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Captopril Inhibits Thioacetamide-Induced Chronic Liver Injury Associated with the Suppression of Inflammation / Hypoxia- Inducible Factor 1-alpha / Profibrogenic Axis-Mediated Hepatotoxicity in Rats

Captopril Inhibe la Lesión Hepática Crónica Inducida por Tioacetamida Asociada con la Supresión de la Inflamación/Factor 1-alfa Inducible por Hipoxia/Hepatotoxicidad Mediada por el Eje Profibrogénico en Rata

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SUMMARY: Liver transplantation is the only available method to treat liver failure induced by chronic liver injury. We sought to determine whether the angiotensin-converting enzyme inhibitor, captopril, can inhibit the development of chronic liver injury induced by the hepatotoxic agent thioacetamide (TAA) in association with the suppression of inflammation (hsCRP, TNF-α, and IL-6) / hypoxia-inducible factor 1-alpha (HIF-1α) / profibrosis (TIMP-1, MMP-9, and α-SMA) axis that mediates liver injury. Therefore, the model group of rats was injected for eight weeks with 200 mg/kg TAA starting at week two. The protective group was pretreated with 150 mg/kg captopril daily for two weeks prior to TAA injections and continued receiving both capropril and TAA agents until being humanely scrificed at week 10. We observed a substantial damage to liver tissue in the model group as demonstrated by a significant ($p<0.0001$) increase in blood and hepatic tissue levels of high sensitivity C-reactive protein (hsCRP), tumor necrosis factor-a (TNF-α), interleukin-6 (L-6), HIF-1α, tissue inhibitor of metalloproteinases-1 (TIMP-1), matrix metalloproteinase-9 (MMP-9), alpha-smooth muscle actin (α-SMA), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). All these parameters were significantly ($p<0.0244$) protected by captopril. Also, a significant ($p<0.0001$) positive correlation was observed between a-SMA (profibrosis) and the serum and tissue levels of hsCRP, TNF-α, HIF-1α, TIMP-1, MMP-9, and ALT. Thus, these findings suggest that the induction of chronic liver injury by the hepatotoxic compound, TAA is associated with the upregulation of inflammation/HIF-1α/profibrosis, with captopril exhibiting beneficial hepatic pleotropic effects.

KEY WORDS: Liver injury; Thioacetamide; Inflammation; HIF-1α; Profibrosis; Rat; Model.

INTRODUCTION

Hepatic fibrosis is part of chronic liver injury complications that can lead to liver failure, which leaves liver transplantation as the only available method of treatment (Bzowej *et al*., 2011). Therefore, regression of hepatic fibrosis could be a potential therapeutic choice to prevent the progression of liver disease to cirrhosis and eventually liver failure (Liedtke *et al*., 2013; Czaja, 2014). Chronic liver insults such as chemicals, toxins, viruses, alcohol abuse, cholestasis, and autoimmune diseases have been associated with the pathophysiology of hepatic fibrosis that could also lead to liver cirrhosis and ultimately liver failure (Friedman, 2003; Czaja, 2014). Thioacetamide (TAA) is a severe hepatotoxic compound that causes cirrhosis of the liver and liver cancer (De Minicis *et al*., 2013) as well as liver fibrosis (Al-Hashem *et al*., 2019).
Inflammation is involved in promoting liver fibrosis via the stimulation of hepatic stellate cells (HSCs) that produce the majority of the fibrogenic extracellular matrix (Robert *et al*., 2016; Luedde & Schwabe, 2011). In addition, knockout mice lacking the inflammatory biomarker TNF-α or the gene for the TNF-α receptor reduced the hepatotoxic effects of the compound carbon tetrachloride, CCl4 (Morio *et al*., 2001). TAA was reported to promote liver fibrosis associated with the activation of HIF-1α, TIMP-1, and α-SMA, ERK1/2, and mTOR protein and gene expression (Zhao *et al*., 2014). Furthermore, cholesterol-induced liver fibrosis in mice is associated with the upregulation of HIF-1α and MMP-9 (Anavi *et al*., 2015). In patients with chronic liver diseases, the renin-angiotensin system was found to be activated, and angiotensin II induced liver fibrosis via the activation of

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HSCs (Yoshiji *et al*., 2001). Thus, this study investigated the potential inhibitory effect of the angiotensin-converting enzyme inhibitor, captopril on TAA-induced chronic liver injury associated with the amelioration of the inflammation/HIF-1α/profibrosis axis-mediated fibrosis.

MATERIAL AND METHOD

Animals. Male Albino rats weighing 180-200 gm were included in the experiment. The study follows the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Ethical Committee at King Khalid University, Rats were housed at a controlled room temperature with 12 hour light/dark cycles, and had free access to food and water.

Experimental design. Rats were separated into 3 groups (n=8 per group) after acclimization. The control group (Control) of rats were non-treated and injected intraperitoneally (i.p.) with the vehicle. The experimental group (TAA) of rats were subjected to i.p. injections with TAA (200 mg/kg, twice per week) for eight weeks, starting at week 3 (Wallace *et al*., 2015). The protective group (Cap+TAA) of rats was given Cap (150 mg/kg) from day one until the end of the experiment, at the 10th week, and received TAA as above for eight weeks. Blood samples were collected under anaesthesia after the completion of the experiment using sodium thiopental (40 mg/kg), and animals were humanely killed by cervical dislocation, and liver tissue specimens were harvested.

Histological examination. Harvested liver specimens were fixed overnight in 10 % formalin and then dehydrated with ascending grades of alcohols. Paraffin blocks were prepared by the standard method, and 5 µm thick sections were deparaffinized and rehydrated. Hepatic sections were then stained with hematoxylin and eosin (H&E) staining.

Immunohistochemistry of a-SMA. Immunohistochemical staining was performed using anti-alpha-smooth muscle actin (a-SMA) (Agilent Dako; cat # M0851) as a marker for HSCs activation. Antigen retrieval was conducted, followed by the application of the primary antibody overnight in a humidity chamber and the secondary antibody for 30 min. Sections were co-stained with Mayer hematoxylin (Sigma-Aldrich; cat # 51275).

Quantitative real-time polymerase chain reaction (qRT-PCR) of TIMP-1 and MMP-9. Total RNA was isolated from rats' livers using the RNeasy Mini Kit (Qiagen Pty, Victoria, Australia) and 1 µg RNA was reverse-transcribed with the complementary DNA (cDNA) synthesis kit (Fermentas, USA). Triplicate cDNA samples and standards were amplified in Master Mix containing SYBR green (Thermo Fisher Scientific Inc, MA, USA) with primers specific for: (i) TIMP-1 (sense, 5'-GGT TCC CTG GCA TAA TCT GA-3'; antisense, 5'-GTC ATC GAG ACC CCA AGG TA-3') (Yoshiji *et al*.), (ii) MMP-9 (sense, 5'-CCTGCGTATTTCCATTCATC-3'; antisense, 5'- GCC TTG GGT CAG GTT TAG AG -3'), and (iii) b-actin. The relative expression was calculated according to the manufacturer's software.

Western blotting analysis of HIF-1a. Proteins from liver tissues were extracted and 20 µg per sample were subjected to Western blot analysis (Al-Ani *et al*., 2010). Membranes were probed with anti-HIF-1a (Thermo Fisher Scientific, MA, USA) at 4 °C overnight. Protein bands were visualized using the enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia, UK). After normalization by β-actin on the ChemiDoc MP Imaging System, relative expression was obtained using image analysis software to read the band intensity of the target proteins against the control sample.

Determination of hsCRP, TNF-a, IL-6, ALT, and AST levels. ELISA kits for blood and tissue determination of hsCRP (Assaypro, St. Charles, MO, USA), TNF-α (Abcam, Cambridge, UK), and IL-6 (Abcam, Cambridge, UK) were measured according to the manufacturer's instructions. Enzymatic kits (Randox Laboratories, Crumlin, UK) were used to determine the blood levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Statistical analysis and morphometry. Analyses of data were conducted utilizing SPSS with version 10.0 (SPSS Inc., Chicago, Ill., USA). Statistical comparisons of data were performed using one-way ANOVA followed by Tukey's *post hoc* test. To detect a probable significance between two different parameters, Pearson correlation was performed. $p \leq 0.05$ was considered statistically significant. Morphometry of the percentage areas of a-SMA positive immunostaining were done using Leica QWin 500 image analyzer (Cambridge, UK) in 10 non-overlapping fields for each group. Data were also analyzed using analysis of variance (ANOVA) as described above.

RESULTS

Induction of chronic liver injury in rats by TAA. In order to test our working hypothesis, we first modelled chronic liver disease in rats. Injection of the experimental group of rats with TAA for 8 weeks caused a profound increase in liver

injury biomarkers (ALT and AST) and hepatic tissue damage (Fig. 1). High blood levels (p<0.0001) of ALT (Fig. 1A) and AST (Fig. 1B) in the experimental group (TAA) compared to normal levels in the control group were detected. Liver sections prepared for basic histology staining (H&E) of TAA injected rats revealed damaged hepatocytes with vacuolated cytoplasm and dark pyknotic nuclei, as well as infiltration of inflammatory cells and dilated congested blood vessels (Fig. 1D) compared to normal liver tissue architecture in the control group (Fig. 1C).

Capropril (Cap) suppresses TAA-induced inflammation / hypoxia (HIF-1α) axis in blood and Liver Tissues. Liver injury is well-known to be induced by inflammation and HIF-1α (Al-Hashem *et al*., 2019), and HIF-1α is located downstream of the inflammatory biomarker TNF-α (Jung *et al*., 2003). To determine whether captopril can inhibit the inflammation/HIF-1α axis in our animal model, one animal group was pre-treated with captopril prior to TAA injections as explained in methods section. In comparison with control groups, TAA caused a sharp increase in blood levels of hsCRP (Fig. 2A) and liver tissue levels of TNF-α (Fig. 2B), IL-6 (Fig. 2C), HIF-1α (Figs. 2E and 2F), as well as blood levels AST (Fig. 2D), which were substantially, but not completely, inhibited by captopril in the Cap+TAA group (Figs. 2A-F).

Captopril (Cap) ameliorates TAA-induced biomarkers of liver fibrosis TIMP-1, MMP-9, and a-SMA in injured liver. To investigate whether captopril treatment used in this study can inhibit the expression of TIMP-1, MMP-9, and a-SMA, which are well-known profibrogenic biomarkers (Al-Hashem *et al*., 2019; Anavi *et al*., 2015), we assessed the levels of these parameters in liver tissue harvested from all rats' group using qRT-PCR analyses for mRNAs expression of TIMP-1 (Fig. 3A) and MMP-9 (Fig. 3B), and immunohistochemistry for α-SMA protein expression (Figs. 3C-F). TAA caused a sharp increase in TIMP-1 and MMP-9 gene expression that were significantly (p<0.0244), but not completely, protected by captopril (Figs. 3A and 3B). Liver tissue sections of the control group revealed few areas of a-SMA positive cells (Control), whereas, many α-SMA positive cells were shown in liver sections of the experimental group (TAA), which were partially inhibited by captopril (Figs. 3C-F).

Correlation between the score of a-SMAand inflammation / HIF-1α axis. The correlation between these parameters (a-SMA, inflammatory biomarkers, and HIF-1α) was evaluated to further support the link between profibrogenic markers and inflammation / HIF-1α axis in chronic liver injury (Fig. 4). A significant (p<0.0001) positive correlation was observed between α-SMA score and hsCRP (r = 0.985) (Fig. 4A), TNF-α (r = 0.985) (Fig. 4B), HIF-1α (r = 0.944) (Fig. 4C), TIMP-1 (r = 0.944) (Fig. 4D), MMP-9 (r = 0.944) (Fig. 4E), and ALT (r = 0.939) (Fig. 4F).

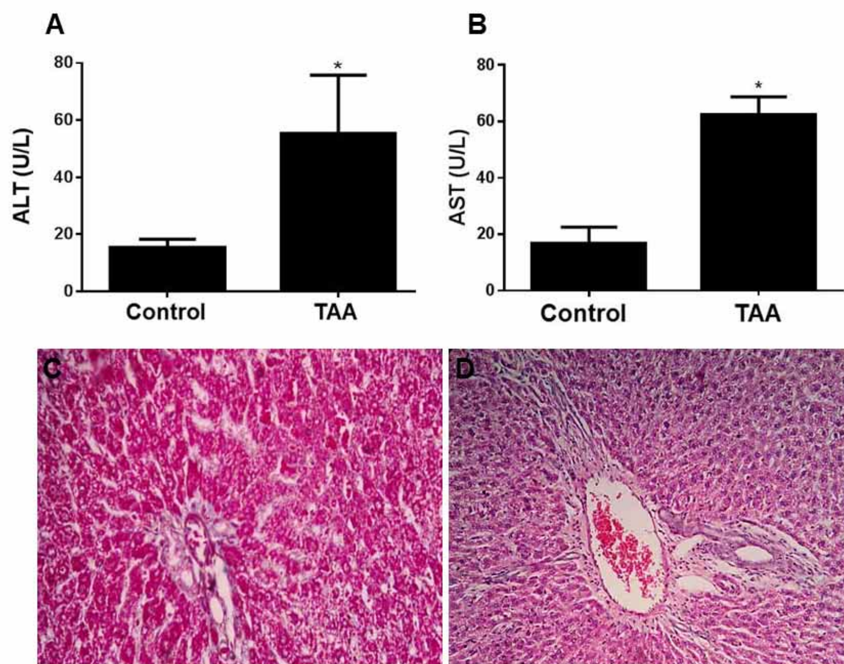

Fig. 1. TAA induces chronic liver injury in rats. Blood levels of ALT (A) and AST (B) were assessed at the end of experiment in the experimental group (TAA) and the control rats (Control). *p<0.0001 versus control. (C and D) H&E stained liver tissue images (x400) obtained from the control (C) and the experimental (D) groups at the end of experiment are shown using light microscopy. ALT: alanine aminotransferase; AST: aspartate aminotransferase; TAA: thioacetamide.

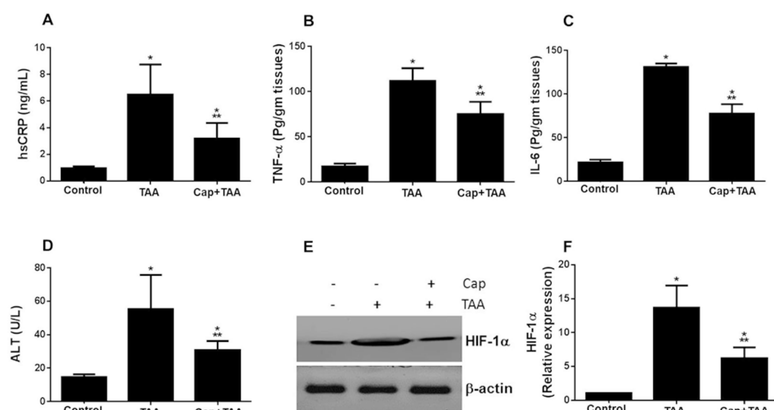

Fig. 2. Inhibition of TAA-induced inflammation, HIF-1α, and AST by captopril (Cap). The inflammatory biomarkers hsCRP (A), TNF-α (B), and IL-6 (C) as well as the liver injury biomarker ALT (D) were measured in all groups of rats at the end of the experiments. (E and F) HIF-1α western blots of liver homogenates prepared from all rats compared with β-actin expression are depicted. Results represent the mean (±SD). Presented p values are all significant; *p≤0.039 versus control, **p≤0.0017 versus TAA. hsCRP: high sensitivity C-reactive protein;TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; ALT: alanine aminotransferase; HIF-1α: hypoxia-inducible factor-1α; TAA: thioacetamide.

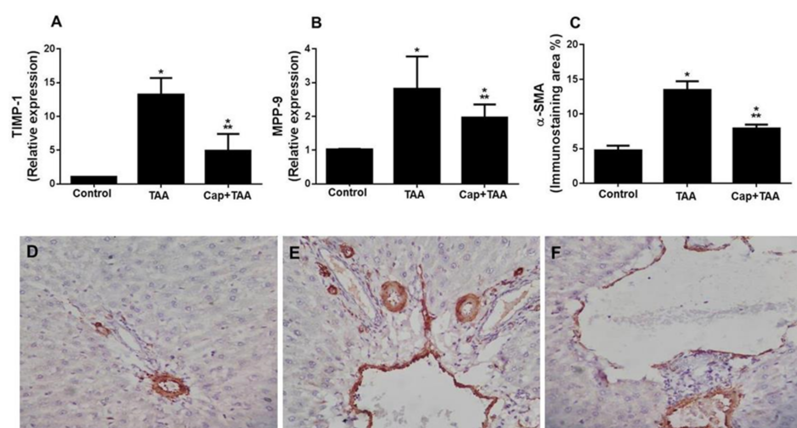

Fig. 3. Inhibition of TAA-induced liver expression of TIMP-1, MMP-9, and α-SMAby captopril (Cap). TIMP-1 (A) and MMP-9 (B) relative gene expression in liver sections from all the rats' groups are shown. α-SMA Immunohistochemistry of liver sections (x400) from the control (D), the TAA (E), and the treated (Cap+TAA) (F) groups of rats are illustrated. Histograms in (C) exemplify a quantitative analysis of α-SMA immunostaining area % in liver sections from groups above. Results represent the mean (±SD). Presented p values are all significant; * p≤0.0127 versus control, **≤0.0244 versus TAA. TIMP-1: tissue inhibitor of metalloproteinases-1; MMP-9: matrix metalloproteinase-9; α-SMA: alpha smooth muscle actin; TAA: thioacetamide.

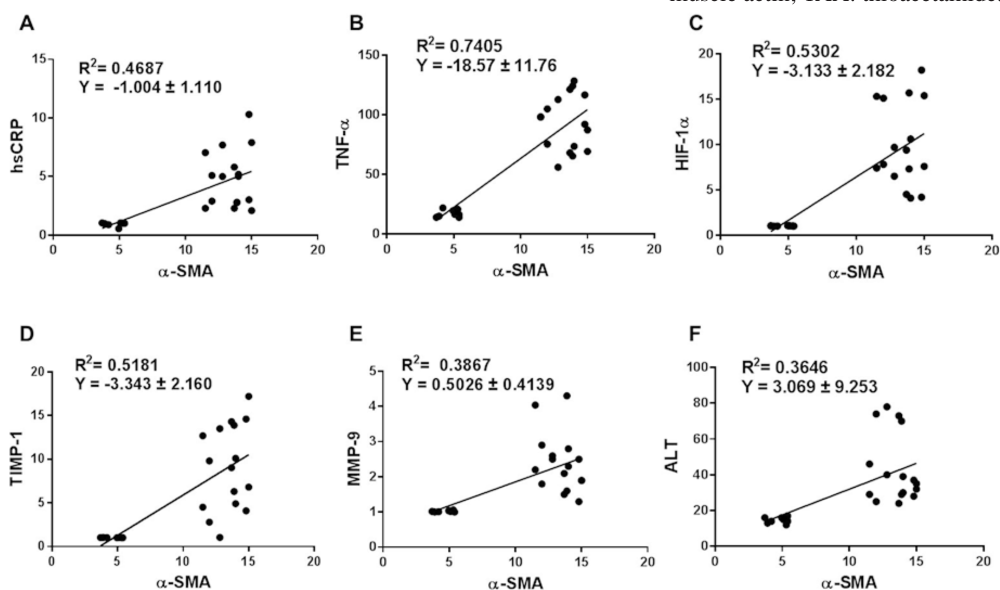

Fig. 4. The a-SMA score correlates with biomarkers of inflammation, liver injury, and hypoxia inducing factor. The degree of α-SMA tissue expression was evaluated at the end of the experiment in all rats and a significant (p≤0.0018) positive correlation was detected between α-SMA versus hsCRP (A), TNF-α: (B), HIF-1α (C), TIMP-1 (D), MMP-9 (E), and ALT (F). hsCRP: high sensitivity C-reactive protein;TNF-α: tumor necrosis factor alpha; HIF-1α: hypoxia-inducible factor-1α; TIMP-1: tissue inhibitor of metalloproteinases-1; MMP-9: matrix metalloproteinase-9; ALT: alanine aminotransferase; α-SMA: alpha smooth muscle actin.

DISCUSSION

The present study investigated the inflammation / HIF-1α / profibrogenic axis-mediated liver injury in rats induced by TAA with and without captopril incorporation using molecular, chemical, immunological, and basic histology staining to address our working hypothesis stated that captopril can protect against TAA-induced chronic liver injury associated with the inhibition of the inflammation / HIF-1α / profibrogenic axis. Here, we demonstrated that TAA intoxication augmented liver tissue inflammation / HIF-1α / profibrogenic axis, and captopril was able to inhibit the effects of TAA. Our results were thus consistent with our working hypothesis. This further corroborate the report that showed captopril inhibited dyslipidemia and systemic hypertension induced by TAA intoxication in rats associated with the amelioration of hepatic mammalian target of rapamycin (mTOR), inflammation, and oxidative stress (Al-Hashem *et al*., 2021). Interestingly, mTOR is located upstream of HIF-1α in cell signalling (Sakamoto *et al*., 2014), and this would extend our investigated axis shown in this work (Figs. 2 and 3) to be blocked by captopril.

The liver is a known target of TAA intoxication that causes hepatic inflammation, fibrosis and cirrhosis (Reif *et al*., 2004; Al-Hashem *et al*., 2019), and the presented data in this study that demonstrated the induction of hepatic inflammatory, profibrogenic, as well as liver injury biomarkers by TAA (Figs. 1-3) are in agreement with the above studies. In addition, our data that point to the induction of MMP-9, TIMP-1, and α-SMA by TAA endorse previous studies showing (i) MMP-9 is involved in liver fibrosis induced by TAA (Lin *et al*., 2017; Yoshiji *et al*., 2001.), (ii) TIMP-1, and α-SMA expression was augmented by TAA (Al-Hashem *et al*., 2019); and (iii) human IL-10 (anti-inflammatory) gene therapy attenuated CCl4-induced TIMP-1, α-SMA, and liver fibrosis in mice (Chou *et al*., 2006).

Previous reports have shown that (i) the peptide hormone angiotensin II, which is known to be inhibited by captopril, can induce liver fibrosis (Yoshiji *et al*., 2001), (ii) in a rat model of bile duct ligation, captopril reduces the progression of liver fibrosis (Jonsson *et al*., 2001), (iii) captopril decreased LPS-induced inflammation and improved liver (Azizi-Malekabadi, 2020), and (iv) captopril decreases angiotensin II-induced HIF-1α in mesenchymal stem cells (Liu *et al*., 2014). These reports corroborate our data displaying significant inhibition of the inflammation / HIF-1α / profibrogenic axis by captopril.

In conclusion, we demonstrated in this study that TAA caused harmful impacts upon liver tissue and inflammation / HIF-1α / profibrogenic axis-mediated fibrosis. Importantly, this effect was inhibited in a rat model of chronic liver injury for duration of 10 weeks by captopril.

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RESUMEN. El trasplante de hígado es el único método disponible para tratar la insuficiencia hepática inducida por una lesión hepática crónica. Buscamos determinar si el inhibidor de la enzima convertidora de angiotensina, captopril, puede inhibir el desarrollo de lesión hepática crónica inducida por el agente hepatotóxico tioacetamida (TAA) en asociación con la supresión de la inflamación (hsCRP, TNF-α e IL-6) / factor inducible por hipoxia 1-alfa (HIF-1α) / profibrosis (TIMP-1, MMP-9 y α-SMA) eje que media la lesión hepática. Por lo tanto, al grupo modelo de ratas se le inyectó durante ocho semanas 200 mg/kg de TAA a partir de la semana dos. El grupo protector fue pretratado con 150 mg/kg de captopril al día durante dos semanas antes de las inyecciones de TAA y continuó recibiendo capropril y agentes TAA hasta que fue sacrificado en la semana 10. Observamos un daño sustancial en el tejido hepático en el grupo modelo, como lo demuestra un aumento significativo (p<0,0001) de los niveles en sangre y tejido hepático de proteína C reactiva de alta sensibilidad (hsCRP), factor de necrosis tumoral-α (TNF-a), interleucina-6 (L-6), HIF-1α, inhibidor tisular de metaloproteinasas-1 (TIMP-1), metaloproteinasa de matriz-9 (MMP-9), actina de músculo liso alfa (α-SMA), alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST). Todos estos parámetros estaban significativamente (p<0,0244) protegidos por captopril. Además, se observó una correlación positiva significativa (p<0,0001) entre α-SMA (profibrosis) y los niveles séricos y tisulares de hsCRP, TNF-α, HIF-1α, TIMP-1, MMP-9 y ALT. Por lo tanto, estos hallazgos sugieren que la inducción de daño hepático crónico por el compuesto hepatotóxico, TAA, está asociada con la regulación al alza de la inflamación/HIF-1α/profibrosis, con captopril exhibiendo efectos pleotrópicos hepáticos beneficiosos.

PALABRAS CLAVE: Lesión hepática; Tioacetamida; Inflamación; HIF-1α; Profibrosis; Rata; Modelo.

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