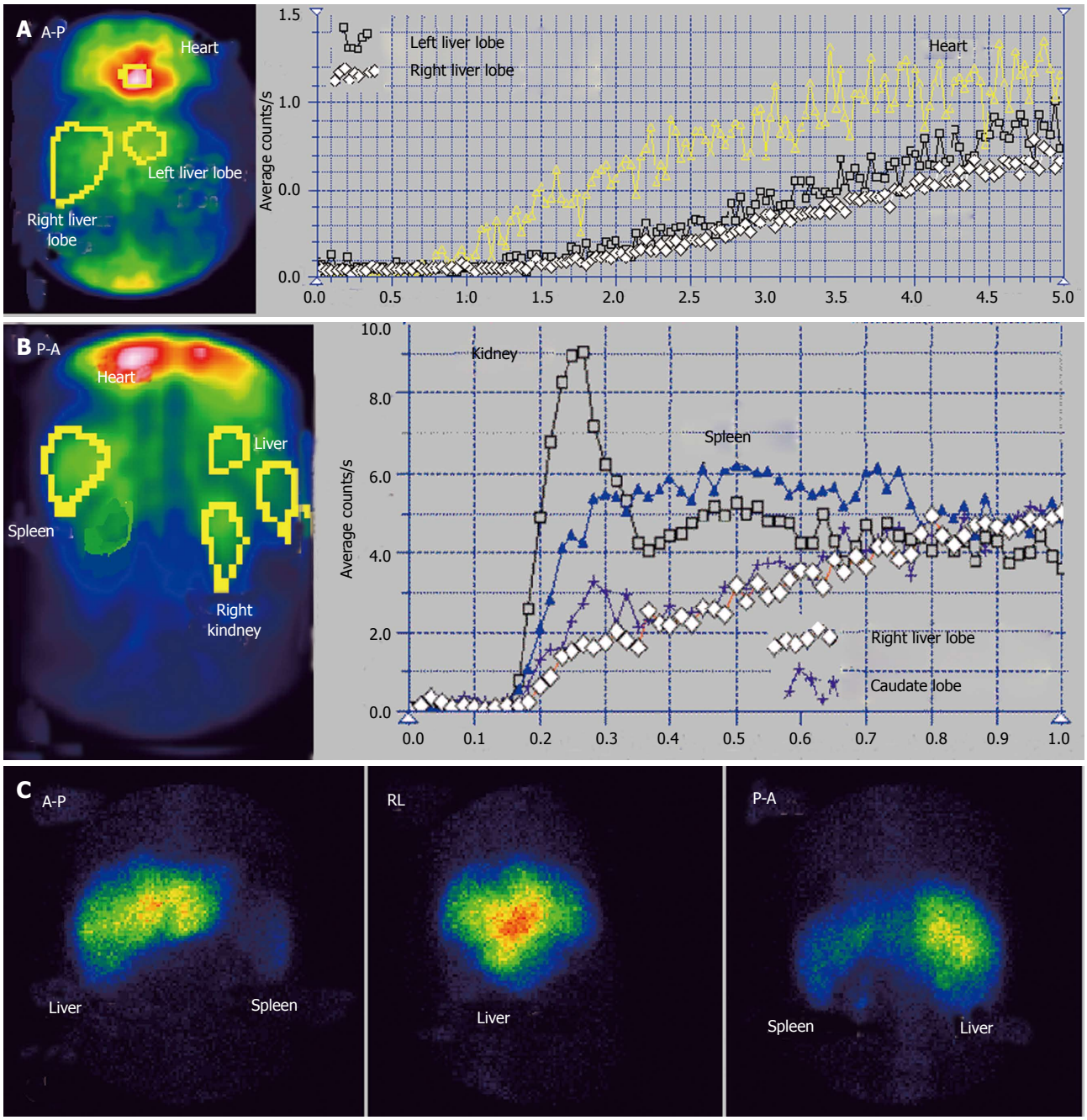

Figure 5 Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins also followed by old obstruction of the left hepatic vein. A: Per-rectal portal scintigraphy; B: Liver angioscintigraphy; C: Liver scan.

the terminal portion of the IVC in both of them. The data shown in Figure 8 belong to a 39-year-old woman with myeloproliferative disease, admitted for recently appeared severe pain in the upper right abdominal quadrant, with vomiting and encephalopathy signs. US and CT scans showed abundant ascites and significant dilation of the HVs.

PRPS highlights a sharp increase of the transit time of the tracer from entering into the liver to reaching the RH, up to values over 50 s. The amount of tracer passing through the heart during the first 30 s after its arrival was very low, with a flattened PRPS cardiac curve. The normal slope of the PRPS curves built on the liver lobes show that the dynamic resistance encountered by the portal flow was not significantly increased. The very late arrival to the RH of a small quantity of tracer was determined by the slow transit through the PSS open to the superior vena cava. PRPS highlights the completely blocked venous outflow on the physiological pathway between the liver and the RH, involving all the HVs. The slow flow through the PSS suggested that the obstruction of the terminal portion of the IVC had been recently installed.

DISCUSSION

We propose a new method to assess the liver hemodynamics

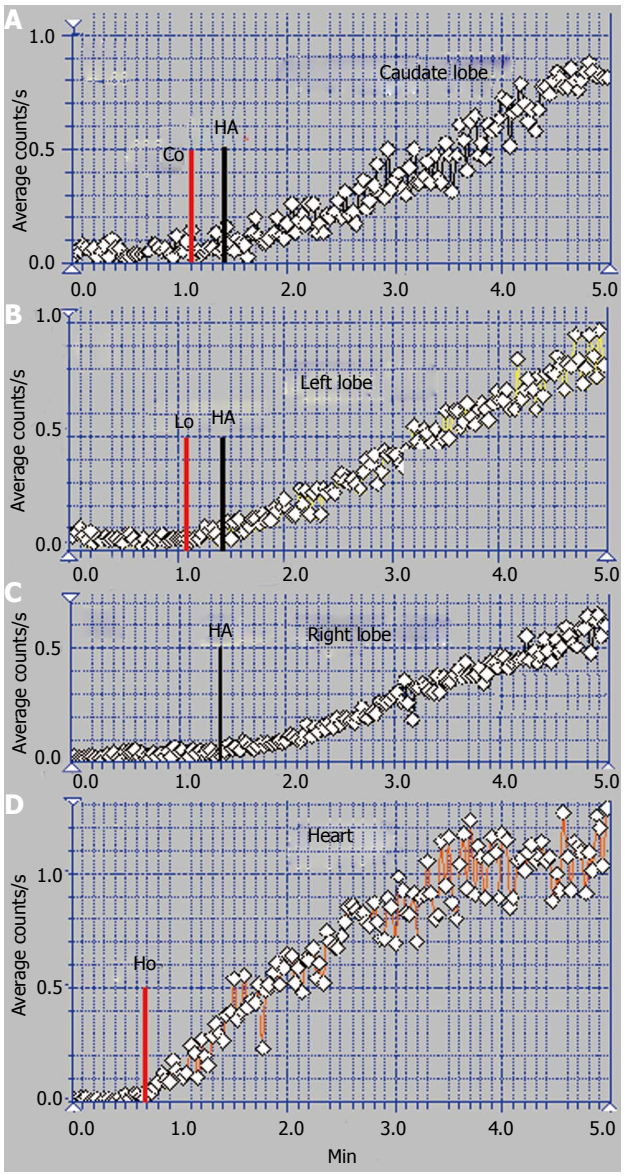

Figure 6 Per-rectal portal scintigraphy in Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins also followed by old obstruction of the left hepatic vein. A: Caudate lobe curve; B: Left lobe curve; C: Right lobe curve; D: Heart curve.

in the BCS by performing the PRPS and LAS, with auxiliary use of the LS. PRPS is the main investigation, based on the parameters introduced by us, LTT and RHLT, offering information about the portal and arterial flows and the effects of the venous outflow obstruction.

The liver areas missing portal inflow with only arterial perfusion are highlighted at PRPS based on the time interval equal to RHLT between the origins of cardiac and liver curves. The increased arterial perfusion of a liver area causes a high slope of the AUI at PRPS and HPI > 45% at LAS. In old obstructions of the HVs, PRPS shows decreased portal and arterial flows of the parenchyma without venous outflow. In recent obstructions of the HVs, PRPS detects small or inverted portal flow, increased arterial inflow and accumulation of radioactivity on the summed image in the affected area. A high quantity of radiotracer appears on the summed PRPS image in the CL of the patients with recent common occlusion of two HVs. Inverted flow in the splenic vein is suggested by a visible spleen on the summed PRPS image.

ASH is useful in the differential diagnosis between the BCS and portal obstructions and also in highlighting arterial-venous shunts and increased resistance opposed to the arterial flow. Reverse portal flow is emphasized by HPI > 100%. LS was performed after ASH, showing increased radioactivity in the CL in particular types of BCS. LS aspect of the liver lobes, spleen and spine is useful when viral or alcoholic CLD is suspected.

Several hemodynamic varieties and stages of the BCS were described by using PRPS and LAS. Liver perfusion status is closely related to the initial number of obstructed HVs and to the lengths of occlusions. The category of the BCS debuted by obstruction of one HV included the patients actually affected by one obstructed HV and the patients affected by an old obstruction of one HV followed by recent obstruction of the other two HVs. For the BCS started by obstruction of two HVs we found different perfusion patterns in acute and chronic stages after the obstruction of the third HV. The BCS with acute onset due to the simultaneous obstruction of all the three HVs is commonly caused by obstruction of the terminal part of the IVC and specifically presents in the acute stage highly prolonged LTT at PRPS. Different hemodynamic patterns of the liver flows related to various types of hepatic venous occlusions underline the autonomous regulation of the perfusion of the two liver lobes.

It is important to know in all the varieties and stages of the BCS if PSS are open. PRPS highlighted that PSS were not open in patients with occlusion of one HV. The CL did not play a significant hemodynamic role in the subacute stage of such patients, suggesting that the blood flow redirected from the area without physiological venous outflow was drained through the unaffected HVs. PSS were not open even in the acute stage after common obstruction of two HVs following an old obstruction of the other HV.

Open PSS were emphasized in our cases with old obstruction of two HVs. This finding suggests that the outflow of two HVs is too high to be redirected in chronic stages only through the unaffected HV and through the CL, with having to leave the liver through extrahepatic PSS.

In acute stages after the occlusion of terminal part of the IVC, the PSS draining the blood to the superior vena cava allowed only a low speed flow. Our data suggest that spontaneous effective drainage through PSS requires at least several weeks to open after the obstruction of two or three HVs.

Hypertrophy of the CL is currently underlined in the diagnosis of the BCS. However, the hemodynamic role of the CL looks to be unimportant or transient in several varieties and stages of the BCS. The CL was not involved as an intrahepatic shunt in asymptomatic pa-

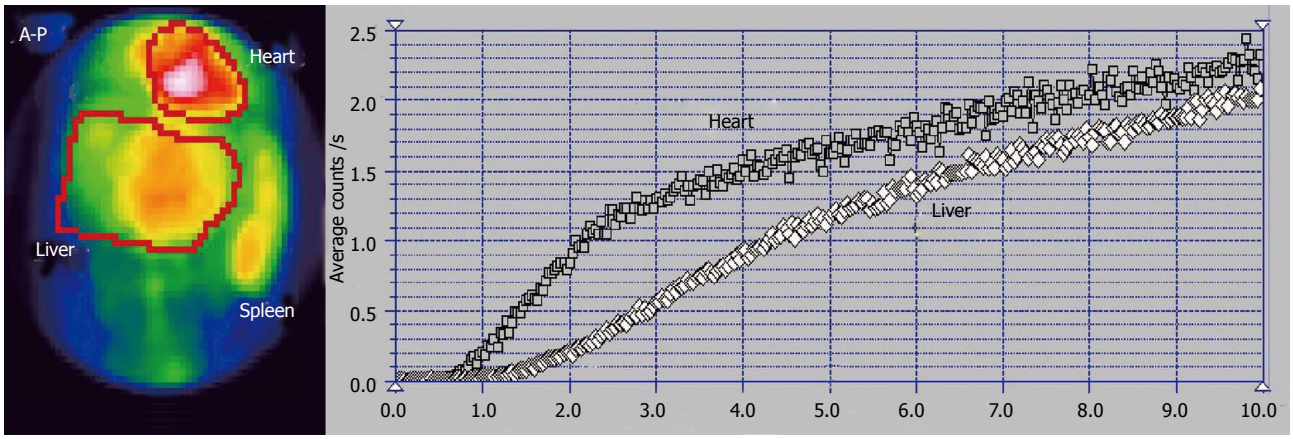

Figure 7 Per-rectal portal scintigraphy of a patient with Budd-Chiari syndrome with old obstruction of the middle and right hepatic veins and recent obstruction of the left hepatic vein.

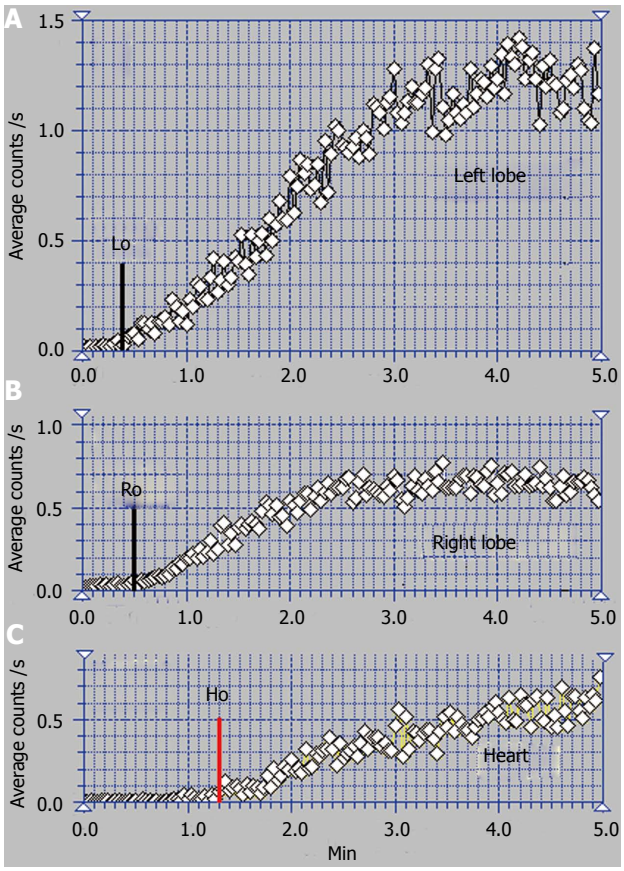

Figure 8 Per-rectal portal scintigraphy in Budd-Chiari syndrome with obstruction of the terminal part of the inferior vena cava. A: Left lobe curve; B: Right lobe curve; C: Heart curve.

tients with obstruction of one HV and in IVC obstructions. In the patients with chronic obstruction of two HVs, the CL had a reduced role of a hemodynamic shunt for part of the arterial flow redistributed from affected areas, presenting arterial-venous collaterals. The CL was highly active in the subacute stage after the obstruction of two HVs following an old occlusion of the third HV. Our study suggests that the CL usually plays an efficient hemodynamic role of intrahepatic shunt to the IVC in acute or subacute stages and in particular varieties of the BCS. The importance in each case of the caliber and morphology of the CL venous drainage for its functioning as hemodynamic shunt has also to be accounted for.

Due to the rarity of the disease, we did not explore several other theoretical varieties of the BCS so our classification will have to be detailed. Common obstruction of the LHV and MHV, acute stage after the debut of the BCS by obstruction of one or two HVs, and chronic stage after the occlusion of the terminal part of the IVC may bring more information about the opening of PSS and the role of the CL in draining the redirected flows.

To conclude, PRPS associated with LAS are able to play a useful role as second line investigations in the BCS, adding important data to the US, CT or MRI findings. These scintigraphic procedures have reliable costs, are non-invasive and easily reproducible. The accuracy of the method, however, is dependent on the operator's expertise.

ACKNOWLEDGMENTS

We are grateful to Professors Sabin Cotul and Doru Dejica, to Dr. Liliana Dina and Crina Briciu for their contribution to the development of PRPS and ASH investigations in our laboratory. We are also grateful to the colleagues from the 3rd Medical and Surgical Clinics of Cluj-Napoca and especially to the Ultrasound department for their diagnosis in the BCS patients.

COMMENTS

Background

Impaired liver perfusion in the Budd-Chiari syndrome is determined by the obstruction of the hepatic blood outflow. Portal and arterial altered flows may be properly explored by combined use of two nuclear medicine dynamic investigations, per-rectal portal scintigraphy and liver angioscintigraphy. Radioisotope techniques allow a more precise diagnosis in different types and stages of the Budd-Chiari syndrome and highlight the changes of perfusion patterns during evolution of the disease.

Research frontiers

Scintigraphy investigations proposed by us to explore the Budd-Chiari syndrome highlight open portosystemic shunts, liver areas without portal inflow, hemodynamic involvement of the caudate lobe, inverted flow in the splenic or

portal vein and length of the obstructions of the hepatic veins or the terminal portion of the inferior vena cava. The authors described three hemodynamic categories of the Budd-Chiari syndrome with several subtypes and stages, based on the finding that perfusion changes depend on the initial number and succession in time of the hepatic veins obstructions.

Related publications

The authors previously described the use of dynamic nuclear medicine investigations to evaluate portal hypertension and portosystemic shunts in chronic liver disease. Clinical applications of the liver angioscintigraphy are commonly related to hepatic tumors evaluation.

Innovations and breakthroughs

The authors introduced a new method of interpretation for the per-rectal portal scintigraphy by proposing two new parameters, the transit time of the portal inflow through the liver and the transit time of the blood from the right heart to the liver. These time parameters allow an accurate description of hepatic hemodynamic changes determined by venous obstructions. The authors used liver angioscintigraphy in the differential diagnosis between the Budd-Chiari syndrome and portal obstructions, highlighting the absence of the hepatic artery buffer response in the Budd-Chiari syndrome. The authors showed that portosystemic shunts are not open after the obstruction of one hepatic vein, while at least several weeks are required in the obstructions of two or three hepatic veins for the spontaneous opening of dynamically efficient portosystemic shunts.

Applications

The hemodynamic data offered by per-rectal portal scintigraphy and angioscintigraphy of the liver are especially important for surgery and TIPS mounting. Diagnosis of the number, length and succession in time of hepatic veins obstructions allow identification of hemodynamic varieties and stages of the Budd-Chiari syndrome and support an adequate therapeutic approach.

Terminology

Per-rectal portal scintigraphy is a dynamic procedure performed by instillation into the rectum of a small quantity of radioactive tracer followed by recording its dynamics through the portal vein, liver and heart areas. Liver angioscintigraphy is performed by rapid antecubital intravenous bolus injection of a small quantity of radiotracer followed by recording its entrance into the liver through the hepatic artery and portal vein, allowing the assessment of an arterial to total liver perfusion (arterial plus portal) ratio. The Budd-Chiari syndrome is a vascular liver disease determined by hepatic venous obstruction localized from the small hepatic veins to the terminal part of the inferior vena cava, resulting in increased sinusoidal pressure, hepatic congestion and portal hypertension.

Peer review

This is a well thought and comprehensive manuscript on the relevance of using PRPS and liver angioscintigraphy techniques to investigate the liver hemodynamics in Budd-Chiari syndrome. The manuscript provides useful and interesting information. It also has potential for clinical application.

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No author given

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Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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