

Suppression of Thioacetamide-Induced Hepatic Injury in Rats treatment with Resveratrol: Role of mammalian Target of Rapamycin (mTOR) Cell Signaling

Supresión de la Lesión Hepática Inducida por Tioacetamida en ratas tratadas con Resveratrol: Rol de la vía de Señalización mTOR (mammalian Target of Rapamycin)

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SUMMARY: Chronic hepatotoxicity is a debilitating and frequently life-threatening disease resulting in progressive liver failure. The toxic chemical, thioacetamide (TAA) is used to evaluate hepatoprotective agents, and the polyphenolic compound, resveratrol was proposed as a novel treatment for diseases with hyperactivation of the mammalian target of rapamycin (mTOR) cell signaling pathway. This analysis sought to investigate the potential protective effect of resveratrol against liver injury induced by TAA via the inhibition of hepatic mTOR. Model group rats received several injections of TAA (200 mg/kg; twice a week for 8 weeks) before being sacrificed at week 10 and the protective group was pretreated with resveratrol (20 mg/kg) daily for two weeks prior to TAA injections and continued receiving both agents until the end of the experiment. Harvested liver tissues were examined using light microscopy and liver homogenates were assayed for biomarkers of inflammation and assessed the levels of mTOR protein in all animal groups. In addition, blood samples were assayed for biomarkers of liver injury enzyme. TAA substantially damaged the hepatic tissue of the model group such as infiltration of inflammatory cells, vacuolated cytoplasm, dark pyknotic nuclei, and dilated congested blood vessel that were effectively protected by resveratrol. Resveratrol also significantly ($p < 0.05$) inhibited TAA-induced mTOR, high sensitivity c-reactive protein (hs-CRP), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in harvested liver homogenates and blood samples. Thus, we conclude that resveratrol effectively protects against TAA-induced hepatotoxicity in rats, possibly due to the inhibition of mTOR and inflammation.

KEY WORDS: Liver injury; Thioacetamide; mTOR; Resveratrol; Inflammation; Rat model.

INTRODUCTION

Chronic liver disease represents a significant challenge to the health sector since it can lead to liver failure as well as the high cost of liver transplantation (Neff *et al.*, 2011). The adverse effects of certain chemicals used in industries such as carbon tetrachloride (CCl₄) and thioacetamide (TAA) on the human body are well documented (Mochizuki *et al.*, 2009). TAA is a severe hepatotoxic agent that causes liver fibrosis (Kadir *et al.*, 2011), cirrhosis and liver cancer (De Minicis *et al.*, 2013),

which depends on the length of exposure of the body to this agent. For example, a single injection of TAA caused acute liver injury in rats in the form of tissue necrosis and severe leukocytes infiltration between 6 – 60 hours following TAA injections; whereas, biomarkers of tissue necrosis, iNOS and NF- κ B assessed in liver tissue peaked at one hour post TAA injection (Chen *et al.*, 2008). Inflammation and necrosis of liver tissue also observed after one week following a single injection of TAA (Luo *et al.*, 2015). On the other hand,

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chronic liver injury model induced by TAA injections twice a week for 6 to 10 weeks caused liver fibrosis and cirrhosis (Wallace *et al.*, 2015; Al-Hashem *et al.*, 2019).

The quiescent, nonproliferative hepatic stellate cells (HSCs) become highly proliferative upon exposing to insults causing these cells producing most of the extracellular matrix (ECM) in the fibrotic liver via the upregulation of mTOR (Neef *et al.*, 2006; Shan *et al.*, 2016). Activated mTOR leads to many downstream cell signalling pathways including the activation of hypoxic transcription factor, HIF-1 α , which participates in promoting liver fibrosis (Zhao *et al.*, 2014) and cancer (Masoud & Li, 2015). The hepatotoxic agents, TAA and CCl₄ are reported to activate the mTOR-HIF-1 α axis in many animal models of liver diseases and HSCs cell lines (Zhao *et al.*; Wu *et al.*, 2016). Furthermore, these agents are also known to augment profibrogenic and inflammatory biomarkers such as α -SMA that upregulates fibroblast activity leading to the production of collagen and other fibers (Nouchi *et al.*, 1991; Zhao *et al.*; Wu *et al.*), and TNF- α (Morio *et al.*, 2001). Induction of TNF- α and IL-6 caused MMP/TIMP imbalance and collagen production (promoting fibrosis) by HSCs (Robert *et al.*, 2016).

Resveratrol is a polyphenolic anti-inflammatory and antioxidant compound found in grapes, berries, and peanuts (Cudmore *et al.*, 2012). It ameliorates several types of liver diseases such as hepatic sinusoidal obstruction, hepatic steatosis (Trepiana *et al.*, 2018), and inhibits HSCs (Kawada *et al.*, 1998). Resveratrol was proposed as a novel treatment for diseases with mTOR pathway hyperactivation such as cancers and tumor syndromes (Alayev *et al.*, 2016). We recently reported that the antidiabetic, anti-inflammatory, and antioxidant drug, metformin, is able to inhibit TAA-induced hepatotoxicity associated with the inhibition of mTOR hyperactivation and inflammation (Al-Hashem *et al.*). Therefore, we speculated that activation of the mTOR and inflammation by TAA in a rat model of liver injury could be inhibited by resveratrol.

MATERIAL AND METHOD

Animals. Albino male rats weighing 180-200 gm were used for the experiments with the approval of Ethical Committee of the college of medicine, King Khalid University, Abha, Saudi Arabia. The animals were obtained from the animal house of the College of Medicine of King Khalid University where they fed with standard rat's pellets and allowed free access to water. They were housed at a controlled ambient temperature of 25 \pm 2 $^{\circ}$ C and 50 \pm 10 % relative humidity, with 12-h light/12-h dark cycles. Experiments were

performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental design. After a one week adaptation period, rats were randomly allocated into 4 groups (n= 6) as follows: Control group (Control): non- treated rats that were injected intraperitoneally (i.p.) with vehicle; resveratrol group (RES): rats treated with resveratrol (20 mg/kg) daily for 10 weeks; TAA-treated group, the model group (TAA): rats were injected i.p. with 200 mg/kg TAA, 2x per week for 8 weeks (week 3 – week 10); the protective group (RES+TAA): rats were treated with resveratrol from week 1 – week 10 and injected with TAA from week 3 – week 10.

Histological Analysis. Liver specimens were immediately fixed in 10 % formal saline for 24 hours. Paraffin blocks were prepared, and 5 mm thick sections were subjected to hematoxylin and eosin (H&E) stain to elucidate the status of hepatic architecture and the structural changes.

Western Blotting Analysis of mTOR. Proteins were extracted from liver tissues, and 20 μ g of protein per sample were immunoblotted as we described previously (Al-Ani *et al.*, 2010). Membranes were probed with anti-mTOR-phospho-S2448 (Thermo Fisher Scientific, MA, USA) at 40C overnight. Proteins were visualized using the ECL detection kit (Amersham-Pharmacia, UK). Relative expression was determined using Image analysis software to read the band intensity of the target proteins against the control sample after normalization by α -actin on the Chemi Doc MP imager.

Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), High Sensitive C-reactive protein (CRP), Tumor Necrosis Factor Alpha (TNF- α), and Interleukin-6 (IL-6) Enzyme-Linked Immunosorbent Assay (ELISA). Serum levels of ALT and AST were measured using an enzymatic kit (Randox Laboratories, UK) according to the manufacturer's instructions. ELISA kits were purchase to determine serum levels of high sensitive C-reactive protein (hsCRP, Cat. No. ERC1021-1) from ASSAYPRO, USA. ELISA kits for determination of liver tissue homogenate levels of Interleukin-6 (IL-6, Cat No. ELR0IL6-001), and tumour necrosis factor alpha (TNF- α , Cat No. R63635), were used according to the manufacturer's instructions.

Statistical analysis. The data was expressed as mean \pm standard deviation (SD). Data was processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for detection of a probable significance between two different parameters. Results were considered significant if $p \leq 0.05$.

RESULTS

Induction of chronic liver injury in rats by TAA. We first sought to characterize the rat model of chronic liver injury induced by the hepatotoxic agent, TAA. Injection of the model group of rats with TAA (200 mg/kg body weight, i.p.) twice a week for 8 weeks caused a sharp increase in biomarkers of liver injury and abnormal liver tissue architecture (Fig. 1). High blood levels of ALT (Fig. 1A) and hs-CRP (Fig. 1B), and H&E images of liver section (Figs. 1C and 1D) confirmed liver injury and abnormal changes to the liver parenchyma in the model group (TAA). H&E-stained liver section from the model group (Fig. 1D) displays ballooning degeneration of hepatocytes, vacuolated cytoplasm, dark pyknotic nuclei, induced the presence of numerous inflammatory cell infiltration and dilated congested blood vessel. However, another H&E image at similar magnification prepared from liver sections of the control rats (Fig. 1C) shows unremarkable liver architecture.

Resveratrol inhibits TAA-induced liver injury enzymes and inflammatory biomarkers. We measured the tissue levels of the inflammatory biomarkers, CRP, TNF- α and IL-6 in all animal groups at week 10. TAA augmented blood levels of CRP and liver tissue homogenates levels of TNF- α and IL-6, and resveratrol treatment (RES+TAA) significantly ($p < 0.05$) inhibited these biomarkers (Table I). However, the inhibition by resveratrol was still significant

($p < 0.05$) when compared with the control groups. This means, a partial inhibition by resveratrol was achieved.

To determine whether resveratrol pre-treatment can reduce the release of the liver injury enzymes ALT and AST in rats injected with TAA, one group of rats was given resveratrol daily prior (for 2 weeks) and post (for 8 weeks) TAA injections. Compared to the control groups, TAA caused a three-fold increase in both ALT and AST that was significantly ($p < 0.05$) inhibited by resveratrol (RES+TAA) (Table I). However, the levels of ALT and AST in the RES+TAA group were still significant ($p < 0.05$) to the control group, which means, a partial inhibition by resveratrol was demonstrated.

Resveratrol inhibits TAA-induced hepatic mTOR activity in rats. Resveratrol was proposed as a novel treatment for diseases with mTOR pathway hyperactivation such as cancers and tumor syndromes (Alayev *et al.*). To test the hypothesis that resveratrol pretreatment can inhibit TAA-induced mTOR liver cell signaling, we assessed the levels of mTOR protein in all animal groups (Fig. 2). TAA strongly augmented mTOR protein levels, which was effectively inhibited by resveratrol (Figs. 2A and 2B). However, the degree of inhibition of mTOR by resveratrol was still significant ($p < 0.05$) to the control rats (Fig. 2B).

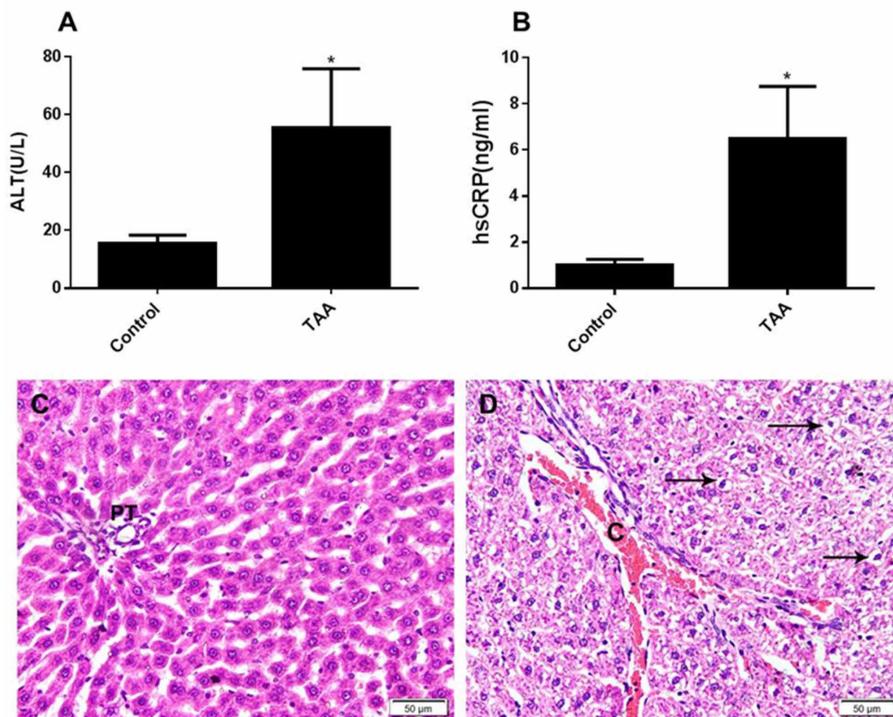


Fig. 1. Induction of chronic liver injury in rats by TAA. Blood levels of ALT (A) and hs-CRP (B) were measured after 10 weeks in the model group (TAA) compared to the control group rats ($n=6$ for each group). Results represent the mean (\pm SD), and experiments were performed in triplicate. * $p < 0.05$ versus control. (C and D). H&E stained images ($\times 400$) of harvested tissues obtained after 10 weeks from the liver of model group (D) compared to control group (C) rats are visualized using light microscopy. Note that arrows point to vacuolated cytoplasm and dark pyknotic nuclei. Abbreviations: PT, portal tract; C, dilated congested blood vessel; TAA: thioacetamide.

Table I. Effects of resveratrol on blood and liver homogenates levels of C-reactive protein (CRP), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), alanine transaminase (ALT), and aspartate transaminase (AST) in all experimental groups of rats.

Animal groups	CRP (ng/ml)	TNF- α (pg/gm)	IL-6 (pg/gm)	ALT (U/L)	AST (U/L)
Control	1.00 \pm 0.29	18.18 \pm 3.015	21.60 \pm 3.81	15.50 \pm 2.81	16.67 \pm 5.89
RES	0.801 \pm 0.18	19.02 \pm 3.15	23.52 \pm 3.6	17.33 \pm 2.42	15.50 \pm 4.41
TAA	6.31 \pm 2.39a	111.7 \pm 14.0a	131.1 \pm 3.75a	55.38 \pm 20.37a	58.50 \pm 11.93a
RES+TAA	3.27 \pm 1.32ab	80.47 \pm 19.82ab	72.60 \pm 19.67ab	28.29 \pm 7.43ab	33.71 \pm 6.31ab

Values are expressed as Mean \pm SD for 6 rats in each group. Values were considered significantly different at P < 0.05. a: Significant in comparison to control; b: Significant in comparison to TAA. TAA: thioacetamide; RES: resveratrol.

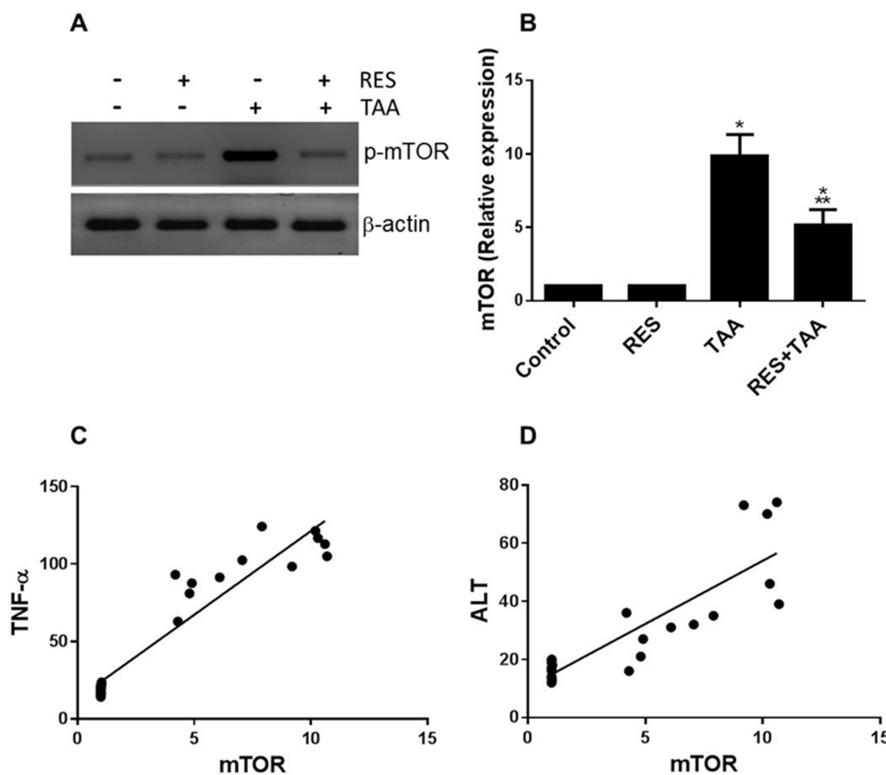


Fig. 2. Resveratrol reduces TAA-induced mTOR signaling protein. Liver tissue lysates prepared from all the groups of rats were examined by Western blotting for phospho-mTOR (p-mTOR) (A and B), and α -actin as a loading control (A). Histograms represent the relative expression of this signaling protein are shown (B). (C and D). Positive correlation between mTOR and TNF- α (C) and mTOR and ALT (D). ALT: alanine aminotransferase; mTOR: mammalian target of rapamycin; TNF- α : tumor necrosis factor- α ; TAA: thioacetamide.

We further determined the correlation between mTOR scoring and the tissue levels of inflammation, and serum levels of liver injury biomarkers in order to confirm and characterize that the role of the resveratrol is stable and an appropriate agent in liver injury in rats, and to further support the link between mTOR and inflammation, and serum levels of liver injury enzymes. As shown in Figs. 2C and 2D, a positive correlation was shown between mTOR and these biomarkers; mTOR versus TNF- α ($r = 0.9476$) ($p < 0.0001$), and mTOR versus ALT ($r = 0.8628$) ($p < 0.0001$).

Resveratrol protects hepatic tissue against TAA-induced liver injuries. We tested the hypothesis that resveratrol can protect liver tissue against damage induced by TAA in rats. Harvested liver tissues obtained from all

animal groups after 10 weeks on resveratrol and 8 weeks on TAA were stained with hematoxylin and eosin (H&E) and then examined under light microscopy (Fig. 3). Representative H&E stained sections of liver from the model group showed that TAA substantially distorted the liver parenchyma with ballooning degeneration of hepatocytes, vacuolated cytoplasm, dark pyknotic nuclei, induced the presence of numerous inflammatory cell infiltration and dilated congested blood vessel (Fig. 3B). Whereas, a normal tissue structure in the control group displaying cords of hepatocytes with acidophilic cytoplasm and vesicular nuclei surrounding portal tract (Fig. 3A). Resveratrol treatment preserved liver architecture against damage induced by TAA (Figs. 3C and 3D). However, small foci of inflammatory cell were noted in some fields.

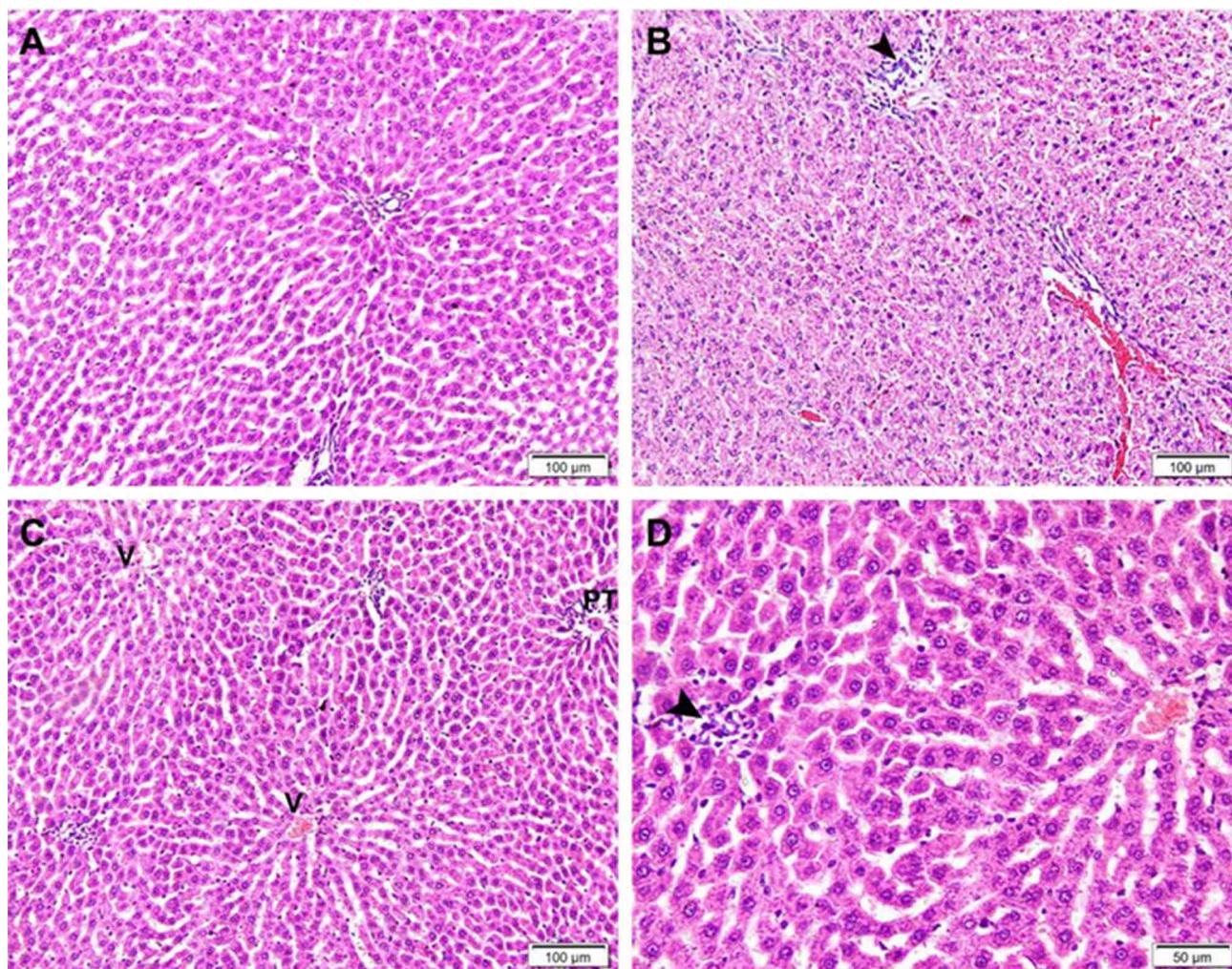


Fig. 3. Resveratrol protects liver architecture against injury induced by TAA. H&E stained images (A,B,C, x100; D, x400) of harvested tissues obtained after 10 weeks from the liver of the control group (A), TAA group (B), and the protective group, RES+TAA (C and D). Note that arrowheads in (B and D) point to the inflammatory cell infiltration. Abbreviations: PT, portal tract; V, central vein. TAA: thioacetamide.

DISCUSSION

In this study, we investigated whether the polyphenolic compound resveratrol can protect the liver from the hepatotoxic agent TAA in a chronic liver injury animal model. Therefore, we induced liver injury in rats using TAA in the presence and absence of resveratrol. Here, we report that resveratrol protected liver parenchyma against damage induced by TAA (Fig. 3), and inhibited biomarkers of inflammation, liver injury enzymes, and fibrosis (Table I and Fig. 2) in liver tissue homogenates and blood. These conclusions are supported by the ELISA, Western blots, and light microscopy data indicating that TAA induced liver tissue damage, inflammation, and mTOR in rats, which were substantially protected by resveratrol. Our results were thus

consistent with our working hypothesis that resveratrol can protect against TAA-induced hepatotoxicity via the inhibition of mTOR hyperactivation and biomarkers of inflammation and liver injury enzyme in rats.

Elevated levels of mTOR in TAA-induced chronic liver injury (Fig. 2) together with other previously published work (Wu *et al.*) that showed TAA caused liver fibrosis and increased levels of mTOR in mice and hepatic stellate cells, further strengthens the link between TAA and liver fibrosis and mTOR cell signaling. Indeed, mTOR was found to increase these profibrogenic biomarkers and hence promote fibrosis (Zhao *et al.*). In addition, inflammation is well-

known inducers of liver fibrosis, and an association between inflammation and mTOR in animal models of liver fibrosis was documented (Wang *et al.*, 2014), which are in agreement with our findings of elevated levels of mTOR, CRP, TNF- α , and IL-6 in TAA-induced liver injury.

Resveratrol was proposed as a novel treatment for diseases with mTOR pathway hyperactivation (Alayev *et al.*), which is in agreement with our data shown in Fig. 2. In addition, previous reports used resveratrol to successfully inhibit liver injuries induced by a chemical insult, CCl₄ in animal models and hepatic cells (Rivera *et al.*, 2008) that is also in agreement with our TAA and resveratrol data (Figs. 2 and 3 and Table I).

In summary, this study demonstrates that TAA induced liver injury, mTOR, and inflammation, which were protected by resveratrol.

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RESUMEN: La hepatotoxicidad crónica es una enfermedad debilitante y potencialmente mortal que produce insuficiencia hepática progresiva. La toxicidad del químico de la tioacetamida (TAA) se utiliza para evaluar los agentes hepatoprotectores y el compuesto polifenólico, resveratrol, se propuso como un nuevo tratamiento para enfermedades con hiperactivación de la vía de señalización celular mTOR (mammalian Target of Rapamycin). Aquí buscamos investigar el posible efecto protector del resveratrol contra la lesión hepática inducida por TAA a través de la inhibición de la vía de señalización mTOR en hepatocitos. Las ratas del grupo modelo recibieron varias inyecciones de TAA (200 mg / kg; dos veces por semana durante 8 semanas) antes de ser sacrificadas en la semana 10 y el grupo protector se trató previamente con resveratrol (20 mg / kg) diariamente durante dos semanas antes de las inyecciones de TAA y continuó recibiendo ambos agentes hasta el final del experimento. Se examinaron los tejidos hepáticos recolectados usando microscopía óptica y se analizaron los homogeneizados hepáticos para detectar biomarcadores de inflamación y se evaluaron los niveles de proteína mTOR en todos los grupos de animales. Además, se analizaron muestras de sangre para detectar biomarcadores de la enzima de lesión hepática. TAA dañó sustancialmente el tejido hepático del grupo modelo, con infiltración de células inflamatorias, citoplasma vacuolado, núcleos picnóticos oscuros y vasos sanguíneos congestionados dilatados que estaban efectivamente protegidos por el resveratrol. El resveratrol también inhibió significativamente ($p < 0.05$) mTOR, proteína C-reactiva (hs-CRP), factor de necrosis tumoral alfa (TNF- α), interleucina-6 (IL-6), alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST) en las muestras de sangre y de hígados

recolectados. En conclusión, el resveratrol protege eficazmente contra la hepatotoxicidad inducida por TAA en ratas, posiblemente debido a la inhibición de mTOR y de la inflamación.

PALABRAS CLAVE: Lesión hepática; Tioacetamida; mTOR; Resveratrol; Inflamación; Modelo de rata.

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