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High-fat diet plus carbon tetrachloride-induced liver fibrosis is alleviated by betaine treatment in rats

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ABSTRACT

Steatosis, the first lesion in non-alcoholic fatty liver disease (NAFLD), may progress to fibrosis, cirrhosis, and hepatocellular carcinoma. Steatosis predisposes the liver to oxidative stress, inflammation, and cytokines. Betaine (BET) has antioxidant, antiinflammatory and hepatoprotective effects. However, the effects of BET on liver fibrosis development are unknown. Rats were treated with high-fat diet (60% of total calories from fat) for 14 weeks. Carbon tetrachloride (0.2 mL/kg; two times per week; i.p.) was administered to rats in the last 6 weeks with/without commercial food containing BET (2%; w/w). Serum liver function tests and tumor necrosis factor-α, insulin resistance, hepatic triglyceride (TG) and hydroxyproline (HYP) levels and oxidative stress parameters were determined along with histopathologic observations. Alpha-smooth muscle-actin (α-SMA), transforming growth factor-β1 (TGF-β1) and type I collagen (COL1A1) protein expressions and mRNA expressions of matrix metalloproteinase-2 (MMP-2) and its inhibitors (TIMP-1 and TIMP-2) were evaluated. BET decreased TG and HYP levels, prooxidant status and fibrotic changes in the liver. α-SMA, COL1A1 and TGF-β1 protein expressions, MMP-2, TIMP-1, and TIMP-2 mRNA expressions diminished due to BET treatment. BET has an antifibrotic effect and this effect may be related to its antioxidant and antiinflammatory actions together with suppression on HSC activation.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of injury from simple fat accumulation (steatosis) to more severe liver diseases such as non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). NAFLD is strongly associated with obesity, diabetes, and insulin resistance, and is accepted to be a hepatic manifestation of metabolic syndrome. The mechanism involved in the development of NAFLD is poorly understood, although several hypotheses have been proposed. One generally accepted theory is the "two-hit" hypothesis. It is accepted that the accumulation of fat in the liver predisposes the liver to secondary stresses such as oxidative stress, inflammation, and cytokines [2,10,17].

Several dietary animal models such as HFD, high fructose diet, and methionine and cholinedeficient diet (MCD) have been used to produce experimental non-alcoholic fatty liver [33]. The feeding of a HFD to normal rodents generally induces insulin resistance, obesity and fatty liver [19,24,37]. Oxidative stress in fatty livers is attributed to enhanced generation of reactive oxygen species (ROS) via multiple intracellular pathways, such as cytochrome P450-mediated ῶ-oxidation of fatty acid, peroxisomal β-oxidation catalyzed by acyl-CoA oxidase, and impaired mitochondrial respiratory chain. Thus, ROS may induce liver damage via lipid peroxidation and inflammation. Therefore, there is growing evidence that fat accumulation in the liver plays a critical role in the initiation and progression of NAFLD [20,24]. It has been accepted that a combination of HFD-induced fatty liver (first hit) and oxidative stress induced by multiple low doses of CCl_4 (second hit) play an important role in fibrotic and cirrhotic changes [23,26,27,33].

NASH and liver fibrosis is known to be reversible; however, cirrhosis and HCC are generally irreversible [16,30]. Therefore, controlling the

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BET, betaine; CAT, catalase; CCl_4, carbon tetrachloride; CHOL, cholesterol; COL1A1, type I collagen; ECM, extracellular matrix; FRAP, ferric reducing antioxidant power; GSH, glutathione; GSH-Px, glutathione peroxidase; GST, glutathione transferase; H and E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HFD, high-fat diet; HOMA, homeostasis model assessment; HSC, hepatic stellate cell; HYP, hydroxyproline; IR, insulin resistance; LDH, lactate dehydrogenase; MDA, malondialdehyde; MMP-2, matrix metalloproteinase-2; MTC, Masson's trichrome stain; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PC, protein carbonyl; ROS, reactive oxygen species; α-SMA, α-smooth muscle-actin; SOD, superoxide dismutase; TG, triglyceride; TGF-β1, transforming growth factor-β1; TIMP-1 and TIMP-2, tissue matrix metalloproteinase inhibitors; TNF-α, tumor necrosis factor-α.

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progression of steatosis to fibrosis and other advanced hepatic lesions may be of great importance. For this reason, preventive potentials of several antioxidants on fibrosis development have been tested [2,10,17].

Betaine (trimethylglycine; BET) is a choline metabolite, formed in liver by choline oxidase. Additionally, vegetables are alimentary sources of BET. It functions as an osmolite and methyl group donor for the methionine-homocysteine cycle. It has antioxidant and antiinflammatory effects [11,35]. Upon these effects, BET is reported to be a hepatoprotective agent. Indeed, BET treatment may be useful in alcoholic [3,18] and non-alcoholic fatty liver [19,24,37], necrotic [1,4,5] and fibrotic [12,22,34] lesions in the liver. However, the effects of BET on liver fibrosis development are unknown.

Fibrosis is an excessive wound healing response to chronic liver injury and characterized by the accumulation of extracellular matrix (ECM) rich in fibrillar collagens (mainly collagen I and III). Activated stellate cell (HSC) is the main fibrogenic cell and HSC activation is crucial in liver fibrogenesis [16,30]. HSCs increase the production of mainly type I and type III fibrillar collagen and are the major cell type for matrix production in damaged liver tissue. In response to liver injury, they undergo morphologic and functional changes through the action of transforming growth factor beta (TGF-β1) and transform to myofibroblastic cells.

In this study, liver fibrosis was created by the administration of a high-fat diet (HFD) together with low doses of carbon tetrachloride (CCl_4). We wanted to examine the effects of BET treatment on fibrosis development and related mechanisms. For this reason, serum liver function tests and tumor necrosis factor-α (TNF-α), insulin resistance (IR), hepatic triglyceride (TG) and hydroxyproline (HYP) levels, and oxidative stress parameters were determined together with histopathologic observations. To examine stellate cell (HSC) activation, α-smooth muscle-actin (α-SMA), TGF-β1, and type I collagen (COL1A1) protein expressions were assayed immunohistochemically. mRNA expressions of matrix metalloproteinase-2 (MMP-2) and its inhibitors (TIMP-1 and TIMP-2) were also evaluated.

2. Materials and methods

2.1. Chemicals

BET was supplied from Alfa Aesar (Germany) and other chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

2.2. Animals

Female Sprague Dawley rats weighing 180–200 g were used in the study. They were obtained from the Experimental Medical Research Institute of Istanbul University. Rats were housed in a light- and temperature-controlled room on a 12/12-h light/dark cycle. The animals had free access to food and water and were kept in wire-bottomed stainless steel cages. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of the Istanbul University.

2.3. Experimental design

In this study, the effect of BET treatment on liver fibrosis was investigated in non-alcoholic fibrosis model. Rats were treated with HFD for 14 weeks and then CCl_4 was administered to rats in the last 6 weeks with/without BET-containing diet. For this purpose, rats were randomly selected and divided into four groups. The experimental design is shown in Fig.1.

1. Control group: Rats were fed with commercial rat chow and injected i.p with an olive oil vehicle twice a week for 6 weeks.
2. BET group: Rats were fed with normal commercial food for 8 weeks and then BET (2%, w/w) containing commercial food diet for 6 weeks together with olive oil injections as a vehicle twice a week.
3. HFD + CCl_4 group: Rats were fed with HFD (60% of total calories from fat) for 14 weeks. The HFD contained 34.3% fat (31% beef tallow, 3.4% corn oil), 27.3% carbohydrate, 23.5% protein, 5% fiber, together with salt and vitamin mixtures. CCl_4, diluted 1:4 in olive oil, was also administered to rats at a dose of 0.2 mL/kg twice a week in the last 6 weeks.
4. HFD + CCl_4 + BET group: Rats were treated with HFD + CCl_4 as described above. Rats were fed with BET (2%, w/w) containing HFD in the last 6 weeks.

2.4. Blood and tissue samples

At the end of the treatment period, all rats were sacrificed by taking blood via cardiac puncture under sodium thiopental anesthesia (50 mg/kg, i.p.). Blood was collected in dry tubes. Livers were rapidly removed, washed in 0.9% NaCl and kept in ice. Serum samples were obtained by centrifugation at 1500 ×g for 10 min. The materials were stored at −80 °C until required for analysis. Liver tissue was homogenized in ice-cold 0.15 M KCl (10%; w/v) and homogenates were centrifuged at 600 ×g for 10 min and this fraction was used for biochemical determinations in the liver.

2.5. Determinations in serum

Serum glucose, cholesterol (CHOL) and TG levels, and alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities were determined in Cobas Integra 800 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum insulin (Rat/Mouse Insulin Kit, Millipore, Missouri, USA) and TNF-α (Rat TNF-α, Diaclone SAS, Besançon, France) levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits. IR was assessed by means of the homeostasis model assessment (HOMA). The HOMA index was calculated as [fasting insulin concentration (μU/mL) × fasting glucose concentration (mg/dL)] / 405.

2.6. Determinations in the liver

2.6.1. Determination of TG levels

Hepatic lipids were extracted with chloroform:methanol (2:1) [13] and TG levels were measured in lipid extracts by using enzymatic procedure.

2.6.2. Determination of HYP levels

Liver HYP levels were measured using the method described by Bergman and Loxley [7]. Briefly, 100 mg of liver tissue was hydrolyzed in 4 mL of 6 N HCl at 108 °C for 16 h in a glass tube with a Teflon stopper. The hydrolysate was filtered and 0.5 mL of hydrolysate was neutralized with approximately 0.4 mL of 6 N NaOH. Then, 0.5 mL of neutral or slightly acid solution was pipetted into clean tubes. Standards and samples were incubated with 1.0 mL of oxidizing reagent containing chloramine T, and blank samples were incubated with the same amount of oxidizing reagent without chloramine T for 5 min. One milliliter of Ehrlich's reagent was added to all tubes, mixed and incubated at 60 °C for 45 min. Absorbances were read at 570 nm using spectrophotometer. Results were calculated from a standard curve generated from known quantities of HYP.

2.6.3. Determinations of ROS formation and malondialdehyde (MDA) and protein carbonyl (PC) levels

ROS generation was determined using a fluorometric assay as described previously with some modifications [36]. Tissue homogenates were incubated with 100 μM 2′,7′-dichlorodihydrofluorescein diacetate at 37 °C for 30 min. The fluorescence of 2′,7′-dichlorofluorescein was determined using a microplate fluorometer and luminometer (Fluoroskan Ascent FL, Thermo Scientific Inc., USA) with an excitation

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