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EDITORIAL

Epigenetic mechanisms in non-alcoholic fatty liver disease: An emerging field

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is an emerging health concern in both developed and non-developed world, encompassing from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer. Incidence and prevalence of this disease are increasing due to the socioeconomic transition and change to harmful diet. Currently, gold standard method in NAFLD diagnosis is liver biopsy, despite complications and lack of accuracy due to sampling error. Further, pathogenesis of NAFLD is not fully understood, but is well-known that obesity, diabetes and metabolic derangements played a major role in disease development and progression. Besides, gut microbioma and host genetic and epigenetic background could explain considerable interindividual variability. Knowledge that epigenetics, heritable events not caused by changes in DNA sequence, contribute to development of diseases has been a revolution in the last few years. Recently, evidences are accumulating revealing the important role of epigenetics in NAFLD pathogenesis and in NASH genesis. Histone modifications, changes in DNA methylation and aberrant profiles or microRNAs could boost development of NAFLD and transition into clinical relevant status. PNPLA3 genotype GG has been associated with a more progressive disease and epigenetics could modulate this effect. The impact of epigenetic on NAFLD progression could deserve further applications on therapeutic targets together with future non-invasive methods useful for the diagnosis and staging of NAFLD.

Key words: Non-alcoholic steatohepatitis; Epigenetics; Diagnosis; Treatment; Non-alcoholic fatty liver disease

Core tip: The interplay of environmental and host factors results in non-alcoholic fatty liver disease (NAFLD) development and influence its progression individually. Nevertheless, the physiopathology of this disease

remains unclear, so this lack of knowledge avoids the development of new therapeutic approaches and non-invasive biomarkers. Epigenetics could be an interesting alternative to cover these issues, considering the amount of evidence accumulated in order to clarify its role on NAFLD.

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EPIDEMIOLOGY

Non-alcoholic Fatty liver disease (NAFLD) is defined as an accumulation of fat in the liver in absence of significant alcohol consumption, hereditary disease or drugs[1]. It constitutes a clinicopathological disease comprising a wide spectrum of disorders, ranging from simple steatosis (SS), initially benign, to non-alcoholic steatohepatitis (NASH), accompanied by inflammation and/or hepatocellular damage. These two entities, SS and NASH, show different natural history, evolution and consequences, through necroinflammation, fibrosis, cirrhosis and even hepatocellular carcinoma. The strongest predictor of fibrosis progression in NAFLD is the presence of steatohepatitis, mainly lobular inflammation and ballooning[2].

Currently, NAFLD is considered the most common chronic liver disease in developed countries[3]. Its worldwide prevalence in general population is estimated to be around 20%-30%[4] in Western countries and 5%-18% in Asia[5], being a common and underdiagnosed condition. The reason for this variability remains unclear, but presumable genetic and epigenetics factors play an important role.

NAFLD is often associated with clinical features of metabolic syndrome, such as central obesity, insulin resistance, type 2 diabetes mellitus, arterial hypertension and dyslipidaemia[6]. Sedentary lifestyles and changes in dietary patterns are responsible for an increased prevalence of obesity and insulin resistance, leading to an increased prevalence of this disease, projected to be the top cause for liver transplantation within the next decade[7]. Furthermore, this disease is related to different systemic disorders, like cardiovascular disease[8,9].

DIAGNOSIS, MANAGEMENT AND TREATMENT

Percutaneous liver biopsy is often recommended in patients with unexplained elevated serum aminotransferases, constituting the gold standard method in the diagnosis of fibrosis and steatohepatitis. It shows inherent limitations, as high costs and associated morbidity, which can lead to major complications (*i.e.*, bleeding, and even death)[10]. Non-invasive methods have been recently developed in order to diagnose non-alcoholic steatohepatitis, such as imaging tests (transient elastography, acoustic radiation force impulse and magnetic resonance elastography) as well as biomarkers (cytokeratin-18 and fibroblast grown factor 21).

NAFLD pathogenesis can be resumed as the excessive accumulation of fat in hepatocytes, leading to increased intracellular vacuoles of fat, lack of capacity for mitochondrial beta-oxidation, oxidative stress, pro-inflammatory mechanisms and hepatocellular apoptosis[11,12]. Since pharmacological treatment for NAFLD remains ineffective[13], the first-line approach for these patients is the reduction on total body weight achieved by decrease of energy consumption and increase of exercise.

EPIGENETIC MECHANISMS

Historically, the term of epigenetic was coined by Conrad Waddington in the 1940s as the branch of biology which study the causal interactions between genes and their products which bring the phenotype into being[14]. Currently, epigenetic modification is defined as phenotypic changes in gene expression that can be inherited through mitosis and/or meiosis caused by an adaptive mechanism unrelated to alteration of primary DNA sequences[15,16].

Epigenetic phenomena are heritable adaptive mechanisms considered reversible, since they are being modulated by environmental stimuli. Disruption of the balance could lead to the development of serious disorders. The most described epigenetic modifications include: (1) histone modification; (2) DNA methylation; (3) microRNAs; and (4) chromatin remodelling. To date, the most intensively studied epigenetic mechanisms in NAFLD are DNA methylation and microRNAs (Figure 1).

HISTONE MODIFICATION

Modifications of the amino-terminal tails histones constitute an important determinant of chromatin structure and gene expression[17]. Aberrant histone modifications promote the development of insulin resistance and consequently, NAFLD[18]. Among the most common modifications acetylation has been reported, associated with gene transcription activation and catalysed by histone acetyltransferase (HAT) and deacetylation, involved in gene repression, and catalysed by histone deacetylase (HDAC).

So far, the most important findings in NAFLD have been described in mice. Imbalance between HAT and HDAC influences gene expression profile on NAFLD, resulting on liver injury[19]. p300 is a transcriptional coactivator that belongs to the HAT family. It has been identified as a key upstream regulator of carbohydrate-responsive element-binding protein activity, an activator

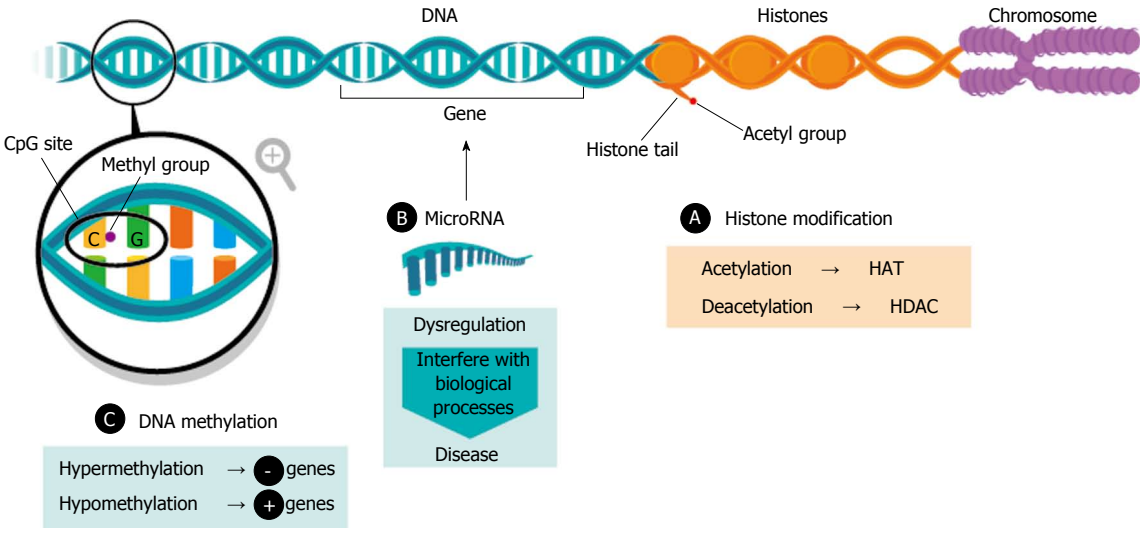

Figure 1 Epigenetic phenomena. HAT: Histone acetyltransferase; HDAC: Histone deacetylase.

of glycolytic and lipogenic genes, so inhibition of its activity may be beneficial for treating hepatic steatosis[20]. Several HDCA also play important roles on NAFLD. HDAC3 displays a circadian rhythm in mouse liver controlling hepatic lipogenesis, and disruption of this mechanism exacerbates metabolic diseases, including obesity and diabetes. Its depletion reroutes metabolic precursors toward lipid synthesis and storage in lipid droplets[21,22]. Sirtuins (SIRT) are master metabolic regulators with protective roles against obesity, glucose and lipid metabolism. Activation of SIRT1 shows potential against NAFLD-related physiological mechanisms, and it has been found increased plasma levels in NAFLD obese patients[23]. SIRT1 could play a dual role, acting as a potential therapeutic target and a noninvasive biomarker on NAFLD[11].

DNA METHYLATION

Many methylations occur in the liver, and hepatic steatosis is often view from the standpoint of the deregulation of one-carbon metabolism, being related to folate deficiency[24]. DNA methylation is the addition of a methyl group on cytosine with guanine as the next nucleotide, also known as CpG site[25].

DNA methylation plays a central role in the regulation of gene expression, representing a level of epigenetic regulation, which is commonly associated with transcriptional repression and chromatin accessibility. Aberrant DNA methylation patterns of genomic stability affect cell homeostasis, such as hypermethylation, associated with gene repression, and hypomethylation, related to gene activation. In mice, kick-off of steatosis is accompanied by alterations in DNA methyltranferases (DNMT) expression in the liver[26]. In humans, hepatic DNMT was found higher in NASH than SS patients and significantly associated with NAS Score[27]. Abnormal DNA methylation is the hallmark of carcinogenesis; this process has been studied in hepatocellular carcinoma (HCC)[28], more specifically in NAFLD-HCC. Metabolites derived from metabolic syndrome, such as insulin, glucose and lipids could perturb epigenetic gene regulation leading to a pro-inflammatory status and disturbing metabolic pathways[29]. Furthermore, DNA methylation signatures can be remodelled by transcriptional factors, so it has also being evaluated after bariatric surgery and the massive loss weight that entails, suggesting that NAFLD-associated methylation changes could be partially reversible[30]. It has been reported that functionally relevant differences in methylation could distinguish between mild and advanced NAFLD in 100 human frozen liver biopsies. Moreover, in patients with advanced *vs* mild NAFLD, 69247 differentially methylated CpG sites (78% hypomethylated, 24% hypermethylated) were described. These findings indicate that differential methylation contributes to differences in expression[31].

MICRORNAS

MicroRNAs (miRNAs) are receiving growing attention because they are commonly deregulated in pathological situations, being the most extensively investigated epigenetic mechanism in NAFLD. MiRNAs constitute a class of small, single-stranded non-coding RNA highly conserved, acting as regulators of gene expression and protein translation. They can interfere in each single aspect of cellular activity, such as differentiation and development, proliferation, metabolism, apoptosis and tumorigenesis[32]. A single miRNA could target multiple genes (multiplicity) and multiple miRNAs could target a just one gene (cooperativity). Taking into account its large potential roles on carcinogenesis, miRNAs could also be categorized as oncogenes (onco-miRNAs) or tumour suppressors[33]. It has been shown their stability in serum, plasma, urine and saliva. Circulating miRNAs, protected from degradation by RNAses contained in body fluids, are currently extensively studied for noninvasive diagnosis of a sort of liver diseases[34], including

Table 1 Dysregulated miRNAs in non-alcoholic fatty liver disease

miR	Modulation	Experimental model
miR-15b[45]	Upregulation	*In vitro*/rats/human
miR-34a[46]	Upregulation	Human
miR-221[47]	Upregulation	*In vitro*
miR-222[47]	Upregulation	*In vitro*
miR-155[48]	Downregulation	Mice
miR-198[37]	Downregulation	Human
miR-451[37]	Downregulation	Human
miR-146b[37]	Upregulation	Human

NAFLD[35]. Thereof, it has been identified several miRNAs in serum/plasma of NAFLD patients that show diagnostic potential for defining different phenotypes of this disease, from simple steatosis to NASH, going through fibrosis[36].

Actually, the major importance or miRNAs on NAFLD stands on the discrimination of NASH and the diagnosis of HCC. In this sense miR-122, the most expressed miRNA in human liver has been reported significantly under-expressed in NASH[37], acting as a tumor-supressor in the liver[29,38]. It has been proposed as a potential therapeutic target in the treatment of hypercholesterolemia[39] or different dyslipidaemia[40].

Besides miR-122, other miRNAs have been demonstrated to be involved in NAFLD. It has been reported a link between liver cell apoptosis, miR-34a/SIRT1/p53 signalling and NAFLD severity[41], and recently, miR-21 seems to regulate triglyceride and cholesterol metabolism *in vivo* and *in vitro*[42]. Moreover, mir-24 is robustly induced in fatty acids treated human hepatocytes, HepG2 cells and high-fat diet mice, revealing the potential role of miR-24 inhibitor as a promising therapeutic target for NAFLD[43]. miR-33a and miR-33b also inhibit genes involved in fatty acids metabolism and insulin signalling in hepatocytes, regulating pathways controlling three risk factors of metabolic syndrome, HDL levels, triglycerides, and insulin[44]. The miR-200 family and others, like miR-155, are also related with NAFLD[33] (Table 1).

Besides, there are still some barriers to the therapeutic use of miRNAs. MiRNAs can be degraded by endogenous RNAses and affect several pathways in different organs, so caution is needed to avoid undesirable adverse effects.

POTENTIAL ROLE OF EPIGENETICS ON NAFLD MANAGEMENT

NAFLD is a complex disease trait where interpatient genetic[49] and epigenetic variations and environmental factors are combined to define development and disease progression. Upsetting the balance of cellular homeostasis, either modifying some of these mechanisms, could trigger disease development. Since epigenetic phenomena are reversible, novel therapies intended to modulate epigenetic abnormalities are trend. Altered epigenetics patterns could distinguish between NAFLD stages, but a better understanding of the molecular mechanisms is mandatory to identify reliable biomarkers and effective treatments. Among epigenetic mechanisms, miRNAs occupy a top position, because their disturbances present potential prognostic and diagnostic, and the ability to be therapeutic targets. Further research is needed to increase knowledge of the role that epigenetics mechanisms could play in determining most aggressive phenotypes of NAFLD. This could lead to disease stratification, from simple steatosis to non-alcoholic steatohepatitis, in order to target therapies, providing new tracks in NAFLD pathogenesis.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis B virus

Recent advances in vaccination of non-responders to standard dose hepatitis B virus vaccine

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Abstract

Hepatitis B virus (HBV) infection is a global health problem. It is estimated there are more than 2 billion individuals exposed to the virus and 250 million are chronically infected. Hepatitis B is the cause of more than 600000 annual deaths due to cirrhosis and hepatocellular carcinoma. An effective vaccine exists and preventative initiatives center around universal vaccination especially in those at highest risk. Effective vaccination algorithms have led to a significant decline in the development of new infections and its devastating consequences. The vaccine is administered intramuscularly in three doses, with 95% showing long lasting serologic immunity. An additional fourth dose or a repeated higher dose three course regimen is given to those that fail to show immunity. Despite these additional regimens, some remain vulnerable to hepatitis B and are deemed non-responders. Individuals with chronic disease states such as kidney disease, liver disease, diabetes mellitus, as well as those with a genetic predisposition, and those on immunomodulation therapy, have the highest likelihood of non-response. Various strategies have been developed to elicit an immune response in these individuals. These include increased vaccination dose, intradermal administration, alternative adjuvants, alternative routes of administration, co-administration with other vaccines, and other novel therapies. These alternative strategies can show improved response and lasting immunity. In summary, HBV vaccination is a major advance of modern medicine and all individuals at risk should be sought and vaccinated with subsequent adequate titers demonstrated.

Key words: Hepatitis B vaccine; Non-responders; Intradermal vaccine; Adjuvants; Oral vaccine

Core tip: Hepatitis B is a major global pandemic. Hepatitis B vaccine has been very effective in eradicating the disease from the world. Despite its efficacy, the standard vaccine fails to produce an immune response in 5% of immunocompetent individuals as well as individuals with chronic diseases and immunosuppressed states. Different modalities have been used to produce an immune response in these non-responders. These include double dosing, more frequent dosing, intradermal vaccine, adjuvant vaccines and recombinant vaccine with variable efficacies. Despite these novel techniques there are still no official guidelines available to vaccinate non-responders.

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INTRODUCTION

Hepatitis B is a major contributor to the burden of infectious disease worldwide. Over 2 billion people have been exposed to the virus, which has resulted in nearly 350 million chronic carriers with 4 million new cases diagnosed yearly worldwide[1]. According to the CDC in United States alone, there were almost 18760 new infections in 2012, contributing to 2.2 million chronic carriers of hepatitis B[2,3]. Hepatitis B is the cause for nearly half of all cirrhosis diagnosis, 80% of hepatocellular carcinomas and 1 million deaths yearly worldwide. This places hepatitis B virus (HBV) infection second only to tobacco as a major carcinogen[1,4,5].

HBV is up to a 100 times more transmissible than human immunodeficiency virus (HIV). This is largely secondary to the fact that it is abundantly found in body fluids, can survive up to seven days on fomites, and most infected carriers are asymptomatic and unaware of their disease[1,3]. As such, development of an effective vaccine and successful vaccination is a major achievement of modern medicine and has the potential to eradicate this infection from humankind.

HBV is an enveloped double stranded DNA virus belonging to the Hepadna family of viruses[6]. Humans are the only known natural host of this virus and the liver is the only organ where it is known to replicate[7]. In developing countries the vast majority of infections are transmitted vertically, of whom nearly 90% develop chronic hepatitis[3,8]. The principle mode of transmission in adults is *via* intravenous (IV) drug use and sexual contact and the vast majority of these cases will clear the virus spontaneously with few if any overt symptoms and only < 5% develop chronic hepatitis[8,9].

In the United States, IV drug use is the major mode of transmission. While the prevalence of chronic HBV infection (HBSAg +ve) is only about 0.5% in general population, it increases to approximately 50% among IV drug users who have been using for at least 1 year and over 90% if injecting drugs for more than 10 years[1,10,11].

Hepatitis B vaccine has been a major breakthrough in the global effort to eradicate the virus. The vaccine is made from the yeast saccharomyces cervisiae and is composed of physiochemically purified non-glycosylated molecule of HepBsAg which is adherent to aluminum hydroxide and preserved with thiamersol[12]. It is highly immunogenic, and dramatically reduces morbidity and mortality related to hepatitis B. For example, the introduction of screening in expectant mothers and subsequent vaccination programs has led to an 80% decline in the incidence of HBV infection between 1987-2004, from 10.7 to 2.1 per 100000[13]. Similarly, in Taiwan, where the prevalence of HBV was inordinately high; introduction of universal HBV in newborns has decreased the rate of hepatocellular cancer by 75%, with the incidence rate declining from 0.70 in 1981-1986 to 0.36 in 1990-1994/100000 children (ages 6-9) and a 68% decline in infant mortality from fulminant hepatitis[14]. The World Health Organization (WHO) now recommends universal vaccination of all neonates and adolescents as well as adults who have not been previously vaccinated[1].

PATHOPHYSIOLOGY

HBV vaccine consists of the highly immunogenic surface antigen (HepBsAg) protein. When administered, it interacts with antigen presenting cells present in the blood (HepBsAg specific B cells) where it is lysed and processed. This epitope coupled with an major histocompatibility complex (MHC)-Ⅱ molecule on the cell surface is then presented to TH-2 cells. The TH-2 cells, when activated, stimulate the differentiation of B-cells to plasma cells. These cells then release hepatitis B surface antibodies (HepBsAb) in large quantities as well as induce development of memory B and T cells. These memory cells then play an important role in long-term protection[15]. Immunogenicity is generally known to last approximately 10-31 years after a primary vaccination, with the duration and degree of immune response depending on the age, body mass index, sex, and smoking status at the time of initial inoculation series[16,17].

It is not entirely clear why the persistence of immunity, as defined by titers of HepBsAb greater than 10 mIU/mL, may last for several decades after a single round of vaccination. One possible explanation of constant antibody response over prolonged periods might be the persistence of antigen on the follicular dendritic cells which may keep up-regulating the B and T cells. Another possibility may be the initial antigen dose. The higher the dose administered initially, the greater the B-cell response. This increases the proportion of

memory B-cells, resulting in longer lasting immunity[18].

VACCINE ADMINISTRATION

The vaccine is typically administered as a 10 mg intramuscular (IM) dose in three doses at 0, 1, and 6 mo. Successful vaccination is documented by an antibody response of more than 10 mIU/mL and is achieved in about 95% of the immune-competent population. A fourth dose can be administered in immunocompromised or individuals at greater risk of exposure to the virus[8]. In high-risk patients the antibody response should be rechecked 1-3 mo after completion of the series and if the antibody titer is less than 10 mIU/mL then the series (40 mg) should be repeated again and the antibody titers should also be rechecked. This usually results in a response in fifty to sixty percent of the non-responder subgroup of patients[8,19]. Those who do not respond to the standard regimen as well as the additional booster or repeated course regimen are labeled as true non-responders[18]. While avoidance of high-risk behavior and prevention of exposure to blood and body fluids remains universally advocated for non-responders, these patients should be monitored for any acute changes in their liver enzymes and aggressively treated if infection is confirmed.

NON-RESPONDERS

Despite the high efficacy of the HBV vaccine, nearly 5% of immunocompetent individuals fail to respond to the primary HBV series. The reason for this non-response is not clear, however certain populations are at high risk including those with genetic predisposition, chronic disease, and immunomodulatory medications. Some interesting observations have been made in these populations. There may be a genetic predisposition for non-response. The human leukocyte antigen (HLA) along with MHC-Ⅱ plays an important role in presentation of the viral peptides to CD-4 T-helper cells and subjects who fail to respond may have a defect in the antigen presentation or the stimulation of T-helper cells. Studies have shown that patients who are homozygous for HLA DRB1*0301, HLA-B8, SC01, DR-3, HLAB44, FC-31, DR-7 have an increased predisposition to non-responsiveness[20,21]. Patients with advanced age, chronic diseases, immune defects or on immunomodulatory medications have a blunted immune response.

In one study of patients more than 60 years old, only 32 of 70 (45.7%) patients developed anti-HBs antibodies[22]. In another study of 106 patients more than 59 years old, only 60% of the subjects had an antibody titer greater than 10 mIU/mL at 7 mo post vaccination[23].

In patients with HIV, the seroconversion rate varies from 18%-72% depending on the immune status of the patient. In patients not receiving HAART therapy, the rate of immune response can vary from 30%-50% while in those receiving HAART the response increases to 60%-70% and is directly proportional to the CD-4 count and inversely proportional to the viral load[19,24,25].

Patients with chronic liver diseases also have a blunted response. In a study done by Mattos *et al*[26], patients with hepatitis C infection who were vaccinated showed only a 55% seroconversion rate. Out of these patients, only 37% had a robust response (Seroconversion was defined as an antibody level greater than 10 mIU/mL while robust response defined as antibody titers > 100 mIU/mL). Interestingly, patients with genotype 1 had a worse response then other genotypes. Additionally, immune response to vaccination was inversely related to advanced liver disease as measured by the MELD score[27,28].

In a study done by Agarwal *et al*[29], evaluating response rates to HBV vaccine in mild (creatinine 1.5 to 3.0 mg/dL), moderate (creatinine 3.0 to 6.0 mg/dL) and severe (creatinine > 6.0 mg/dL) chronic kidney disease, the seroconversion rates after 3 doses of 40 μg HBV vaccine were 87.5%, 66.6% and 35.7%; respectively. Rates improved significantly after a 4th dose was administered to 100%, 77% and 36.4%, respectively. Multiple studies have demonstrated patients with low glomerular filtration rate, higher creatinine (late stage kidney disease), diabetes, and old age are less likely to seroconvert[28].

STRATEGIES TO VACCINATE PATIENTS WHO DO NOT RESPOND TO STANDARD THERAPY

Increased dose

Considerable data exists on increased dose vaccination as well as accelerated frequency to elicit an immune response in high-risk individuals. Bonazzi *et al*[30] showed 68% response rate with double dosing (40 μg IM) at 0, 1 and 6 mo in pre-transplant patients. Forty-one percent of their patients had a robust response with an anti-HBs level > 1000 IU/mL. In another study, Wiedmann *et al*[31] showed a seroconversion rate of 80% in patients with chronic hepatitis C who had not responded to a primary vaccine just after giving a single (40 μg) high dose booster. Ramzan and colleagues also showed that higher dose and shorter interval (40 μg/mo for 3 mo) produced seroconversion in 72% of the patients with chronic liver disease as compared to 92% response in controls. Response was lower in cirrhotics as compared to non-cirrhotics (54% *vs* 80%) but after an additional booster dose of 80 mg, response increased to 74% and 88%, respectively[32].

INTRADERMAL ADMINISTRATION OF THE VACCINE

Multiple studies have exploited the fact that a large number of antigen presenting dendritic cells reside in the skin, specifically the dermis. These cells then activate the immunogenic cells in the corresponding lymph

node where they drain. This presentation of antigen into the dermis enhances the potential for activation of the immune cascade and development of protective antibodies[33-35].

Rahman *et al*[36] compared the efficacy of intradermal vaccine and showed that it resulted in a significantly higher immune response as compared to IM form. Both T and B cell response were higher with intradermal form as compared to IM form, suggesting that the intradermal presentation of the antigen to Langerhans cells in the dermis might result in trapping of the antigen in the skin resulting in a more robust and more sustained humoral and cell mediated response.

Forty-two chronic liver disease patients who had not responded to standard 40 mg three doses and booster doses were treated by Dhillon *et al*[37] using 40 mg intradermally (20 μg in each arm) for a maximum of three doses and seroconversion was seen in 29 (69%) of the 42 patients in their study with 15 (51%) of the patients developing a robust response.

In patients on hemodialysis who were primarily non responsive to standard dosing of hepatitis B vaccine, Barraclough *et al*[38] showed seroconversion rate of 79% in intradermal *vs* 40% in IM when a weekly 5 μg dose was injected intradermally for 8 wk as compared to 40 μg IM dose at 1 and 8 wk. The response in the intradermal group was more robust than IM group with titers being 239 IU/L *vs* 78 IU/L respectively[38].

No significant complications have been reported with intradermal vaccination in the studies mentioned above. Discoloration, itching, and nodule formation at the site of injection were the most commonly noted side effects and typically resolved spontaneously. The intradermal vaccine requires a certain skill set for proper inoculation in the dermis and its enhanced effectiveness. This coupled with general lack of knowledge regarding its efficacy has led to its limited adoption in non-responders[39].

IMPROVED IMMUNOGENICITY

Much research has focused on improving the immunogenicity by adding pre-S1, pre-S2 particle or nucleocapsids containing core antigen (HBcAg) to the S-protein to enhance efficacy of the vaccine. There are several reports citing an increase response in non-responders by using this technique[40-43]. In a study done by Zuckerman *et al*[43] on 100 non-responsive health care workers to standard vaccine who failed to seroconvert after 3 doses plus booster vaccine; a single dose of the triple S recombinant produced seroconversion in 69 patients. Similarly, seroconversion rates of 65% and 71% were reported after a 3rd and 4th dose of recombinant pre-S1 and pre-S2 containing hepatitis B vaccine; in 17 non-responders with underlying chronic renal failure[44].

USE OF ADJUVANTS

Currently, the HBV vaccine uses aluminum as an adjuvant to enhance immune response. Other more immunogenic compounds have been identified. 3-deacylatedmonophosphoryl lipid A (3D-MPL) combined with aluminum has shown to produce more immunogenicity then aluminum alone in unresponsive subjects, with immune response seen in up to 98% of the patients one month after receiving three doses[45]. Another polysaccharide adjuvant, Delta inulin: Advax™, has shown to enhance immunogenicity (strong CD-4 And CD-8 T cell response) with a robust response in pre-clinical trials on mice and pigs when compared with the traditional aluminum based vaccine[46].

OTHER NOVEL THERAPIES

Phase 3 clinical trials are underway for HEPLISAV-B™, a toll like receptor (TLR) agent in which HepBsAg is combined with immunostimulatory TLR 9 agonist to enhance response on a 2 dose regimen over 1 mo compared to the current 6 mo 3 dose regimen. It has shown earlier and higher seroconversion rates than the standard vaccine in those at risk for blunted or non-response. In 218 subjects that were divided in two groups (179 HEPLISAV B and 39 Energix B) the sero-protection rates at 12 and 52 wk post immunization for HEPLISAV B was 79% and 82% respectively as compared to 61% and 11% in the standard vaccine group[47].

ALTERNATIVE MECHANISM

Akbar and colleagues compared antibody production between HepBsAg and HepBcAg pulsed dendritic cells from spleen and liver of HBV infected transgenic mice. They showed while the surface antigen stimulated cells resulted in production of only surface antibodies, core antigen stimulated cells produced both core and surface antibodies with higher titers ($P < 0.05$). Thus, the use of core antigen is yet another fertile area of research in the development of the next generation of vaccines against HBV[48].

ROUTES OF ADMINISTRATION

While intramuscular and intradermal administration have been commonly used and extensively studied, other routes are also being actively sought.

NASAL VACCINE

A nasal based vaccine, Nasvac, which is a combination of HBV surface and core antigen has shown good efficacy in healthy as well as chronic HepB carriers possibly by stimulating naive human B cells[49]. In Phase 1 trials of NASVAC, a mixture of 50 mcg of HBsAg and HBcAg were administered *via* nasal spray to healthy adults (age 18-45) in five doses at 0, 7, 15, 30 and 60 d. It showed anti-HBc seroconversion in 100% of patients as early as day 30 with anti-HBs titers > 10 IU/L in 75% of the patients at day 90 with no major side effects[50].

ORAL VACCINE

A once daily Oral preparation, V-5 Immunitor™, has shown efficacy both in development of protective antibody as well as normalization of liver function tests in chronically infected individuals. When administered to ten patients with chronic hepatitis B, it resulted in normalization of liver enzymes in 100% of the patients (112.4 to 44.4 U/L for aspartate aminotransferase and 118.8 to 46.1 U/L for alanine aminotransferase) while half of the patients became HBsAg negative at the end of one month[51]. The preventative and therapeutic potential for such a compound would be a major breakthrough in the study of this infection.

COMBINATION VACCINES

Another technique employs both HepBcAg and HepBsAg to elicit humoral/cell mediated response which could be employed in controlling infection *via* CD-8 T cells as well as antibody production by stimulating memory B-cells. Vaccine development trials are underway which would stimulate both humoral and cellular immunity and help in controlling infection *via* CD-8 T cells as well as stimulating memory cells in producing antibodies. A novel vaccine consisting of HepBsAg and HepBcAg on a saponin-based ISCOMATRIX™ adjuvant has proven to be effective. In mice with chronic HBV infection it produces HBsAg specific and HBcAg specific CD-8 T-cells as well as stimulates plasma cells to produce high titers of antibodies against both antigens[52]. These early trials are encouraging but more clinical trials are needed in humans to document efficacy in the future. In another study, Cardell *et al*[53] gave three doses of IM combined hep A, hep B vaccine (TWIN-RIX) to 44 patients, who had been previously non-responsive to 4 doses of intradermal vaccine. Approximately 95% (42 patients) showed an immune titer > 10 mIU/mL, with 35 of these patients developing an antibody titer of > 100 mIU/mL. This suggests hepatitis A antigen may act as an adjuvant and enhance immune response globally.

In another study, hepatitis B vaccine was combined with HPV 16/18 and given to previously seronegative women. One month after the third dose, there was no difference in immune response in two groups (96.4% *vs* 96.9%). This confirms that co-administration of vaccine does not affect immunogenicity of either vaccine[54].

CONCLUSION

HBV is a global medical problem. While much morbidity and mortality is attributed to the disease, vaccination against this virus is both efficacious and readily available. Control of this infection *via* vaccination has markedly decreased the rates of new infection as well as hepatocellular cancer and chronic liver disease worldwide. Typically the vaccine shows a 95% response rate with durable and long-lasting immunity. Multiple novel methods have been developed to address those who do not respond to the regular vaccine schedule. Every effort should be made in high-risk populations (IV drug users, healthcare workers, patients with chronic diseases such as diabetes mellitus and chronic kidney disease) to vaccinate against this virus and the antibodies should be checked to ensure immunity. Both long-lasting immunity and therapeutic potential has been demonstrated with the various vaccines mentioned.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis C virus

Liver fibrosis in human immunodeficiency virus/hepatitis C virus coinfection: Diagnostic methods and clinical impact

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Abstract

Several non-invasive surrogate methods have recently challenged the main role of liver biopsy in assessing liver fibrosis in hepatitis C virus (HCV)-monoinfected and human immunodeficiency virus (HIV)/HCV-coinfected patients, applied to avoid the well-known side effects of liver puncture. Serological tests involve the determination of biochemical markers of synthesis or degradation of fibrosis, tests not readily available in clinical practice, or combinations of routine tests used in chronic hepatitis and HIV/HCV coinfection. Several radiologic techniques have also been proposed, some of which commonly used in clinical practice. The studies performed to compare the prognostic value of non-invasive surrogate methods with that of the degree of liver fibrosis assessed on liver tissue have not as yet provided conclusive results. Each surrogate technique has shown some limitations, including the risk of over- or under-estimating the extent of liver fibrosis. The current knowledge on liver fibrosis in HIV/HCV-coinfected patients will be summarized in this review article, which is addressed in particular to physicians involved in this setting in their clinical practice.

Key words: Human immunodeficiency virus/hepatitis C virus coinfection; Liver fibrosis; Liver biopsy; Fibroscan; Liver ultrasonography

Core tip: The extent of liver fibrosis is a marker of disease progression influencing the clinical and therapeutic decisions to be made for human immunodeficiency virus (HIV)/hepatitis C virus (HCV)-coinfected patients. The international guidelines suggest anti-HCV therapy for HIV/HCV-coinfected patients with histological fibrosis score $\geqslant$ 2 in the Metavir scoring system since they have an increased risk of liver failure. Due to the high clinical impact of liver fibrosis and of the well-known limitations of liver biopsy, surrogate, non-invasive technologies have been researched. The pros and cons of liver biopsy and surrogate technologies in detecting liver fibrosis in HIV/HCV-coinfected patients will be discussed in this review article.

Sagnelli C, Martini S, Pisaturo M, Pasquale G, Macera M, Zampino R, Coppola N, Sagnelli E. Liver fibrosis in human immunodeficiency virus/hepatitis C virus coinfection: Diagnostic methods and clinical impact. *World J Hepatol* 2015; 7(24): 2510-2521 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i24/2510.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i24.2510

INTRODUCTION

Nearly 7 million people are chronically coinfected with human immunodeficiency virus (HIV) and hepatitis C virus (HCV), *i.e.*, around 20% of the entire HIV-positive population worldwide[1-3].

HCV liver-related mortality is among the leading causes of death of HIV-positive patients, despite the introduction of new potent antiretroviral regimens that have resulted in recent years in a consistent reduction in hepatic decompensation and mortality. Liver-related mortality remains higher in HIV/HCV-coinfected patients than in those with HIV or HCV monoinfection[4], most probably because HIV infection promotes HCV replication and speeds up the progression of liver fibrosis to its more severe stages[5-7].

An accurate assessment of liver fibrosis is fundamental for monitoring the disease progression and for the therapeutic decisions to be made in HIV/HCV coinfection. According to the current international guidelines, HIV/HCV-coinfected patients with chronic hepatitis and significant fibrosis (grade F2 or more in the Metavir scoring system) should be considered for anti-HCV therapy[8,9], especially those with a controlled HIV infection[9,10].

Liver biopsy, an invasive method entailing side effects or complications in a minority of cases[11-16], remains the gold standard for a morphological assessment of chronic liver diseases, particularly in patients with chronic hepatitis of dubious etiology. However, clinicians need repeatable, non-invasive procedures to monitor liver fibrosis in patients with HIV/HCV coinfection. Two types of non-invasive procedures have been developed: The radiologic assessment of liver morphology and the comprehensive evaluation of surrogate serum markers that in any way correlate with the extent of liver fibrosis. Each technique shows some limitations, including the risk of an over- or under-estimation of the extent of liver fibrosis.

This review presents the invasive and non-invasive techniques currently available to assess liver fibrosis in HIV/HCV-coinfected patients, and offers the physicians who have these patients in care an evaluation of the pros and cons of each method[10].

HIV INFECTION AND LIVER FIBROSIS IN HCV-RELATED CHRONIC HEPATITIS: A PATHOGENETIC APPROACH

Several factors possibly accelerating the progression of HCV-related liver fibrosis to its more severe stages have been investigated, namely the direct action of HIV, immune deregulation, an alteration in the cytokine pattern towards a pro-fibrotic state, HIV-related depletion of gut $CD4^+$ cells and consequent microbial translocation, oxidative stress, and hepatocyte apoptosis[1]. It has been suggested that HIV promotes liver fibrosis by acting on CCR5 and CXCR4 co-receptors for HIV-1 on hepatocytes and other liver cells[1]. In addition, experiments using *in vitro* models have suggested that the HIV-1 viral envelope glycoprotein gp120 may directly promote hepatocyte apoptosis[17] and high viral loads by inducing transforming growth factor (TGF)-β, a cytokine that alters the immune response and promotes liver fibrosis and hepatocyte transformation towards HCC[18,19]. Alterations and immune deregulation of the cytokine network may further promote the loss of $CD4^+$ cells induced by HIV, and a deregulated $CD4^+$ cell function may lead to a reduction in the anti-fibrotic activity of natural killer cells, possibly resulting in an accelerated progression of liver fibrosis[20]. The marked deregulation of peripheral and intrahepatic cytokine networks and the altered balance between $CD4^+$ and $CD8^+$ in HIV infection may play an important role in accelerating liver fibrosis. In fact, the predominant $CD8^+$ cell response is characterized by an increased production of cytokines such as interleukin-4 (IL-4), IL-5 and TGF-α, which promote collagen deposition by fibroblasts. It has also been observed that the acceleration of liver fibrosis is more pronounced when the peripheral blood $CD4^+$ cell count is consistently decreased[21-25], an observation possibly accounting for the reduction in the secretion of interferon-gamma (a cytokine with anti-fibrotic action) by $CD8^+$ cells following a decline in the number of $CD4^+$ cells[26-28].

There is also some evidence that HIV and HCV infections may promote hepatic fibrosis through an

increase in microbial translocation. Recent studies have shown that massive $CD4^+$ cell depletion in lymphoid tissue at the gastrointestinal level leads to an increased microbial translocation through a disrupted epithelium[29,30], and microbial products, like lipopolysaccharide (LPS), enter the bloodstream[31]. Of note, experiments in animal models have shown that LPS increases hepatic fibrosis and may activate Kupffer cells to promote fibrosis[32]. A further contribution to fibrosis progression may also come from an HIV- and/or HCV-induced inflammatory activity in the liver tissue that, by increasing the susceptibility of intrahepatic lymphocytes, hepatocytes, and/or hepatic stellate cells to apoptosis, may give rise to a continuous cycle of cell death and regeneration in the lymphocytes and hepatocytes that may promote fibrogenesis[1,33].

Metabolic factors like insulin resistance and non-alcoholic liver steatosis have also been proposed as factors involved in the pathogenesis of HCV-related liver disease[34], and in turn HCV and chronic inflammation of the liver may contribute to the development of these metabolic syndromes. In addition, HIV infection also induces metabolic abnormalities, including glutathione deficiency, which could predispose T cells to apoptosis through enhanced susceptibility to oxidative stress[5,35].

In conclusion, HIV infection accelerates hepatic fibrosis progression either by its direct viral action or indirectly through a dysfunction of the immune system, favoring a pro-fibrotic cytokine pattern, an increase in bacterial translocation from the gut to the bloodstream and an enhancement of apoptosis and oxidative stress.

Liver biopsy

Liver histopathology is still considered the gold standard to assess the degree of liver fibrosis, necroinflammation and steatosis[36,37]. The degree of liver fibrosis has been used as a predictive factor of disease prognosis and a guide for treatment of HCV infection[38-44], both in HCV-monoinfected and HIV/HCV-coinfected patients. Liver biopsy, usually performed with a 1.6 mm needle, has some limitations, including sampling errors and intra/inter-observer variations (approximately 24% false-negative rate in the diagnosis of liver cirrhosis)[38,45], infrequent but potentially severe complications and the difficulty to obtain multiple determinations[12,14-16,38,46]. Safety in liver biopsy has always been considered a main issue and important improvements have been obtained over time. In 1986, a nationwide Italian survey considering 68272 percutaneous needle biopsies[14] registered a mortality rate of 9/100000. In this study, ultrasound assistance to liver biopsy was available only for a small percentage of patients and the six patients who died had liver cancer or liver cirrhosis. From then on, the routine use of ultrasound-guided liver biopsy and the improved skills of the clinicians in selecting patients have greatly reduced the incidence of complications and the mortality rate following percutaneous liver biopsy.

The transjugular liver biopsy (TJLB), proposed for patients with coagulation disorders and massive ascites, allows the procurement of a liver specimen even in patients with advanced liver diseases. TJLB is a safe technique that provides good-quality specimens with a low rate of complications[47], and is highly recommended also for patients with coagulation disorders[48]. Another main issue is the representativeness of the liver specimen, which correlates with the number of portal tracts observed at microscopy and consequently, at least in part, with its size. In a recent study, Komemushi *et al*[49] compared the weight of liver specimens obtained in ten bovine livers using either an aspiration-type semiautomatic cutting biopsy needle, or an aspiration-type semiautomatic biopsy needle without aspiration, or a normal-type semiautomatic biopsy needle. The weights of the specimens were 6.80 ± 0.615 mg, 5.62 ± 0.843 mg, and 4.19 ± 0.140 mg, respectively, suggesting that, at least in bovine livers, heavier specimens can be obtained using an aspiration-type semiautomatic cutting biopsy needle.

The liver specimen is formalin-fixed and paraffin embedded. Four microns-thick sections are stained with hematoxylin-eosin or with trichrome stain. Liver fibrosis should be evaluated using the Metavir scoring system[50] or the Ishak[51] scoring system.

Several Authors assessed the degree of fibrosis on a specimen obtained by liver biopsy to predict fibrosis progression and cirrhosis development both in HCV-monoinfected patients[52,53] and in those with HIV/HCV coinfection[54]. Comparative studies showed that HIV infection accelerates the progression of fibrosis[55-57] and that fibrosis is more severe and cirrhosis development more rapid in HIV/HCV-coinfected patients with a $CD4^+$ value < 200 cells/mm^3[58-60].

It has also been demonstrated that a high degree of liver steatosis speeds up the progression of fibrosis to its more severe forms in HIV/HCV-coinfected patients[41-43,61,62].

At present, the examination of a liver specimen is still considered the gold standard to assess liver fibrosis, necroinflammation and steatosis in chronic hepatitis of all etiologies, and to diagnose autoimmune hepatitis, primary biliary cirrhosis, diseases related to iron or copper deposits, alcoholic diseases, genetically induced liver damage and toxicity- and drug-induced liver illness.

RADIOLOGIC TECHNIQUES

Morphological procedures

Conventional ultrasound: Ultrasound (US) examination of the liver, the first non-invasive repeatable procedure used to diagnose liver cirrhosis, remains the first step in the management of chronic hepatitis. Liver fibrosis is detected through US signs such as a coarse or nodular parenchymal feature, hepatomegaly, caudate lobe hypertrophy or irregular liver edges[63]. US cannot differentiate between the different degrees of liver fibrosis, but it allows the assessment of some signs of compensated or decompensated cirrhosis such as portal vein diameter, the velocity of flow, flow

reversal, ascites and splenomegaly[64]. Hepatic surface nodularity, especially as detected by a linear probe, has been shown to be the most direct sign of advanced fibrosis[65].

Contrast-enhanced US can be used for a more accurate detection of cirrhosis[66], but it should be remembered that the "arrival time" of the contrast medium into the hepatic vein is reduced in patients with cirrhosis. In addition, contrast-enhanced US requires additional expertise and entails added costs, factors which may limit its use in routine clinical practice.

As there is a higher risk of an early development of liver cirrhosis and hepatocellular carcinoma (HCC) in HIV/HCV coinfection than in HCV monoinfection[67], conventional US is of great clinical value for an early detection of liver cirrhosis in HIV/HCV coinfection, since it is cheap, easy to perform and safe and, consequently, repeatable. It should be repeated at a 12-mo interval in HIV/HCV-coinfected patients without cirrhosis and at a 6-mo interval in those with liver cirrhosis or an advanced stage of liver disease[68].

Computed tomography: Morphological signs of liver cirrhosis and portal hypertension observed at computed tomography (CT), a technique of high sensitivity but moderate specificity, have been used to diagnose liver cirrhosis. CT allows an examination of the entire abdomen and shows high sensitivity in detecting small varices at various typical locations. Some parameters, obtained from multiple measurements during dynamically-enhanced CT studies and proposed as markers of liver fibrosis[69], have not as yet been validated in multicenter trials.

Of note, some important factors limit the use of CT to assess liver cirrhosis in clinical practice, namely, its cost and the exposure of patients to ionizing radiations and to intravenous contrast medium. As it is not routinely repeatable, it is of little use in HIV/HCV coinfection.

Magnetic resonance electrography: Magnetic resonance electrography (MRE) uses a vibration device to induce a shear wave in the liver. This process involves applying a probe to the back of the patient which generates continuous low-frequency vibrations (60 MHz). Transmitted into the body, the acoustic vibrations produce a shear-wave motion within the liver that is measured through the magnetic resonance imaging (MRI) spin echo sequence. A calculator analyzes the wave images with an inversion algorithm to obtain a quantitative image of shear stiffness (elastogram). MRE has a higher sensitivity than the elastographic methods in defining mild fibrosis and a better reproducibility. A meta-analysis of five trials comparing MRE to liver biopsies showed a sensitivity of 94% and specificity of 95% in differentiating F0-F1 from F2-F4, as well as a sensitivity of 98% and specificity of 94% in differentiating F0-F3 from F4[70]. It is also possible to use MRI techniques to quantify liver fibrosis using diffusion-weighted MRI and contrast-enhanced MRI to evaluate the slow washout of intravenous contrast in fibrotic areas[71]. The use of these techniques is limited by their high cost and by the high degree of expertise required. As they are not routinely repeatable, they are of little use in HIV/HCV coinfection.

Elastography techniques

Fibroscan (transient elastography): Transient elastography (TE, Fibroscan) is a technique used for the non-invasive assessment of liver fibrosis using a transducer on the end of a US probe that transmits 50-MHz pressure waves through the liver tissue. The velocity of the resulting "shear wave", measured by US, correlates with the liver stiffness and provides an estimate of liver fibrosis. Liver stiffness is expressed in kilopascal (kPa) and is measured on a section of liver tissue 100 times bigger than the biopsy sample, ensuring more representative information. The result is the median of at least 10 valid measurements performed in a single session. The system considers valid only the shear waves with a stable velocity. The result of liver stiffness has been correlated with the degree of fibrosis as detected by the Metavir staging system. For HCV-related chronic hepatitis a value lower than 7 kPa reflects fibrosis stage F0-F1, from 7 to 10 kPa stage F2, from 10 to 14 kPa stage F3 and over 14 kPa stage F4, a sign of liver cirrhosis. The TE technique, evaluated for different etiologies of chronic liver disease[72] has a pooled sensitivity and specificity for the diagnosis of cirrhosis of 83% and 89%, respectively. It is easy to perform, repeatable, and well tolerated, but it necessitates expensive equipment and is less reliable in detecting the intermediate levels of fibrosis. In addition, the diagnostic accuracy of transient elastography (TE) is lower in obese patients[73], but a specific probe has been developed to improve the accuracy in these cases[74]. Studies performed on patients with chronic hepatitis B or C[75] have shown that the score of liver stiffness increases in patients with elevated aspartato aminotransferase (ALT) serum levels, indicating a reduced accuracy of TE in detecting liver fibrosis in these cases. Of note, the consumption of a meal before TE can increase the scores by as much as 27%[76].

At present, TE is the procedure used most to assess liver fibrosis as it is well-validated and non-invasive both in HCV monoinfection and HIV/HCV coinfection. Its limitations, however, should be taken into consideration when interpreting the results. De Lédinghen *et al*[77] studied 72 consecutive HIV/HCV-coinfected patients who underwent both liver biopsy and liver stiffness measurement by transient elastography. Liver stiffness values ranged from 3.0 to 46.4 kPa and a value $\geqslant$ 14.5 kPa for the diagnosis of cirrhosis showed good specificity and a positive predictive value.

Acoustic radiation force impulse: This technique uses conventional US to generate a shear wave directly within the liver for the estimation of liver stiffness. The propagation velocity of the shear wave is reported in meters per second and correlates with the liver stiffness.

Due to the direct generation of shear waves within the liver, the distortion of waves induced by chest, abdominal wall and ascites is avoided. Acoustic radiation force impulse (ARFI) has an excellent accuracy in the diagnosis of liver cirrhosis, with 84% sensitivity and 92% specificity. The location of a region of interest allows ARFI to estimate liver stiffness accurately. Measurements made 1-2 cm below the liver capsule offer the best results. The region of interest in ARFI (1-2 cm) is smaller than in TE (5 cm)[78], but placing the region of interest directly in the liver tissue avoids distortions. As regards obesity, ARFI has the same limitations as Fibroscan, providing unreliable results when the body mass index (BMI) is over 30[79]. The accuracy of ARFI has been compared to that of standard TE in HIV/HCV-coinfected patients. In particular, Frulio *et al*[80] studied 46 HIV/HCV-coinfected patients who underwent both ARFI and TE within 6 mo. The agreement between the two methods was defined as very good in predicting severe fibrosis (F $\geqslant$ 3) and moderate in predicting significant fibrosis (F $\geqslant$ 2). Morphological ultrasound analysis concomitant to ARFI detected HCC in two cases, indicating that, at least in this study, ARFI was more useful than TE.

Supersonic shear wave imaging: Like ARFI, supersonic shear wave imaging (SSWI) is a real-time shear wave elastography technique that generates shear waves directly within the liver and uses the Mach cone of supersonic US waves. It uses conventional US and at the same time displays the image of the liver, measures the velocity of the shear wave and calculates hepatic stiffness. Compared to TE, SSWI shows more accuracy in assessing mild fibrotic stages and a similar performance in detecting liver cirrhosis[81]. Based on a single excitation, SSWI analyzes the transversal propagation of the wave outside the region of excitation (ROE), whereas ARFI gives a local measurement of the ROE and a qualitative measurement of liver stiffness. There are no data on the application of this technique in HIV/HCV coinfection, but it seems reasonable that, as it is similar to ARFI, it merits validation also in this setting.

SEROLOGICAL MARKERS OF LIVER FIBROSIS

The assessment of liver fibrosis and the presence of liver cirrhosis based on the results of serological biomarkers have been investigated in several studies. Overall, the association of two or more biomarkers can be considered a good indicator of the presence or absence of severe fibrosis or cirrhosis, but their use in distinguishing between the intermediate stages of fibrosis or evaluating the progression of fibrosis needs further investigation and validation. A combined use of some biomarkers and radiologic techniques might afford a more accurate assessment of liver fibrosis. Two large categories of biomarkers have been established: The direct and indirect. Indirect biomarkers are correlated with the liver function, whereas the direct biomarkers reflect the turnover of the extracellular matrix.

INDIRECT BIOMARKERS

The aspartate aminotransferase/alanine aminotransferase ratio index

The aspartate aminotransferase/alanine aminotransferase ratio (AAR) index, also called aspartate aminotransferase/alanine aminotransferase ratio (AST/ALT) ratio, is one of the oldest markers used in clinical practice for an approximate determination of disease etiology and extent of liver fibrosis. This ratio is usually over 2.0 in alcoholic liver diseases and below 1.0 in patients with long-lasting cholestatic syndromes and in those with virus-related chronic hepatitis without cirrhosis. A significant correlation between this ratio and the presence of liver cirrhosis was documented in a retrospective study on 252 HCV-monoinfected patients, where an AST/ALT ratio of 1.0 or higher was more frequently detected in a subset of 63 patients with cirrhosis than in those without ($P < 0.001$)[82]. In this study the AAR index showed 81.3% sensitivity and 55.3% specificity in identifying cirrhotic patients, but 16 patients died within 1 year of follow-up. There are no data on the application of this index in HIV/HCV coinfection.

The aspartato aminotransferase/platelet ratio index

This test is based on the ratio between AST and platelet count: Aspartato aminotransferase/platelet ratio index (APRI) = [(AST/upper normal limit) × 100/platelet count]. APRI values increase in the case of portal hypertension because of the decline in the platelet count. APRI has been extensively validated in chronic hepatitis C. In a meta-analysis of 18 studies[83], an APRI value > 2 had a specificity of 94% for the diagnosis of cirrhosis and an APRI value > 0.5 showed a sensitivity of 81% and a specificity of 55% for the diagnosis of fibrosis (n = 28 studies). APRI has also been studied in non-alcoholic fatty liver disease (NAFLD)[84], but has not been validated for other etiologies. In a multicenter study, Castera *et al*[85] evaluated the reliability of APRI, Fibrotest, TE, and two algorithms combining TE and fibrotest (FT), or APRI and Fibrotest in a cohort of 116 HIV/HCV-coinfected patients. They observed that for F $\geqslant$ 2, both TE and FT had a better diagnostic performance than APRI ($P < 0.005$) and for F4, TE had a better performance than FT ($P = 0.005$) or APRI ($P = 0.025$). In HIV/HCV-coinfected patients, the performance of APRI and FT might be affected by HIV-induced thrombocytopenia[86] or by drug-related toxicity, *e.g.*, bilirubin elevation caused by atazanavir or gamma glutamyl transpeptidase abnormalities caused by non-nucleoside reverse transcriptase inhibitors.

FT and actitest

FT is a biomarker panel containing 5 biochemical

markers and 2 clinical parameters[87]: Alpha-2 macroglobulin, haptoglobin, total bilirubin, apolipoprotein-A, gamma glutamyl transferase (GGT), age and gender. The results of these biomarkers are combined in a formula yielding a numerical value between 0.0 and 1.0 and the resulting score correlates with the METAVIR fibrosis stages. FT was originally developed in 205 HCV-monoinfected patients and validated in 134 patients. FT was subsequently validated in numerous patients with cirrhosis of different etiologies. Poynard *et al*[88] conducted a meta-analysis of 30 studies (n = 6378 patients) including patients with chronic hepatitis C, chronic hepatitis B, alcoholic liver disease (ALD), and NAFLD. This study demonstrated that FT was moderately accurate in distinguishing between adjacent fibrosis stages in all etiologies investigated. The combination of FT with transient elastography was assessed[89] in a study on 183 HCV-monoinfected patients who underwent FT, TE and liver biopsy. When FT and TE results were concordant, liver biopsy confirmed the diagnosis of cirrhosis in 94% of patients, suggesting that the combination of these two tests can be used instead of liver biopsy in a large proportion of patients. Poynard *et al*[90] studied the progression of fibrosis in 2472 patients with chronic liver disease of various etiologies and found that FT and liver biopsy had a high degree of concordance in estimating fibrosis progression. Vermehren *et al*[91] assessed liver fibrosis using FT and TE in 202 consecutive HIV-infected patients, 35 of whom with HIV/HCV coinfection. A combination of TE and FT indicated significant fibrosis in 8% of patients (31% in HIV/HCV-coinfected and 3% in HIV-monoinfected individuals). The Actitest, an evolution of FT that considers the same panel of biotests plus the ALT values and correlates with liver necroinflammation, has been validated for the diagnosis of cirrhosis in chronic hepatitis C.

The Fibrosis 4 score

The Fibrosis 4 score (FIB4) is a biomarker panel using age, AST, platelet count and ALT [FIB4 = (age × AST)/(platelets × ALT)][92]. This marker was originally developed and validated in a study on 832 HIV/HCV-coinfected patients, where FIB4 > 3.25 had a specificity of 97% for the diagnosis of cirrhosis; the authors estimated that 71% of liver biopsies could be avoided using FIB4[93]. FIB4 was subsequently validated in 592 HCV-monoinfected patients, where a value > 3.25 correlated with cirrhosis, while a value < 1.45 had a sensitivity of 74% in excluding severe fibrosis[94].

Forns index

The Forns Index uses a panel of common parameters: Age, GGT, cholesterol and platelet count but requires a complex calculation of the score. A score lower than 4.25 has a negative predictive value of 96% for excluding significant fibrosis (≥ F2), whereas a score greater than 6.9 has a positive predictive value of 66% for significant fibrosis. This index is therefore useful to identify patients with a low risk of significant fibrosis but does not reliably predict the more advanced stages of fibrosis or liver cirrhosis. The Forns Index should not be used for patients with HCV-genotype-3 infection and liver steatosis since they frequently show a high cholesterol serum level that may affect the score. In addition, the administration of drugs reducing the plasma level of lipids may compromise the test results[95]. The Forns Index is considered useful to diagnose liver cirrhosis, but of lower efficacy in detecting advanced fibrosis. In addition, in HIV/HCV coinfection its diagnostic accuracy is affected by the $CD4^+$ cell count and ALT levels[10].

NAFLD fibrosis score, Fibroindex

The NAFLD fibrosis score is a panel of parameters comprising impaired fasting glucose (diabetes), age, AST, ALT, platelets, BMI and albumin[96]. Fibroindex[97] is a score based on the platelet count, AST and GGT. These tests, considered of some clinical value in detecting liver fibrosis in HCV monoinfection, have not yet been used in HIV/HCV coinfection.

FibroMax

The FibroMax test is a panel of 5 different hepatic tests that allows the assessment of liver fibrosis by a complex sophisticated algorithm. This procedure is based on the Fibro-test, ACTI-test, Steato-test, ASH-test and NASH-test. FibroMax, which has shown similar efficacy to that of histopathology in assessing liver fibrosis in HCV monoinfection, but being non-invasive and repeatable, it might be particularly useful for long-term management and treatment monitoring. It can also measure liver steatosis and/or steatohepatitis. There are no data, however, on its application in HIV/HCV coinfection.

DIRECT BIOMARKERS

Hyaluronic acid

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan in the extracellular matrix that is produced by hepatic stellate cells. Elevated HA levels may be due to an increased production within a fibrotic liver or to a reduced clearance. Serum HA concentration has been found to correlate with both liver inflammatory activity and the fibrotic stage. In a study on 486 HCV-monoinfected patients, those with cirrhosis had significantly higher serum HA levels than those without (382 mcg/L *vs* 110 mcg/L)[98]. In this study, an HA level < 60 mcg/L excluded cirrhosis (sensitivity 98%), while a score > 110 mcg/L showed 78% specificity for cirrhosis. HA has also been combined with indirect markers (bilirubin, GGT, alpha-2 macroglobulin), age and gender to formulate the Hepascore, a panel validated in 221 HCV-monoinfected patients[99]. Resino *et al*[100] studied HA as a possible marker of liver fibrosis in HIV/HCV coinfection in 201 patients naïve for anti-HCV therapy who underwent a liver biopsy. In this study the serum HA levels correlated with the degree of hepatic fibrosis on the liver biopsy, in particular for F4 (Metavir score).

PIIINP

PIIINP (amino-terminal propeptide of serum type Ⅲ procollagen) is a serum marker of collagen turnover indicating tissue repair and fibrosis. Although associated with liver cell necrosis and high aminotransferase serum values, it has been studied as a non-invasive marker of liver fibrosis. First studied in primary biliary cirrhosis (PBC)[101], PIIINP values were found to correlate with the histological stage of PBC and with the levels of cholestasis. In patients with chronic viral hepatitis, PIIINP was identified as an independent predictor of liver cirrhosis[102]. There are no data, however, on the application of PIIINP in HIV/HCV coinfection.

Tissue inhibitor of metalloproteinase-1

Tissue inhibitors of metalloproteinase are a family of enzymes that inactivate collagenase and metalloproteinases. The development of hepatic fibrosis causes an imbalance between collagen production and collagen degradation, which entails decreased levels of serum collagenase. The levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) were found to be higher in alcoholic patients with significant fibrosis and cirrhosis than in those with steatosis alone[103]. TIMP-1 was also studied in a cohort of 194 HCV-monoinfected patients[104] and found to significantly correlate with the stage of fibrosis. A cutoff value of 1300 ng/mL showed 75% sensitivity and 70% specificity for the diagnosis of extensive fibrosis. Of note, also in HBV-related chronic hepatitis TIMP-1 correlated significantly with both liver inflammatory activity and fibrosis[105]. Macías *et al*[106] analyzed the changes in the mediators of fibrogenesis as a non-invasive marker of liver fibrosis in HIV/HCV-coinfected patients starting MVC-based antiretroviral therapy. Twenty-four patients were enrolled and TGF-β1, matrix metalloproteinase-2 and the TIMP-1 were measured in serum samples obtained at baseline and 6 mo after starting maraviroc (MVC)-based therapy. Serum mediators of liver fibrogenesis and fibrosis did not change significantly in HIV/HCV-coinfected patients treated with MVC. Since the TGF-β1 levels have been found to increase in HIV/HCV coinfection in relation to the increase in fibrosis[103], this deterioration was considered to have been prevented by MCV therapy.

YKL-40

YKL-40 (Chondrex) is a member of the bacterial chitinase enzyme family thought to play a role in extracellular matrix remodeling. In alcoholic liver diseases, the levels of YKL-40 were found to correlate with the presence of fibrosis[107] and with a lower survival. In patients with HCV monoinfection the YKL-40 technique showed 80% sensitivity and 71% specificity in the diagnosis of cirrhosis[108]. YKL-40 was investigated in a cohort of 95 HIV/HCV-coinfected patients at the Johns Hopkins HIV Clinic to evaluate its efficacy in the assessment of liver fibrosis: Patients with a Metavir score ⩾ F3 had significantly higher serum levels of YKL and hyaluronic acid than those with a lower fibrosis score ($P < 0.05$)[109].

ASSOCIATION OF DIRECT AND INDIRECT BIOMARKERS

Hepascore

The Hepascore, also known as the FibroScore, includes specific and non-specific parameters to assess liver fibrosis: Age, sex, total bilirubin, GGT, alpha-2-macroglobulin, and hyaluronic acid serum levels; a Hepascore is generated using a very complex equation model. Values ⩽ 0.2 are negative predictive values excluding fibrosis in 98% of cases, whereas values ⩾ 0.8 are positive predictive values for cirrhosis in 62% of cases. Given the good negative predictive value of a low Hepascore, this method is useful to exclude significant fibrosis but is not indicated to predict liver cirrhosis. Calès *et al*[110] compared 5 non-specific tests, APRI, FIB-4, Fibrotest, Hepascore, FibroMeter, and 2 new specific blood tests, FibroMeter HICV (human immunodeficiency and C virus) and HICV test, in detecting liver fibrosis in 467 HIV/HCV-coinfected patients. These tests, originally designed for HCV monoinfection were found to be less effective in HIV/HCV coinfection (the Hepascore in particular), while FibroMeter HICV and HICV test proved to be acceptably reliable in identifying the different stages of fibrosis.

Enhanced liver fibrosis score

The enhanced liver fibrosis score (ELF) score was developed in a cohort of 1021 patients with chronic liver disease[111]. It combined age, HA, TIMP-1 and PIIINP. This test identified liver cirrhosis with 90% sensitivity and 69% specificity and showed high efficacy in ALD and NAFLD. The ELF score to detect liver fibrosis has also been validated in chronic hepatitis C and B[112,113]. A modified ELF (not including age) was validated as a predictor of severe fibrosis in patients with NAFLD[114]. There are no data, however, on the application of ELF in HIV/HCV coinfection.

CONCLUSION

The assessment of liver fibrosis in HIV/HCV-coinfected patients is invaluable for an accurate evaluation of the clinical condition and therapeutic decisions to be made[113-125]. In fact, the main risk for patients with chronic hepatitis is the development of liver cirrhosis and an associated HCC, clinical events entailing liver transplantation and a high mortality rate. Although liver biopsy is considered the gold standard for the assessment of liver fibrosis because it offers a direct view of the liver lesions, it is an invasive procedure with complications in a limited number of cases, although seldom life-threatening, and is not readily accepted by patients and not easily repeatable.

The use of non-invasive radiologic techniques and direct and indirect serological biomarkers to assess

liver fibrosis has gained popularity with clinicians. Non-invasive techniques have been found to be quite sensitive and specific in detecting liver cirrhosis, but less accurate in differentiating between the intermediate stages of fibrosis. Different combinations of radiologic techniques and direct and indirect serological biomarkers to assess liver stiffness are currently under evaluation.

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REVIEW

Dietary approach in the treatment of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has been identified as one of the most prevalent chronic liver disease in adults and children populations. NAFLD is usually associated with the metabolic syndrome (MS), which is chiefly related to insulin resistance and its consequences. Insulin resistance has a crucial role in the pathogenesis of hepatic steatosis and potentially nonalcoholic steatohepatitis (NASH). Because of the contemporary epidemics of MS and obesity, the burden of NAFLD is also expected to rise. Unhealthy diets, such as the so-called western diet, are enriched in fructose, trans-fatty acids and saturated fat and seem to be associated with the development of NAFLD. In human studies, certain dietary sugars, particularly fructose, are used as a substrate for lipogenesis leading to hepatic fatty infiltration, inflammation, and possibly fibrosis. Other investigations have shown that fat consumption especially cholesterol and trans/saturated fatty acids are also steatogenic and seem to increase visceral adiposity. The identification of specific dietary components that favor the development of NASH could be important for the management of this disorder. This review focuses on the effects of different dietary approaches to prevent and treat NAFLD emphasizing the macronutrients and energy composition.

Key words: Fatty liver; Dietary carbohydrates; Dietary

fats; Dietary fructose; Energy intake

Core tip: Nonalcoholic fatty liver disease (NAFLD) has been identified as one of the most prevalent chronic liver disease. Its pathogenesis is not fully elucidated, and until now there is no effective treatment for this condition. Evidence supports that dietary pattern may be related to the development of NAFLD. Furthermore, dietary intervention could be beneficial in NAFLD treatment. However, there is no consensus regarding the best dietary intervention to treat NAFLD. In this context, we conducted a systematic review about recent advances in the effects of different diets in the development of NAFLD in humans, and also in the dietary treatment approach of this disorder.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has been considered the most common chronic liver disease in the Western World[1]. NAFLD defines a spectrum of liver diseases that can progress from steatosis (nonalcoholic fat liver) to nonalcoholic steatohepatitis (NASH), hepatic fibrosis/cirrhosis, and also to hepatocellular carcinoma[2]. The prevalence of NAFLD is growing fast because of the increasing prevalence of obesity and diabetes[1,2]. It is expected, by this year, 2015, that the number of overweight subjects exceeds 2.3 billion. More than 20%, 60% and 90% of the Western population, diabetic individuals, and morbidly obese patients, respectively, will present steatosis. Furthermore, up to 15% of the Western population, 25%-30% of subjects with either obesity or type-2 diabetes mellitus, and over 35% of the severely obese individuals will develop NASH[2].

NAFLD is strongly associated with insulin resistance (IR), visceral obesity, and dyslipidemia; therefore, it was early recognized as the hepatic manifestation of the metabolic syndrome (MS). Currently, NAFLD is considered a multifactorial condition that causes a rise in the rate of complications and death due to liver disorders, and increases the chances of becoming type-2 diabetic and developing cardiovascular diseases[3].

The pathophysiology of NAFLD is complex involving mechanisms not completely understood. However, based on the theory proposed by Day, in 2002, which is widely accepted, IR is crucial element in initiating lipid accumulation in the liver and, possibly, NASH[4]. Several metabolic pathways are involved in the development of NAFLD such as high flux of free fatty acids (FFA) from adipose tissue to the liver due to increased lipolysis in visceral and subcutaneous adipose tissue; enhanced FFA supply to the liver as a consequence of a high-fat diet (HFD); the impairment of the β-oxidation of FFA in the liver; high hepatic *de novo* lipogenesis (DNL); and diminished export of FFA from the liver due to reduced synthesis or secretion of very low density lipoprotein (VLDL)[5-7].

In this context, it seems that the dietary composition is related to NAFLD pathogenesis since it can influence IR, FFA cell influx, DNL, and oxidative stress in the liver[8,9]. Furthermore, the NAFLD/NASH patients seem to have a dietary pattern characterized by a higher consumption of saturated fats (SF) and cholesterol, and lower ingestion of polyunsaturated fats (PUFA), fibers and antioxidants (vitamin C and E)[10,11]. Fructose consumption is likely to be more elevated in subjects with NAFLD than in control patients without NAFLD[12-14]. In subjects with NAFLD, the consumption of fructose per day was associated with more extensive fibrosis[15].

Non-pharmacological interventions are the first clinical approaches aiming at correcting unhealthy life-style, treat the clinical manifestations of the MS, and therefore, are an effective therapeutic option for patients with NAFLD[16]. Lifestyle changes include acquiring healthy dietary pattern, increasing physical exercise and losing weight[1,16]. Although weight loss is recommended in NAFLD treatment, certain diets such as very low-carbohydrate diet (VLCD) or HFD in spite of causing weight loss, can induce IR and, thus, may cause or exacerbate the hepatic disorder. Indeed, modifications of the macronutrient composition of the diet, such as reducing fat or carbohydrate intake, can improve NAFLD without any changes in body weight[8].

This comprehensive review aims to analyze the available clinical trials that evaluated different dietary approaches in NAFLD treatment, and their relationship with intra hepatocellular lipids, hepatic DNL and serum levels of liver enzymes. We also discuss the chief aspects on the role of diet in NAFLD development.

RESEARCH

The systematic review was conducted in the PubMed database using the following terms: "Fatty Liver" AND "Dietary Carbohydrates" OR "Dietary Fats" OR "Diet, Fat-Restricted" OR "Dietary Sucrose" OR "Diet, Mediterranean" OR "Energy Intake" OR "Ketogenic Diet" OR "Diet, High-Fat" OR "Diet, Carbohydrate-Restricted" OR "Feeding" OR "Hyperphagia" OR "Food Consumption" OR "Eating" OR "Food Composition" OR "Portion Size" OR "Food" AND "non-alcoholic".

From the 1422 articles initially selected, the publication date 2004-2015; the English, Portuguese and Spanish languages; and adult age (adult, mild aged and

elderly) were added as filters. The search brought up 147 articles; then, we selected the clinical trials. From the 48 clinical trials, we selected those that evaluated dietary intervention as the unique NAFLD treatment, excluding the studies in which the diet was associated with physical exercises, drugs, herbal medicines, supplements as probiotics, multivitamins or whey protein. The only supplements that we included in the review were n-3 PUFA and fibers. Twenty-five clinical trials were considered for the present review. Additional articles were manually selected from the reference lists of the published systematic reviews and some cross sectional studies, based on their relevance.

Thus, we selected controlled clinical trials in which different dietary approaches were used for treating NAFLD or NASH diagnosed by imaging methods and/or histological evaluation, regardless of sex and ethnic origin of the participants.

THE ROLE OF DIET IN THE DEVELOPMENT OF NAFLD

It is increasingly recognized that hepatic steatosis occurs when there is a combination of IR and permanent excess of fatty acids delivered to the liver[9]. The sources that supply fatty acids to the liver are the endogenous fat deposits, hepatic DNL, and dietary fat intake. Approximately 90% of the FFA originate from adipocyte lipolysis and are released by the action of lipoprotein lipase on adipose tissue and other tissues being transported such as circulating triglyceride (TG)-containing lipoproteins. The second major source of liver fatty acids is their synthesis inside the hepatocytes through DNL, which uses carbohydrates as the major substrate. Although DNL usually represents only 5% of fatty acid in the liver, this percentage can reach 30% in patients with NAFLD[17].

The mechanism involving fat-induced hepatic IR is not fully understood. It is likely that as a consequence of a HFD, there is an accumulation of fat metabolites, which in turn stimulate the secretion of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). These cytokines trigger several signal transduction pathways, for example the serine/threonine kinases [protein kinase-C (PKC)], c-JUN NH2-terminal kinase-1 (JNK), and kappa B-kinase inhibitor, causing hepatic IR[6]. The PKC binds to the insulin receptor, inhibits its tyrosine kinase activity and interfere with the ability of insulin to phosphorylate the insulin receptor substrate-2[18,19]. Furthermore, abundant dietary fat consumption increases FFAs delivered to the liver. FFAs stimulate the hepatocytes, leading to intracellular translocation of the protein Bax to the lysosome and as a consequence release cathepsin B. Capthepsin B induces the nuclear factor-κB translocation which enhanced the secretion of TNF-α inhibiting the action of insulin. Indeed, cathepsin B causes alteration in the mitochondrial function culminating in death of the liver cells and evolution from hepatic steatosis to NASH[20]. Evidence suggests that patients with NASH present impaired postprandial TG response, which may increase TG uptake by the liver, promoting hepatic fatty accumulation[19]. Patients with NASH show an abnormality of hepatic fatty metabolism, which increases lipogenesis. Compared to healthy controls, these patients produce 3 times more TG by hepatic lipogenesis[21].

The intake of simple carbohydrates such as fructose and sucrose has increased over the past decades. Fructose is a monosaccharide that exists in natural foods such as fruits, some vegetables and honey. These natural foods contain small amounts fructose, which are absorbed slowly. However, nowadays, fructose is mostly consumed through commercial and industrial products such as sweetened beverages, soft drinks, and high-fructose corn syrup (HFCS). A high fructose diet decreases the hepatic lipid oxidation by reducing the function of the peroxisome proliferator-activated receptor α (PPARα) leading to hepatic steatosis in experimental models[22]. Furthermore, not only fructose, but also glucose stimulates lipogenic genes in the liver. Diets with high content of fructose or sucrose lead to metabolic alterations related to endoplasmic reticulum stress in the liver by activating JNK, which contributes to liver fat deposition and posterior inflammation[23]. Fructose could affect the metabolism in the liver at the transcriptional level, increasing the proinflammatory transcription factor NFkB and oxidative stress[22]. A high-fructose diet decreases PPARs protein in the liver and increases forkhead boxO1, a transcription factor that stimulates the apolipoprotein apoCIII production, which results in increased VLDL-TG production[24]. The carbohydrate responsive element-binding protein, another transcription factor, participates in the regulation of lipid metabolism in the liver, as carbohydrates bind to it stimulating lipogenic gene expression[25].

In addition to role of the monosaccharides, the effects of high glycemic-index carbohydrate have been explored. The high-glycemic index carbohydrate has a close relationship with obesity, IR and increased plasma and hepatic TGs[26,27]. Dietary glycemic index seems to be associated with the grade of hepatic steatosis, regardless of total energy or carbohydrate intake[28]. The underlying mechanism is not fully elucidated, but some hypotheses have been proposed. Foods with high-glycemic index enhance the hepatic influx of glucose. The excess of hepatic glucose exceeds the ability of glycogen production; therefore, this carbohydrate will be used for the synthesis of new TG through DNL within the hepatocytes. An elevated-glycemic index food might augment oxidative stress, which can contribute to NASH development[29].

Based on the exposed data, it is reasonable to conclude that over-consumption of fat and carbohydrates may promote hepatic steatosis. In the following sections, we will discuss the clinical trials on different diets in the development and treatment of NAFLD.

EFFECTS OF DIETARY CARBOHYDRATE INTAKE IN INTRAHEPATOCELLULAR LIPIDS, DNL AND LIVER ENZYMES - EVIDENCE FROM CLINICAL TRIALS OF "HEALTHY" HUMANS

In only a few clinical trials the effects of excessive carbohydrate intake on the liver were investigated in health humans[30-51].

Intrahepatocellular lipids

Evidence from human studies suggests that in a high simple carbohydrate diet the liver quickly accumulates fat. Sevastianova *et al*[46] evaluated 16 overweight subjects that in addition to their usual diet ingested 1000 kcal/d from simple carbohydrate (candy, pineapple juice, sugar-sweetened soft drinks) for 3 wk, and, thereafter, were placed on a hypocaloric diet for 6 mo. It was observed that carbohydrate overfeeding during 3 wk caused a 10-fold higher relative increase in hepatic fat content (27%) than in body weight (2%). The augment in hepatic steatosis was proportional to the rise in *de novo* lipogenesis. When the patients lost weight, they restored liver fat to normal[46].

Considering the simple carbohydrates, the effects of fructose on liver fatty have been explored in many studies. A group of researchers from Switzerland published 5 studies comparing the effects of high fructose and energy diet with a isocaloric diet on intrahepatocellular lipids (IHCLs) in healthy, male adults[39,40,42,49,51]. In the first study, there were no changes in IHCL [measured by magnetic resonance spectroscopy (MRS)] and insulin sensibility in both hepatic and adipose tissue, as well as in the whole body insulin sensitivity, after a 4-wk fructose overfeeding (1.5 g fructose per kilogram body weight per day, which corresponds to the fructose content of 2 L of soda) in 7 lean healthy males. However, it was observed increased plasma concentrations of triacylglycerol, VLDL-triacylglycerol, leptin and fasting glucose after the intervention[39]. The lack of effects on liver fat accumulation after a fructose overfeeding could have been influenced by the reduced sample size of this earliest study. The results of the following 4 studies from the Switzerland group were not consistent with this finding[40,42,49,51]. They were evaluated in a recent random-effects meta-analysis, which leads to the conclusion that short term (1 wk) hypercaloric (35% of energy above the requirement) fructose diets (3 or 3.5 g fructose per kilogram fat free mass per day) compared with an isocaloric diet increased IHCLs by an average of 54% in a total population of 74 healthy adult males[52].

Moreover, in the context of hypercaloric diets, the effects of high-fructose or high-glucose intake were compared in some interventional studies including different healthy populations[36,42,47,48]. In all those studies, the subjects consumed fructose or glucose dissolved in water, 3 or 4 times per day, with the main meals. The results showed that both hypercaloric diets - high-fructose or high-glucose - increased IHCL, and these effects were not different between the 2 monosaccharides[36,42,47,48].

Fructose and glucose are likely to increase IHCL when they are consumed within a hypercaloric diet. However, the studies that examined their effects when they are intake in an isocaloric diet showed controversial results. In the study by Johnston *el al*[36], fructose or glucose were consumed in an isocaloric diet for 2 wk (25% of daily caloric need from glucose or fructose) by healthy, but centrally overweight men, and both monosaccharide diets did not alter IHCLs[36]. In a controlled, randomized double-blinded study, involving 24 adolescents (with hepatic fat > 8% on imaging) received fructose or glucose beverages (with the same energetic value), during 4 wk (3 servings of 8 fluid ounces bottle of study-provided beverage each day, with 33 g of glucose or fructose), no significant changes in hepatic fat (measured by MRS), liver enzymes and body weight were observed[53]. Finally, in a randomized control trial that included 64 healthy subjects, the authors compared the effects on steatosis [measured by computed tomography (CT)] of a HFCS sweetened beverage with sucrose-sweetened low-fat milk at 8%, 18% or 30% of the caloric needs for maintenance of body weight, and observed no significant changes in liver fat despite the kind or quantity of the beverage[32]. These findings support that if monosaccharides are ingested in a normocaloric diet in usually ingested sweeteners, such as sucrose or HFCS, hepatic steatosis is not an expected finding. However, the study by Maersk *et al*[41], which evaluated the results on hepatic fat deposition (measured by MRS) of 47 healthy that consumed 1 L/d of sucrose sweetened regular cola (Coca Cola®; 106 g sucrose/day = 53 g bound fructose/day; 430 kcal), aspartame-sweetened diet cola (Coca Cola®; 4 kcal), semi-skimmed milk (Arla Foods®; 451 kcal), or mineral water (Aqua D'Or®; 0 kcal), during 6 mo, demonstrated significant increase in IHCL in the sucrose sweetened regular cola group, without any changes in whole caloric consumption or body weight[41].

Hepatic de novo lipogenesis

There are few data from human studies about the effects of carbohydrate intake in hepatic DNL. A hypercaloric high-fructose diet (3 g per kilogram body weight plus balanced diet to keep body weight) rose the DNL in the liver by 7.8% (95%CI: 5.8%, 9.8%) in 7 male subjects without any disease[35]. When compared to a hypercaloric high-glucose diet, only the hypercaloric high-fructose (additional 25% of daily energy, during 10 wk) elevated DNL, caused dyslipidemia, reduced insulin sensitivity, and augmented visceral adiposity in subjects who are overweight or obese[50]. On the other hand, the addition of the non-digestible carbohydrate inulin to a diet with 55% of total energy from carbohydrate, for 3 wk, decreased lipogenesis in the liver and blood triglyceride concentrations in 8 healthy subjects examined

in a double-blind, randomized, placebo-controlled cross-over study[54].

Liver enzymes

The liver enzymes outcomes after hypercaloric high-fructose diet are distinct among the studies. After a high-fructose diet (200 g/d) during a 2-wk period, 74 years old male subjects, without any disease, presented increase in all liver enzyme concentrations[43]. In 2 randomized control trials, the authors demonstrated that a diet rich in fructose (3.5 g fructose per kilogram fat free mass daily; 30%-35% of calories above the energy requirement) was associated with increase only in the alanine aminotransferase (ALT) concentrations when compared to the consumption of a weight maintaining diet in healthy people[33,40]. However, in another randomized control trial, the authors did not observe any alteration in this parameter with the same intake of fructose[42]. Sobrecases *et al*[49] assessing the respective effects of high-fructose, high-saturated fat and the association of these diets in young men, without any disease or obesity, verified that the high-fructose diet alone did not change the ALT serum levels; however, the high-fructose associated with high-saturated fat diet increased the concentrations of this enzyme[49].

A random-effects meta-analysis on randomized control trials[30,34,36,42] that compared the outcome of the liver enzymes between hypercaloric high-fructose and hypercaloric high-glucose diets (range 40 g/d to 3.5 g fructose per kilogram fat free mass per day) did not show any significant differences in the ALT and aspartate aminotransferase (AST) levels, regardless of the tested monosaccharide[52]. Only one study demonstrated that high consumption of fructose, but not of glucose, increased gama-glutamyl transpeptidase (GGT) activity[34]. Unexpectedly, an energy balanced diet, with 25% of the caloric need per day from fructose or glucose, was associated with a slight reduction in the liver enzymes concentrations in centrally overweight men[36].

The effects of a hyperenergetic high-sucrose diet on liver function tests have also been investigated. A hyperenergetic (double energy requirement) high-sucrose diet (32% of caloric requirement from sucrose), compared with a standard isocaloric diet in 12 healthy male subjects, increased the blood concentrations of alkaline phosphatase, ALT, AST, GGT, and bilirubin[45]. In agreement with these findings, Porikos *et al*[44] demonstrated that a hypercaloric sucrose containing food-supplemented diet (25%-30% kcal) also raised the ALT and AST levels. However, comparing high-sucrose diet with high-glucose diet, it was not observed any differences in the ALT and AST levels[30,37].

The chief limitations of all intervention studies presented above are the following: (1) small sample size; (2) most studies included only men, and the effects in women may differ due to differences in fructose metabolism mediated by hormonal and anthropometric mechanisms; (3) evaluation of the effects of the disaccharides or monosaccharides on the liver in short term; (4) monosaccharides were provided as their constituent powders as opposed to either incorporated into the matrix of a foodstuff, or as a constituent of sucrose in usual diet; (5) use of large amounts of monosaccharide, which were higher than the levels usually consumed; and (6) fructose or sucrose consumption seems to have been confounded with the excess of caloric ingestion in some studies. Regardless the limitations it is reasonable to conclude that the consequences of fructose consumption on glucose and lipid metabolism seems to be dose-dependent. High-fructose, -glucose and -sucrose diets influence on the amount of fat in the liver, but fructose seems to cause DNL in a higher magnitude. However, the effects of long term dietary intervention on hepatic DNL were not evaluated. Further studies are needed to evaluate if the energy overfeeding changes are monosaccharide specific, and to assess the outcomes of low monosaccharide intakes in patients with NAFLD. Evidence regarding the association between carbohydrate intake and the development of NAFLD comes from observational studies and only a few data result from interventional studies as we will discuss in the following topics.

CARBOHYDRATE INTAKE AND CARBOHYDRATE DIETARY INTERVENTION IN THE TREATMENT OF NAFLD

Some observational studies demonstrated association between high carbohydrate intake or increased simple sugars (sucrose or fructose) consumption and the development of NAFLD[12-14,55-58]. The intake of simple sugars appears to have been associated with the amount of fat in the liver and to the severity of the disease[55,57]. A cross-sectional study demonstrated that high intake of fructose was associated with increased GGT concentrations in 38 subjects under 19 years old who were overweight or obese and presented NAFLD[59]. Based on these findings, some clinical trials were conducted with the aim of understanding the effects of carbohydrate restriction in improving NAFLD.

Intrahepatocellular lipids

In a controlled clinical trial including 18 patients with NAFLD (14 of them biopsy proven), the authors evaluated the effectiveness of a 2-wk administration of VLCD (20 g/d, 9 patients) *vs* calorie restriction (1200-1500 kcal/d, 9 patients) at reducing hepatic TGs measured before and after the intervention by MRS. Weight loss was similar between the groups. Liver TG decreased significantly with weight loss, but the reduction was more intense in the low-carbohydrate subjects compared with in the low-calorie group[60].

In order to evaluate the effects of an even more restricted carbohydrate diet (< 20 g/d of carbohydrate)

on liver histology, a pilot study including 5 NAFLD obese subjects (confirmed by liver biopsy) were instructed to follow a ketogenic diet associated with nutritional supplementation for 6 mo. Post-treatment liver biopsies were performed in 4 of those patients and showed improvement in steatosis, inflammatory grade, and fibrosis. Additionally, after treatment, the patients lost weight (mean weight loss 12.8 kg; range 0-25.9 kg)[61].

Among the simple carbohydrates, the effects on IHCLs of a fructose reduction diet were evaluated in a before-after clinical trial including 10 overweight adults with NAFLD. After 6 mo of the intervention, the subjects diminished their fructose intake in approximately 61% associated with significant reduction in total energy consumption per day, total fat, and SF (224%), which resulted in a decreased of IHCL content (236%). In addition, the patients presented a reduction in their body weight and body mass index (BMI)[62].

Liver enzymes

To evaluated the effects on liver enzymes of different proportion of carbohydrates in a hypocaloric diet, Ryan *et al*[63], randomized 52 subjects with obesity and IR (high possibility of developing NAFLD) to receive a normal carbohydrate (60% carbohydrate, 25% fat or both) or moderate restricted carbohydrate (40% carbohydrate, 45% fat) diet during 16 wk. The two dietary interventions lead to a similar decrease in body weight, daily insulin requirement and plasma ALT levels; however, the 40% moderate restricted carbohydrate intervention was associated with more significantly decrease in IR, and in serum levels of insulin and ALT. The reduction of the ALT concentrations was associated with increasing in insulin sensitivity and decreasing in daily insulin requirement[63]. On the other hand, a 2-wk on VLCD (20 g/d) or calorie restriction (1200-1500 kcal/d) correlated with a reduction in the serum levels of AST, but not ALT, in patients with NAFLD[60].

The specific effect of a low-fructose diet was evaluated in both children and adults with NAFLD. Obese children with NAFLD received a restricted-fructose diet (no intake of beverages with sugar neither any type of food sweetened with HFCS) or a restricted-fat diet [according to the American Heart Association (AHA) guidelines] associated with instructions about the diet, during 6 mo. The comparison between the 2 groups demonstrated no improvement in ALT and AST concentrations in either group at the end of the intervention. Likewise, children's BMI Z scores demonstrated no significant improvement[64]. On the other hand, in an adult overweight population with NAFLD, fructose-restricted diet during 6 mo led to weight loss, normalization of AST and ALT levels, and reduction in GGT concentrations in 7 out of 10 patients[62].

Studies using indigestible carbohydrates as dietary fiber supplementation have attracted interest of researchers due to its several physiological benefits. After treatment with soluble fibers (10 g/d) for 3 mo, 75% of the NAFLD patients presented normalization of the liver enzymes (AST, ALT and GGT), and 100% of them showed reduction in BMI, waist circumference and IR index[65]. Corroborating these findings, Daubioul *et al*[66], in a randomized double-blind crossover investigation, studied the effects of daily ingestion of oligofructose (OFS), a kind of soluble fiber, in 7 patients with NASH (biopsy proven). The subjects were randomly assigned to intake 16 g of OFS or maltodextrine (placebo) daily during 8 wk. OFS decreased serum AST after 8 wk, and insulin concentrations after 4 wk[66]. In a randomized controlled trial, the authors evaluated the effects of beta glucan-containing oat cereal (n = 16), another soluble fiber, *vs* placebo (n = 18) in overweight subjects during 12 wk. The consumption of oat reduced the levels of AST and ALT, body weight, BMI, percentage of body fat, and waist-to-hip ratio. However, the anatomic changes were not observed on ultrasound examination[67].

EFFECT OF DIETARY LIPIDS INTAKE IN INTRAHEPATOCELLULAR LIPIDS IN HUMANS WITHOUT FATTY LIVER

HFD is involved in the pathogenesis of IR[68]. Dietary fat and oxidative stress are likely to play a role in NAFLD pathogenesis and its evolution to NASH. The effects of dietary fat content on hepatic TG (assessed by MRS), body fat distribution [evaluated by magnetic resonance imaging (MRI)], biomarkers of inflammation (serum concentrations of IL-6, IL-12, TNF-α, interferon-γ), and oxidative stress (assessed by urinary F2-α isoprostanes) were evaluated in overweight or obesity patients without glucose intolerance. The subjects ingested a control diet (35% fat, 12% saturated fat and 47% carbohydrate) during 10 d, and then, they consumed a low fat [(20% fat wherein 8% as saturated fat) and 62% carbohydrate; n = 10] or a HFD [55% fat (25% saturated fat) and 27% carbohydrates; n = 10] for 4 wk. After the intervention, both groups remained with body weight stable. In the low-fat diet group, compared to the control diet, the hepatic TG decreased, but, in the HFD patients, the hepatic TG presented no alteration. In both diets, intra-abdominal fat did not change; however, the subcutaneous abdominal fat increased in the HFD group. The inflammatory markers, fasting metabolic parameters and urinary F2-α isoprostanes did not demonstrate any changes[69]. Contrary to these findings, the consumption of HFD by 10 healthy subjects, during 4 d, increased IHCLs by approximately 90%[70].

The effects of an isocaloric restricted-fat, restricted-saturated fat (LSAT) and restricted-glycaemic index (GI) diet [LSAT: 23% fat (7% saturated fat), GI < 55; n = 20] on liver fat (without weight loss) were compared with the effects of a high-fat, high-saturated fat (HSAT) and high-GI [HSAT: 43% fat (24% saturated fat) GI > 70; n = 15] diet in an old population. In the LSAT group the IHCL (measured by MRI) decreased significantly while in the HSAT there were no changes in this parameter. The LSAT diet also reduced total cholesterol,

high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and fasting glucose, and increased TG levels, and the HSAT intervention had no influence on HDL-c or glucose and raised LDL-c and total cholesterol. The Matsuda index of insulin sensitivity got better on the LSAT intervention, but fasting insulin and homeostasis model of insulin resistance (HOMA-IR) did not demonstrated any improvement as a result of both dietary interventions[71].

Several important questions about the effects on hepatic steatosis remain unanswered, including the effects of the different types of fat: monounsaturated fatty acids (MUFA), PUFA, and SF acid (SFA). In a randomized, parallel-group study, the authors compared the effects of PUFAs and SFA on hepatic steatosis, systemic inflammation, and metabolic disorders in 61 subjects with abdominal obesity (15% with type-2 diabetes). The subjects received a 10-wk isoenergetic diet (without altering the macronutrient intake), high in vegetable n-6 PUFA (PUFA diet) or in SFA mainly from butter (SFA diet). In the PUFA diet group, hepatic steatosis (measured using MRI and MRS), TNF-α receptor-2, and IL-1 receptor antagonist levels reduced, whereas blood insulin tended to increase in the SFA dietary intervention patients. The n-6 PUFAs diet compared with the SFA diet intake modestly improved the metabolic status and decreased liver fat regardless of weight loss. Indeed, a high n-6 PUFA diet was not associated with any signs of oxidative stress or inflammation[72]. Considering the suppressor action of long-chain PUFAs on DNL in the liver, it could believe that fish oil supplements might be useful in the treatment of NAFLD[35]. This subject will be discussed further.

FAT INTAKE AND FAT DIETARY INTERVENTION IN THE TREATMENT OF NAFLD

Some observational studies were performed to evaluate fat intake by individuals with NAFLD. According to the investigation by Musso *et al*[11], the usual dietary pattern of subjects with NAFLD without hyperlipidemia, diabetes and obesity was rich in SF and cholesterol, and poor in PUFAs. SF intake correlated with insulin sensitive index, features of the MS, and increased postprandial TG[11]. Compared to the healthy controls, patients with NAFLD have been shown to have a lower dietary intake of omega-3 fatty acids and to have an increased n-6/n-3 ratio in their diet[57,73]. The elevated n-6/n-3 ratio in the food has been associated with a pro-inflammatory state[74,75] and increased lipogenesis causing steatosis in animal models[76,77]. On the other hand, it has been demonstrated that omega-3 PUFAs inhibits sterol regulatory element binding protein 1c and upregulates PPARα favoring fatty acid oxidation and improving steatosis[78]. NASH patients seem to have impairment in glutathione metabolism towards an oxidant status, and this alteration has been associated with a high intake of dietary SF and a low consumption of carbohydrates[79]. Based on this finding, some clinical trials were designed to investigate the modulation of dietary fat in the treatment of NAFLD.

Intrahepatocellular lipids

Chan *et al*[80], studied 9 NAFLD subjects who underwent a weight loss program through a low fat diet (LFD) and the results showed a decrease in body weight, BMI, hepatic steatosis, amount of visceral and subcutaneous fat, HOMA-IR score, TGs, VLDL-apoB-100 levels, and VLDL-apoB-100 secretion rate. The amount of steatosis reduction correlated significantly with a decrease in the rate of secretion of VLDL-apoB-100 and visceral fat[80].

The modulation of the different types of fat as a dietary approach of NAFLD has been examined. The effects of the Mediterranean diet (MD), known as a high content in MUFA diet, on IHCL (measured by MRS) and insulin sensitivity were evaluated in 12 biopsy proven NAFLD subjects without diabetes, in a randomized, crossover investigation comprising 6 wk of diet intervention. All participants receive, in a random order, both MD and a control diet [low fat, high-carbohydrate diet (LF/HCD)], with a wash-out period of 6 wk between the dietary interventions. The results demonstrated that although the patients did not present weight loss, the MD was associated with a reduction in the hepatic fat content and also improved insulin sensitivity in the patients with NAFLD and glucose intolerance, in comparison with the subjects from the LF/HCD group[81].

Nigam *et al*[82], compared, in NAFLD patients, the effects of different types of vegetal oil: Canola oil (61% MUFAs, 7% saturated fatty acids and n-6/n-3 ratio nearly 2/1), olive pomace oil (70% MUFAs, 15% saturated fatty acids and n-6/n-3 ratio nearly 9/1), and control oil group - soybean or safflower (15%-24% MUFAs, 12%-16% saturated fatty acids, 50%-60% PUFAs and n-6/n-3 of 7/1 for soya oil and higher than 100 for safflower oil). Ninety-three patients, matched by age and BMI, were randomly assigned into 3 groups to intake not more than 20 g daily of olive oil, canola oil, or soyabean/safflower oil (control; n = 30) and lifestyle counseling, for 6 mo. Compared to the control oil group, the olive oil patients decreased weight and BMI. Furthermore, these subjects presented reduction in fasting insulin level, HOMA-IR, HOMA denoting b-cell function, and disposition index, when compared to the canola oil individuals. Pre- and post-intervention analysis demonstrated that the olive oil group presented increase in HDL-c levels; the canola oil group presented reduction in fasting blood glucose and TG; and both groups (olive and canola oils) presented improvement in the grade of fatty liver and liver span[82].

In several studies, the effects of the intake of n-3 PUFA supplements were investigated as a complementary treatment of NAFLD[83-89]. In general, these studies demonstrated a reduction in liver fat measured by different imaging methods, with the doses raging from 0.83 to 6 g/d, and duration therapy ranging from

8 wk to 18 mo[83-87]. A supplementation of 2 g of n-3 PUFA 3 times per day, for 24 wk, was associated with a complete fatty liver regression in almost 20% of the NAFLD patients and some reduction in 53% of them. In contrast, in the placebo group, 7% fatty liver completely regressed and 35% presented certain reduction in IHCL (measured by ultrasound)[87]. Similar effects were observed using a supplementation daily dose of 4 g of n-3 fatty acids, for 8 wk, on liver fat (measured by MRS) in women with polycystic ovary syndrome. N-3 PUFA diminished liver steatosis, TGs levels, and systolic and diastolic blood pressures[83] while these effects were not observed in the placebo group. Using the same dose of supplementation (4 g/d) of docosahexaenoic acid (DHA) plus eicosapentaenoic acid (EPA), NAFLD subjects were randomized in a clinical trial, double blind and placebo-controlled to receive n-3 therapy (n = 51) or placebo (n = 52) during 15-18 mo. Liver fat percentage was measured by MRS in 3 liver zones and liver fibrosis was evaluated by 2 validated scores. The supplementation adherence and the presence of contaminants as DHA and EPA in the placebo group were investigating by verifying erythrocyte percentage of DHA and EPA enrichment. The median liver fat at the beginning was 21.7% in the placebo group and 23% in the intervention group, and they changed to 19.7% and 16.3%, respectively. The adjusted multivariable regression model demonstrated a trend towards a reduction in liver fat following treatment with the DHA + EPA; however, there was variable adherence to the intervention and evidence of contamination in the placebo group. DHA enrichment was independently associated with a reduction in hepatic steatosis in the regression analysis. There was no improvement in the fibrosis scores. According to the results, erythrocyte DHA enrichment with DHA + EPA supplementation could be associated with less hepatic steatosis. The authors concluded that increased percentage of erythrocyte DHA enrichment can cause a reduction in hepatic steatosis in NAFLD subjects[84].

These findings were corroborated by the results of another study, in which a smaller dose (2 g/d) of PUFA associated with AHA recommended diet, for 6 mo, were evaluated. About one third (33.4%) of the NAFLD patients presented a complete fatty liver regression, and 50% demonstrated some reduction. In contrast, no patient achieved complete regression in the group that received only the AHA diet, and only 27.7% of them presented some reduction in the steatosis[86]. Another study employing low dose of n-3 PUFA supplementation (0.83 g/d, of which 0.47 g of EPA and 0.24 g of DHA) in olive oil demonstrated improvement in the liver echotexture (evaluated by ultrasound) and in the hepatic Doppler perfusion index, after 12 mo of treatment of NAFLD patients, compared to a control group of individuals also with NAFLD[85].

Although the investigations based on imaging methods demonstrated a reduction in liver fat as commented above, the studies that investigated the role of n-3 PUFA in NAFLD treatment using liver histology to evaluate the results have shown controversial results[88,89]. Tanaka *et al*[89] prescribed highly purified EPA (2700 mg/d) to 23 NASH (biopsy-proven) patients during 12 mo and the effects were measured by biochemical parameters and hepatic biopsy. The outcome showed a reduction in AST, FFA, soluble TNF-α receptors 1 and 2, ferritin, and thioredoxin serum concentrations, which may be the result of oxidative stress in the liver. Body weight, blood glucose, insulin and adiponectin concentrations did not changed. Post-treatment liver biopsy was performed in 7 out of the 23 NASH patients and demonstrated improvement in liver fat, fibrosis, ballooning in liver cells, and lobular inflammation in 6 subjects[89]. However, in a larger phase 2b multicenter, placebo-controlled trial, these findings were not confirmed[88]. Sanyal *et al*[88], compared the effects of different doses of EPA-E, a synthetic PUFA, in the liver histology of 243 NASH patients with NAFLD activity score $\geqslant$ 4 (minimum score of 1 for steatosis and inflammation, along with either ballooning or at least stage 1a fibrosis). The patients were randomized into three groups which received low-dosage EPA-E (1800 mg/d; n = 82), high-dosage EPA-E (2700 mg/d; n = 86) or placebo (n = 75) and were followed for 12-mo. Liver biopsy was performed 2 wk after the end of the treatment. The primary efficacy end point was NAFLD activity score $\leqslant$ 3 without fibrosis worsening; or a reduction in NAFLD activity score $\geqslant$ 2 with contribution from at least 2 variables, without worsening of fibrosis, 1 year after the end of the intervention. The percentages of patients in the groups that achieved the main end point were similar: 40% in the placebo group, 37% in the low-dose, and 35.9% the high-dose group. EPA-E was not associated with any improvement in the histological parameters of NASH (liver fat, inflammation, ballooning, or fibrosis scores)[88].

Liver enzymes

In some of the studies the effects of n-3 PUFA supplementation were also assessed by the analysis of the liver enzymes[85,87-89]. In an investigation, patients with NAFLD that consumed olive oil plus 0.83 g of n-3 PUFA, during 1 year, presented reduction in liver enzymes (ALT, AST and GGT) and TGs serum levels, and increased in adiponectin serum concentrations, compared with the control group that received similar package of olive oil without addition of n-3 PUFA[85]. In the study in which the NAFLD subjects consumed a higher dose of n-3 PUFA supplementation (2 g/d) associated with the AHA diet or received solely the AHA regular diet, during 6 mo, it was observed a decrease in the ALT levels after the treatment with the n-3 supplementation plus diet, while the other enzymes remained unchanged. These patients also presented a reduction in the TGs and TNF-α level, and in the HOMA-IR score. No alterations in the evaluated parameters were verified in the control group[86]. Different findings were observed with the use of EPA in a dose of 2.7 g/d in the NASH (biopsy-proven) patients, for 12 mo. Among the liver enzymes, only the AST levels were reduced[89]. Corroborating the results of Tanaka *et al*[89], it was demonstrated that in a higher

EPA dose (6 g/d), the NAFLD subjects demonstrated more expressive reduction in the ALT concentrations and also in total symptom scores and serum TG levels when compared to a placebo group. In both groups there were no changes in body weight, fasting glycemia, renal function and blood cell counts, but there was a tendency toward improvement in AST, GGT, total cholesterol and HDL-c levels, without significant differences between the groups[87].

The possible benefit of EPA supplementation on liver enzymes was not supported in a large population of patients with biopsy proven NASH. Both EPA-E 1800 mg/d and EPA-E 2700 mg/d used for 12 mo in NASH patients had no effects on liver enzymes concentrations, IR, adiponectin, keratin 18, high-sensitivity C-reactive protein, or hyaluronic acid levels, but the elevated dose of EPA-E was associated with a reduction of TG levels, without any serious adverse events[88].

CLINICAL TRIALS COMPARING CARBOHYDRATE *VS* FAT DIETARY INTERVENTIONS

Liver enzymes

The results of the studies comparing the effects of low carbohydrate diet (LCD) with LFD on liver amino-transferases in NAFLD patients, allow concluding that despite of macronutrient restriction both diets lead to favorable results when associated with a reduction in body weight[90,91]. In one of these studies, obese subjects were included in the group Ⅰ (n = 112) if normal levels of ALT, or group Ⅱ (NAFLD, n = 30) if increased ALT concentrations (≥ 43 UI/L); then, they were randomly allocated to receive LFD or LCD, for 3 mo. After outcome analysis, the authors concluded that weight reduction following either hypocaloric diets - LFD or LCD - was associated with improvement in ALT concentrations and IR in subjects with NAFLD[90]. Likewise, Rodríguez-Hernández *et al*[91] randomized 59 obese women with NAFLD to receive either LCD (n = 31) or LFD (n = 28), for 6 mo. At end of the intervention, both groups presented weight loss (5.7% and 5.5% in the LCD and LFD, respectively) and decrease in AST and ALT serum levels, but without differences between the groups[91].

Based on the above data, some authors investigated the effects of energy restriction diet without modulation in the proportion of the macronutrients in the treatment of patients with NAFLD, as will be discussed in the next topic.

ENERGY RESTRICTION ON DIETARY INTERVENTION IN THE TREATMENT OF NAFLD PATIENTS

According to the American guidelines for the diagnosis and management of NAFLD, loss of at least 3%-5% of the initial weight through hypocaloric diet (alone or associated with increased physical activity) reduces liver fat, however more expressive weight loss (up to 10%) might be necessary to determine improvement in necroinflammation[1]. In this context, Elias et *al*[92] evaluated the effects of a 6-mo hypocaloric diet (reduction of 500-1000 calories per day) with 15% protein, 55% carbohydrates and 30% fat, on IR, biochemical parameters of the MS, and grade of liver fat in 31 NAFLD subjects. The participants were called adherent (group 1; n = 17), if they showed weight loss higher than 5% of the initial weight, or non-adherent (group 2; n = 14), if they do not reach 5% weight loss from the initial weight. Group 1 patients improved all the anthropometric parameters, and presented a reduction in ALT and GGT levels, HOMA-IR, visceral fat and hepatic density on computed tomography, along with increase on HDL cholesterol levels. These subjects reduced the total caloric consumption and total and saturated fats intake. Group 2 presented only a significant decrease in BMI and waist circumference. Based on these results, the authors concluded that the dietary intervention as the only therapeutic approach but leading to a weight loss of at least 5% from the initial body weight is effective in improving NAFLD[92].

A group of Taiwanese researchers compared the effects of 2 different VLCDs (450 or 800 kcal/d) in 132 subjects presenting obesity (83 with NAFLD). The patients were randomized to 2 VLCD groups aimed to weight loss in 12 wk. During the 12-wk intervention period, the VLCD-450 group showed a loss of 9.14% of the initial body weight and the VLCD-800 group lost 8.98% of the initial weight as revealed by the intention-to-treat analysis. A total of 40.9% subjects from the VLCD-450 group and 43.9% from the VLCD-800 group lost at least 10% of the body weight at the end of the intervention. In both groups, it was observed improvement in the following parameters: body weight, waist circumference, hip circumference, fat mass, blood pressure, TG and glycemia, without difference between the 2 groups. NAFLD was resolved in 41.5% of the cases in the VLCD-450 group and in 50.0% in the VLCD-800 group[93].

Until nowadays, there is no specific recommendation regarding the amount of calorie restriction necessary to improve NAFLD. As demonstrated by Elias *et al*[92] and Lin *et al*[93], the reduction of 500 to 1000 kcal/d in the usual energy intake or even a more restrictive diet (450 or 800 kcal/d) could be effective and safety to treat obesity and to improve NAFLD; without any additional benefit in recommending a more restrictive dietary intervention.

CONCLUSION

Regardless of weight loss, restriction and modulation of dietary carbohydrate (*e.g.*, restriction of simple carbohydrate and high glycemic carbohydrate) and fat (*e.g.*, restriction of total and saturated fat and

increase in MUFAs and n-3 PUFAs) seem to improve metabolic parameters such as IR, decrease the liver enzymes levels and/or reduce the grade of steatosis in NAFLD patients. Contrary, weight loss, independently of restriction of carbohydrate or fat improves the liver parameters. Therefore, in some studies, the effects of the restriction of one macronutrient *per se* could have been confused with the effects of a restriction of energy from the diet. Finally, data demonstrating improvement in liver histology associated with different dietary approaches are scarce. In this context, long-term studies are needed to elucidate the detailed dietary approach of NAFLD, before getting to clinical practice.

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MINIREVIEWS

Roles of lipoprotein receptors in the entry of hepatitis C virus

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Abstract

Infection by hepatitis C virus (HCV), a plus-stranded RNA virus that can cause cirrhosis and hepatocellular carcinoma, is one of the major health problems in the world. HCV infection is considered as a multi-step complex process and correlated with abnormal metabolism of lipoprotein. In addition, virus attacks hepatocytes by the initial attaching viral envelop glycoprotein E1/E2 to receptors of lipoproteins on host cells. With the development of HCV model system, mechanisms of HCV cell entry through lipoprotein uptake and its receptor have been extensively studied in detail. Here we summarize recent knowledge about the role of lipoprotein receptors, scavenger receptor class B type I and low-density lipoprotein receptor in the entry of HCV, providing a foundation of novel targeting therapeutic tools against HCV infection.

Key words: Lipoprotein receptors; CD81; Scavenger receptor class B type I ; Hepatitis C virus entry; Low-density lipoprotein receptor

Core tip: As cirrhosis and hepatocellular carcinoma caused by hepatitis C virus (HCV) is one of the major health problems in the world, the investigation of HCV infection becomes more and more important. HCV entry is the initial step to start infection and is a multiple process involved in abnormal metabolism of lipid. Hence, here we summarize recent knowledge about the role of lipoprotein receptors for better understanding of HCV.

Lyu J, Imachi H, Fukunaga K, Yoshimoto T, Zhang H, Murao K. Roles of lipoprotein receptors in the entry of hepatitis C virus.

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INTRODUCTION

Hepatitis C virus (HCV) mainly affects liver and causes infectious disease hepatitis C in the world[1]. As a RNA virus, HCV infects about 2%-4% of people all over the world and induces kinds of liver diseases, including about 343000 deaths due to liver cancer from HCV occurred in 2013 up from 198000 in 1990 and an additional 358000 in 2013 occurred due to cirrhosis[2]. Different from hepatitis A virus and hepatitis B virus, there is no available vaccine against HCV until now, and current therapy for HCV infection is based on direct-acting antivirals with or without peginterferon plus ribavirin[3,4]. Hence, knowing the mechanism of HCV infection is becoming more and more important.

HCV AND ITS STRUCTURE

The HCV belongs to the family Flaviviridae and is a kind of the genus hepacivirus. Based on the differences of nucleotide sequence, which is 30%-35% varying over the complete genome, it is classified into seven genotypes[5]. Sixty percent of all cases are caused by subtypes 1a and 1b and both types of HCV are able to be found all around the world. For further study on HCV entry and the elucidation of the mechanisms of HCV infection, HCV-like particles (HCV-LP), HCV pseudotyped particles (HCVpp) and HCV cell culture (HCVcc) system is widely developed recently[6-8]. HCV-LPs, production of baculovirus expression systems, can infect both hepatoma cells and human primary hepatocytes by the mediation of its receptors[9]. However, they are lack of reporter to reflect the earliest stages of infection[10]. The disadvantage of HCV-LPs is made up by HCVpp, which is produced by lentiviral particles incorporating unmodified HCV glycoproteins into the lipid envelope[11,12]. HCVpp mimics the very early stage of cell entry by carrying a marker gene. Only one deficiency of HCVpp system, they cannot be associated with lipoproteins, because it is lack of lipoproteins in the producing cells, 293T kidney cells[10]. Following, HCVcc system is developed to represent the complete replication cycle of virus and to release the production of authentic virus particles that are able to infect *in vitro* and *in vivo*[13,14]. Until now, HCVcc system is the only model system which can completely mimic a natural HCV infection, although its recipient cells are limited to two specific cell lines, LH86 and Huh-7[10]. Unfortunately, there are few suitable small animal models for the research of HCV and only used for certain aspects of HCV infection *in vivo*[15,16].

In a typical HCV particle, a core of genetic material (a positive single-standard RNA), which consists of a single open reading frame of 9600 nucleotide bases long, is surrounded by a protective shell of nonstructural protein (NS2, NS3, NS4A, NS4B, NS5A and NS5B), and further encased in a lipid envelope[10]. There are two kinds of viral envelope glycoproteins, E1 and E2, which are embedded in the lipid envelope. Since non-structural proteins play important roles in viral self-replication, both envelope proteins are necessary and serve as the fusogenic subunit during the process of HCV entry; particularly, E2 acts as the receptor binding protein[17,18]. Based on this, soluble form of recombinant E2 glycoprotein (sE2) was synthesized to study the receptors of HCV in cell entry[19,20].

HCV INFECTION

Infecting a target cell by HCV entails an orchestrated process which can be described into several steps starting from the binding of the viral particles to receptors with co-receptors[21]. Usually, the interaction of glycoproteins (E1 and E2) on the viral surface and specific receptors on the surface of target cell determines the association of a HCV with a target cell. Here, we define the process of viral entry into cells into a three-step process (Figure 1). Initially, HCV recognizes a target cell by binding to the mannose-binding lectins L-SIGN, which is mainly expressed on the endothelium of liver and DC-SIGN, which is expressed on dendritic cells. Both of the cell surface proteins are believed to function as HCV capture receptors[22]. Laterly, the viral glycoproteins interacts with the CD81 tetraspanin[23] and lipoprotein receptors[24-26], transferring the virus from the surface to side gradually. Finally, tight junction proteins may be utilized to help HCV entry by inducing clathrin-mediated endocytosis.

ROLE OF CD81 TERASPANIN IN THE ENTRY OF HCV

During the process of HCV cell entry, CD81 teraspanin, which contains a small extracellular loop, a large extracellular loop (LEL), four transmembrane domains and intracellular N- and C-terminal domains, plays an important role. It was firstly reported that CD81 interacted with a soluble HCV glycoprotein E2 and blockade of CD81 by a specific antibody or silencing of CD81 inhibited the HCV entry and decreased HCV infectivity, demonstrating that CD81 is necessary for the entry of HCV[19,27,28]. In more detailed, the initial step of HCV binding to CD81 is actually the linking between HCV glycoprotein E2 and the LEL of CD81[29], showing LEL served directly in HCV entry. Subsequent research pointed out the relation between CD81 expression on cell surface and membrane lipid composition, that ceramide enrichment of the plasma membrane strongly inhibited the expression of CD81. As lipids organization on the membrane of host cells is essential for HCV entry, internalization of CD81 induced by ceramide inhibited HCV entry[30]. In HepG2 cells and Huh-7, which are derived from hepatoma, CD81 was also demonstrated to affect the susceptibility to HCV infection and the efficiency of HCV entry[31-33]. In addition, the dynamic

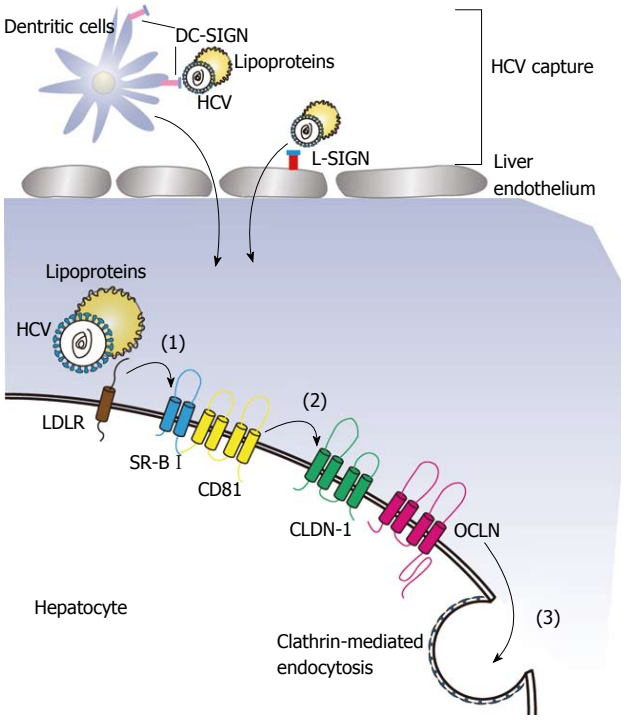

Figure 1 Process of hepatitis C virus cell entry. After being captured by DC-SIGN and L-SIGN, virions with apolipoprotein may first attach a host cell by interacting with LDLR on the cell surface (1), following by binding to CD81 and SR-B Ⅰ (2), and finally by a later utilization of the tight junction protein CLDN1 and OCLN (3). HCV: Hepatitis C virus; SR-B Ⅰ: Scavenger receptor class B type Ⅰ; LDLR: Low-density lipoprotein receptor; CLDN-1: Claudin-1; OCLN: Occluding.

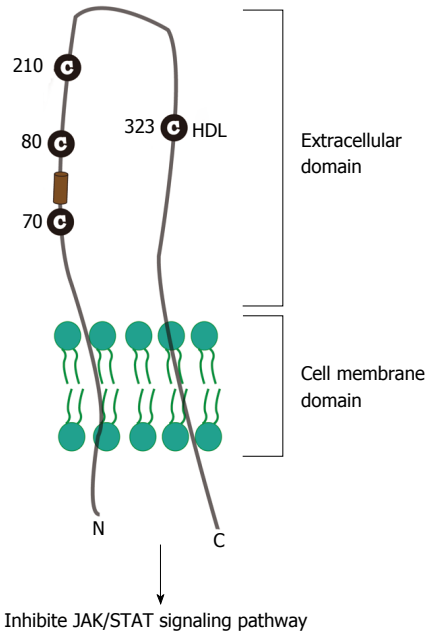

Figure 2 Model of scavenger receptor class B type Ⅰ topology and its relevance for hepatitis C virus entry. The SR-BI regions comprise cytoplasmic C-terminal and N-terminal domains separated by a large extracellular domain. Cholesterol uptake and HCV entry is mainly mediated by extracellular domain. Particularly, C323 is critical for SR-BI-mediated cholesterol ester uptake. Amino acids 70-87 and the single residue E210 of SR-BI are required for E2 recognition in HCV entry. SR-B Ⅰ: Scavenger receptor class B type Ⅰ; HCV: Hepatitis C virus; HDL: High-density lipoprotein; JAK/STAT: Janus kinase/signal transducer and activator of transcription.

of CD81, which is dependent on the hepatocytes polarization, could regulate HCV infection[34] and the trafficking of CD81 on the host cell membrane promoted claudin-1-dependent HCV particle internalization[35]. Recently, it was demonstrated that the expression of CD81 also modulated HCV RNA replication[36], suggesting that the HCV life-cycle also requires CD81.

Recent study pointed out that multiple RTKs could mediate HCV entry by regulating CD81-claudin-1 and viral glycoprotein-dependent membrane fusion[37]. Liu *et al*[38] also found that HCV transiently activates the phosphatidylinositol-3-kinase/AKT pathway to facilitate its entry. These findings may contribute to a new approach to prevention and treatment of HCV infection.

ROLE OF LIPOPROTEIN RECEPTORS IN THE ENTRY OF HCV

The metabolism of apolipoproteins, lipids and lipoproteins is mainly regulated by the liver and HCV attacks liver, leading to abnormal serum lipoproteins and accumulation of lipids in hepatic cells in a chronic mode[39-41]. In recent years, the relationship between cholesterol metabolism and fatty acid biosynthetic pathways in target cells and HCV infection has gained much attention. As a result, the role of lipoprotein receptor in the HCV entry is extensively investigated in detail. Hence, we will focus on roles of the two lipoprotein receptors, scavenger receptor class B type Ⅰ (SR-B Ⅰ) and low-density lipoprotein receptor (LDLR) in this review.

SR-B Ⅰ

SR-B Ⅰ, as a 509 amino acid glycoprotein, is an integral membrane receptor with cytoplasmic C-terminal and N-terminal domains separated by a large extracellular domain (Figure 2); and is found in numerous cell types and tissues, including the liver and adrenal. There are evidences that SR-B Ⅰ selectively mediates uptake of high-density lipoprotein (HDL) cholesterol ester (CE) into transfected Chinese hamster ovary cells[42] and C323 of SR-B Ⅰ is critical for SR-B Ⅰ-mediated cholesterol ester uptake[43]. Previous study also proves that the human homologue of SR-B Ⅰ, CD36 and LIMPII Analogous-1 (hSR-B Ⅰ/CLA-1), serves as a receptor of HDL and regulates cholesterol efflux to HDL during the process of reverse cholesterol transport[44-47].

Recent reports indicate that HDL promoted HCV entry and this enhancement was mediated by the formation of SR-B Ⅰ, HDL and HCV envelope glycoproteins complex[20,48,49]. Many groups demonstrated that the glycoprotein E2 could bind SR-B Ⅰ in hepatoma cells: Scarselli *et al*[20] demonstrated that extracellular domain of SR-B Ⅰ interacts with E2 hypervariable region 1 (HVR1)[20,26]; Catanese *et al*[50] found out that amino acids 70-87 and the single residue E210 of SR-B Ⅰ are required for E2 recognition, raising a possibility for new therapeutic strategies targeting virus/SR-B Ⅰ recognition.

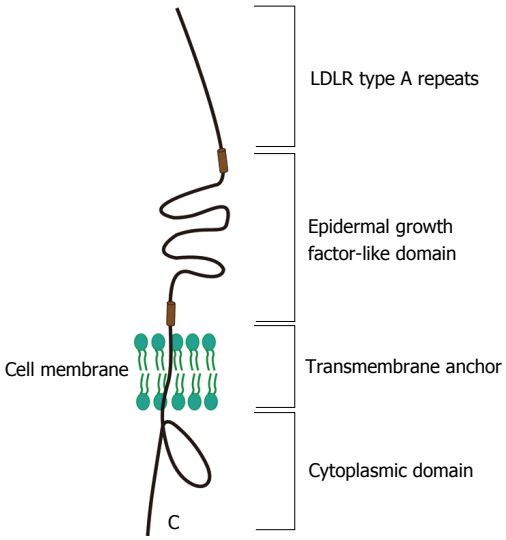

Figure 3 Structure of low-density lipoprotein receptor and its relevance for hepatitis C virus entry. LDLR is structurally composed by four motifs: LDLR type A repeats, which is the main binding site for ligand; an epidermal growth factor-like domain, which is response to the change of pH to release ligand; a transmembrane anchor and a cytoplasmic domain, which mediates clustering of the receptors into the clathrin-coated pit. LDLR: Low-density lipoprotein receptor.

Based on the detailed internship between virus and SR-B Ⅰ, Murao *et al*[25] point out that interferon alpha decreases the efficiency of HCV infection by down regulating the binding of SR-B Ⅰ with both synthesized E2 region-Ⅰ (4931) and E2 region-Ⅱ (4938) peptides in HepG2 cells. Vercauteren *et al*[51] recently found a new anti-SR-B Ⅰ antibody, small molecule inhibitors monoclonal antibody1671 (mAb1671), significantly inhibited infection of hepatoma cells with wild-type HCV by inhibiting the function of SR-B Ⅰ, suggesting that mAb1671 could be used as a therapeutic antibody.

SR-B Ⅰ not only acts as a binding receptor of HCV, but also plays a critical role in the post-binding steps. Catanese *et al*[50] and Zeisel *et al*[52] showed that the susceptibility of human hepatoma cells to HCVcc infection is markedly reduced by silencing of SR-B Ⅰ with specific siRNA, SR-B Ⅰ specific antibody or mutation of SR-B Ⅰ and the effect is independent of lipoprotein, pointing out the role of SR-B Ⅰ in the post-binding process.

HCV particles associated with plasma lipoproteins like HDL can be found in the viral particles isolated from patients and the abnormal metabolism of lipid influences the HCV infection, suggesting that HCV entry might also potentially involve the interactions with SR-B Ⅰ ligands, HDL. Although HCVpp is not able to interact with HDL apolipoprotein, the increase of HDL still markedly induces the enhancement of HCVpp entry, while inhibition of the transfer of HDL CE reduces the entry of HCVpp into cells[49,53]. However, the ability of HDL to facilitate HCV entry is largely in a SR-B Ⅰ -dependent manner since silencing of this receptor cancelled the effect of HDL on enhancement of viral entry[49].

Another goal of recent researches was to examine the signaling pathways involved in the HCV infection in more detail. After binding of glycoprotein E2 to SR-B Ⅰ, multiple signaling pathways will be inactivated to facility HCV entry in host cells. There is a report points out that HCV selectively decreased the abundance of signal transducer and activator of transcription 1 (STAT1) and reduced the phosphorylation of STAT1 in the nucleus by binding its core protein to STAT1 in a proteasome-dependent manner to defense the immunity induced by JAK/STAT pathway[54]. In turn, the treatment with interferon alpha was proved to phosphorylate STAT1 to protect the host cells from infection of HCV[25]. STAT3 activation in human hepatocytes was also confirmed to resist an attack from HCV infection *in vitro*[55]. Clinically, treatment with interferon alpha and ribavirin is one of the therapies for chronic HCV infection. Type 1 interferon is a production from host cells infected with virus and constitute the primary defense mechanism against viral infection and replication[56]. Secreted interferon acts through an autocrine and paracrine loop that requires intact interferon receptor and JAK/STAT pathways involving STAT family members[57].

In summary, all of these evidences point out the critical role of SR-B Ⅰ in enhancing HCV entry into hepatic cells and the complicated process requires the complex between lipoproteins, SR-B Ⅰ, and HCV envelope glycoproteins. The *SR-B Ⅰ* gene is able to transcript into two mRNA splice variants, SR-B Ⅰ and SR-B Ⅱ and the two variants are different from their C-termini. Although there is evidence that HCV soluble envelope glycoprotein E2 is able to interact with not only human SR-B Ⅰ but also SR-B Ⅱ[58], the role of SR-B Ⅱ in the HCV entry is rarely reported.

In the family of scavenger receptors, there is another member named scavenger receptor class A (SR-A), which is mainly expressed in macrophage. It is composed of a cytosol domain, a transmembrane domain, a spacer domain, an alpha-helical coiled-coil domain, a collagen-like domain and a cysteine-rich domain and has two different types, SR-AI and SR-AII[59]. Different from the SR-B, the main function of SR-A in innate immunity is defense of bacteria. Recently, it was reported that SR-AI could bind to the non-structural protein NS3 of HCV in dendritic cells, pointing out that SR-A may serve as endocytic innate receptors in NS3 recognition[60].

LDLR

LDLR is another potential lipoprotein receptor involved in HCV infection of hepatocytes. LDLR (Figure 3), an 893 amino acids transmembrane protein, is a cell surface receptor that mediates uptake of cholesterol-rich low-density lipoprotein (LDL)[61,62]. When the main ligand cholesterol-LDL binds to the receptor, it is transferred into hepatic cells by clathrin-mediated endocytosis and then the receptor will release the bound LDL particle because of the conformational change induced by change in pH. Accumulation of serum LDL directly leads to the development of atherosclerosis.

Since Agnello *et al*[63] firstly suggested the role of LDL-R in HCV entry in 1999, most studies focus on its role as a receptor of HCV or facilitating initial attachment to cell surface[10]. André *et al*[64] reported that lipo-viro-particles isolated from patients with hepatoma infect hepatic cells in an LDLR-dependent manner, indicating the important role of LDLR in HCV infection. In coincidence with this report, LDLR is confirmed to take part in an early stage in infection of normal human hepatocytes by serum-derived HCV virions *in vitro*[65]. A human study that LDLR expression of 68 patients with HCV chronic infection was significantly associated with HCV-viral load, supplies the evidence that the LDLR may be one of the receptors implicated in HCV replication[66].

While HDL facilities the entry of HCV into hepatic cells, LDL could significantly inhibit the cell entry of serum HCV and HCVpp *via* LDLR. Some studies also reported that ApoE-containing very LDL, as a ligands of LDLR, mediates the HCV entry *in vitro*. Recently, Ficolin-2, as a lectin-complement pathway activator, inhibited the chronic HCV infection by inhibition the function of LDLR and SR-B Ⅰ and this effect was blocked by ApoE3-mediated immune escape[67]. It was confirmed by a recent report that ApoE3 and ApoE4 rescue the production of infectious virus and it requires both the LDLR and SR-B Ⅰ [68]. By contrast, Prentoe *et al*[26] found in the process of HCV entry, the function of LDLR is in an ApoE-independent but E2 HVR1-dependent manner. Although there are lots of evidences to prove that LDLR, the same as SR-B Ⅰ and CD81, plays a critical role in the initial step of HCV entry, one of studies suggested that LDLR is not necessary for HCV entry and implied the physiological function of LDLR in HCV replication[69]. Recently, it was demonstrated that HCV upregulates the expression of LDLR *via* SREBPs and PCSK9 at both transcriptional and posttranslational level to increase the uptake of lipid and to promote viral proliferation[70]. Until now, there is no doubt that LDLR is able to mediate the HCV infection. However, the detailed mechanism how it really works during this complex process needs to be further investigated.

ROLES OF TIGHT JUNCTION PROTEINS IN THE LATER PHASE OF HCV ENTRY

By using screening cDNA library, two kinds of tight junction proteins, claudin-1 (CLDN-1) and occludin (OCLN), were identified as factors that are able to affect the HCV entry in the later phase[71,72]. Either CLDN-1 or OCLN contains four transmembrane domains and two extracellular loops with the N-terminus and C-terminus in the cytoplasm. Interestingly, there is no evidence to confirm that there is direct interaction between CLDN-1 or OCLN and HCV particles. However, it was proved that CLDN-1 directly interacts to CD81 and the association increases the virus entry in the later phase[73]. Laterly, Krieger *et al*[74] produced CLDN-1 specific antibody and found it inhibited HCV infection by reducing the binding of E2 with host cell surface and disrupting the formation of CD-81-CLDN-1 complex. OCLN is also able to interact directly with E2, and silence of CLDN-1 and OCLN by specific siRNA reduced both HCVpp and HCVcc cell entry[75].

OTHER FACTORS ON CELL SURFACE INVOLVED IN HCV ENTRY

Besides the receptors we talked above, there are some other factors on host cell surface, which are believed to be functional in HCV entry. Lupberger *et al*[37] pointed out the important role of epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) as cofactors in HCV entry. EGF accelerated HCV entry by activating signaling pathways and inhibition of EGFR or EphA2 activity reduced CD81-CLDN1 association. Following, Diao *et al*[76] confirmed that EGFR internalization and activation are critical for HCV entry and firstly identified a hitherto-unknown association between CD81 and EGFR by using HCVcc system. Based on these theories, Meyer *et al*[77] recently supposed a model that interferon-α inducible protein 6 inhibits HCV entry by impairing EGFR mediated CD81/CLDN1 interactions. Niemann-Pick C1-like 1 (NPC1L1), as a cholesterol uptake receptor was firstly identified as an HCV entry factor by Sainz *et al*[78] and they also proved clinically available FDA-approved NPC1L1 antagonist ezetimibe potently blocks HCV uptake *in vitro via* a virion cholesterol-dependent step, discovering a new antiviral target and potential therapeutic agent. Furthermore, transferrin receptor 1 has also been reported as a receptor for HCV entry[79]. However, the roles of these new factors in HCV entry remain to be determined in detailed.

CONCLUSION

The process of HCV entry is a multi-step process and the major steps have already been described as the combination of HCV glycoprotein and targeting cell-surface molecules, such as CD81 and lipoprotein receptor SR-B Ⅰ and LDLR. With the development of HCV model system, the role of lipoprotein and its receptor in HCV infection is more and more detailed understood. However, since all the model system has their own limitations, the results obtained by using system in vitro do not completely reflect the *in vivo* situation. Further studies are required, especially by using engineering new animal models, for HCV infection, and a detailed understanding of the mechanism of HCV entry will give a sufficient groundwork for the development of new therapeutic drugs and tools.

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Era of direct acting antivirals in chronic hepatitis C: Who will benefit?

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Abstract

In the era of highly effective direct acting antiviral (DAA) drugs for the treatment of chronic hepatitis C (CHC) infection, where eradication is almost ensured with minimal side effects, all hepatitis C carriers should benefit theoretically. In the real world setting however, only a small proportion will benefit at this time point due to the multiple barriers to accessing therapy. Given that universal treatment is unlikely, treatment with DAAs will likely be restricted to those with the highest health benefits, and for those who can afford the high expense of a treatment course. Those with the highest unmet needs include those who have failed previous interferon-based therapy or who are interferon-ineligible with evidence of active disease, those with advance liver disease, and those with recurrence of hepatitis C after liver transplantation. In the future, the focus should be on increasing access to treatment for those infected with CHC.

Key words: Treatment; Direct acting antivirals; Benefit; Unmet needs; Cirrhosis; Liver transplantation; Chronic hepatitis C

Core tip: Chronic hepatitis C has become an easily curable disease with new direct acting antivirals (DAAs). However, due to multiple barriers to therapy, only those with highest unmet clinical needs including those with prior treatment failure, cirrhosis, and post-liver transplant, will likely receive therapy. DAAs have been shown to be highly efficacious in these groups.

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INTRODUCTION

An estimated 170 million people worldwide are

chronically infected with the hepatitis C virus (HCV), affecting 2-3 percent of the world population, and constituting a major health burden globally[1,2]. For those with chronic hepatitis C (CHC), approximately 20% will progress to the development of liver cirrhosis[3]. This process usually takes several decades to occur although disease progression can be accelerated by the presence of co-existing liver disease[4], co-infection with other viruses such as human immunodeficiency virus[5-8], and also with alcohol intake[9-11]. Once cirrhosis is established, further complications may occur with liver decompensation and the development of hepatocellular carcinoma (HCC)[12]. In fact, the history of HCV is relatively short, with the discovery of the blood-borne virus in 1989 just over 25 years ago. Major routes of transmission include transfusion of unscreened contaminated blood products[13], and persons who inject drugs using contaminated drug paraphernalia[14].

EVOLUTION OF ANTIVIRAL THERAPY FOR CHC

In the early 1990s, standard interferon (IFN) alpha-2b was used to treat CHC infection. However, the curative rate with standard IFN monotherapy was dismal, with only approximately 15% achieving a sustained virological response (SVR)[15,16]. The addition of ribavirin enhanced the SVR to 33% and 41% for 24 and 48 wk of therapy respectively[17-19]. The subsequent introduction of pegylated IFN (Peg-IFN) in combination with ribavirin (RBV) improved the SVR rate to an estimated 50%[20,21]. The length of treatment ranged from 24-48 wk, and together with the SVR, was dependent on the genotype of the HCV[22]. Despite significant side effects, the modest SVR rate, the need for parenteral injections, and prolonged duration of therapy, Peg-IFN and RBV (PR) remained the standard of care for over a decade from the turn of the new millennium.

Over the last several years, there has been an exponential increase in therapeutic agents approved for CHC infection. These agents, collectively known as direct acting antivirals (DAAs), work by inhibiting specific stages of the HCV replication cycle. Classes of drugs include NS3/4A protease inhibitors, non-nucleoside polymerase inhibitors, NS5B nucleos(t)ide polymerase inhibitors, NS5A inhibitors, and cyclophilin inhibitors. In 2011, boceprevir and telaprevir were approved for the treatment of GT1 patients, improving the SVR to 68%-75% for treatment-naive patients[23,24], and 51%-59% in treatment-experienced patients[25,26]. Despite the modest improvement, these first generation DAAs still required the use of PR, resulting in a complicated treatment regimen and potential for significant side effects. In 2013, sofosbuvir and simeprevir were approved. The use of simeprevir or sofosbuvir with PR therapy for 12 wk achieved an SVR of approximately 80% and 90% respectively[27-29]. An all-oral combination of simeprevir and sofosbuvir achieved an overall SVR12 rate of 92%, proving evidence of the efficacy of an all-oral regimen[30]. In 2014, 3 all-oral combination regimens were approved, including sofosbuvir + simeprevir, sofosbuvir + ledipasvir, and ombitasvir + paritaprevir + ritonavir + dasabuvir. All 3 regimens were demonstrated to be highly effective, with SVR rates approaching 100%[31-35].

Within a space of a few years, the treatment of CHC has seen a dramatic shift in paradigm. CHC infection has emerged from a disease that has been difficult to cure with prolonged parenteral therapy to one that is easily curable with a short duration of oral antiviral therapy with minimal side effects.

WHO WILL BENEFIT?

Given the high SVR rates achieved, and the favorable side effects profile of DAAs, it stands to reason that all CHC carriers should benefit from these newly approved all-oral combination DAA therapies. Moreover, with the high cure rates observed, for the first time, there is a glimpse of opportunity to eradicate HCV completely. In reality however, only a small fraction of CHC patients will likely receive DAAs at this time point, as shown in Figure 1. This is due to the fact that there are multiple barriers to HCV treatments that currently exist, precluding patients from receiving the best treatment available[36,37]. The barriers most inherent to DAAs include the availability of these new agents and the high cost associated with a treatment course[38]. Until these become widely available, and at an affordable cost, the benefits of DAAs will likely be restricted to those with the highest unmet needs. These include patients who have failed previous IFN-based therapies with evidence of disease progression, those who are ineligible for IFN therapy with progressive disease, those with established cirrhosis, those on the liver transplant waiting list, and those who have had a liver transplant (Figure 1).

Patients who have failed previous IFN-based therapies

For those who have failed previous IFN-based therapies, combination DAAs offers the best chance of achieving SVR. In the COSMOS trial of 80 HCV GT1 patients who were null responders treated with simeprevir and sofosbuvir +/- ribavirin for 24 wk, the SVR achieved was 90%[30]. A trial (the SAPPHIRE 2 study) of 297 HCV GT1 patients without cirrhosis who had failed PR therapy and treated with 12 wk of ritonavir-boosted paritaprevir + ombitasvir + dasabuvir + RBV resulted in an overall SVR rate of 96.3%[39]. In the ION-2 study, 440 HCV GT1 previously treated patients given sofosbuvir + ledipasvir +/- RBV Ⅰ for 12-24 wk resulted in a SVR rate of $\geqslant$ 94%[31]. Despite previously failing to respond to IFN-based regimens, combinations DAAs have been demonstrated to be highly effective for this group of patients.

Patients ineligible for IFN therapy

There may be many different reasons as to why patients may be ineligible for IFN-based treatment. These include

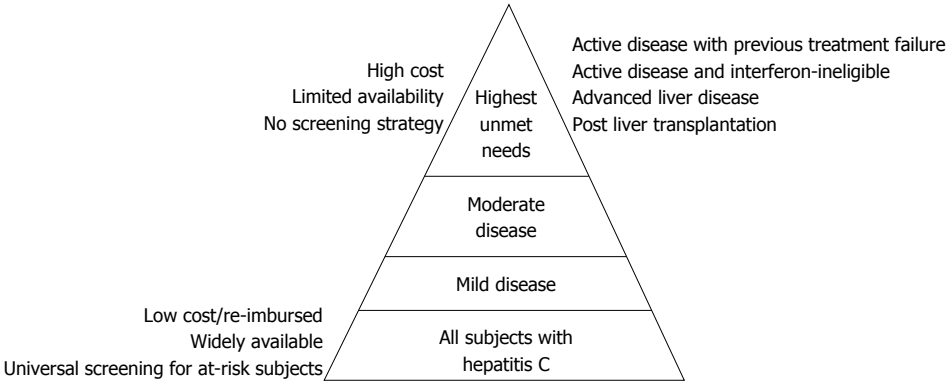

Figure 1 Current treatment model in the era of direct acting antivirals for chronic hepatitis C with respect to the patients who will benefit.

those who are intolerant to IFN or have hypersensitivity to polyethylene glycol, have underlying autoimmune hepatitis or other severe autoimmune disorders[40,41], history of significant psychiatric disorder such as depression[42], and pre-existing cardiac disease[43]. Those patients with significant anemia, neutropenia, or thrombocytopenia may also be contra-indicated, as therapy with IFN can potentially compound the pre-existing cytopenic state[44-46]. Patients with established severe liver disease are also contra-indicated for IFN-based therapy, as there is a risk of decompensation and liver failure with the use of IFN[47].

Therefore, for those with evidence of active hepatitis and disease progression, and whom IFN is contra-indicated or tolerated poorly, the use of combination DAAs is the only therapeutic option in this setting.

Patients with established cirrhosis

As mentioned previously, those patients with established cirrhosis, especially with evidence of advance liver disease (Child Pugh B or C), are not eligible for IFN-based therapy as there is an increase risk of precipitating liver decompensation and failure[48]. Prior to the availability of all-oral combination of DAAs regimen, there was no specific antiviral therapy available for this group. Management of these patients with evidence of active hepatitis and disease progression was often frustrating for clinicians where the only outcome was either decompensation leading to death or liver transplantation. Ironically, this group of patient perhaps is the group that would need effective antiviral therapy the most.

Several studies have shown that DAAs are highly effective in patients with established cirrhosis. In the TURQUOISE Ⅱ trial of 380 compensated cirrhotic patients infected with HCV genotype 1 using ritonavir-boosted paritaprevir + ombitasvir + dasabuvir + RBV, the SVR rate was noted to be 92% and 96% with 12 and 24 wk of treatment respectively[49]. A multicenter double blind study using ledipasvir + sofosbuvir for 24 wk and ledipasvir + sofosbuvir + RBV for 12 wk in compensated HCV genotype 1 patients, the SVR12 rates were 97% and 96% respectively[50]. In the SOLAR-2 study of 108 decompensated patients with HCV GT 1/4, treatment with ledipasvir + sofosbuvir + RBV for 12 and 24 wk were associated with a SVR rate of 87% and 89% respectively[51].

Patients on the liver transplant waiting list

As hepatitis C recurrence is universal for those patients who are viremic at the time of liver transplantation, there is enormous impetus to eradicate or treat HCV prior to transplantation[52-54]. The indications for liver transplantation for CHC patients include those with decompensated cirrhosis and those with HCC[55]. For those with decompensated cirrhosis, as discussed in the previous section, treatments using DAAs has been shown to be effective. There are several rationales for treating wait-listed patients. Firstly, there is a theoretical opportunity that for those that achieve SVR, liver synthetic function may be restored sufficiently to the point whereby liver transplantation is no longer required (similar to that observed for patients with chronic hepatitis B)[56,57]. Secondly, achieving SVR or complete viral suppression will prevent or reduce the rate of disease progression and further decompensation[58,59]. Thirdly, by achieving viral suppression or SVR prior to liver transplantation, the recurrence rate after liver transplantation will be significantly improved, and even prevented for those who achieve SVR prior to their liver transplant[60]. In a phase Ⅱ open-label study of 61 patients with HCV of any genotype and compensated cirrhosis with HCC were treated up to 48 wk of sofosbuvir + RBV before liver transplantation. Of these, 43 had undetectable HCV RNA at the time of transplantation, of which 30 (70%) had virological response at 12 wk post transplant. The recurrence was related inversely to the number of consecutive days of undetectable HCV RNA before transplantation[61]. There has also been reported case of decompensated patient improving to the point of being delisted after treatment with sofosbuvir and ribavirin for 24 wk[62].

Patients after liver transplantation

For patients with recurrent hepatitis C after liver transplantation, the disease progression is accelerated, with approximately 20% developing graft cirrhosis by 5 years[63-66]. The use of long-term immunosuppression or large doses of pulse steroid during episodes of acute

cellular rejection may increase the severity of graft hepatitis[63]. Treatment with combination Peg-IFN and RBV is often poorly tolerated after transplantation, and has been associated with poor SVR rates of approximately 30%[67]. For those who do not tolerate therapy with evidence of active hepatitis and disease progression, those who do not respond to therapy with evidence of disease progression, and those with severe fibrosing cholestatic hepatitis, the rate of graft failure is high, leading to graft decompensation and death or retransplantation[68,69].

Treatment with DAAs has been shown to improve significantly the SVR rates in post transplant patients. The use of early DAAs including telaprevir and boceprevir in combination with Peg-IFN and RBV improved the SVR rate to 41%-51%[70]. Not surprisingly, there were significant side effects observed with these regimens. Furthermore, significant drug interactions occur between telaprevir/boceprevir with the immunosuppressive therapy[71,72]. All-oral combination DAAs have recently been shown to be highly effective in post transplant patients. In the CORAL-Ⅰ trial of ritonavir-boosted paritaprevir + ombitasvir + dasabuvir with RBV, the SVR12 rate was 97% after 24 wk of therapy[73]. Significant dose reduction of immunosuppression must also be undertaken when using this combination because of significant drug interactions. In another study using sofosbuvir and RBV after liver transplantation for 24 wk, the SVR12 rate was 70%[74]. In the SOLAR-1 trial using ledipasvir + sofosbuvir + RBV for 12 or 24 wk, the SVR rates was 96% and 98% respectively for non-cirrhotic patients, and 96% for those with compensated cirrhosis[75].

One key aspect of using DAAs after transplantation is that treatment should be given early at the time of hepatitis recurrence[76]. Despite the excellent virological response, the clinical benefit of DAAs when administered late at the time of advanced disease with graft decompensation may be negated. The SVR rates have been shown to be lower for these patients, and despite complete viral suppression, patients may still succumb due to the poor general condition with high susceptibility to infections[51,74].

IS THE THERAPY COST-EFFECTIVE?

With SVR rates approaching 100% with all-oral combination DAAs, there is no doubt that therapy is highly effective. Due to the high replicative rate of the HCV, and coupled with a lack of proofreading mechanism of the RNA-polymerase enzyme, a large number of variants are produced[77,78]. Treatment with DAAs may select out those pre-existing resistance-associated variants with lowered susceptibility to the drugs, resulting in potential treatment failure[79-81]. Although treatment failure rates are low, as evident by the consistently high SVR rates achieved with combination DAAs, the development of resistance may potentially be a significant problem in the future as increasing number of patients are being treated.

Due to the high cost of DAAs, questions are raised regarding the cost-effectiveness of these new regimens. There are now several studies demonstrating that the new DAAs are cost-effective across a wide range of patients, including those with early disease[82-84]. However, in the real world, with millions of eligible patients for antiviral therapy, the cost of therapy would be prohibitive. The previous sections have identified subsets of chronic HCV carriers with perhaps the highest unmet needs currently, and with the highest health benefit to cost ratio. For this group of patients, the cost of managing decompensated liver cirrhosis or liver transplantation far exceeds the cost of a course of DAAs.

CONCLUSION

The introduction of DAAs over the last several years has revolutionized the treatment landscape for CHC infection. Currently, SVR can be achieved in almost all patients treated with combination all-oral regimens with minimal side effects. Therefore, these newly approved DAAs will certainly improve almost every aspect of patient outcome, but only for those who have access to treatment. Although the high costs remain a major hurdle for many, other barriers exist. These barriers include the lack of an effective screening strategy to identify all chronic HCV carriers, medical eligibility for therapy, stigmatization of HCV carriers, and also the attribution of the patient and health care provider. The possibility of treatment being available to all CHC subjects with complete eradication of HCV remains a distant prospect, and can only be achieved with universal screening of at-risk subjects, and making treatment widely available and affordable to all (Figure 1). In the current review, those infected the CHC with the highest unmet needs are discussed. These are patients for whom treatment is likely to incur the highest health benefit. As treatment becomes increasingly affordable and more widely available, more patient groups will stand to benefit. In the era of highly effective DAAs where treatment efficacy is no longer an issue, the focus should now be on increasing access and removing barriers to therapy.

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ORIGINAL ARTICLE

Basic Study

Role of pentoxifylline in non-alcoholic fatty liver disease in high-fat diet-induced obesity in mice

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Abstract

AIM: To study pentoxifylline effects in liver and adipose tissue inflammation in obese mice induced by high-fat diet (HFD).

METHODS: Male swiss mice (6-wk old) were fed a high-fat diet (HFD; 60% kcal from fat) or AIN-93 (control diet; 15% kcal from fat) for 12 wk and received pentoxifylline intraperitoneally (100 mg/kg per day) for the last 14 d. Glucose homeostasis was evaluated by measurements of basal glucose blood levels and insulin tolerance test two days before the end of the protocol. Final body weight was assessed. Epididymal adipose tissue was collected and weighted for adiposity evaluation. Liver and adipose tissue biopsies were homogenized in solubilization buffer and cytokines were measured in supernatant by enzyme immunoassay or multiplex kit, respectively. Hepatic histopathologic analyses were performed in sections of paraformaldehyde-fixed, paraffin-embedded liver specimens stained with hematoxylin-eosin by an independent pathologist. Steatosis (macrovesicular and microvesicular), ballooning degeneration and inflammation were histopathologically determined. Triglycerides measurements were performed after lipid extraction in

liver tissue.

RESULTS: Pentoxifylline treatment reduced micro-steatosis and tumor necrosis factor (TNF)-α in liver (156.3 ± 17.2 and 62.6 ± 7.6 pg/mL of TNF-α for non-treated and treated obese mice, respectively; $P < 0.05$). Serum aspartate aminotransferase levels were also reduced (23.2 ± 6.9 and 12.1 ± 1.6 U/L for non-treated and treated obese mice, respectively; $P < 0.05$) but had no effect on glucose homeostasis. In obese adipose tissue, pentoxifylline reduced TNF-α (106.1 ± 17.6 and 51.1 ± 9.6 pg/mL for non-treated and treated obese mice, respectively; $P < 0.05$) and interleukin-6 (340.8 ± 51.3 and 166.6 ± 22.5 pg/mL for non-treated and treated obese mice, respectively; $P < 0.05$) levels; however, leptin (8.1 ± 0.7 and 23.1 ± 2.9 ng/mL for non-treated and treated lean mice, respectively; $P < 0.05$) and plasminogen activator inhibitor-1 (600.2 ± 32.3 and 1508.6 ± 210.4 pg/mL for non-treated and treated lean mice, respectively; $P < 0.05$) levels increased in lean adipose tissue. TNF-α level in the liver of lean mice also increased (29.6 ± 6.6 and 75.4 ± 12.6 pg/mL for non-treated and treated lean mice, respectively; $P < 0.05$) while triglycerides presented a tendency to reduction.

CONCLUSION: Pentoxifylline was beneficial in obese mice improving liver and adipose tissue inflammation. Unexpectedly, pentoxifylline increased pro-inflammatory markers in the liver and adipose tissue of lean mice.

Key words: Pentoxifylline; Steatosis; Obesity; Adipose tissue; Adipokine; Tumor necrosis factor-α

Core tip: Pentoxifylline is prescribed to patients with severe alcoholic hepatitis, which suggest that this drug could also be beneficial to non-alcoholic steatohepatitis (NASH) patients. However, experimental results with pentoxifylline have shown conflicting data depending on the NASH model employed. Considering that obesity is strongly associated with the development of NASH, our study evaluated the effects of pentoxifylline in a high-fat diet induced obesity model. Our results showed that pentoxifylline was beneficial in obesity-associated NASH improving liver and adipose tissue inflammation. Unexpectedly, pentoxifylline treatment resulted in undesirable effects in adipose tissue and liver inflammatory markers in lean mice.

Acedo SC, Caria CRP, Gotardo ÉMF, Pereira JA, Pedrazzoli J, Ribeiro ML, Gambero A. Role of pentoxifylline in non-alcoholic fatty liver disease in high-fat diet-induced obesity in mice. *World J Hepatol* 2015; 7(24): 2551-2558 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i24/2551.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i24.2551

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of hepatic disorders including steatosis or uncomplicated fatty liver and non-alcoholic steatohepatitis (NASH). NAFLD is frequently associated with metabolic syndrome establishment and it occurs more often in males than in females and primarily affects the middle aged and the elderly[1].

Several factors are associated with the development of fatty liver, and NAFLD diagnosis requires the exclusion of secondary etiologies, including alcohol consumption, drug usage, hepatitis B and C[2]. Although simple steatosis is considered benign, NASH can progress to end-stage liver disease, such as fibrosis, cirrhosis and hepatic cancer[3]. The mechanism of steatosis progression to more severe liver injuries is not fully understood, but it is associated with several risk factors, including elevated serum transaminases, inflammation upon liver biopsy, old age, diabetes mellitus, high body mass index ($\geqslant$ 28 kg/m^2), presence of ballooning plus Mallory hyaline or fibrosis upon biopsy and increased visceral adipose tissue[2,4].

The growing epidemic of obesity and an aging population have led to an important demand for a medical therapy for NAFLD, but several decades of pharmacological research have resulted in very few options[2]. As NAFLD is considered a hepatic manifestation of a metabolic syndrome, the first treatment approach is a lifestyle change, including dietary alterations and increased physical activity to reduce adiposity and body weight[5]. Therapeutic drugs are an adjunctive approach to lifestyle changes. Statins are used to control dyslipidemias, metformin and glitazones are used to control diabetes mellitus, and angiotensin receptor blockers are used to control inflammatory cell recruitment and hepatic fibrosis development in addition to their anti-hypertensive effects. In total, these drugs aim to control the symptoms of the metabolic syndrome[1,2,6].

Pentoxifylline is a non-selective phosphodiesterase inhibitor that has been reported to have antioxidant activity and decrease tumor necrosis factor (TNF)-α gene transcription. Pentoxifylline treatment improved the 6-mo survival rate of patients with severe alcoholic hepatitis compared with placebo[7]. Recent studies have shown that pentoxifylline may be a promising drug therapy for NASH treatment[8-10]. Experimental results with pentoxifylline have shown conflicting data depending on the model employed. In NAFLD induced by a choline- and methionine-deficient diet, pentoxifylline treatment was beneficial because it decreased hepatic inflammation and alanine aminotransferase (ALT) levels[11]. However, in a genetic obesity model, pentoxifylline worsened fatty liver in *ob/ob* mice because it increased intestinal glucose absorption, and thus, hyperglycemia[12].

Considering that obesity is strongly associated with the development of NAFLD and one of the main causes of epidemic obesity is a hyperlipidic and hypercaloric

Table 1 Diet composition

	Control (AIN-93)		HFD	
	g/kg	kcal/kg	g/kg	kcal/kg
Cornstarch (QSP)	397.5	1590	1155	462
Casein	200	800	200	800
Sucrose	100	400	100	400
Dextrinated starch	132	528	132	528
Soybean oil	70	630	40	360
Lard	-	-	312	2808
Cellulose	50	-	50	-
Mineral mix	35	-	35	-
Vitamin mix	10	-	10	-
L-cystine	3	-	3	-
Choline	2.5	-	2.5	-
Total	1000	3948	1000	5358

HFD: High-fat diet.

diet, our study evaluated the effects of pentoxifylline in a high-fat diet (HFD)-induced obesity model. Metabolic parameters, hepatic inflammation and adipose tissue alteration were studied after 2 wk of pentoxifylline treatment in mice after 12 wk of a HFD.

MATERIALS AND METHODS

Animals, diets and treatment

Specific pathogen-free, 4-wk-old male Swiss mice were obtained from CEMIB (State University of Campinas, Campinas, São Paulo, Brazil). All experiments were performed in accordance with the principles outlined by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) and received approval from the Ethics Committee of São Francisco University, Bragança Paulista, SP, Brazil (Protocol CEA/USF 00.02.11). The animal protocol was designed to minimize pain or discomfort to the animals.

The animals were individually housed and acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. After random selection, 6-wk-old mice were introduced to control AIN-93 or HFD ad libitum for 12 wk (Table 1). The mice received 100 mg/kg per day ip pentoxifylline (Sigma Aldrich, Co. - Saint Louis, Missouri, United States) diluted in 0.9% NaCl during weeks 10-12 before being sacrificed. Each experimental group used 5 animals.

Blood glucose levels and insulin tolerance tests

Twenty-four hours before the end of the protocol, mice were fasted for 6 h, and blood samples were collected from the tails. Glucose was measured using the glucose oxidase method. Insulin (1.5 U/kg) was administered by intraperitoneal injection, and blood samples were collected for serum glucose determination at 0, 10, 15, 20 and 30 min. The rate constant for glucose disappearance during an insulin tolerance test (kITT) was calculated using the formula 0.693/t1/2. The glucose t1/2 was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase.

Necropsy and sample collection

At the end of protocol, mice were fasted for 12 h and euthanized by xylasine/ketamine overdose (0.1 mL/30 g body weight of 1:1 *v/v* of 2% xylasine and 10% ketamine), and blood samples were collected in tubes by portal vein or cardiac puncture. Liver was perfused with 15 mL phosphate buffered saline (PBS), collected and weighed. Samples were immediately processed or stored at -80 ℃ for further analysis.

Hepatic enzyme analysis

Aspartate aminotransferase (AST) and ALT serum levels were determined using a commercial kit (LABORLAB, Sao Paulo, Brazil).

Cytokine and chemokine analysis in the liver and adipose tissue

Liver and adipose tissue biopsies were homogenized in solubilization buffer containing 100 mmol/L Tris (pH = 7.6), 1% Triton X-100, 150 mmol/L NaCl, 0.1 mg aprotinin, 35 mg/mL PMSF, 10 mmol/L Na_3VO_4, 100 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$ and 4 mmol/L EDTA to extract total protein. Liver and adipose tissue extract supernatants were collected and used in ELISA kits (R and D Systems, Inc, Minneapolis, MN, United States) or Multiplex Assay kits (Millipore, Billerica, MA, United States), respectively, according to the manufacturer's protocol.

Liver histology

Hydrated 4.0 mm sections of paraformaldehyde-fixed, paraffin-embedded liver specimens were stained with hematoxylin-eosin to evaluate liver histology. Additional sections were stained with Masson's trichrome for fibrosis analysis. For each group, six to nine mouse livers were prepared and stained. An expert pathologist evaluated the stained samples in a blinded fashion. Steatosis, ballooning degeneration and inflammation were histopathologically determined. The percentage of steatotic cells (macrovesicular and microvesicular) was determined and graded as follows: (1) 0: absent; (2) 1: < 25%; (3) 2: 26%-50%; (4) 3: 51%-75%; or (5) 4: > 75% of the parenchyma. Hyperemia, inflammation and fibrosis were evaluated as either present or absent.

Measurement of triglycerides in the liver

Liver tissues were homogenized with in chloroform and methanol (2:1 *v/v*) and an aqueous solution of NaCl was added[13]. The chloroform layer was dried under N_2, the total extract ressupended in PBS and triglycerides were determined using commercial enzymatic kit (LaborClin, Pinhais, PR, Brazil).

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons

Table 2 Metabolic and anthropometric parameters of lean (control) and obese (high-fat diet) mice treated with pentoxifylline

	Control		HFD	
	NT	PTX	NT	PTX
Final BW (g)	34.2 ± 1.0	37.0 ± 2.0	51.6 ± 1.5[a]	52.0 ± 2.7
Liver (g)	1.7 ± 0.2	1.7 ± 0.0	2.6 ± 0.2[a]	1.7 ± 0.0[c]
Liver (% of BW)	4.1 ± 0.0	4.6 ± 0.1[c]	4.8 ± 0.2[a]	3.3 ± 0.0[c]
Visceral adipose tissue (g)	0.8 ± 0.1	0.9 ± 0.2	1.8 ± 0.2[a]	2.7 ± 0.1[c]
Visceral adipose tissue (% of BW)	2.2 ± 0.2	2.4 ± 0.4	3.4 ± 0.3[a]	5.4 ± 0.4[c]
Blood glucose (mg/dL)	120.6 ± 6.8	131.0 ± 3.0	178.6 ± 10[a]	141.0 ± 16
kITT	4.0 ± 0.6	3.6 ± 0.3	2.1 ± 0.1[a]	1.6 ± 0.3
Insulin (ng/mL)	0.4 ± 0.1	0.4 ± 0.2	4.4 ± 2.0[a]	4.5 ± 1.2
AST (U/L)	19.4 ± 4.6	9.4 ± 2.3[a]	23.2 ± 6.9	12.1 ± 1.6[c]
ALT (U/L)	3.12 ± 0.9	9.0 ± 2.0[a]	2.25 ± 0.9	4.2 ± 1.5

[a]$P < 0.05$ *vs* control NT group; [c]$P < 0.05$ *vs* paired NT group. HFD: High-fat diet; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PTX: Pentoxifylline; BW: Body weight; NT: Non-treated; kITT: Rate constant for glucose disappearance during an insulin tolerance test.

Table 3 Liver histological score of lean (control) and obese (high-fat diet) mice treated with pentoxifylline

	Control		HFD	
	NT	PTX	NT	PTX
Macrosteatosis	0 (0-0)	0 (0-0)	2.0 (1-3)	2.0 (2-3)
Microsteatosis	0 (0-0)	0 (0-0)	1.8 (1-3)	1.3 (1-2)[a]
Hyperemia	0 (0-0)	0 (0-0)	0.8 (0-1)	0.3 (0-1)[a]
Inflammation	0 (0-0)	0 (0-0)	0.6 (0-1)	0.3 (0-1)[c]
Fibrosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)

[a]$P = 0.06$; [c]$P < 0.05$ *vs* the matched NT group. HFD: High-fat diet; NT: Non-treated; PTX: Pentoxifylline.

Table 4 Hepatic cytokines in lean (control) and obese (high-fat diet) mice treated with pentoxifylline

	Control		HFD	
	NT	PTX	NT	PTX
TNF-α (pg/mL)	29.6 ± 6.6	75.4 ± 12.6[c]	156.3 ± 17.2[a]	62.6 ± 7.6[c]
IL-10 (pg/mL)	27.2 ± 3.6	37.3 ± 8.3	27.5 ± 5.2	29.4 ± 8.2
MCP-1 (pg/mL)	34.9 ± 4.9	56.2 ± 12.3	89.1 ± 10.8[a]	112.3 ± 22.4

[a]$P < 0.05$ *vs* control NT group; [c]$P < 0.05$ *vs* the matched NT group. HFD: High-fat diet; NT: Non-treated; PTX: Pentoxifylline; TNF-α: Tumor necrosis factor α; IL-10: Interleukin-10; MCP: Monocyte chemoattractant protein-1.

among groups of data were made using a one-way ANOVA test followed by the Dunnett multiple comparisons test. Non-parametric data (scores) are expressed as the median (range) and were analyzed using the Mann-Whitney test. An associated probability (P value) of 5% was considered statistically significant.

RESULTS

Metabolic and anthropometric parameters after pentoxifylline treatment

Animals on a HFD for 12 wk presented significant alterations in body weight. However, pentoxifylline-treated mice showed no change in body weight compared with the matched controls. Pentoxifylline treatment decreased liver weight in obese mice, but the depot of visceral adipose tissue significantly increased. We evaluated blood glucose levels and insulin tolerance at the end of the treatment and did not find any differences between treated animals and untreated animals. AST levels decreased after pentoxifylline treatment, but ALT levels did not change (Table 2).

Liver histological analysis and triglycerides content

The livers from HFD mice presented pronounced macrosteatosis, microsteatosis, hyperemia and inflammation, features that were not observed in lean mice. We did not observe fibrosis in any of the groups. Pentoxifylline treatment did not alter the livers of lean mice, but it reduced inflammation, and we observed a trend to reduce microsteatosis and hyperemia in HFD mice (Figure 1 and Table 3). However, triglycerides measurement revealed a tendency to reduction in livers from lean mice but not from obese mice (Figure 1).

Inflammatory markers in liver and adipose tissue

We evaluated TNF-α, interleukin (IL)-10 and monocyte chemoattractant protein (MCP)-1 protein levels in the livers of untreated obese mice or obese mice treated with pentoxifylline. A high-fat diet increased TNF-α and MCP-1 levels but did not affect IL-10 expression. Pentoxifylline treatment reduced TNF-α level but did not modify hepatic MCP-1 or IL-10 levels (Table 4). Adipose tissue analysis revealed that total plasminogen activator inhibitor (PAI)-1, MCP-1 and leptin levels increased in obese mice. Pentoxifylline treatment significantly decreased TNF-α and IL-6 levels in obese adipose tissue, but increased leptin and PAI-1 in lean adipose tissue (Table 5).

DISCUSSION

NAFLD is currently considered a consequence of obesity, and its prevalence in obese subjects is very high. Sedentary life style and consumption of foods with high-fat and high-caloric content are the main contributing

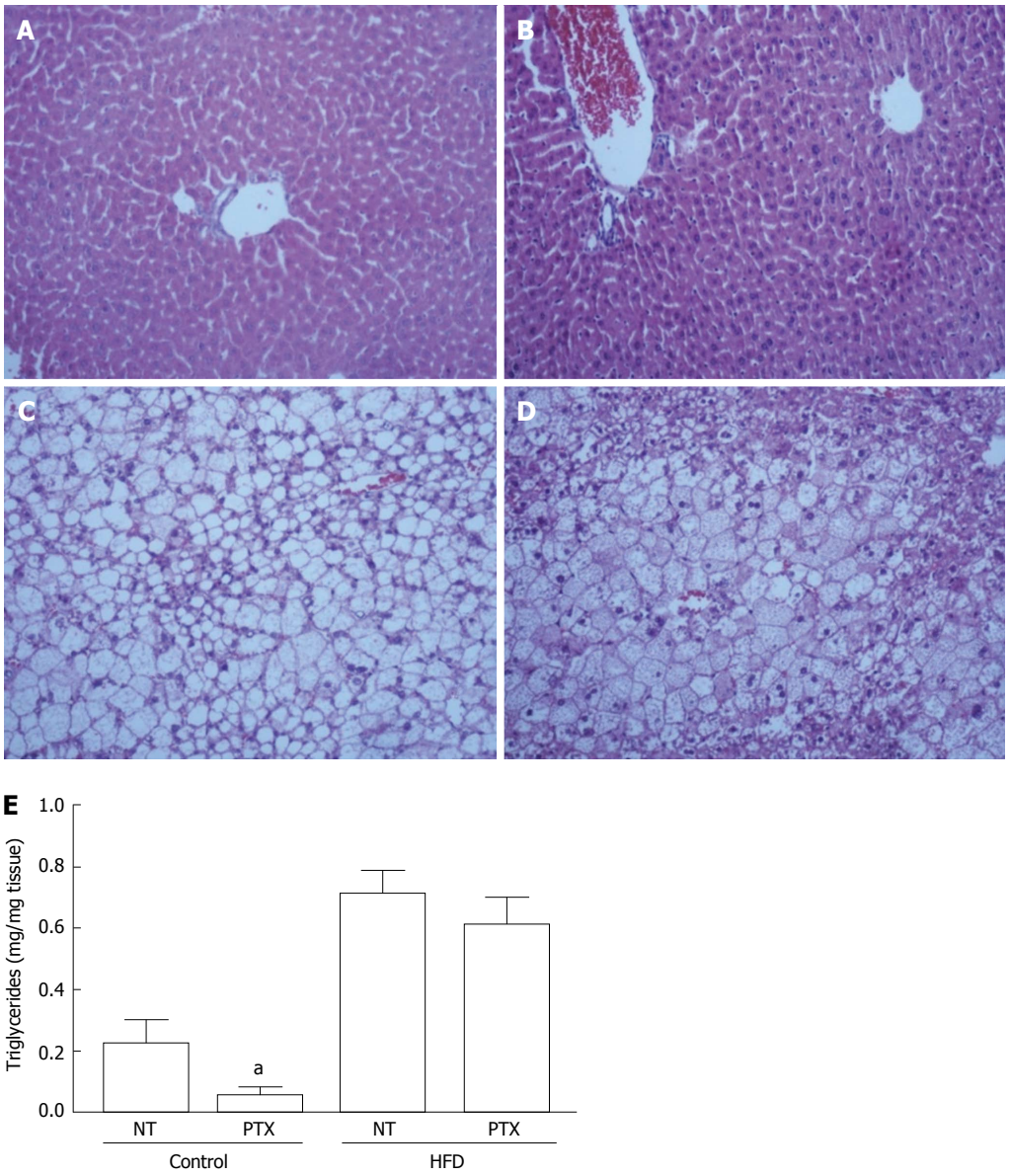

Figure 1 Pentoxifylline effects on non-alcoholic fatty liver disease in mice. A: Liver of lean mice and pentoxifylline-treated lean mice; B: A normal histology; C: Liver of mice on a high-fat diet for 12 wk; D: Treated with pentoxifylline shows pronounced steatosis. Hematoxylin-eosin staining of 4.0 μm sections of livers. Magnification: 200 ×; E: Hepatic triglycerides content was determined and expressed as mg/mg of liver tissue of all groups. Data are shown as mean ± SEM of 4 mice per group, $^{a}P = 0.07$. NT: Non-treated; PTX: Pentoxifylline; HFD: High-fat diet.

Table 5 Adipokine profile of lean (control) and obese (high-fat diet) mice treated with pentoxifylline

	Control		HFD	
	NT	PTX	NT	PTX
TNF-α (pg/mL)	87.2 ± 2.8	75.5 ± 3.9	106.1 ± 17.6	51.1 ± 9.6^{c}
IL-6 (pg/mL)	227.9 ± 23.3	178.5 ± 30.9	340.8 ± 51.3	166.6 ± 22.5^{c}
PAI-1 (pg/mL)	600.2 ± 32.3	1508.6 ± 210.4^{c}	2646.3 ± 755.1^{a}	4434.5 ± 1400.1
MCP-1 (pg/mL)	287.8 ± 17.1	253.8 ± 13.3	721.5 ± 112.8^{a}	563.8 ± 109.7
Leptin (ng/mL)	8.1 ± 0.7	23.1 ± 2.9^{c}	26.6 ± 3.0^{a}	20.5 ± 3.7
Adiponectin (ng/mL)	109.1 ± 0.5	102.8 ± 0.6	106.8 ± 1.7	102.8 ± 5.9

$^{a}P < 0.05$ *vs* control NT group; $^{c}P < 0.05$ *vs* the matched NT group. HFD: High-fat diet; NT: Non-treated; PTX: Pentoxifylline; TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6; MCP: Monocyte chemoattractant protein-1; PAI: Plasminogen activator inhibitor-1.

factors to obesity[14]. The reduction of body weight and lifestyle changes are the primary recommendations to

control NAFDL, and pharmacological interventions aim to induce weight loss (*e.g.,* orlistat and sibutramine), to improve the antioxidant response (*e.g.,* vitamin E and C, ursodeoxycholic acid) or to ameliorate insulin resistance and glucose and lipid metabolism (*e.g.,* metformin, thiazolidinediones)[15]. Pentoxifylline has been considered an alternative treatment to control NAFDL, as it has been recommended in alcoholic fatty liver disease by acting as an anti-inflammatory drug[16]. Pentoxifylline is a methylxanthine derivative that acts as a nonspecific phosphodiesterase inhibitor to promote an increase in cyclic AMP levels and inhibit *TNF-α* gene transcription[17,18].

A high-fat diet obesity model is suitable to study metabolic and liver disease associated with adipose tissue expansion and to study potential therapeutics to control obesity. Our results show that Swiss mice fed a HFD for 12 wk present increased body weight, increased adiposity, adipose tissue inflammation, insulin resistance, hyperglycemia, steatosis, inflammation and increased TNF-α and MCP-1 levels in the liver. Pentoxifylline treatment did not change the final body weight but did decrease the liver weight. Visceral (epididymal) adipose tissue increased after pentoxifylline treatment, which may explain why we did not observe a decrease in body weight. Pentoxifylline treatment did not improve glucose homeostasis, but some NAFLD features improved, such as hepatic steatosis, inflammation, TNF-α levels, and serum AST levels. Our results are consistent with a previous study, where Sprague-Dawley rats fed a HFD for 16 wk were treated with pentoxifylline for 4 wk (16 mg/kg per day). The results showed decreased AST levels but not ALT, and improvements in basal glucose but not HOMAIR index[19]. Additionally, in a previous study of Sprague-Dawley fed HFD for 10 wk and treated with pentoxifylline for 6 wk (50 mg/kg per day), hepatic steatosis and plasma levels of TNF-α were reduced[20]. In our work, we evaluated both hepatic and adipose tissue TNF-α levels and found that pentoxifylline treatment reduced both. However, glucose homeostasis did not improve, but TNF-α and IL-6 levels decreased. A balance between leptin and adiponectin have been suggested to have a role in metabolic syndrome and type 2 diabetes[21]. Pentoxifylline treatment could not reverse the alterations in the obesity-induced leptin/adiponectin ratio.

Interestingly, we observed increased TNF-α in the liver and increased PAI-1 and leptin in adipose tissue of lean mice after pentoxifylline treatment. A systematic review of pentoxifylline data in patients with NAFLD revealed that AST and ALT plasma levels and liver histological scores were improved in several studies using pentoxifylline. However, pentoxifylline treatment did not inhibit plasma cytokines levels such as IL-6 in all studies[22]. Although pentoxifylline has been shown to inhibit TNF-α, Zein *et al*[9] reported that pentoxifylline treatment did not inhibit TNF-α plasma levels in NASH patients. The authors suggested that TNF-α plasma levels may not be related to hepatic levels of this cytokine because they observed histological improvement. Both the adipose tissue and liver of lean mice increased pro-inflammatory cytokine production in response to pentoxifylline treatment, an unexpected result that should be further studied. Interestingly, triglycerides levels presented a tendency to reduction in liver of lean mice. Several findings suggested that triglycerides per se are not toxic, on contrary; they protect liver from lipotoxicity by buffering the accumulation of fatty acids. Triglycerides synthesis inhibition improves steatosis but stimulates oxidizing systems that increase hepatic oxidative stress and liver damage[23]. Although, pentoxifylline is able to decrease oxidative stress and to inhibit lipid peroxidation in patients with NASH[24], we did not rule out the possibility that lean mice had an increase in oxidative response due pentoxifylline treatment. We hypothesize that metabolic status, liver metabolism, adiposity or inflammation degree can interfere with the pentoxifylline response, which could explain the controversial data obtained in different clinical studies of NAFLD patients. In this line of reasoning, pentoxifylline is effective in states of hyperinflammation because relevant anti-inflammatory effects can be achieved only in the presence of sufficient adenosine concentrations[25]. Metabolic stress, hypoxia and inflammation are conditions related to increase adenosine extracellular concentrations[26], and thus, could interfere with the pentoxifylline response.

In conclusion, our results showed that pentoxifylline was beneficial in an obesity-associated NAFLD model by improving liver inflammation and adipose tissue inflammation, but it was not able to improve obesity-induced metabolic disturbances. Unexpectedly, pentoxifylline treatment increased pro-inflammatory markers in the liver and adipose tissue of lean mice.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of hepatic disorders including steatosis and non-alcoholic steatohepatitis that can progress to end-stage liver disease, such as fibrosis, cirrhosis and hepatic cancer. NAFLD is frequently associated with metabolic syndrome establishment and obesity. Several decades of pharmacological research have resulted in very few options for NAFLD management; therefore, new therapeutic approaches need to be researched.

Research frontiers

Pentoxifylline is a non-selective phosphodiesterase inhibitor that has been reported to have antioxidant activity and decrease tumor necrosis factor (*TNF*)-*α* gene transcription. Pentoxifylline is prescribed to patients with severe alcoholic hepatitis, which suggest that this drug could also be beneficial to NAFLD. In this study, authors described pentoxifylline effects upon NAFLD using an experimental model of high-fat diet induced obesity in mice. Pentoxifylline was beneficial in obesity-associated NAFLD reducing liver microsteatosis and TNF-α, as well as, serum aspartate aminotransferase levels. However, pentoxifylline treatment in lean mice resulted in pro-inflammatory cytokine production in the liver and adipose tissue, suggesting that pentoxifylline effects could be dependent of additional conditions, as metabolic status, liver metabolism, adiposity or inflammation degree.

Innovations and breakthroughs

NAFLD has reached epidemic proportions nowadays. The current therapy is

limited to suggestions of lifestyle changes and metabolic alterations control. Here, authors described beneficial pentoxifylline effects upon NAFLD using an experimental model of high-fat diet induced obesity in mice. Interestingly, the same treatment protocol in lean mice resulted in non-desirable effects upon inflammatory parameters in liver and adipose tissue.

Applications

By demonstrating that pentoxifylline has a protective role in liver from obese mice but not from lean mice, this study contributes to a better understanding of conflicting results provides by clinical studies using this therapeutic for NAFLD.

Terminology

NAFLD defines a spectrum of hepatic disorders including steatosis, non-alcoholic steatohepatitis, liver fibrosis, cirrhosis and hepatic cancer. Pentoxifylline is a non-selective phosphodiesterase inhibitor prescribed to patients with severe alcoholic hepatitis, which suggest that this drug could also be beneficial to NAFLD.

Peer-review

In the current manuscript, Acedo *et al* reported that administration of pentoxifylline was able to reduce the fat accumulation in liver of obese mice fed by high-fat diet. This study is helpful to better understand the mechanism of pentoxifylline on NAFLD.

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CASE REPORT

Unusual case of drug-induced cholestasis due to glucosamine and chondroitin sulfate

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Abstract

Glucosamine (GS) and chondroitin sulfate (CS) are common over-the-counter (OTC) supplements used in the treatment of osteoarthritis. These medications are seemingly safe, but there are increasing reports of hepatotoxicity with these supplements. We reported a unique case of drug-induced cholestasis caused by GS and CS in a combination tablet. The etiology of the jaundice was overlooked despite extensive investigations over a three-month period. Unlike drug-induced hepatocellular injury, drug-induced cholestatic jaundice with GS and CS has only been reported twice before. This case emphasizes the importance of a complete medication history, especially OTC supplements, in the assessment of cholestasis.

Key words: Glucosamine; Chondroitin; Hepatotoxicity; Cholestasis; Jaundice

Core tip: Glucosamine and chondroitin sulfate are common over-the-counter medications available in North America and other countries in the treatment of osteoarthritis. We report a unique case of drug-induced cholestatic injury caused by this combination tablet. The etiology of this patient's new jaundice went undiagnosed despite extensive investigations over three months. Only after careful questioning of his medication history and review of his liver biopsy was the correct diagnosis obtained. This case adds to the increasing reports of hepatoxicity related to this supplement. Furthermore,

it highlights the importance of a complete medication history, especially over-the-counter medications, in the assessment of cholestatic jaundice.

Ip S, Jeong R, Schaeffer DF, Yoshida EM. Unusual case of drug-induced cholestasis due to glucosamine and chondroitin sulfate. *World J Hepatol* 2015; 7(24): 2559-2562 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i24/2559.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i24.2559

INTRODUCTION

In North America and other countries, glucosamine (GS) and chondroitin sulfate (CS) are common over-the-counter (OTC) supplements marketed for supporting the structure and function of joints, especially in treating osteoarthritis (OA). Although clinical trials have suggested that these medications have minimal to no significant clinical benefit in OA[1,2], they continue to be utilized, which may be attributed to their reputation as "natural products", easy tolerability, and low side-effect profile[3]. A rare complication related to GS and/or CS use is hepatotoxicity especially classic hepatocellular drug-induced liver injury (DILI)[3-5]; however, purely cholestatic injury is less documented[4,6]. We report a unique case of drug-induced cholestasis caused by GS and CS. The etiology of the jaundice was missed despite exhaustive medical investigations over three months. This case adds to the growing literature of hepatotoxicity related to this supplement. Furthermore, it highlights the importance of a complete medication history, especially OTC supplements, in the assessment of cholestatic jaundice.

CASE REPORT

A 78-year-old man originally presented with a three-month history of jaundice of unknown etiology at his home hospital. He was subsequently transferred to a tertiary centre for further investigations. His past medical history was significant for a subarachnoid hemorrhage and OA. He was previously well until he reported a three-month history of pruritus, fatigue, nausea, vomiting, and a thirty-pound weight loss with no abdominal pain. He had no history of alcohol abuse or intravenous drug use, no recent travel history, no mushroom ingestion, and no family history of similar jaundice or any liver disease. According to records from his home hospital, he had not taken any other medications except acetaminophen intermittently and vitamin D, which he had been consuming for many years. On direct questioning, however, he disclosed that he had taken GS and CS approximately two months prior to the onset of jaundice. This was his first exposure to this supplement, and he took three tablets per day for his joint pain (maximum daily dose outlined on the bottle). When he started to become jaundiced three months ago, he discontinued the supplement.

On exam, he was clearly jaundiced with no asterixis. His body mass index was 28 kg/m^2. He had no other stigmata of chronic liver disease. His abdominal exam was benign with no appreciable hepatosplenomegaly.

His bloodwork drawn at the tertiary centre revealed a total bilirubin of 470 mol/L (normal is < 18 mol/L), direct bilirubin of 383 mol/L (normal is < 5 mol/L), alkaline phosphate of 136 U/L (normal is 30-135 U/L), gamma-glutamyl transferase of 59 U/L (normal is 0-80 U/L), aspartate transferase of 46 U/L (normal is 10-80 U/L), and alanine transferase (ALT) of 52 U/L (normal is 10-80 U/L). This was virtually unchanged from his previous bloodwork at his home hospital (Table 1). His international normalized ratio and albumin levels were near normal. The remainder of his bloodwork was non-contributory. Liver enzymes and liver function tests were previously normal.

At his home hospital, he had had extensive investigations for his jaundice. Serology for hepatitis A, B and C were negative. Antinuclear antibody, antimitochondrial antibody, and antineutrophil cytoplasmic antibody were negative. Ceruloplasmin and alpha-antitrypsin levels were normal, and anti-tissue transglutaminase was negative with a normal IgA level. A skin biopsy was negative for vasculitis. He had had multiple abdominal ultrasounds that showed cholelithiasis, a somewhat inhomogeneous liver with no discrete lesions, but specifically no evidence of intra or extrahepatic bile duct dilatation. He had two endoscopic retrograde cholangiopancreatographies (ERCPs), which revealed no biliary tract abnormalities. A sphincterotomy and stent insertion were completed in hopes of relieving any kind of obstruction with no success. A subsequent magnetic resonance cholangiopancreatography (MRCP) showed no evidence of duct dilatation. Two liver biopsies showed non-specific signs of acute cholestasis (Figure 1). Upon further review with a pathologist at the tertiary centre, these biopsies were most consistent with drug-related cholestasis. Temporally, GS and CS fit as the likely culprit.

DISCUSSION

GS is an aminosaccharide that is important for proteoglycan formation, which helps preserve cartilage integrity of joints[7] while CS is an essential part of aggrecan, a component of the cartilage structure[8]. Both supplements are frequently taken together in the treatment of OA. Previous systemic reviews have shown that these supplements are safe with no significant side effects[8,9]; however, reports of hepatotoxicity have been documented[3-5]. We report a unique case of drug-related cholestasis that adds to the expanding literature of this mechanism of liver injury associated with GS and CS.

There have been two other similar reports regarding cholestatic DILI with GS and/or CS. Ossendza *et al*[6] describe a case of hepatitis with significant ALT

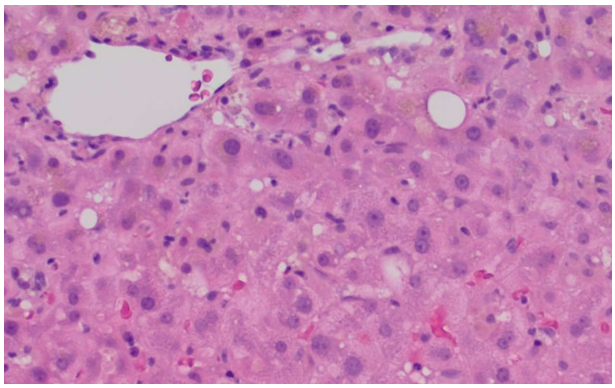

Figure 1 Histologic micrograph of liver biopsy. This H and E photomicrograph shows a representative area of the adequate core needle biopsy, depicting overall a very mild lobular lymphocytic hepatitis with areas of mild cholestasis with canalicular plugs and focal hepatocyte bile pigmentation. The hepatic architecture is preserved with a normal relationship of central veins and portal tracts and no evidence of fibrosis.

and bilirubin elevation, approximately 6- and 10-fold respectively, in a patient taking therapeutic doses of GS four months prior to presenting with pruritus and jaundice. This patient recovered with discontinuation of the supplement. Smith *et al*[4] describe a case of elevated cholestastic liver enzymes and normal bilirubin that return to normal after stopping GS and CS. Our case is unique in that the patient presented with purely hyperbilirubinemia with mild liver enzyme derangement. An exhaustive search for a cause was undertaken including multiple investigations (*e.g.*, ERCPs, MRCP, *etc.*), highlighting the importance of a complete medication history in the evaluation of new onset jaundice.

The mechanism by which GS and/or CS causes hepatotoxicity is unclear. An allergic mechanism has been proposed given the presence of rash and/or eosinophilia in previous case reports[3]. Furthermore, GS is derived from the exoskeleton of shellfish, which would theoretically worsen in those with known seafood allergies. No cases of hepatotoxicity, however, have been recorded in patients with known shellfish allergy[10]. The purity of the supplement may be another factor in causing hepatotoxicity. In Europe, GS and/or CS is classified as a medication, but in North America and other countries, they are available as an OTC supplements, which is not under regulative scrutiny regarding purity or efficacy. In our case, our patient appeared only to be taking therapeutic doses suggested by the manufacturer.

In conclusion, we report a rare case of cholestatic injury related to GS and CS, adding to increasing reports of hepatotoxicity of this supplement. Although this supplement may be thought to be harmless, clinicians need to consider this supplement in the evaluation of liver enzyme derangement and/or jaundice.

COMMENTS

Case characteristics

A 78-year-old man presents with 3 mo of painless jaundice.

Table 1 Pattern of liver enzymes

	Presentation at home hospital	Presentation at tertiary centre
Total bilirubin (mol/L) Normal: < 18 mol/L	476	440
AST (U/L) Normal: 10-80 U/L	45	40
ALT (U/L) Normal: 10-80 U/L	47	48
GGT (U/L) Normal: 0-80 U/L	59	136
Alkaline phosphatase (U/L) Normal: 30-135 U/L	136	136

AST: Aspartate transferase; ALT: Alanine transferase; GGT: Gamma-glutamyl transferase.

Clinical diagnosis

The patient clearly had scleral icterus but no other stigmata of chronic liver disease. His abdominal exam revealed no masses.

Differential diagnosis

Medication (*e.g.*, over-the-counter), malignant (*e.g.*, pancreatic cancer), benign obstruction (*e.g.*, gallstones), autoimmune hepatitis.

Laboratory diagnosis

His bloodwork revealed significant hyperbilirubinemia of 470 mol/L (normal is < 18 mol/L) with relatively preserved liver enzymes and liver function tests.

Imaging diagnosis

He has multiple normal abdominal ultrasounds, endoscopic retrograde cholangiopancreatographies and a magnetic resonance cholangiopancreatography.

Pathological diagnosis

The liver biopsy was consisted with drug-related cholestasis.

Treatment

His glucosamine and chondroitin sulfate supplements were stopped.

Related reports

There have been only two reports of drug-related cholestasis with glucosamine and/or chondroitin sulfate.

Term explanation

Glucosamine and chondroitin sulfate are commonly available over-the-counter supplements in the treatment of osteoarthritis.

Experiences and lessons

A complete drug history, especially over-the-counter medications, are important in the evaluation of liver enzyme derangement and/or jaundice.

Peer-review

This is a good instructive case which will benefit readers.

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