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Theophylline, an old drug with multi-faceted effects: Its potential benefits in immunological liver injury in rats

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ABSTRACT

Aim: A low dose of theophylline enhances histone deacetylase activity leading to inhibition of proinflammatory transcription, and inhibits lung fibroblast proliferation. The present work investigated the effect of low dose theophylline on biochemical and histological pictures of liver tissues in rats with immunological hepatic injury induced by concanavalin A (Con A).

Main methods: Rats were assigned to control vehicle, model (Con A) and theophylline groups. Half of the animals in each group were sacrificed at the end of the 4th week and the other half were sacrificed at the end of the 8th week.

Key findings: There was a time-dependent increase in the liver injury parameters by the end of the 4th and 8th weeks in the Con A treated group. Theophylline (20 mg/kg/day), produced a significant decrease in serum liver enzymes (ALT, AST), serum interferon gamma (IFN-γ) levels and the hepatic transforming growth factor-β (TGF-β) level. A significant decrease in liver tissue hydroxyproline content together with reduction in portal hypertension at the end of the 8th week was detected compared to the Con A group. Theophylline treated rats exhibited a significant decrease in hepatic vacuolation, apoptosis, leucocyte infiltration, and accumulation of collagen fibers in comparison to the Con A group. In addition, significant decreases in the area percentage of fibrosis and the area percentage of caspase +ve cells were reported compared to the Con A group.

Significance: Theophylline effectively reduced the inflammation of liver tissues and alleviated the liver damage by decreasing IFN-γ and TGF-β in liver tissues of rats with immunological hepatic injury.

1. Introduction

Liver fibrosis and its end-stage sequel cirrhosis are major causes of morbidity and mortality worldwide [29]. For years, liver fibrosis has been considered irreversible. However, histological assessment of biopsies from either patients with chronic liver diseases or animals with various etiologies of liver fibrosis, whom have been successfully treated, indicated that fibrosis is a dynamic, bidirectional process in which recovery with remodeling of scar tissue is possible [56]. Chronic hepatitis C virus (HCV) infection is one of the major causes of liver fibrosis [10]. HCV stimulates the effector cells of innate immunity including phagocytes and antigen-presenting cells. The dispersed signals that emanate from the innate immune response trigger the components of the adaptive immune response that stimulate T and B lymphocytes [38].

In studying immune system-mediated liver injury the concanavalin A (Con A)-induced hepatitis model has been widely used [18]. The pathogenesis of this hepatitis model resembled chronic HCV infection in humans to a certain extent [27,33]. Con A induces hepatic activation of CD_4 T cells, natural killer T (NKT) cells and Kupffer cells which secrete large amounts of hepatotoxic cytokines such as interferon-γ (IFN-γ) [11].

Hepatocyte apoptosis is a common feature in the HCV-infected liver [7]. Engulfment of apoptotic bodies by hepatic stellate cells (HSCs) triggers a profibrogenic response that stimulates transforming growth factor-β (TGF-β) expression, which induces collagen. Indeed, phagocytosis of apoptotic bodies by quiescent HSCs facilitates their phenotypic transformation to myofibroblasts [9]. Engulfment of apoptotic bodies by Kupffer cells enhances the expression of pro-fibrogenic genes (e.g. TGF-β) and of death ligands, which initiate independent intracellular signaling cascades that further amplify liver injury [8].

Hydroxyproline is a major component of collagen and plays key roles for collagen stability [35] and its resistance to degradation [21]. Hydroxyproline quantification is considered the most common method for evaluating tissue fibrosis/collagen deposition [50]. Histopathologically,

Abbreviations: Hyp, hydroxylproline; PP, portal venous pressure; Con A, concanavalin A.

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the decrease in hydroxyproline content approximately paralleled decreases in the fibrosis stage [32].

Some drugs that are currently in use for other human diseases such as theophylline may have antifibrotic effects. Theophylline is a phosphodiesterase enzyme (PDE) inhibitor and adenosine-receptor blocker [46]. Theophylline is one of the histone deacetylase (HDAC) activators. The low dose of theophylline, devoid of its major side effects, has the ability to enhance HDAC activity [22], with its subsequent inhibitory effect on proinflammatory transcription factors [54]. Indeed Yano et al. [53] demonstrated that theophylline inhibited lung fibroblast proliferation.

The present study was undertaken to detect the effects of theophylline on liver fibrosis progression in Con A induced immunological liver fibrosis in Wistar rats as regards changes in liver biochemical and histological markers in an attempt to find out the effects of the tested drug. In addition, the changes in portal venous pressure were measured.

2. Materials and methods

2.1. Experimental animals

Forty eight adult white male Wistar rats (body weight: 150–200 g) were purchased from the National Research Institute (Cairo, Egypt). All animal studies were according to the Institutional Animal Ethics of Ain Shams University. Male Wistar rats were housed in wire mesh cages under standard experimental conditions with temperature at about 22 °C and lighting (12 h light–dark cycle) control.

2.2. Chemicals & drugs

Concanavalin A was purchased from Bio-Research Products, Inc., USA. It was supplied as white powder and was dissolved in saline. Theophylline was a generous gift from MEDICO, Cairo, Egypt and was dissolved in saline. Ketamine and xylazine hydrochloride were purchased from Sigma, Cairo, Egypt. They were supplied as solution.

2.3. Experimental procedures

2.3.1. Induction of liver fibrosis and study design

To establish a chronic hepatitis induced liver fibrosis model, 20 mg/kg Con A was intravenously injected into the rat's tail veins once a week for either 4 weeks or for 8 weeks [24]. Theophylline was administrated in 20 mg/kg/day by gastric gavage from the beginning of the induction of liver injury [26]. At the end of the 4th and 8th weeks of the experiment, blood samples were collected for biochemical analysis and livers were removed for histological and immune-histochemical analysis. Portal venous pressure was measured only at the end of the 8th week.

2.3.2. Animal groups (16 animal/group)

Group I (normal control group): Rats received IVI of saline in the tail vein once a week and saline orally by gastric gavage daily.

Group II (Con A group): Rats received IVI of Con A 20 mg/kg in the tail vein weekly and saline orally by gastric gavage daily.

Group III (theophylline group): Rats received Con A as in group II and were concomitantly treated with theophylline (20 mg/kg) by gastric gavage daily.

Half of the animals in each group were sacrificed at the end of the 4th week and the other half were sacrificed at the end of the 8th week.

2.4. Biochemical measurements

2.4.1. Serum ALT and AST levels

Serum ALT and AST levels were measured according to the method described by Reitman [42] using kits purchased from Diamond Diagnostic (Cairo, Egypt).

2.4.2. Serum IFNγ level

The serum IFNγ level was measured by an ELISA kit purchased from RayBiotech, Inc. according to the protocol provided by the manufacturer. In brief, this assay employed an antibody specific for the IFNγ coated well plate. IFNγ present in a sample was bound to the wells by the immobilized antibody. An anti-rat IFNγ antibody was added. After washing away the unbound biotinylated antibody, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Tetramethylbenzidine (TMB) substrate solution was added to the wells and color developed in proportion to the amount of IFNγ bound. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.4.2.1. Liver sample preparation. After rat sacrifice, livers were rapidly removed at the end of the 4th and 8th weeks of the experiment. The right lobe of the liver was removed, cut into thin slices and kept in 10% formalin for histological and immunohistochemical examination. Part of the other lobe was frozen for the assessment of hepatic hydroxyproline content and hepatic TGF-β level. The temperature for freezing should be −80 °C.

2.4.3. Measurement of hydroxyproline (Hyp) content

Liver Hyp was determined by an ELISA kit purchased from Uscn, Life Science Inc. Hyp content was expressed as ng/g tissue. Liver samples were centrifuged for 20 min at 1000 × *g*. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Hyp. Samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for Hyp. Next, Avidin conjugated to HRP was added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain Hyp, the biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme–substrate reaction is terminated by the addition of sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm.

2.4.4. Measurement of the liver TGF-β level

Levels of hepatic TGF-β in rats were determined by using a corresponding ELISA kit purchased from Sigma-Aldrich Co. Hepatic TGF-β was expressed as ng/g tissue. Hepatic TGF-β was measured in the prepared sample as discussed before in Hyp content measurement.

2.5. Histological and immunohistochemical studies

Five μm thickness sections were stained with hematoxylin and eosin (H&E) for detection of vacuolation and mononuclear cellular infiltration and Mallory's triple [49] for detection of fibrosis. Immunohistochemical staining by caspase-3 was used for detection of apoptosis [3].

2.5.1. Immunohistochemical study

The caspase-3 technique (ready to use) was used for detection of apoptosis in the hepatocytes. Paraffin sections of the liver and a positive control (tonsils) were cut at 5 μm thickness on positive charged slides, and were incubated in a 42 °C oven for 24 h. Sections were deparaffinized in xylene (1 h), then hydrated in descending grades of alcohol. They were incubated in hydrogen peroxide (5 min). Sections were washed twice in phosphate-buffered saline (5 min each). Ultra V block was applied (10 min). A ready to use primary antibody was applied to sections and then incubated (1.5 h). Sections were then washed twice in phosphate-buffered saline (5 min each). A secondary antibody