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Nicotinic acid prevents experimental liver fibrosis by attenuating the prooxidant process

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ABSTRACT

Liver fibrosis is the excessive accumulation of extracellular matrix proteins that occurs in most chronic liver diseases. Nicotinamide treatment has been shown to prevent collagen accumulation and fibrogenesis in a bleomycin model of lung fibrosis. In this study, we evaluated the effects of nicotinic acid (NA) on experimental liver fibrosis and investigated its underlying mechanism.

Methods: Fibrosis was induced by chronic TAA administration and the effects of co-administration with NA for 8 weeks were evaluated, including control groups.

Results: TAA administration induced liver fibrosis, which was prevented by nicotinic acid. NA prevented the elevation of liver enzymes and prevented hepatic glycogen depletion. Liver histopathology and hydroxyproline levels were significantly lower in the rats treated with TAA plus NA compared with TAA only. NA demonstrated antioxidant properties by restoring the redox equilibrium (lipid peroxidation and GPx levels). Western blot assays showed decreased expression levels of TGF-β and its downstream inductor CTGF. Additionally, NA prevented hepatic stellate cell activation due by blocking the expression of α-SMA. Zymography assays showed that NA decreased the activity of matrix metalloproteinases 2 and 9.

Conclusions: NA prevents experimental fibrosis; the mechanisms of action are associated with its antioxidant properties and the reduction in TGF-β expression. The decrease in TGF-β levels may be associated with the attenuation of the oxidative processes, thus resulting in a reduction in HSC activation and ECM deposition. The findings suggest a possible role for NA as an antifibrotic agent for liver injury.

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1. Introduction

Cirrhosis is the end-stage consequence of hepatic parenchyma fibrosis and results in nodule formation and altered hepatic function. Fibrosis and cirrhosis result from a sustained wound-healing response to chronic liver injury from a variety of causes including viral, autoimmune, drug-induced, cholestatic and metabolic diseases [1]. Liver fibrosis is initiated by mechanisms that lead to inflammation, which activates a wound-healing response due to the production of the fibrogenic cytokine transforming growth factor-β (TGF-β) [2]. TGF-β appears to be a key cytokine/growth factor mediator in human fibrogenesis because it activates hepatic stellate cells (HSCs) to increase the production and accumulation of extracellular matrix (ECM) [3]. One protein that has shown potential as a downstream mediator of TGF-β signalling in fibroblastic cells is the cysteine-rich peptide connective tissue growth factor (CTGF) [3,4]. CTGF has been suggested to be an important downstream modulator of TGF-β activity and is capable of amplifying the TGF-β profibrogenic action in the liver and in other tissues. TGF-β is not only mitogenic and chemotactic in fibroblasts, but it also stimulates the synthesis of at least two extracellular matrix components: (1) type I collagen and (2) fibronectin [5,6].

Nicotinamide is the amide form of vitamin B3 (niacin) and is obtained via synthesis in the body or as a dietary source and supplement [7]. Nicotinic acid (NA) is the other form of the water-soluble vitamin B3 (Fig. 1). Over the years, NA has been used to treat various diseases such as schizophrenia and type I diabetes [8]. NA has beneficial effects on plasma lipoproteins and has demonstrated clinical benefits in reducing cardiovascular events and atherosclerosis progression. Moreover, NA also exerts anti-inflammatory actions that may be beneficial to patients with inflammatory skin diseases [9]. The side effects of NA have limited its use in general clinical practice. Serious hepatic toxicity has been reported at doses above 3 g/day [7]; these side effects are rare when NA is prescribed at lower doses. Therefore, it is generally considered safe as a food additive and as a component in cosmetics and

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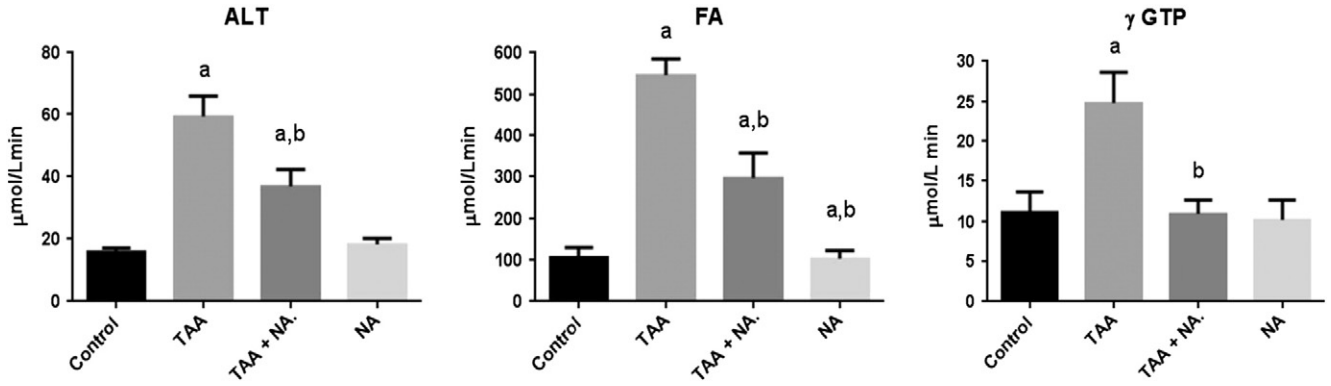

Fig. 1. ALT, AP and γ-GTP activities were determined in serum from control rats following chronic TAA administration for 8 weeks. Control (CONTROL), TAA-treated (TAA), TAA plus nicotinic acid (TAA + NA), and nicotinic acid (NA) rats. Data represent the mean values of the experiments performed in triplicate ± S.E. (n = 6). An "a" denotes a significant difference from the control ($P < 0.05$) and a "b" denotes a significant difference from the TAA-treated rats ($P < 0.05$).

medicines [10]. Evidence from several studies suggests that NA is a potential antifibrotic agent. Treatment with NA was found to attenuate collagen accumulation and lung fibrosis in a bleomycin hamster model [11–13]. In vitro, NA can induce apoptosis in hepatic stellate cells and reduce the expression of collagen I and proinflammatory cytokines [8]. Hepatotoxins, like TAA, initially damage the centrilobular regions of the liver, where there are high levels of cytochrome P450 oxidase that mediate their conversion to toxic intermediates, followed by the production of reactive oxygen species (ROS), lipid peroxidation, and the release of pro-inflammatory cytokines [14]. P450 2E1 enzymes located in the microsomes of liver cells, which convert TAA to a highly reactive toxic intermediates known as thioacetamide sulphur dioxide ($TASO_2$) through oxidation [15,16], inducing hepatotoxicity in experimental animals and different grades of liver damage, including nodular cirrhosis, production of pseudolobules, proliferation of hepatic cells, and necrosis of parenchyma cells [17].

It has been reported that NA inhibits liver fibrosis in rats intoxicated with TAA by suppressing DNA synthesis and enhancing apoptosis of hepatic stellate cells [18]. In CCl_4-induced liver injury, NA has shown protective effects since 1967 [19], it prevents liver necrosis by restoring mitochondrial ability for Ca^{2+} uptake [20], also NA prevents CCl_4-induced liver toxicity in sheep [21]. The molecular mechanisms by which NA exerts these effects have not been identified. In this study, we aimed to evaluate the potential antifibrotic properties of NA on liver injury induced by repeated thioacetamide (TAA) administration to rats and to explore if the action mechanism is associated with attenuation of oxidative stress and downregulation of TGF-β and CTGF.

2. Materials and methods

2.1. Chemicals

Nicotinic acid, sodium thiosulfate, anthrone, thiobarbituric acid, chloramine-T, p-dimethylaminobenzaldehyde, γ-glutamyl-p-nitroanilide, L-γ-glutamyl-p-nitroaniline, p-nitrophenyl phosphate, bovine serum albumin and thioacetamide were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Sodium hydroxide, glacial acetic acid, hydrochloric acid, sulphuric acid, ethanol, methanol, toluene, and formaldehyde were obtained from J.T. Baker (Xalostoc, Mexico City, Mexico). All of the reagents were of analytical quality.

2.2. Study design

Wistar male rats initially weighing 100–110 g and fed a Purina chow rat diet ad libitum were used in the study. Four or five animals were housed per polycarbonate cage under controlled conditions (22 ± 2 °C, 50–60% relative humidity and 12 h light–dark cycles). Cirrhosis was induced by i.p. administration of TAA (Sigma Chemical Company St. Louis, MO, USA) (200 mg/kg of body weight) dissolved in saline three times a week for 8 weeks. In order to determine the capacity of NA (Sigma Chemical Company St. Louis, MO, USA) to prevent liver fibrosis, four groups were formed and treated for 8 weeks. Group 1 (n = 8) consisted of the control animals receiving the vehicle only (saline, i.p.); group 2 (n = 15) was administered TAA; group 3 (n = 15) received TAA plus NA 50 mg/kg dissolved in a saline solution, p.o., daily; and group 4 (n = 8) received NA only. All of the animals were killed

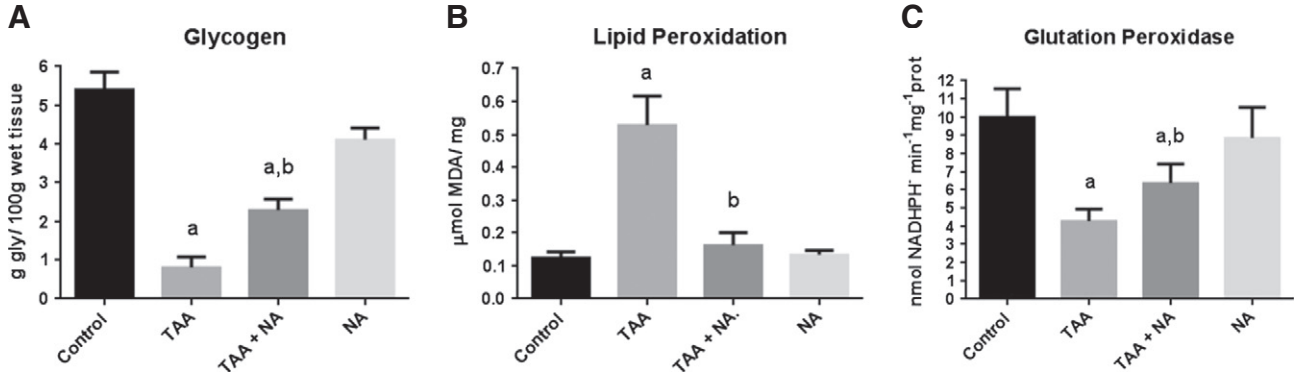

Fig. 2. (A) Liver glycogen content, (B) glutathione peroxidase and (C) lipid peroxidation determined in the livers from control rats following chronic TAA administration for 8 weeks. Control (CONTROL), TAA-treated (TAA), TAA plus nicotinic acid (TAA + NA), and nicotinic acid (NA) rats. An "a" denotes a significant difference from the control ($P < 0.05$) and a "b" denotes a significant difference from the TAA-treated rats ($P < 0.05$).

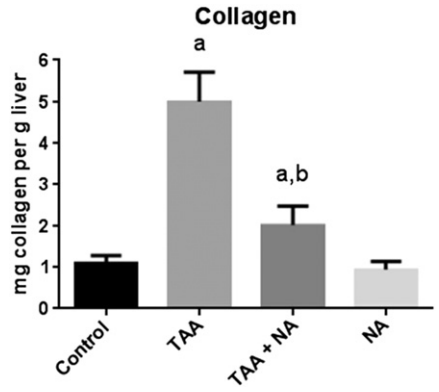

Fig. 3. Liver collagen as measured by hepatic hydroxyproline content in the livers from rats following chronic TAA administration for 8 weeks. Control (CONTROL), TAA-treated (TAA), TAA plus nicotinic acid (TAA + NA), and nicotinic acid (NA) rats. Data represent the mean values of experiments performed in triplicate ± S.E. (n = 6). An "a" denotes a significant difference from the control ($P < 0.05$) and a "b" denotes a significant difference from the TAA-treated rats ($P < 0.05$).

under light anaesthesia. Blood was collected by cardiac puncture and the liver was rapidly removed. All of the samples were kept on ice until analysis. Animals received humane care according to the institution's guidelines and the Mexican Official Norm (NOM-062-ZOO-1999) regarding technical specifications for the production, care, and use of laboratory animals.

2.3. Serum enzyme activities

Samples were centrifuged at 1200 *g* for 15 min to obtain the serum. Then, the level of the level of liver damage was determined by measuring the activities of alanine aminotransferase (ALT) [22], alkaline phosphatase (AP) [23] and γ-glutamyl transpeptidase (γ-GTP) [24].

2.4. Assessment of lipid peroxidation

The extent of lipid peroxidation was determined in liver homogenates by measuring the formation of malondialdehyde (MDA) using the thiobarbituric acid method [25]. Protein concentration was determined according to a Bradford assay using bovine serum albumin as the standard [26].

2.5. Glutathione peroxidase (GPx) activity in the liver

GPx 1, the most abundant version activity, was assayed with cumene hydroperoxide (Sigma Chemical Company St. Louis, MO, USA) as the substrate according to Lawrence and Burk [27]. An aliquot of 1.5 mL of the 10% liver homogenate with 75 mM potassium phosphate buffer (pH 7.0) was filtered through muslin cloth and centrifuged at 900 *g* for 5 min at 4 °C. The reaction mixture contained 200 μL of the homogenate supernatant, 2.0 mL of 75 mM potassium phosphate buffer (pH 7.0), 50 μL of 60 mM glutathione, 0.1 mL of 30 U mL^{-1} glutathione reductase, 0.1 mL of 15 mM EDTA, 0.1 mL of 3 mM β-nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma Chemical Company St. Louis, MO, USA), and 0.3 mL of water. The reaction began with the addition of 0.1 mL 45 mM cumene hydroperoxide. Oxidation of NADPH was recorded at 340 nm for 4 min, and the enzyme activity was calculated as nmol of NADPH oxidised min^{-1} mg^{-1} of protein, using a molar extinction coefficient of 6.22×10^6 M^{-1} cm^{-1}.

2.6. Collagen determination

Fresh liver samples (100 mg) were placed in ampoules with 2 mL of 6 N HCl. The samples were sealed and hydrolysed at 100 °C for 48 h. Next, the samples were evaporated at 50 °C for 24 h and resuspended in 3 mL of sodium acetate–citric buffer, pH 6.0. Half a gram of activated charcoal was added, the mixture was stirred vigorously for 1 min, and then it was centrifuged at 5000 *g* for 15 min. One millilitre of chloramine T (Sigma Chemical Company St. Louis, MO, USA) was added to 1 mL of the supernatant. The mixture was maintained at room temperature for 20 min and the reaction was stopped by the addition of 0.5 mL of 2 M sodium thiosulfate with 1 mL of 1 N sodium hydroxide. The aqueous layer was then transferred to test tubes. The hydroxyproline oxidation product was converted to a pyrrole by boiling the samples. The pyrrole-containing samples were incubated with Ehrlich's reagent for 30 min, and absorbance was read at 560 nm. Recovery of known amounts of standards was carried out using the same procedure on similar liver samples to provide a basis for quantification [28].

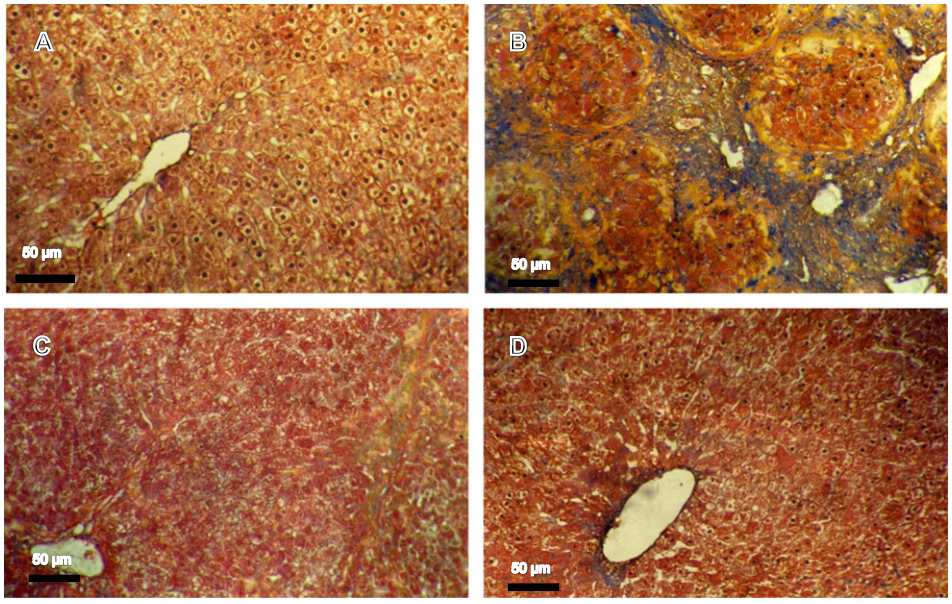

Fig. 4. Representative Mallory's trichromic stain of liver sections obtained from rats treated with vehicle (panel A), TAA (panel B), and TAA plus NA (panel C). Collagen can be visualised as grey in colour. The mean quantitative amount of collagen was determined by measuring amount of liver hydroxyproline.

2.7. Glycogen determination

Small pieces of liver (0.5 g) were separated from the whole for glycogen determination using the anthrone reagent according to Seifter et al. [29].

2.8. Histology

Liver samples were taken from all of the animals and fixed with 10% formaldehyde in phosphate-buffered saline for 24 h. Tissue pieces were washed with tap water, dehydrated in alcohol, and embedded in paraffin. Five-micrometre sections were mounted on glass slides, covered with silane, and stained with haematoxylin and eosin (H&E) and Masson's trichromic stain.

2.9. Western blot assays

To carry out western blot assays, the TriPure reagent (Roche Diagnostics, Indianapolis, IN, USA) was used to isolate total protein from the sample liver tissues. Fresh tissue was homogenised in 1 mL of the TriPure reagent. Next, 0.2 mL of chloroform was added to the homogenates, and the lower phase was treated with isopropanol to precipitate the total protein. Samples were centrifuged at 12 000 rpm for 10 min at 4 °C and then washed three times with 0.3 M guanidine hydrochloride in 95% ethanol. After a final wash was performed with 100% ethanol, the samples were centrifuged as previously described and the pellet was resuspended in 1% SDS. Volumes equivalent to 50 μg of proteins (determined using the Lowry method) were transferred onto a 12% polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred onto an Immuno-Blot™ PVDF membrane (BIO-RAD, Hercules, CA, USA). The blots were blocked with 7% skim milk with 0.05% Tween 20 for 60 min at room temperature and then independently incubated overnight at 4 °C with specific antibodies against TGF-β (MAB1032, from Millipore Corp. Billerica, MA, USA), IL-10 and CTGF (SC-57245 and SC-14939, respectively, Santa Cruz Biotechnology, INC.), and α-SMA (A5691 Sigma Aldrich). The following day, the membranes were washed and then exposed to a secondary peroxidase-labelled antibody (Zymed, San Francisco, CA, USA) in the blocking solution for 1 h at room temperature. Blots were washed and developed using the western lightning™ Plus-ECL Enhanced Chemiluminescence detection system (NEN Life Sciences Products, Elmer LAS Inc., Boston, MA, USA). The blots were stripped and incubated with a monoclonal antibody directed against β-actin [30], which was used as a control to normalise cytokine protein expression levels. To strip the membranes, the blots were first washed four times with phosphate–saline buffer pH 7.4 (0.015 M, 0.9% NaCl). Then, the blots were immersed in the stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate and 62.5 mM Tris–HCl, pH 6.7) for 30 min at 60 °C with gentle shaking. Then, the membranes were then washed five times with 0.05% Tween-20 in phosphate–saline buffer. Images were digitalised using the BioDoc-It System (UVP, Upland, CA, USA) and then analysed densitometrically using the Lab Works 4.0 Image Acquisition and Analysis software.

2.10. Zymography

Proteolytic activity was assayed using gelatine-substrate gels as previously described [31]. Briefly, the same volume of non-heated samples was mixed with sample buffer (2.5% SDS, 1% sucrose and 4 μg mL^{-1} phenol red) without reducing agent and loaded into 8% acrylamide gels copolymerised with 1 mg mL^{-1} gelatine. Following electrophoresis at 72 V for 2 h, the gels were rinsed twice in 2.5% Triton X-100 to remove the SDS and then incubated in assay buffer (50 mM Tris–HCl, pH 7.4 and 5 mM $CaCl_2$) at 37 °C for 48 h. The gels were then fixed with 0.25% Coomassie Brilliant Blue G-250 (Sigma Chemical Company St. Louis, MO, USA) in 10% acetic acid and stained with 30% methanol. Proteolytic activity was detected as clear bands against the background stain of the undigested substrate in the gel. The positive control for MMP-9 and MMP-2 secretion was obtained according to the modified method of

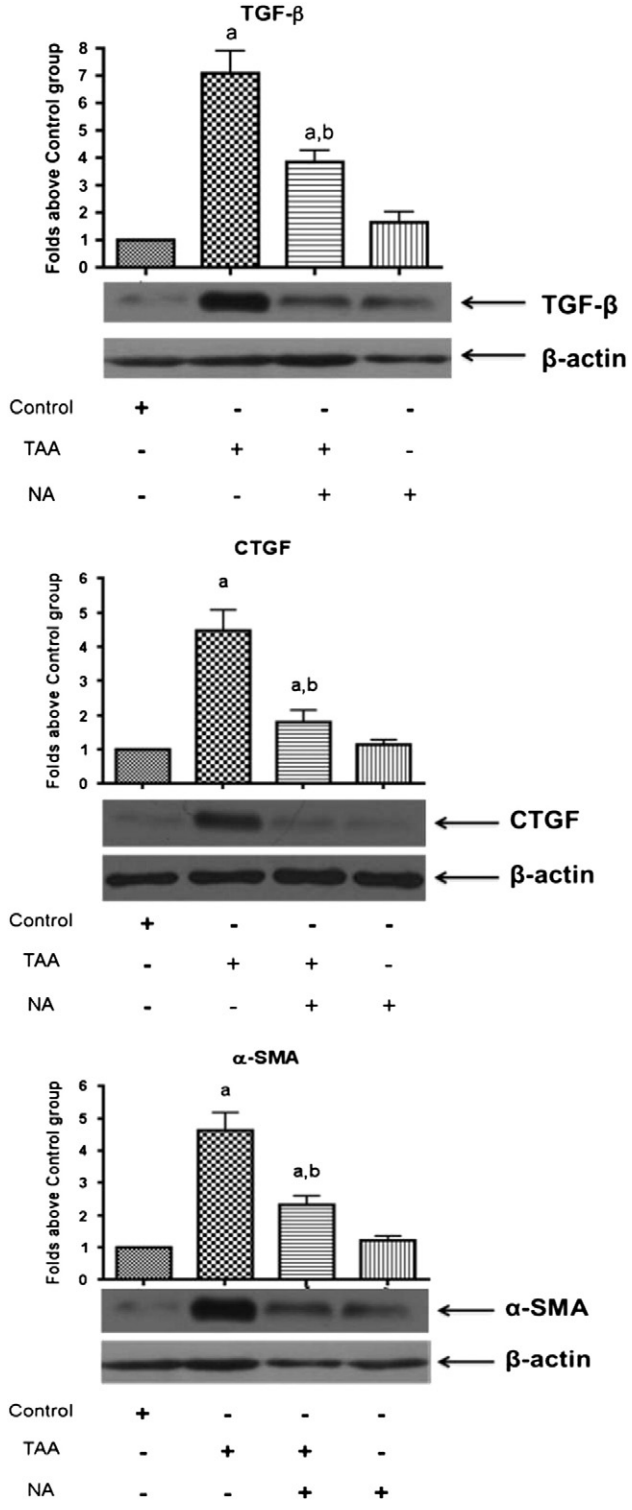

Fig. 5. Expression of transforming growth factor (TGF-β), connective tissue factor (CTGF) and alpha-SMA (α-SMA) were determined by western blot analysis from hepatic extracts. A representative western blot is shown. Control (CONTROL), TAA-treated (TAA), TAA plus nicotinic acid (TAA + NA) and nicotinic acid (TAA) rats. The figure also shows the calibrator, β-actin. Values are expressed as the fold increase of relative IOD normalised to the control group values (control = 1). Each bar represents the mean values from 3 rats ± S.E. in triplicate. An "a" denotes a significant difference from the control ($p < 0.05$) and a "b" denotes a significant difference from the TAA group ($p < 0.05$).

Etique et al. [32] using the non-tumorigenic breast epithelial cell line MCF10A. MCF10A cells were cultured in DMEM/F12 (3:1) medium supplemented with 5% FBS, 10 μg mL^{-1} insulin, 0.5 μg mL^{-1} hydrocortisone, 20 ng mL^{-1} recombinant EGF and antibiotics, in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Briefly, the confluent MCF10A cultures were grown in DMEM without FBS, insulin, hydrocortisone and EGF for 12 h before treatment, then they were washed twice with DMEM without serum, equilibrated in the same medium at 37 °C for at least 30 min, and then treated with 400 mg dl^{-1} of ethanol for 25 h. The supernatant was collected and concentrated.

3. Results

3.1. NA co-administration prevented the induction of liver fibrosis

The enzyme activities of serum ALT, γ-GTP and AP serve as useful tools to evaluate cell death or the increase in plasma membrane permeability. An increase in ALT activity was observed in the TAA-treated group (Fig. 1); similarly, an elevation in the γ-GTP and AP activities were also found in the same group. These increases are associated with hepatocyte necrosis and cholestatic damage [1]. In the rats, NA significantly prevented the ALT, γ-GTP and AP activities caused by TAA administration.

Glycogen is the principal storage form of glucose in animal and human cells; therefore, it is the main source of energy. Chronic intoxication by TAA resulted in a depletion of hepatic glycogen (Fig. 2a). However, NA partially prevented this decrease.

Oxidative stress is an important damage mechanism in this experimental model; the measurement of MDA, a product of lipid peroxidation, is useful to assess this process. Treatment with TAA led to a significant increase in MDA levels. NA completely prevented the increase in MDA (Fig. 2b). The oxidative stress level was also determined by quantifying the GPx activity. Consistent with lipid peroxidation, GPx also indicated oxidative stress by decreasing significantly in the TAA group (Fig. 2c). While NA prevented lipid peroxidation induced by TAA, it also increased GPx activity.

The collagen content is the most important parameter used in evaluating liver fibrosis because it is the main component of the connective tissue accumulated in this disease. We observed a significant increase in the collagen content in the TAA-treated group during the 8-week experiment; in contrast, NA prevented collagen deposition in the liver (Fig. 3). In order to corroborate the biochemical findings of collagen content, we decided to stain the liver slices with Masson's trichromic stain. The normal parenchyma liver is shown in Fig. 4, panel A. The TAA-treated group displayed an altered parenchyma architecture and severe necrosis due to the accumulation of collagen fibres (Fig. 4, panel B). NA maintained the normal architecture because the liver slices in the group treated with TAA and NA showed fewer necrotic areas and less collagen fibres compared with the TAA-treated group (Fig, 4, panel C). This histological approach is in agreement with the ALT enzymatic activity (Fig. 1) and the liver collagen content (Fig. 4).

TGF-β is an important cytokine in the progression toward liver fibrosis. We evaluated the effect of NA on TGF-β expression. Chronic intoxication with TAA produced a significant increase in TGF-β levels, but this effect was prevented by the simultaneous treatment with NA (Fig. 5). In contrast, it has been suggested that CTGF is an important downstream modulator of TGF-β, capable of amplifying the profibrogenic action of TGF-β in the liver and in other tissues [33]. NA has important effects on the expression of TGF-β. TAA administration increased the expression of CTGF, whereas NA prevented this effect (Fig. 5).

Alpha smooth muscle actin (α-SMA) expression by hepatic stellate cells reflects their activation to myofibroblast-like cells and has been directly related to experimental liver fibrogenesis and indirectly related to human fibrosis in chronic liver disease [34]. We found that α-SMA expression was increased in the TAA-treated group, but when NA was administered, the expression of this protein decreased significantly (Fig. 5).

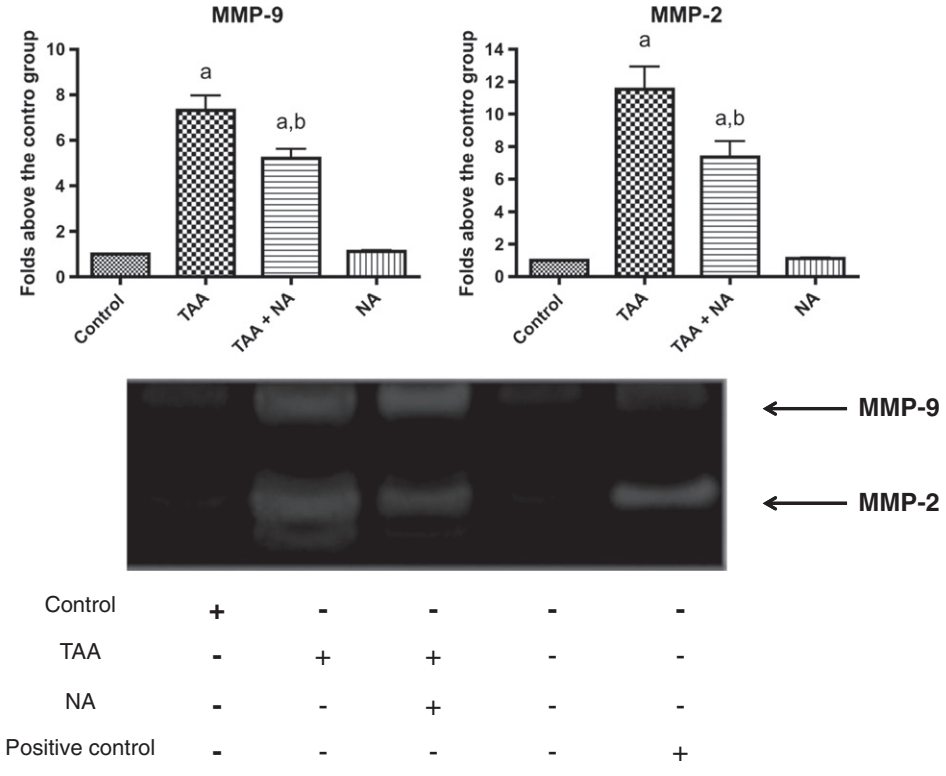

Fig. 6. MMP-9 and MMP-2 activities were analysed by zymography using gelatine-substrate gels. Liver samples from control rats, TAA-treated rats (TAA), TAA plus NA-treated rats (TAA + NA), and rats administered with NA alone (NA) were analysed. The positive control for zymography was obtained from confluent cultures of MCF10A cells treated with 400 mg dl^{-1} of ethanol for 25 h. Each bar represents the mean values from 3 rats ± S.E. in triplicate. An "a" denotes a significant difference from the control ($p < 0.05$) and a "b" denotes a significant difference from the TAA group ($p < 0.05$).

Metalloproteinases (MMPs) are enzymes capable of degrading the components of the ECM. Because MMP-2 and -9 are expressed in TAA-induced liver fibrosis, we evaluated their proteolytic activity using a gelatine zymography assay. Fig. 6 shows that TAA increased the secretion of MMP-2 and MPP-9, but NA decreased it.

4. Discussion

Nicotinamide, also known as nicotinic acid amide or the amide of nicotinic acid (vitamin B3/niacin), exerts anti-inflammatory actions that may be beneficial to patients with inflammatory skin diseases [9]. NA has also been used to treat various diseases such as pellagra, psoriasis, schizophrenia, and type I diabetes [8]. Previous studies have suggested that NA is a potential antifibrotic agent for lung fibrosis [11,12]. This study was performed to evaluate the antifibrotic properties of NA in a liver damage model induced by repeated TAA administrations in male Wistar rats. We found that treatment with NA at a concentration of 50 mg/kg prevented experimental fibrosis. This effect may be associated with the ability of NA to down regulate the fibrogenic cytokine TGF-β and its antioxidant properties; however, other actions cannot be ruled out.

The main causes of hepatic fibrosis/cirrhosis include alcohol abuse, hepatitis B or C infections, cholestasis, and non-alcoholic steatohepatitis. Cirrhosis is the end-stage consequence of hepatic parenchyma fibrosis and is characterised by nodule formation and altered hepatic function. Liver fibrosis results from chronic damage. Over time, the ability of liver to regenerate fails and the hepatocytes are replaced by ECM [1].

A major change in both the quantity and composition of the ECM is a characteristic of most types of chronic liver diseases. TGF-β1 is a growth factor that effects extracellular matrix homeostasis via the following mechanisms: (1) increasing the synthesis and secretion of matrix proteins; (2) increasing the transcription, translation, and processing of receptors for the extracellular matrix–cell adhesion proteins; (3) decreasing the synthesis of matrix-degrading proteases; and (4) increasing the synthesis of specific inhibitors of matrix-degrading proteases [35].

NA partially, but significantly, prevented the increase in TGF-β induced by chronic exposure to TAA, and as a consequence prevented liver fibrosis. TGF-β induced CTGF expression in hepatocytes and fibrogenesis [6]. Via intracellular activation of the kinase domain of the Alk5-receptor, it triggers Smad2, but not Smad3, phosphorylation leading to nuclear translocation with the common mediator Smad4 and subsequent CTGF expression. CTGF has been suggested to be an important downstream mediator of TGF-β, capable of amplifying

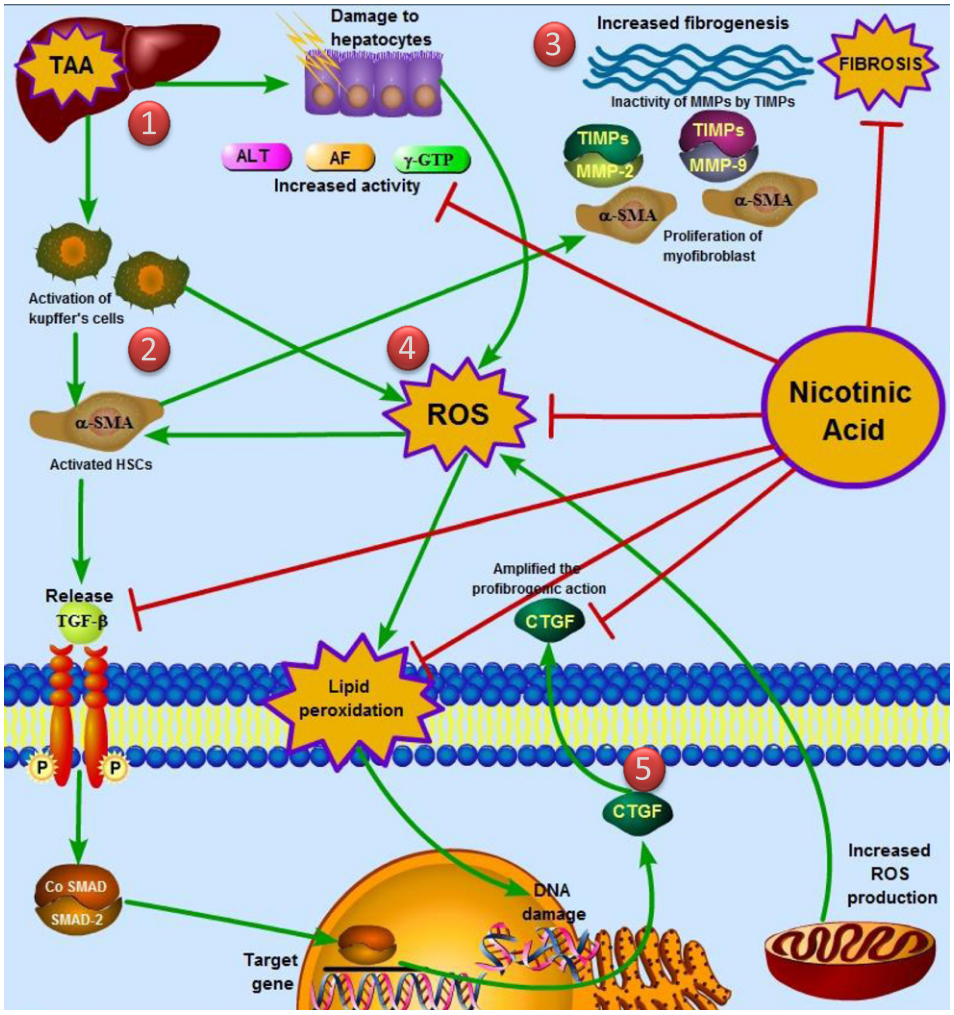

Fig. 7. Schematic diagram summarising the pathogenetic sequence of fibrogenic activation of hepatic stellate cells (HSCs) that leads to fibrosis and cirrhosis. (1) In response to liver injury, (2) the Kupffer cells are activated and lead to dramatic phenotypic changes in HSCs to potent fibrogenic cell-type myofibroblasts, which are capable of autocrine stimulation and paracrine activation of resting HSCs. (3) In activated HSCs, the expression of TIMP-1 is upregulated leading to the inhibition of MMP activity and subsequent accumulation of EMC in the extracellular space. (4) Reactive oxygen species (ROS) mediate many of the fibrogenic effects of transforming grown factor (TGF-β). (5) Connective tissue factor (CTGF) has been suggested as an important downstream mediator of TGF-β, amplifying the profibrogenic action of this cytokine in the liver. The antioxidant properties of NA prevent oxidative stress, and reduce the expression of TGF-β. This decrease in TGF-β results in a lower expression of CTGF, which results in a reduction of HSC activation into myofibroblasts and, as consequence, less ECM deposition.

the profibrogenic action of this cytokine in the liver and in other tissues [6,33]. This crucial role of CTGF in fibrogenesis is shown by the significant up-regulation of ECM in fibrotic livers. Our results show that NA administration decreases CTGF levels by blocking the elevation of the profibrogenic cytokine TGF-β. This decrease results in a lower CTGF expression level and less ECM deposition. In fact, NA decreased the hydroxyproline content; this was demonstrated both biochemically and histologically. Interestingly, another study reported that taurine and niacin inhibit the expression of procollagen types I and III at the level of gene transcription in a bleomycin (BL) hamster model of lung fibrosis [13].

HSCs are located in the space of Disse, between the sinusoidal endothelial cells and hepatic epithelial cells, and account for 5%–8% of the cells in the liver. In a healthy liver, stellate cells are quiescent and contain numerous vitamin A lipid droplets, constituting the largest reservoir of vitamin A in the body [36]. Moreover, in response to liver injury, HSCs undergo an 'activation' process characterised by proliferation and myofibroblastic transformation mainly induced by TGF-β and oxidative stress. HSCs play an unequivocal yet main role in the excessive production and accumulation of extracellular matrix in liver fibrosis. The substantial change in ECM composition is due to the deposition of collagens, mainly fibril-forming types I, III, and IV, which increase in fibrotic ECM up to tenfold. In response to their activation, HSCs show an increase in cytoplasmic α-SMA [36,37]. α-SMA is an actin isoform and a specific marker for smooth muscle cell differentiation. Therefore, α-SMA expression has been used to identify activated HSCs that show a myofibroblastic phenotype [38]. Our results show an increase in the expression of α-SMA in the TAA-treated group. In contrast, NA was able to significantly reduce the levels of this protein, suggesting a decrease in HSC activation.

The matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in matrix remodelling in physiological and pathological processes. MMP activities are regulated by TIMPs, which bind in substrate- and tissue-specific manners to MMPs and block their proteolytic activity [39]. MMPs are synthesised and secreted, in most cases, as proenzymes that are then activated by proteinases such as plasmin. For example, pro-MMP-2 is activated by MT1-MMP and/or plasmin, and pro-MMP-9 can be activated by MMP-3 and plasmin. Because matrix turnover is tightly regulated, activation of pro-MMPs to active MMPs is strictly controlled by complex formation with TIMPs. MMPs are expressed in activated HSCs, or hepatocytes, whereas TIMP-1 and TIMP-2 are expressed only in normal HSCs [40]. In the fibrotic liver, an imbalance occurs between excess synthesis and/or a decrease in the removal of ECM with consequent scarring. In activated HSCs, the expression of TIMP-1 is upregulated leading to the inhibition of MMP activity and subsequent accumulation of matrix proteins in the extracellular space [41,42]. It was reported that TAA could cause an increase in the expression of MMPs and TIMPs [43]. Zymography assays show that the group receiving NA showed a decrease in the secretion of MMP-2 and MMP-9; this effect was significant compared with the TAA-treated group. Thus, the reduced MMP activity may be linked to a decrease in HSC activation.

Oxidative stress, resulting from an imbalance between ROS generation and the antioxidant defence capacity of the cell, affects major cellular components including lipids, proteins, and DNA [44,45]. Thannickal et al. [46] reported that TGF-β1 stimulated ROS production through activation of the cell membrane-associated oxidase, which led to an increased release of H_2O_2 to the extracellular space in human lung fibroblast and bovine pulmonary artery endothelial cells. Other studies reported that TGF-β1 increased ROS production in the mitochondria of rat hepatocytes [47]. Using different inhibitors, Albright et al. [48] demonstrated that mitochondria and microsomes were the major source of ROS in TGF-β1-treated rat hepatocytes. Cells have defence mechanisms against high levels of free radicals and thus prevent oxidative stress, for example, by using endogenous antioxidants such as catalase and GPx. The TAA-treated group showed decreased GPx activity. However, by introducing NA, we observed an increase in GPx activity. Additionally, NA also inhibits lipid peroxidation, thus showing a reduction in membrane oxidative stress (i.e. lipid peroxidation). These data suggest that NA, by preventing oxidative stress, could partially prevent TGF-β1 expression.

In this work, cirrhosis was induced experimentally by repeated TAA administration. This model induces fibrosis/cirrhosis associated with hepatocyte death and the activation of Kupffer cells (KC) and HSCs. In addition, cirrhosis is characterised by the elevation of hepatic enzymes such as ALT, AP and γ-GTP. Interestingly, NA administration significantly prevents hepatocytes death, as seen by the reduced ALT activity. Moreover, NA prevents cholestatic damage as was demonstrated by the decrease in the levels of AP and γ-GTP activities.

5. Conclusions

Our results show some hepatoprotective effects of NA in a rat cirrhosis model. It seems likely that the antioxidant properties of NA are linked to the prevention of oxidative stress, this effect may be associated which reduction of the expression of TGF-β and CTGF. It can also be suggested that this effect may decrease HSC activation into myofibroblasts and as consequence in less deposition of ECM components (Fig. 7). In contrast, NA has beneficial effects on plasma lipoproteins and it has been shown to reduce cardiovascular events and atherosclerosis progression. NA now appears to be a promising therapeutic alternative to treat hepatic diseases.

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