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Melatonin mitigates thioacetamide-induced hepatic fibrosis via antioxidant activity and modulation of proinflammatory cytokines and fibrogenic genes

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ABSTRACT

Aims: The potential antifibrotic effects of melatonin against induced hepatic fibrosis were explored.
Main methods: Rats were allocated into four groups: placebo; thioacetamide (TAA) (200 mg/kg bwt, i.p twice weekly for two months); melatonin (5 mg/kg bwt, i.p daily for a week before TAA and continued for an additional two months); and melatonin plus TAA. Hepatic fibrotic changes were evaluated biochemically and histopathologically. Hepatic oxidative/antioxidative indices were assessed. The expression of hepatic proinflammatory cytokines (tumor necrosis factor-α, and interleukin-1β), fibrogenic-related genes (transforming growth factor-1β, collagen I, collagen, III, laminin, and autotaxin) and an antioxidant-related gene (thioredoxin-1) were detected by qRT-PCR.
Key findings: In fibrotic rats, melatonin lowered serum aspartate aminotransferase, alanine aminotransferase, and autotaxin activities, bilirubin, hepatic hydroxyproline and plasma ammonia levels. Melatonin displayed hepatoprotective and antifibrotic potential as indicated by mild hydropic degeneration of some hepatocytes and mild fibroplasia. In addition, TAA induced the depletion of glutathione, glutathione s-transferase, glutathione peroxidase, superoxide dismutase, catalase, and paraoxonase-1 (PON-1), while inducing the accumulation of malondialdehyde, protein carbonyl (C = O) and nitric oxide (NO), and DNA fragmentation. These effects were restored by melatonin pretreatment. Furthermore, melatonin markedly attenuated the expression of proinflammatory cytokines and fibrogenic genes via the upregulation of thioredoxin-1 mRNA transcripts.
Significance: Melatonin exhibits potent anti-inflammatory, antioxidant and fibrosuppressive activities against TAA-induced hepatic fibrogenesis via the suppression of oxidative stress, DNA damage, proinflammatory cytokines and fibrogenic gene transcripts. In addition, we demonstrate that the antifibrotic activity of melatonin is mediated by the induction of thioredoxin-1 with attenuation of autotaxin expressions.

1. Introduction

Hepatic fibrosis and cirrhosis are consequences of chronic liver injuries, e.g., hepatic viral diseases, alcoholic hepatitis, biliary diseases, hemochromatosis or chemical-induced hepatic disorders [1]. The pathogenesis of liver fibrosis results from the activation of myofibroblasts, causing an imbalance between the synthesis and degradation of the extracellular matrix (ECM) and resulting in the replacement of parenchymal tissues with connective tissues [2]. The foremost mediators of myofibroblasts are a group of proinflammatory cytokines and growth factors that contribute to the proliferation and enhancement of ECM production [3]. Among these cytokines, transforming growth factor-β1 (TGF-β1) stimulates hepatic satellite cells (HSCs) to produce high amounts of ECM and prevents HSC degradation and the resulting liver fibrosis [4]. Moreover, TGF-β1, the most potent fibrogenic cytokine produced by multiple types of cells in the liver, promotes the transcription of collagen type I and III, which enhance liver fibrosis via the SMAD3 signaling pathway [5].

Thioacetamide (TAA; C_2H_5NS), an organosulfur fungicide, can induce liver fibrosis and cirrhosis in experimental animals [6]. TAA-induced liver injuries, regenerative nodules, and fibrosis in rats are similar to those of human liver fibrosis [7]. Thioacetamide-S-oxide, a

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Nomenclature

ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATX	Autotaxin
C = O	Protein carbonyl
CAT	Catalase
CDNB	1-Chloro-2, 4-dinitrobenzene
Col1a1	Collagen I-α1
Col3a1	Collagen III-α1
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
Enpp-2	Ectonucleotide pyrophosphatase/phosphodiesterase-2
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HSCs	Hepatic satellite cells
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LN	Laminin
MDA	Malondialdehyde
PDGF	Platelet-derived growth factor
PON1	Paraoxonase-1
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAA	Thioacetamide
TBRAS	Thiobarbituric acid-reactive substances
TCA	Trichloroacetic acid
TGF-β1	Transforming growth factor-β1
TIMPs	Metalloproteinase
TNF-α	Tumor necrosis factor-α
Txn-1	Thioredoxin

potential metabolite of TAA, has been incriminated in hepatic necrosis through changes in cell permeability and Ca^{+2} uptake and the subsequent enlargement of nucleoli and inhibition of mitochondrial activity [8]. Damaged hepatocytes produce a wide range of proinflammatory cytokines, which subsequently activate HSCs to secrete more cytokines and induce liver fibrosis through the above-mentioned mechanism [9]. It is well established that TAA can generate reactive oxygen species (ROS) in different tissues. ROS induce the production of proinflammatory cytokines, which are associated with chronic inflammatory diseases such as fibrosis, cirrhosis, and hepatocellular carcinoma [10].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone synthesized from the amino acid tryptophan in the pineal gland of humans and animals in response to various physiological stimuli [11]. Melatonin plays a pivotal role in the regulation of sexual behavior and energy metabolism and the immune, reproductive, cardiovascular, and neuropsychiatric systems [12]. Further, melatonin has anticancer and antiosteoarthritic activities [13]. Interestingly, melatonin functions as an antioxidant through the inhibition of lipid peroxidation and inactivation of the preformed hydroxyl radicals, nitrogen oxide, singlet oxygen, and hydrogen peroxide [14] and by stimulating the formation and regeneration of reduced glutathione [15]. Moreover, melatonin has an inhibitory effect on proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and TGF-β1 [16]. Recently, it has been suggested that melatonin may regulate different molecular pathways, giving it a potential use in the prevention and treatment of liver injuries induced by various factors [13].

This study focused on the antifibrotic and hepatoprotective potential of melatonin against TAA-induced liver fibrosis. Mechanistically, the interconnection of different pathways involved in this activity will be evaluated through (a) an oxidative stress and antioxidant system, (b) expression of proinflammatory cytokines and growth factors, (c) profibrogenic gene expression, and (d) the activity of autotaxin (ATX), a novel protein that plays a critical role in the pathogenesis of liver fibrosis and cancer [17].

2. Methods

2.1. Animals, housing conditions, and experimental protocol

Forty adult male Wistar rats, weighing 150–170 g, were obtained from the Animal Breeding Unit at the Higher Institute of Graduate Studies and Research, Alexandria University. The rats were housed in clean metal cages in an environmentally controlled room (21 °C, 55% RH, and a 12-h dark/light cycle) and provided with free access to animal chow and water. The ethics committee at the Faculty of Veterinary Medicine, Alexandria University, approved the animal experiment. Following an acclimatization period, rats were randomly divided into four groups (10 rats/group): placebo; TAA (200 mg/kg bwt dissolved in 0.9% NaCl and injected i.p, twice weekly); melatonin (5 mg/kg bwt dissolved in dimethyl sulfoxide [DMSO; $(CH_3)_2SO$] solution and injected i.p. daily one week before TAA and along with TAA); and melatonin together with TAA (with the same doses and routes of administration). Rats in the placebo group were injected i.p with DMSO. TAA, melatonin, and DMSO were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). After two months, rats were anesthetized by i.p injection of ketamine/xylazine solution, and blood samples were collected into two clean tubes for the separation of serum and plasma. The rats were then euthanized by the approved protocol. Livers were collected, washed with ice-cold phosphate-buffered saline, and divided into two parts: one part was preserved at − 20 °C for biochemical analysis, and the second part was preserved in liquid nitrogen at the time of collection and kept at − 80 °C for gene expression analysis.

2.2. Serum biochemical analyses

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, bilirubin content and plasma ammonia levels were measured using commercially supplied bio-diagnostic kits (Vitro Scient Co., Egypt) according to the manufacturer's instructions. Serum ATX or ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp-2) activity was determined by ELISA [18].

2.3. Estimation of hydroxyproline in liver tissue

Hydroxyproline levels were determined in liver tissue following the procedures reported by Bergman and Loxley [19]; Medugorac [20]. The assay is based on the acidic hydrolysis of hydroxyproline and its oxidation into pyrrole. The product is then coupled with *p*-dimethyl-amino benzaldehyde, forming a red color that is measured at 456 nm. The hydroxyproline concentration was calculated using the concentration obtained from the standard curve and expressed as μg/g tissue.

2.4. Oxidative stress indices

To determine the percent of DNA fragmentation, liver tissues were homogenized in lysis buffer [10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.5% TritonX-100] and centrifuged at 3000 rpm for 15 min to obtain supernatants of smaller DNA fragments (F) and pellets of intact DNA (I).

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