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Protective effect of Thymic Humoral Factor on porcine serum-induced hepatic fibrosis and liver damage in Wistar rats

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ABSTRACT

Introduction. Immunomodulatory drugs have been reported to have anti-inflammatory and anti-fibrotic properties. Thymic Humoral Factor (THF), a peptide produced in the thymus, causes a potent immunomodulatory effect on different components of the immune system. **Objective.** To evaluate the effect of THF on different stages of liver damage and fibrosis induced in rats through the administration of porcine serum (PS). **Material and methods.** PS-induced liver fibrosis models serve as a primarily immunological mechanism in the development of liver damage and fibrosis. **Results.** The intraperitoneal administration of THF in rats with PS-induced liver damage produced a reduction of ALT and AST after 60 days. Histopathological changes in liver sections showed an improved histological appearance and lower % of fibrosis after 60 days in liver damaged rats that received THF treatment. Serum IL-6 levels were visibly reduced by THF administration after 60 days and in comparison with rats that did not receive the treatment. This was due to an increment in serum IL-10 levels caused by the administration of THF, which appears to reduce the inflammatory process by decreasing immune response. **Conclusion.** THF had beneficial effects in combating liver damage and fibrosis processes in an autoimmune model of PS-induced liver fibrosis in rats.

Key words. Hepatitis. IL-6, IL-10. Autoimmune. THF. Fibrosis.

INTRODUCTION

Liver fibrosis is a worldwide major medical problem and is associated with significant morbidity and mortality.[1] Hepatic fibrosis is the wound-healing response of a liver subjected to continuous liver injury and is associated with chronic inflammation and a variety of chemical factors commonly found in most chronic liver diseases.[2,3]

There are several immunological mechanisms involved in liver fibrosis, including inflammatory cell infiltration, activation of hepatic stellate cells (HSC) and production of cytokines such as TNF-α, TGF-β and IL-6 at early stage,[4,5] the latter being a key element in local regulation of the fibrogenic response.[6,7] At an advanced state, cytokines such as TNF-α and TGF-β, growth factors like PDGF or chemokines such as MCP-1 are produced in high quantities and are involved in the progression of fibrosis and liver failure.[8-10]

Different kinds of liver fibrosis animal models have been developed, but most of them are post-necrotic hepatic fibrosis models. The porcine serum (PS) induced hepatic fibrosis model is characterized by minor hepatocyte damage but intense immune response given the chronic administration of the heterogeneous serum. This immunological response is regulated by MHC class II molecules and inflammatory cells, which activate HSC-producing liver fibrosis.[3,11,7] These immunologic mechanisms make this an appropriate model for the evaluation of immunomodulatory drugs, since immune response is the key factor in the development of liver fibrosis.

It has been reported that some immunomodulatory agents are able to modify liver injury from different

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etiology. It has been shown that AM3, a biological response modifier, reverses the concurrent inflammatory system activation in peripheral blood and liver during experimental cirrhosis. This leads to a reduction of hepatic fibrosis, portal hypertension and peripheral vasodilatation.[12] Other agents, like PI3Kγ inhibitor, a member of the class 1 PI3 kinases family, interact with phospho-Akt and PIP3 at cell membrane level and prevent phosphorylation, which leads to downstream cell signaling pathways associated with inflammation and immune functions.[13]

Thymic humoral factor (THF), a peptide produced in the thymus, has a potent immunomodulatory effect on different components of the immune system. It has been found that THF restores T cell growth factor[14] and can modify IL-2 levels on human umbilical cord blood lymphocytes.[15] THF has been used, along with α-interferon or mitogen phytohaemagglutinin in patients with hepatitis B[16,17] and as monotherapy in hepatitis D[18] without adverse reactions; THF also increases anti-viral activity against cytomegalovirus in animal models.[19] Because of all this, we hypothesized THF had a potential therapeutic role in controlling liver damage and fibrosis.

OBJECTIVE

To evaluate THF effect during different stages in an immunological model of PS-induced rat liver injury and fibrosis.

MATERIALS AND METHODS

Reagents and animals

THF was synthesized by New England Peptide, Gardner, MA, USA. PS was purchased from Sigma-Aldrich, USA. Commercial kits were used for determining alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ-GT); all kits were obtained from ELITech Group (France). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure IL-6 and IL-10. All kits were obtained from BD Biosciences (San Diego, California).

Seventy-two male Wistar rats (110-130 g) were purchased from Harlan Mexico, S.A. de C.V. Animals were randomly housed in groups of six per cage and kept under controlled conditions (24 ºC and 58% humidity) with alternating 12 h dark/light cycles. Animals were fed with a Rodent Laboratory Chow diet and had free access to food and water. Before the study, animals were subjected to 1-week acclimation. All procedures were approved by the Institutional Animal Care and Use Committee of the Veterinary Medical School at the National Autonomous University of Mexico. The experiments were conducted in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals.[20]

Animal model

Liver injury and fibrosis were induced in male Wistar rats following Paronetto's method.[21] Briefly, 3.5 mL/kg of sterile PS were administered twice per week via intraperitoneal injection (ip) for eight weeks. This model is characterized by a substantial immune response in the liver, causing little hepatocyte damage and liver fibrosis after 8 weeks of PS administration.

Pharmacological treatments and sample collection

Animals were randomly distributed into the following groups (Figure 1): groups 1, 2, 3 and 4 were evaluated at 30 days:

- ***1. Control.*** 0.5 mL of saline ip twice per 7 days for 30 days.
- ***2. THF.*** 50 ng/kg ip three times per 7 days for 15 days (last 15 days of 30).
- ***3. Liver damaged.*** 3.5 mL/kg of porcine-serum ip twice per 7 days for 30 days.
- ***4. Liver damaged + THF.*** Doses used in groups 2 and 3.

The groups 5, 6, 7 and 8 were evaluated at 60 days:

- ***5. Control.*** 0.5 mL of saline ip twice per 7 days for 60 days.
- ***6. THF.*** 50 ng/kg ip three times per 7 days for 30 days (last 30 days of 60).
- ***7. Liver damaged.*** 3.5 mL/kg of porcine-serum ip twice per 7 days for 60 days.
- ***8. Liver damaged + THF.*** Doses used in groups 6 and 7.

Finally, groups 9, 10, 11 and 12 were evaluated at 90 days:

- ***9. Control.*** 0.5 mL of saline ip twice per 7 days for 90 days.

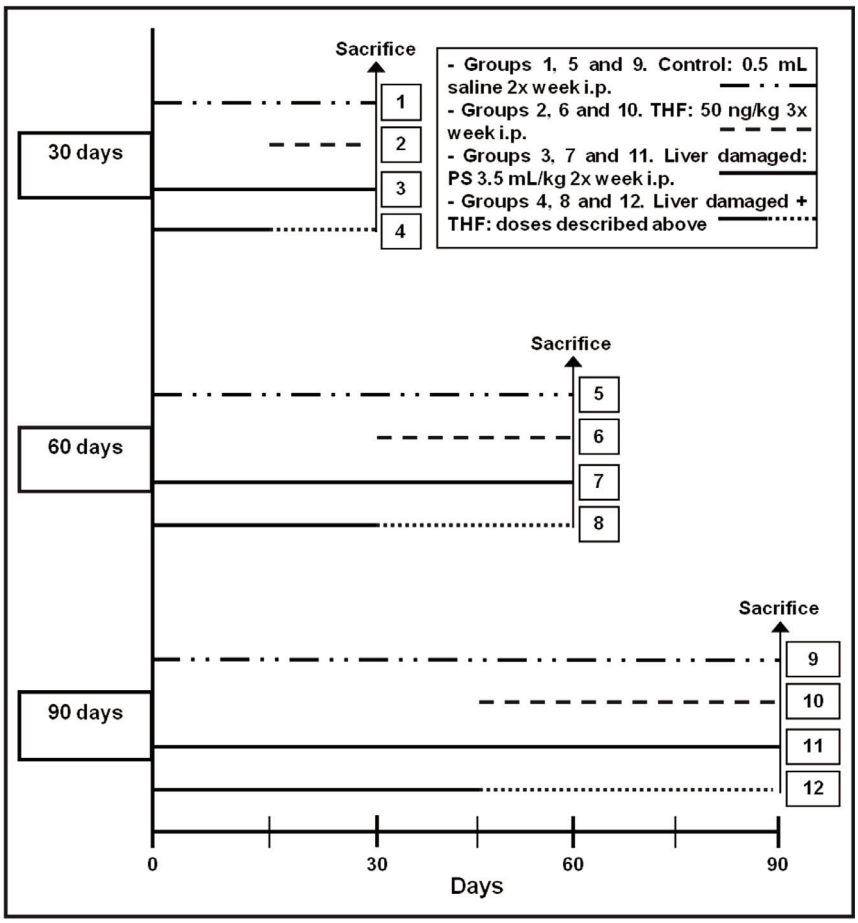

Figure 1. *Protocol for experimental groups: 1. Control: rats received 0.5 mL of the vehicle saline solution via ip injection twice per week. 2. THF: rats were treated with THF 50 ng/kg three times per week via ip injection. 3. LD (liver damaged): animals received PS 3.5 mL/kg twice per week via ip injection; and 4. LD treated with THF: animals were treated with PS (3.5 mL/kg twice per week) and THF (50 ng/kg three times per week).*

- ***10. THF.*** 50 ng/kg ip three times per 7 days for 45 days (last 45 days of 90).
- ***11. Liver damaged.*** 3.5 mL/kg of porcine-serum ip twice per 7 days for 90 days.
- ***12. Liver damaged + THF.*** Doses used in groups 10 and 11.

Rats were weighed and drug doses were adjusted accordingly once a week.

Groups of six rats each were euthanized under ether anesthesia after 30, 60 and 90 days. Blood samples were collected from the inferior vein cava and centrifuged for 10 min at 2,000 rpm. The serum was preserved at -80 °C until analysis. The liver was excised and preserved in different ways for further evaluation.

Biochemical analysis in serum

The serum activity of ALT, AST, ALP and γ-GT was determined using a commercially available kit according to manufacturers' instructions. Briefly, 20 μL (for ALT and AST), 10 μL (ALP) or 5 μL (γ-GT) of serum were mixed with 0.2 mL of assay solution (ALT, AST and ALP) or 0.25 mL of γ-GT, then measured in an absorbance microplate reader (EL x 800 BioTek) with the software Gen5 v.1.07.5, following the supplier's protocol. ALT, AST, ALP and γ-GT activity were expressed as an international unit per liter (U/L).

Quantification of IL-6 and IL-10 in serum

IL-6 and IL-10 levels were evaluated by ELISA kit in accordance with the manufacturer's protocol. Wells were coated with 100 μL of diluted capture antibody per well and incubated overnight at 4 °C. Wells were aspirated and washed 5 times. Then, wells were blocked with 200 μL of assay diluents and incubated for 1 h at room temperature. After intensive washing, 100 μL of sample or standard were added to each well and incubated for 2 h at room temperature; followed by washing and secondary an-

tibody incubation (100 μL) for 1 h at room temperature. After washing, 100 μL of diluted SAv-HRP were added to each well for detection and incubated for 30 min at room temperature. TMB substrate solution (Sigma-Aldrich, USA) was added to each well and incubated for 30 min in the dark. An absorbance microplate reader (ELx800 BioTek) and Gen5 v.1.07.5 software were used to read at 450 nm with λ correction 570 nm.

Histopathological and morphometric analysis

Liver tissue fragments were fixed in 10% formaldehyde solution, dissolved in phosphate-saline buffer (pH 7.4), dehydrated in alcohol and embedded in paraffin. Four-micrometer paraffin sections were stained with hematoxylin and eosin (HE) or Masson trichromic (MT) and subjected to histopathological examination.

Fibrosis was scored under light microscopy following the criteria reported elsewhere.[22]

- Grade 0 is normal.
- Grade I stands for light fibrosis: collagen fibers extend from the portal triad or central vein to the peripheral region.
- Grade II is mild fibrosis: mild collagen fibers with extension are present, but there is no nodules formation.
- Grade III is moderate fibrosis: collagen fibers form incomplete nodules.
- Grade IV is severe fibrosis: well constituted nodules limited by collagen fibers.

Each sample was observed at x 20 magnification and every specimen analyzed contained a centrilobular vein. The degree of fibrosis was expressed as the mean of 10 different fields in each slide. In order to quantify fibrosis, we also performed automated morphometric analysis to determine the percentage of fibrosis. A JVC TK-C1380 camera was used to digitalize and analyze the MT sections, studying 40-80 random selected fields at x 20 magnification. We calculated peri-hepatocellular and luminal fibrosis using the following formula:

$$\% \text{ Fibrosis} = \frac{\text{Fibrosis area}}{(\text{parenchymal area-luminal area}) \times 100}$$

Measured using Leica QWin Standard v2.2 software.

Another morphometric analysis was undertaken to determine the percentage of normal hepatocytes and changes related to regeneration (binucleation), apoptosis (condensed nuclei with well preserved cytoplasm) and necrosis (nuclei fragmentation with cytoplasm dissolution). Images from each HE stain were digitalized (JVC TK-C1380) at x 100 magnification. 200 cells were randomly selected using the Leica QWin Standard v2.2 program and the formula:

$$\% \text{ hepatocytes} = \frac{\text{Number of cells} \times 100}{200}$$

Statistical analysis

Data are reported as means ± standard deviation of three independent experiments conducted in quadruplicate. Statistical analysis was performed using a non parametric ANOVA. Group differences were analyzed using Tukey's test. Significant differences were established at $p < 0.05$. The results were analyzed by SPSS 15.0 software.

RESULTS

Evaluation of biochemical parameters

An analysis of serum ALT, AST, γ-GT and ALP activity was carried out to evaluate the amount of liver injury. After 30 days, the group of liver damaged showed significantly higher levels of ALT, AST and γ-GT than those of the control ($p < 0.05$) (Table 1). No significant differences were observed in ALT, AST and γ-GT levels between the liver damaged group and the liver damaged + THF group after 30 days. No significant differences in ALP levels were detected in any group after 30 days.

After 60 days, ALT, AST, γ-GT and ALP levels were significantly higher in the liver damaged group than in the control ($p < 0.05$) (Table 1). THF administration significantly decreased the serum levels of ALT and AST in rats with PS-induced liver damage ($p < 0.05$) (Table 1). No significant γ-GT and ALP level changes were observed in the groups with liver damage and THF-treated liver damage.

After 90 days, there was a slight increase in ALT and AST levels in the liver damaged group in comparison with the control group. However, γ-GT and ALP levels had significantly increased in the first group when compared with the control ($p < 0.05$) (Table 1). No significant difference was found between the liver damaged group and the THF-treated liver damaged group.

There was no significant difference in ALT, AST,

Table 1. Effect of THF on serum levels of ALT, AST, γ-GT and ALP after 30, 60 or 90 days of evaluation.

	ALT (U/L)	AST (U/L)	γ-GT (U/L)	ALP (U/L)
• 30 days				
Control	54.2 ± 3.4	81.5 ±6.5	1.8 ± 0.7	127.7 ± 9.6
THF	53.1 ± 3.4	85.7 ± 6.9	1.6 ± 0.6	115.9 ± 10.7
LD	70.4 ± 8.6*,†	103.9 ± 10.7*,†	5.5 ± 0.9*,†	126.9 ± 10.1
LD + THF	64.9 ± 4.4*,†	101.8 ± 5.9*,†	4.8 ± 1.2*,†	124 ± 10.7
• 60 days				
Control	54.6 ± 10	73.3 ± 9.8	3.2 ± 1.4	135.1 ± 20.1
THF	67.8 ± 11.9	78.7 ± 9	4.6 ± 1.9	150.1 ± 20
LD	138.6 ± 54.3*,†	160.6 ± 25.6*,†	14.3 ± 1.1*,†	375.4 ± 68.1*,†
LD + THF	70.3 ± 12.2‡	96.9 ± 17.7‡	12.2 ± 1.8*,†	370.5 ± 14.1*,†
• 90 days				
Control	54.9 ± 10.9	74.6 ± 7.1	2.2 ± 0.8	139 ± 13.4
THF	57.2 ± 8.9	70.2 ± 11.5	2.8 ± 0.9	146.2 ± 21
LD	86.1 ± 10.3*,†	107.7 ± 20.5*,†	16.9 ± 3.2*,†	362.7 ± 65.1*,†
LD + THF	74.9 ± 15	95.5 ± 20.4	16.2 ± 2.4*,†	285.2 ± 73.4*,†

Values are presented as mean ± S.D. from 6 animals in each group. *$p < 0.05$ compared with the control group in its respective days. †$p < 0.05$ compared with the THF group in its respective days. ‡ $p < 0.05$ compared with the LD (liver damaged) group in its respective days.

γ-GT and ALP levels between THF-treated groups and the control group after 30, 60 and 90 days.

Effect of THF on serum IL-6 and IL-10 levels

Pro-inflammatory cytokines have been shown to be critical mediators in hepatocellular injury. We measured serum IL-6 levels given that it is one of the major pro-inflammatory cytokines, and also determined IL-10 levels considering it is an anti-inflammatory cytokine.

The liver damaged group showed an increase in both IL-6 and IL-10 serum levels ($p < 0.05$) (Figure 2A) when compared with the control after 30 days. THF treatment in animals with PS-induced liver damage showed a significant reduction in the serum levels of both cytokines when compared with the liver damaged group ($p < 0.05$) (Figure 2A); IL-6 levels, however, did not reach those of the control group. By itself, THF resulted in an increase in the serum levels of both cytokines when compared with the control ($p < 0.05$) (Figure 2A).

After 60 days, the liver damaged group showed very high levels of IL-6 and IL-10 when compared with the control ($p < 0.05$, Figure 2B). THF induced a significant reduction of IL-6 and IL-10 in animals with liver damage when compared with the PS-induced liver damaged group ($p < 0.05$) (Figure 2B). Rats treated exclusively with THF showed no significant change in IL-6 and IL-10 levels when compared with the control.

After 90 days, there was a significant increase in IL-10 levels in the liver damaged group when compared with control group ($p < 0.05$) (Figure 2C). THF treatment of rats with liver damage produced a significant reduction in IL-10 when compared with the liver damaged group ($p < 0.05$) (Figure 2C). No differences in the IL-6 and IL-10 serum levels of the THF and control groups were observed.

Histopathological findings

Figures 3 and 4 show representative histological changes in the livers of the different groups. By day 30, the animals that received PS showed mild mononuclear inflammatory infiltrate in the portal areas with scarce hepatocyte damage and lack of evident fibrosis (Figure 3C). Sections from animals with PS-induced liver damage and treated with THF did not show significant differences when compared to the liver damaged group (Figure 3D). By day 60, animals with PS-induced liver damage showed intense chronic inflammatory infiltrate in portal areas with proliferation of biliary ducts; scattered necrotic and regenerative hepatocytes were also seen (Figure 3E). MT stains showed variable size nodules limited by thick fibrotic trabecula (Figure 4E). Rats treated with liver damaged and treated with THF showed a striking reduction of inflammation, hepatocytes damage and fibrosis, but this varied

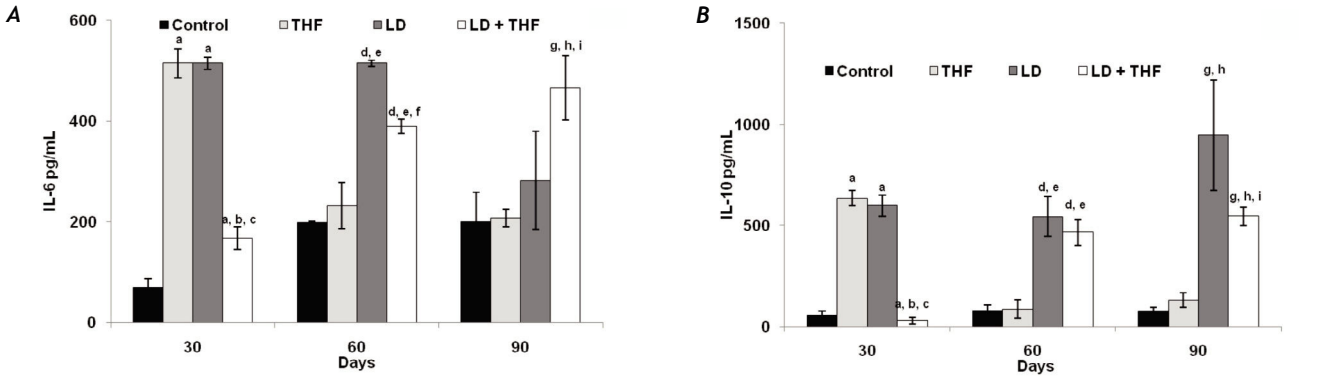

Figure 2. *Effect of THF on serum IL-6 and IL-10 levels. Serum levels for groups at 30, 60 and 90 of:* ***A.*** *IL-6.* ***B.*** *IL-10. The results are presented as mean ± S.D. from 6 animals in each group.* [a] *p < 0.05 compared with the control at 30 days.* [b] *p < 0.05 compared with the THF at 30 days.* [c] *p < 0.05 compared with LD (liver damaged) at 30 days.* [d] *p < 0.05 compared with the control at 60 days.* [e] *p < 0.05 compared with the THF at 60 days.* [f] *p < 0.05 compared with LD at 60 days.* [g] *p < 0.05 compared with the control at 90 days.* [h] *p < 0.05 compared with the THF at 90 days.* [i] *p < 0.05 compared with LD at 90 days.*

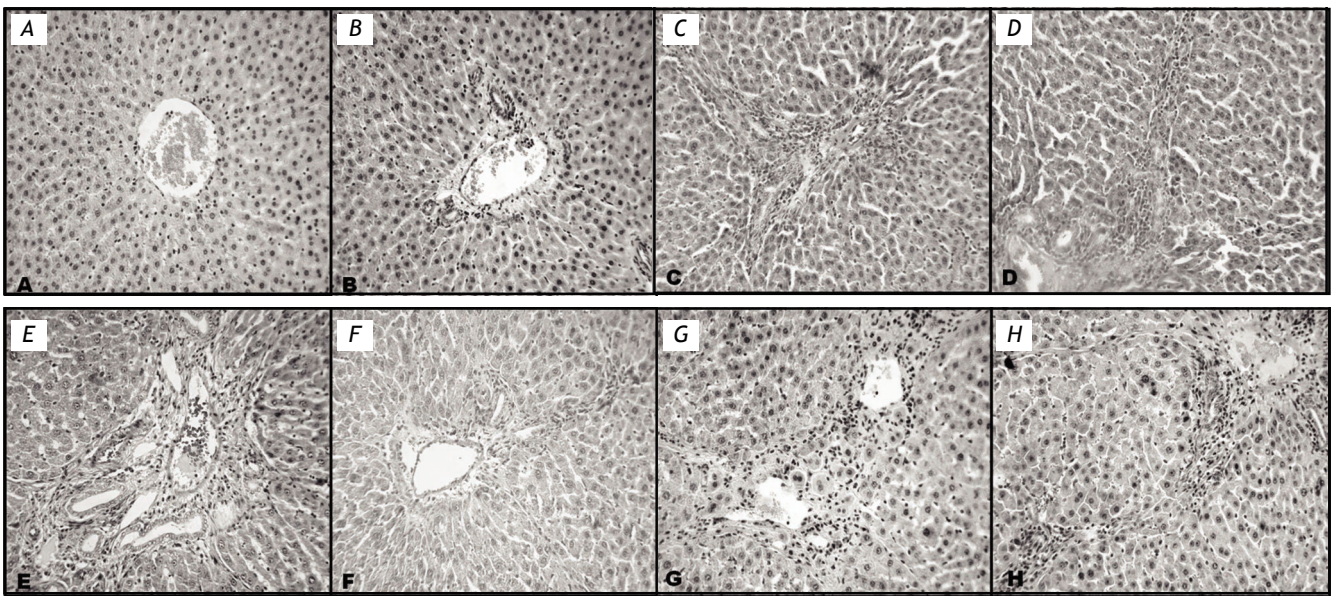

Figure 3. *Effect of THF on liver histopathology. Liver slice:* ***A.*** *Control.* ***B.*** *THF.* ***C.*** *LD (liver damaged) after 30 days.* ***D.*** *LD + THF after 30 days.* ***E.*** *LD after 60 days.* ***F.*** *LD + THF after 60 days.* ***G.*** *LD after 90 days.* ***H.*** *LD + THF after 90 days. Hematoxylin and Eosin staining, magnification x 20.*

among the animals. Four of the six rats examined showed evident improvement while in the other two did not (Figure 3F and 4F). After 90 days, the liver damaged group and the liver damaged group treated with THF showed scattered nodules limited by collagen fibers, mild chronic inflammation in portal areas with numerous biliary ducts and hepatocytes damage and regeneration expressed in binucleation (Figure 3G-3H and 4G-4H). No evident histological abnormalities were seen in the liver of animals treated with THF (Fig 3B and 4B). Grades of fibrosis are reported in table 2.

There was a good correlation between the morphological (Figure 4) and morphometrical (Table 3) analysis. Liver fibrosis was observed 60 days after PS administration, showing a 200-fold increase when compared with the control group ($p < 0.05$) (Table 3). Animals with PS-induced liver damage and treated with THF showed an important reduction in fibrosis (59%) after 60 days when compared with the liver damaged group ($p < 0.05$) (Table 3). After 90 days, fibrosis had decreased and there was no difference between PS liver damage and animals with liver damage treated with THF ($p < 0.05$)

Table 2. Effect of THF on the pathologic grading of hepatic fibrosis in rats administered PS, n = 6.

	Degree of hepatic fibrosis				
	0	I	II	III	IV
• 30 days					
Control	6	0	0	0	0
THF	6	0	0	0	0
LD	6	0	0	0	0
LD + THF	6	0	0	0	0
• 60 days					
Control	6	0	0	0	0
THF	6	0	0	0	0
LD	0	0	3	3	0
LD + THF	2	3	1	0	0
• 90 days					
Control	6	0	0	0	0
THF	6	0	0	0	0
LD	0	0	2	3	1
LD + THF	1	2	2	1	0

LD: Liver damaged.

Table 3. Effect of THF on % fibrosis after 30, 60 or 90 days.

	Fibrosis (%)		
	30 days	60 days	90 days
Control	0.24 ± 0.02	0.26 ± 0.11	0.23 ± 0.09
THF	0.22 ± 0.02	0.2 ± 0.07	0.24 ± 0.08
LD	0.23 ± 0.04	23.89 ± 3.21*,†	11.74 ± 1.46*,†
LD + THF	0.24 ± 0.01	9.78 ± 3.28*,†,‡	10.01 ± 2.68*,†

Values are presented as mean ± SD from 6 animals in each group. * $p < 0.05$ compared with the control group in its respective days. † $p < 0.05$ compared with the THF group in its respective days. ‡ $p < 0.05$ compared with the LD (liver damaged) group in its respective days.

(Table 3). There was no fibrosis in the THF groups after 30, 60 and 90 days (Table 3).

Individual hepatocytes analysis was performed to determine the percentage of normal, regenerative, apoptotic and necrotic hepatocytes according to morphological criteria. After 30 days, the liver damaged group had a decrease in % of normal hepatocytes, and an increase in the % of regenerative, apoptotic and necrotic hepatocytes when compared with the control ($p < 0.05$). The liver damaged, THF-treated group showed a reduction in the % of cell regeneration when compared with the liver damaged group ($p < 0.05$) (Figure 5A).

After 60 days, the liver damaged group showed a decrease in % of normal hepatocytes and a % increase in regenerative, apoptotic and necrotic hepatocytes when compared to the control ($p < 0.05$) (Figure 5B). The THF-treated liver damaged group showed an increase in % of normal hepatocytes and a lesser % of apoptotic and necrotic cells than those observed in the liver damaged group ($p < 0.05$) (Figure 5B).

After 90 days, normal cells continued to be at a low level and the % of regenerative and apoptotic hepatocytes had increased in the liver damaged group when compared with the control ($p < 0.05$) (Figure 5C). There was no difference between the liver damaged and the liver damaged and THF-treated groups.

No differences were observed between the THF group and the control group after 30, 60 and 90 days.

DISCUSSION

In the past decade there have been significant advances in the design of new antifibrotic agents, mostly due to an improved understanding of the cellular and molecular mechanisms associated with the deve-

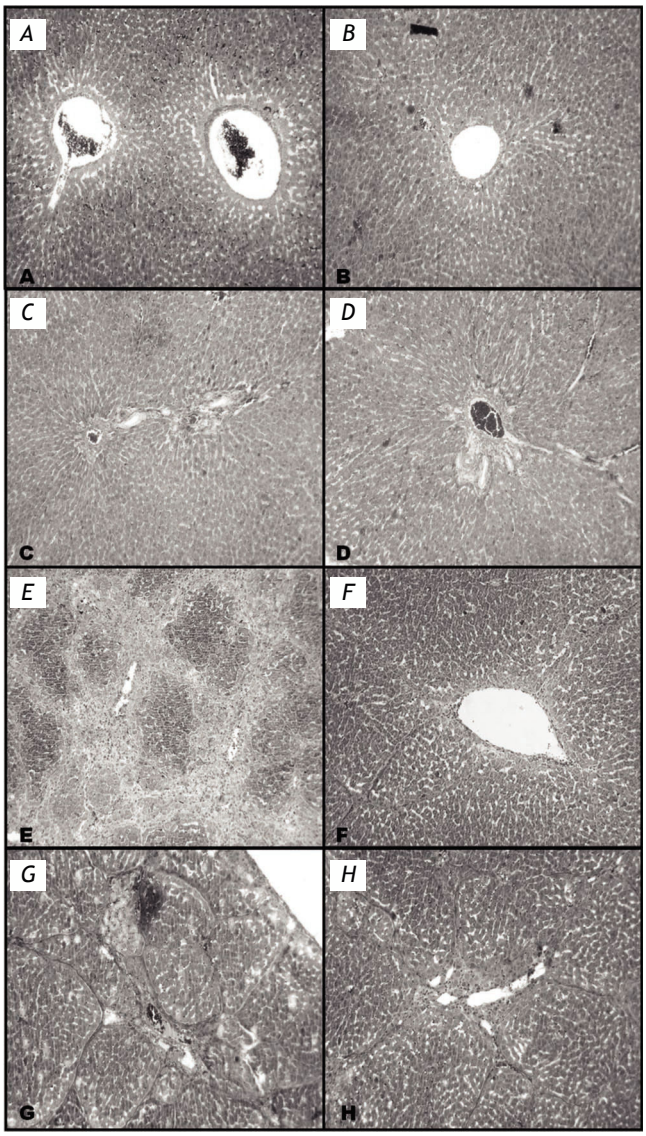

Figure 4. *Effect of THF on liver fibrosis. Liver slice:* ***A.*** *Control.* ***B.*** *THF.* ***C.*** *LD (liver damaged) after 30 days.* ***D.*** *LD + THF after 30 days.* ***E.*** *LD after 60 days.* ***F.*** *LD + THF after 60 days.* ***G.*** *LD after 90 days.* ***H.*** *LD + THF after 90 days. Masson's trichrome staining, magnification x10.*

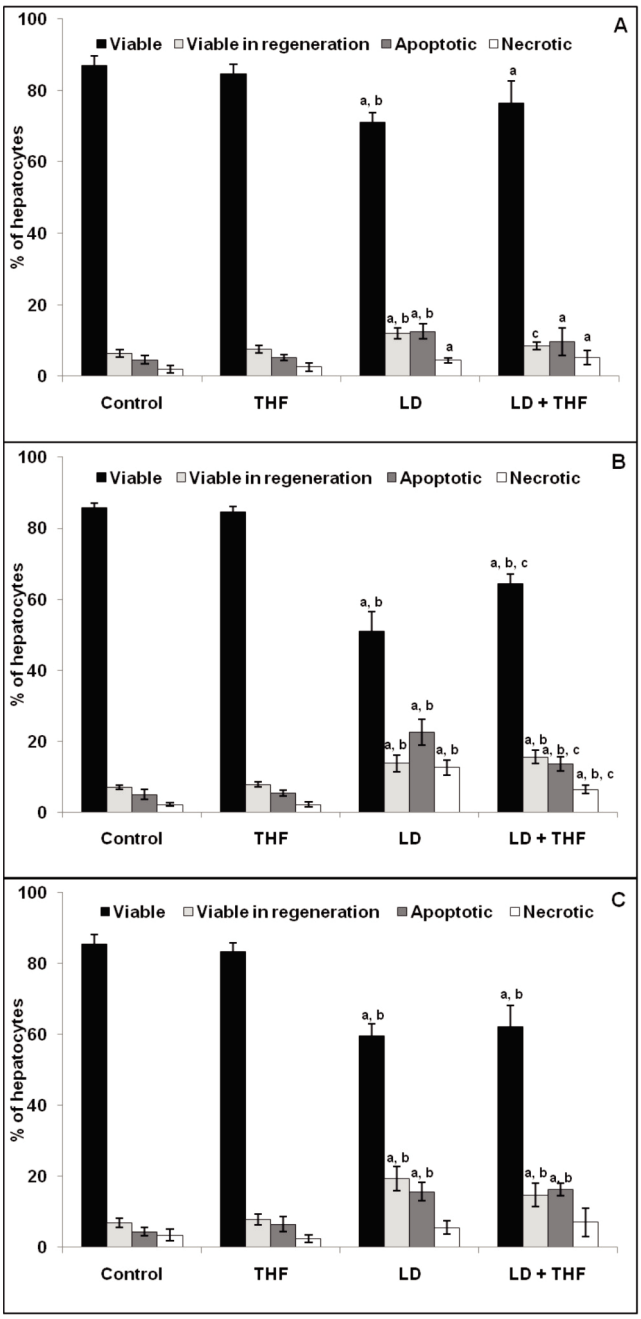

Figure 5. *Effect of THF on hepatocyte status. % of normal, normal in regeneration, apoptotic and necrotic hepatocytes for groups in:* ***A.*** *30 days.* ***B.*** *60 days.* ***C.*** *90 days. The results are presented as mean ± S.D. from 6 animals in each group.* [a] *p < 0.05 compared with the control in its respective days.* [b] *p < 0.05 compared with THF in its respective days.* [c] *p < 0.05 compared with LD (liver damaged) in its respective days.*

lopment of liver cirrhosis. But only a few agents have reached the clinical trial phase and a complete and successful pharmacological treatment for liver cirrhosis is not yet available. Here we examined the effect of THF in hepatic fibrosis during different stages of development using an immunologically-induced liver fibrosis model.

Most hepatic fibrosis models, such as thioacetamide, carbon tetrachloride (CCl_4) and bile-duct ligation, are established by inducing so-called post-necrotic hepatic fibrosis. PS-induced hepatic fibrosis is mainly the product of intense immune responses characterized by slight hepatic damage and a slow development of fibrosis.[23] The histopathological changes of this model are characterized by mono-

nuclear cell infiltration and a fibrotic response that mostly takes place in the periportal area.[3]

It has been reported that, in a PS-induced liver damage model, fibrosis is produced after 60 days of PS administration in Wistar rats. We studied the inflammatory process during stages of early liver damage and prior to the development of fibrosis (30 days), as well as during post-chronic damage and liver fibrosis stages (90 days).

ALT and AST enzymatic levels were measured in conjunction, given that they are indicative of liver damage. ALT and AST levels increased after 30 to 60 days in rats receiving PS. These increments were not as high as those observed in other models but were statistically significant when compared with the control group. Therefore, the current results indicate the presence of hepatocellular damage and disease progression, which is in agreement with other studies.[24] THF-treated animals with PS-induced liver damage showed a reduction in ALT and AST levels, showing the positive effect of THF on a damaged liver. Few studies have documented PS-induced liver injury and fibrosis after 60 days, showing low or null increase in enzymatic levels.[3] Likewise, we did not observe any changes in the ALT or AST levels of animals with PS-induced liver fibrosis after 90 days, which suggests spontaneous resolution after 60 or more days, as in the case of other immunological models such as the collagen-induced arthritis one.[25] Indeed, the liver damaged, THF-treated group showed no changes after 90 days, probably as a consequence of spontaneous resolution.

It has been suggested that this model is autoimmune because of the deposition of gammaglobulins and immune complexes,[21,23,26] which is similar to the bile duct and portal tract damage observed in human autoimmune hepatitis.[27-29] For this reason, we evaluated γ-GT and ALP levels, as these enzymes are indicative of hepatocyte damage and bile duct injury.[30] γ-GT levels increased after 30 days and remained high until day 90, whereas ALP levels increased after 60 and 90 days in animals with liver damage and fibrosis. These results suggest that hepatocyte and bile duct damage progressively increases after PS administration. THF treatment did not alter high ALP and γ-GT levels, suggesting that, while THF might reduce (30 days) or prevent (60 days) hepatocytes damage; it cannot prevent bile duct or portal tract damage in this model.

We observed significant abnormalities in the histological study that satisfactorily correlated with enzyme levels during days 30 and 60. We found an increase in the portal tracts area due to extensive inflammatory cell infiltration and striking bile duct proliferation. THF treatment reduced the inflammatory cell infiltration in portal tracts in the time points observed. It has been previously reported that THF may modify IL-2 levels[15] and that this cytokine acts as an anti-inflammatory by modulating IL-1, a potent inflammatory cytokine that increases inflammatory response.[31] The changes in inflammatory response and the alterations in liver architecture could be due to the fact that THF treatment is modulating the immune response to a certain degree.

After 30 days of PS administration, morphometric evaluation showed an increase in necrotic and apoptotic cells, but not as high as those observed in other post-necrotic models.[23,26] It has been observed that, by itself, PS does not produce hepatocytes damage but induces changes in the micro-environment, affecting their functioning. Autoimmune hepatitis is characterized by a release of pro-inflammatory cytokines (like TNF-α, IL-1 or IL-6), which leads hepatocytes to apoptosis.[32-35] Necrosis has also been associated to intense autoimmune responses; patients with high auto-reactivity show elevated percentages of centrilobular necrosis.[36,37] After 60 days of liver damage due to PS administration, we observed a higher percentage of apoptotic and necrotic hepatocytes than on day 30. Those changes correlated with increased ALT and AST levels. THF treatment reduced the percentage of necrotic and apoptotic hepatocytes, particularly after 60 days. Moreover, fibrosis was also decreased by THF treatment, suggesting that it might improve the inflammatory and immunological micro-environment in the liver.

After 30 and 60 days, rats with liver damage showed a higher increase in serum IL-6 levels. It is well known that IL-6 is a pro-inflammatory cytokine that participates in the inflammatory response, but it also plays a key role in matrix extracellular production by regulating the activity of MMP-13 and TIMP-1.[5,38,39] Moreover, IL-6 is also a cytotoxic cytokine released by Kupffer cells and is considered to be one of the mediators that stimulate target cells (inflammatory cells, hepatocytes, epithelial cells and HSC) at the beginning of the liver damage process.[5,7] This study found that liver-damaged rats showed an increase in serum IL-6 levels with progressive inflammation and liver fibrosis after 30 and 60 days. After 90 days, rats treated with PS showed normal IL-6 levels, which suggest the spontaneous resolution of the inflammatory/immune response.

Animals with liver damage treated with THF

showed a reduction in serum IL-6 levels along with a decrease in the inflammatory response at 30 and 60 days of THF treatment. Human liver myofibroblasts can produce IL-6[40] and this cytokine induced the proliferation of quiescent HSCs.[7,41] By decreasing IL-6 production *in vivo*, THF might reduce the rate of HSC proliferation and activation, thus decreasing liver fibrosis. By itself, THF was able to induce production of IL-6 at 30 days, but no alterations in liver function and morphology were found, indicating that THF alone does not induce liver damage. However there are no reports in the literature of THF producing damage, and the increase of IL-6 (and IL-10) suggest an inflammatory process. More experiments need to be performed in order to clarify this observation.

The reduction of the inflammatory response observed in THF-treated animals with liver damage and fibrosis suggests an anti-inflammatory response. IL-10 has been reported to have anti-inflammatory effects in some liver diseases.[42,43] We observed that THF increased IL-10 levels in the liver damaged group after 30 days. This could be explained for the increase of IL-6 levels, observed in this group of liver damage after 30 days.[44] As mentioned above, more experiments, including evaluation in other tissues and blood parameters need to be performed in order to understand this observation. After 60 days, serum IL-10 levels showed a striking increase in the THF-treated liver damaged group, probably in response to higher IL-6 levels. After 90 days we observed higher IL-10 levels in the liver damaged group, along with low IL-6 levels. In fact, several studies have reported the role of IL-10 as a protective cytokine working against hepatic injury in diseases such as diet-induced insulin resistance in liver; hepatitis C virus infection, and fatty liver disease.[45-47] In this model, THF may be acting having an effect on $CD4^+CD25^+FoxP3^+$ T_{reg} cells, a subtype that regulates autoimmune/inflammatory process, like the one in this model, via IL-10 production. Other thymus extracts, like thymic stromal lymphopoietin, are able to induce the generation of $FoxP3^+$ regulatory T cells by activating plasmoacytoid dentritic cells.[48] THF could be inducing a Th1 cytokines, like IL-10,[49] and suppressing Th2, like IL-6. This effect has been reported in other thymus extracts.[50,51]

CONCLUSION

Our findings suggest that THF effectively reduced the development of liver fibrosis in a model of PS-induced liver fibrosis in rats. The mechanism might be associated to THF-induced IL-6 down-regulation, probably by induction of $CD4^+CD25^+FoxP3^+$ T_{reg} cells or dendritic cells, which suppresses inflammatory response and, presumably, reduces the activation of HSCs. Although more experiments are needed in order to understand the effect and security of THF, these results suggest that THF could be considered a new class of potential anti-hepatic fibrosis drug.

ABBREVIATIONS

- **HSC:** Hepatic stellate cell.
- **TGF-β:** Transforming growth factor-beta.
- **IL-6:** Interleukin 6.
- **PS:** Porcine serum.
- **THF:** Thymic humoral factor.
- **ALT:** Alanine aminotransferase.
- **AST:** Aspartate aminotransferase.
- **ALP:** Alkaline phosphatase.
- **γ-GT:** Gamma-glutamyltransferase.
- **IL-10:** Interleukin 10.
- **ip:** Intraperitoneal.

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