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Effective Inhibition of TNF-α/mTOR Axis-Mediated Liver Inflammation and Fibrosis Induced by TAA Using a Combination of Metformin and Resveratrol in Association with the Inhibition of the Profibrotic Gene and Protein Expression

Inhibición Efectiva de la Inflamación Hepática Mediada por el Eje TNF-α/mTOR y la Fibrosis Inducida por TAA Utilizando una Combinación de Metformina y Resveratrol en Asociación con la Inhibición del Gen Profibrótico y la Expresión de Proteínas

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SUMMARY: The response of the immune system to harmful stimuli leads to inflammation, and the adverse effects of the toxic hepatitis chemical, thioacetamide (TAA) on the human body are well documented. This article investigated the degree of protection provided by the combined pleotropic drug, metformin (Met) and the plant polyphenolic and the antiinflammatory compound, resveratrol (Res) on liver tissue exposed to TAA possibly via the inhibition of the inflammatory cytokine, tumor necrosis factor-α (TNF-α) / mammalian target of rapamycin (mTOR) axis-mediated liver fibrosis, as well as amelioration of profibrotic gene and protein expression. Rats were either given TAA (200 mg/kg via intraperitoneal injection) for 8 weeks beginning at the third week (experimental group) or received during the first two weeks of the experiment combined doses of metformin (200 mg/kg) and resveratrol (20 mg/kg) and continued receiving these agents and TAA until experiment completion at week 10 (treated group). A considerable damage to hepatic tissue in the experimental rats was observed as revealed by tissue collagen deposition in the portal area of the liver and a substantial increase ($p<0.0001$) in hepatic levels of the inflammatory marker, tumor necrosis factor-α (TNF-α), as well as blood levels of hepatocellular injury biomarkers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). TAA also augmented hepatic tissue levels of the signalling molecule that promotes liver fibrosis (mTOR), and profibrogenic markers; alpha-smooth muscle actin (α-SMA) protein, tissue inhibitor of metalloproteinases-1 (TIMP-1) mRNA, and matrix metalloproteinase-9 (MMP-9) mRNA. All these parameters were protected ($p\leq0.0016$) by Met+Res. In addition, a significant correlation was detected between liver fibrosis score and inflammation, liver injury enzymes, mTOR, and profibrogenesis markers. Thus, these findings suggest that Met+Res effectively protect the liver against damage induced by thioacetamide in association with the downregulation of the TNF-α/mTOR/fibrosis axis.

KEY WORDS: Thioacetamide; Inflammation; mTOR; Profibrosis; Liver fibrosis; Metformin; Resveratrol.

INTRODUCTION

Complications of liver injury like hepatic fibrosis can result in the failure of the organ that leaves liver transplantation from a donor as the only offered treatment protocol (Bzowej *et al*., 2011). Thus, reversion of liver fibrosis may provide a possible therapeutic option to avoid the advancement of the pathogenesis of liver fibrosis to cirrhosis and eventually, end-stage liver disease (Liedtke *et al*., 2013). Continuous hepatic insults with toxic agents like

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heavy alcohol use, chemicals, virus hepatitis, toxins, and some medications have been associated with the change in liver structure and function resulting in hepatic fibrosis, cirrhosis, hepatocarcinoma, and liver failure (Czaja, 2014; Chrostek & Panasiuk, 2014; Castaneda *et al*., 2021). Thioacetamide (TAA) is a toxic hepatitis chemical that was reported to induce liver fibrosis, cell necrosis, cirrhosis, hepatocarcinoma, and intrahepatic cholangiocarcinoma (Low *et al*., 2004; Guest *et al*., 2014).

Inflammation is implicated in stimulating liver fibrosis due to the stimulation of hepatic stellate cells (HSCs) that make most of the extracellular matrix components responsible for fibrosis (Robert *et al*., 2016). In addition, the adverse effect of the toxic hepatitis chemical, carbon tetrachloride was reduced in mouse models deficient of TNF-α or the TNF-α receptor gene (Morio *et al*., 2001), and liver fibrosis was reduced in 57 % to 79 % of patients treated with anti-inflammatory agents (Czaja, 2014). Carbon tetrachloride also described to promote hepatic fibrosis linked with the upregulation of hypoxia, mTOR and α-SMA expression (Zhao *et al*., 2014). Furthermore, an association with the augmentation of nitrosative stress and MMP-9 in mice with liver fibrosis induced by a high cholesterol diet was described (Anavi *et al*., 2015). In clinical and research, metformin and resveratrol are widely used for (i) liver protection (Zhang *et al*., 2021); (ii) inhibition of hepatic steatosis (Trepiana *et al*., 2018); (iii) inhibition of carbon tetrachloride-induced hepatic fibrosis (Yu *et al*., 2019); and (iv) a combination of metformin with atorvastatin added more protection to the liver in diabetic rats with dyslipidemia, which further decreased hepatic markers of inflammation and oxidative stress (Matafome *et al*., 2011). Accordingly, this study examined the extend of inhibition of TAA-induced hepatic fibrosis by a combination of metformin and resveratrol in rats in association with the suppression of the inflammation/mTOR axis-mediated fibrosis.

MATERIAL AND METHOD

Animals. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the Scientific Ethics Committee of Princess Nourah bin Abdulrahman University (Protocol Number: 20-0342). All surgery was performed under sodium pentobarbital anesthesia and every effort was made to minimize suffering.

Experimental design. 24 Albino rats (170-200 g) were split into 3 equal groups after five-day acclimization. The control (Control) group: rats were injected with the vehicle (intraperitoneal route). The TAA group: from week 3, this group received a number of injections of the hepatotoxic agent TAA (200 mg/kg, 2 per week) for a period of 8 weeks (Al-Hashem *et al*., 2019). The protective group (Met+Res+TAA): rats were given combined doseas of metformin (200 mg/kg) plus resveratrol (20 mg/kg) during the whole experimental period (2.5 months), and given TAA simillar to the experimental group; dosage and period. Blood was drawn under anaesthesia after 10 weeks, and rats were sacrificed, then liver tissue samples were processed for further examinations.

Histological examination. Liver tissue samples were fixed for 15 h in formalin (10 %) followed by alcohol dehydration. A standard method was used to prepare paraffin blocks. Paraffin wax was removed from the prepared sections (5 μm) and rehydrated. Liver sections were then stained with Sirius red to evaluate levels of collagen deposition (fibrosis). Morphometry of the areas percentage of collagen deposition were achieved by screening ten fields for each group using the image analyzer.

α-SMA immunohistochemistry of the liver. Processed tissue sections from the paraffin blocks were incubated with the primary antibody, anti-alpha-smooth muscle actin (α-SMA) (Cat # M0851, Dako, Santa Clara, CA, USA) for 14 hours in a humidity chamber after the antigen retrieval. Then, the secondary antibody was added for half hour. Sections were co-stained with Meyer hematoxylin.

TIMP-1 and MMP-9 qRT-PCR. RNeasy Mini Kit purchased from Qiagen Pty, Clayton, Victoria, Australia was used to isolate total RNA from liver homogenates. Complementary DNA (cDNA) prepared from the isolated RNA by the reverse transcriptase was amplified with primers specific for TIMP-1 (sense, 5'-GGT TCC CTG GCA TAA TCT GA-3'; antisense, 5'-GTC ATC GAG ACC CCA AGG TA-3'); MMP-9 (sense, 5'-CCT GCG TAT TTC CAT TCA TC-3'; antisense, 5'- GCC TTG GGT CAG GTT TAG AG -3'); and) β-actin using qRT-PCR kit (Thermo Fisher Scientific Inc, MA, USA). The relative expression was estimated based on the manufacturer's software.

mTOR western blotting analysis. 20 μg per sample of extracted proteins from liver tissues were used for immunoblotting analysis previously described (Al-Ani *et al*., 2010). Blotting membranes were incubated at 4 ºC overnight with anti-mTOR-phospho-S2448 obtained from Thermo Fisher Scientific, MA, USA. The ECL kit (Amersham-Pharmacia, UK) was used to visualize protein bands. The relative level of mTOR was acquired employing the Image analysis software after standardization with the loading control for western blotting, β-actin.

Assessment of liver and blood levels of inflammation and liver damage. ELISA kits purchased from Abcam, Cambridge, United Kingdom were used to evaluate tissue levels of TNF-α and IL-6 according to the instructions given by the manufacturers. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) blood levels were estimated using the enzymatic kits acquired from Randox Laboratories, Crumlin, UK.

Statistical analysis. The analyses of collected data were executed using version 10 of the SPSS. One-way ANOVA then Tukey's post hoc test was used to achieve statistical comparisons of data. Pearson correlation was done to find a probable significance between two dissimilar parameters. $p \leq 0.05$ was accepted to be statistically significant.

RESULTS

Induction of hepatic injury by the toxic hepatitis chemical TAA in rats. The first task was to model the disease (chronic hepatic injury) in rats in order to execute our investigation. TAA Administration to the experimental group for eight weeks triggered a significant ($p<0.0001$) rise in markers of liver damage (ALT and AST) and hepatic biomarker of fibrosis α-SMA (Fig. 1). Augmented ALT (Fig. 1A) and AST (Fig. 1B) blood levels in the TAA group compared to normal values in control rats was obtained. Liver tissue sections used for assessing α-SMA expression by immunohistochemistry approach revealed in the control rats (Fig. 1C) unremarkable levels of the protein expression compared to a profound augmentation of α-SMA expression in the TAA group of rats (Fig. 1D), which support the establishment of a chronic liver injury animal model.

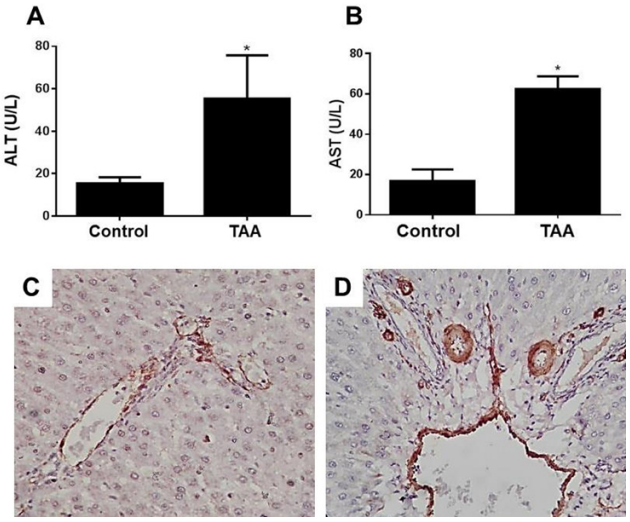

Fig. 1. TAA causes hepatic damage in rats. Liver injury enzymes, ALT (A) and AST (B) were evaluated after 2.5 months in the control group (Control) and the TAA group. *$p<0.0001$ versus control. (C and D) α-SMA immunohistochemistry stained images of liver sections from the control (C) and the TAA (D) groups are displayed using light microscopy.

Metformin (Met) plus resveratrol (Res) suppress TAA-induced inflammation as well as liver injury enzymes. Liver injury is well-known to be associated with the augmentation of inflammation (Robert *et al*., 2016). Therefore, these parameters were assessed in rats with and without Met+Res incorporation. In comparison with the control rats, TAA significantly ($p<0.0001$) augmented liver tissue and blood levels of TNF-α (Fig. 2A), IL-6 (Fig. 2B), ALT (Fig. 2C), and AST (Fig. 2D), which were markedly protected by Met+Res to levels comparable to the control

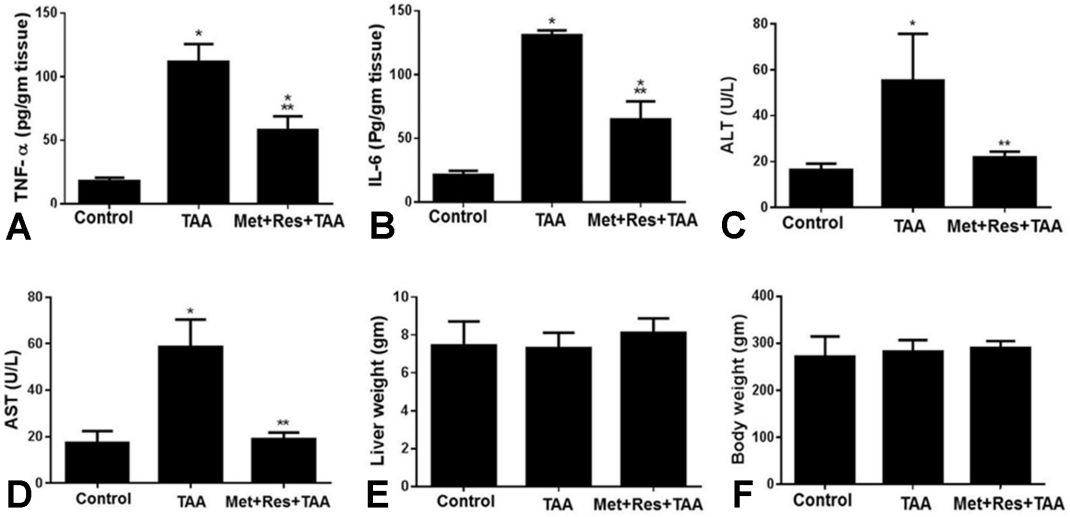

Fig. 2. TAA-induced inflammation and liver injury enzymes are inhibited by metformin (Met) plus resveratrol (Res). Biomarkers of inflammation TNF-α (A) and IL-6 (B) as well as biomarkers of liver damage ALT (C) and AST (D) were assessed at week 10 in all rats' group. (E and F) Liver and body weights were measured for all rats at week 10. *$p \leq 0.028$ versus control, **$p<0.0001$ versus TAA.

rats in (C and D). However, assessment of liver weight (Fig. 2E) and body weight (Fig. 2F) in all rats displayed no significant change between the animal groups ($p \geq 0.2756$).

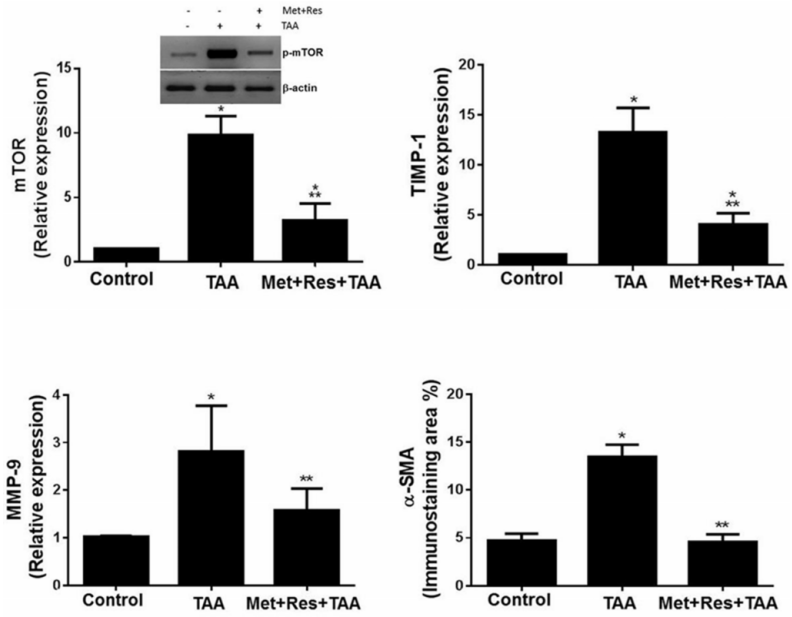

Fig. 3. Metformin (Met) plus resveratrol (Res) are associated with the amelioration of TAA-induced hepatic mTOR, TIMP-1, MMP-9, and α-SMA. Liver lysates prepared from the animal groups were immunoblotted for phospho-mTOR and the housekeeping gene protein α-actin (inset of A). - -, represent the Control; - +, represent TAA group; and + +, represent the treated group (Met+Res+TAA). The bar graph in (A) represents the relative expression of p-mTOR. The bar graph in (B and C) shows the relative gene expression of TIMP-1 (B) and MMP-9 (C) in all studied groups. A quantitative analysis of α-SMA immunostaining area percent in liver sections from all the rats' groups is displayed in (D). * p≤0.0026 versus control, **p≤0.0016 versus TAA.

Metformin (Met) plus resveratrol (Res) suppress TAA-induced hepatic mTOR and biomarkers of fibrosis in injured liver. In cell signaling pathway, mTOR is placed upstream of tissue fibrosis (Dawood *et al*., 2022) and the monoclonal antibody against the inflammatory biomarker TNF-α reduced mTOR expression in patients with a chronic inflammatory skin diseases (Balato *et al*., 2019). Therefore, hepatic tissue levels of mTOR and fibrosis markers (α-SMA, TIMP-1, and MMP-9) were evaluated at week 10 with and without Met+Res incorporation using Western blots, immunohistochemistry and qRT-PCR analyses (Fig. 3). TAA initiated an upsurge in mTOR (Fig. 3A), TIMP-1 (Fig. 3B), MMP-9 (Fig. 3C), and α-SMA (Fig. 3D) protein and gene expressions that appeared to be substantially protected by Met+Res to levels equivalent to the control in (C and D).

Metformin (Met) plus resveratrol (Res) inhibit liver fibrosis caused by TAA. We then assessed the extent of protection provided by Met+Res against TAA-induced collagen deposition (fibrosis) at week 10. Staining harvested liver tissues with Sirius red revealed weak collagen deposition in the control rats (Fig. 4A) compared with a strong positive fibrosis in the experimental rats (Fig. 4B) that showed coarse collagen deposition in the portal area of the liver. Met+Res treatment completely ($p = 0.809$) protected against liver fibrosis by inhibiting collagen deposition produced by TAA (Figs. 4C and 4D).

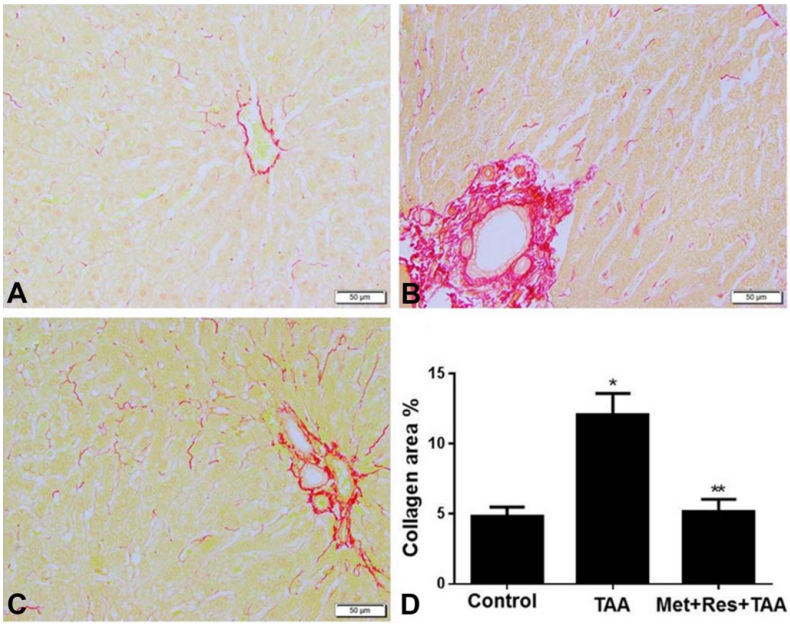

Fig. 4. TAA-induced hepatic fibrosis is blocked by metformin (Met) plus resveratrol (Res). Sirius red stained images (x200) of liver sections from the control (A), TAA (B), and Met+Res +TAA (C) groups are illustrated. (D) Degree of hepatic fibrosis (percent collagen deposition) gathered from Sirius red stain. Error bars indicate the mean (±SD). *p<0.0001 versus control, **p<0.0001 versus TAA.

Correlation between the score of liver fibrosis and the parameters associated with the induction of liver injury. To draw a connection between the pathogenesis of TAA-induced liver injury, the correlation between the score of liver fibrosis and the levels of protein and gene expression of TNF-α, IL-6, mTOR, TIMP-1, MMP-9, α-SMA, ALT, and AST was determined. This also endorses that the role of Met+Res are effective agents in liver injury. Figures 5A-5H presents a significant ($p<0.0001$) association between liver fibrosis score and the following parameters: TNF-α($r = 0.907$), IL-6 ($r = 0.860$), mTOR ($r = 0.925$), TIMP-1 ($r = 0.960$), MMP-9 ($r = 0.765$),α-SMA ($r = 0.974$), ALT ($r = 0.783$), and AST ($r = 0.847$).

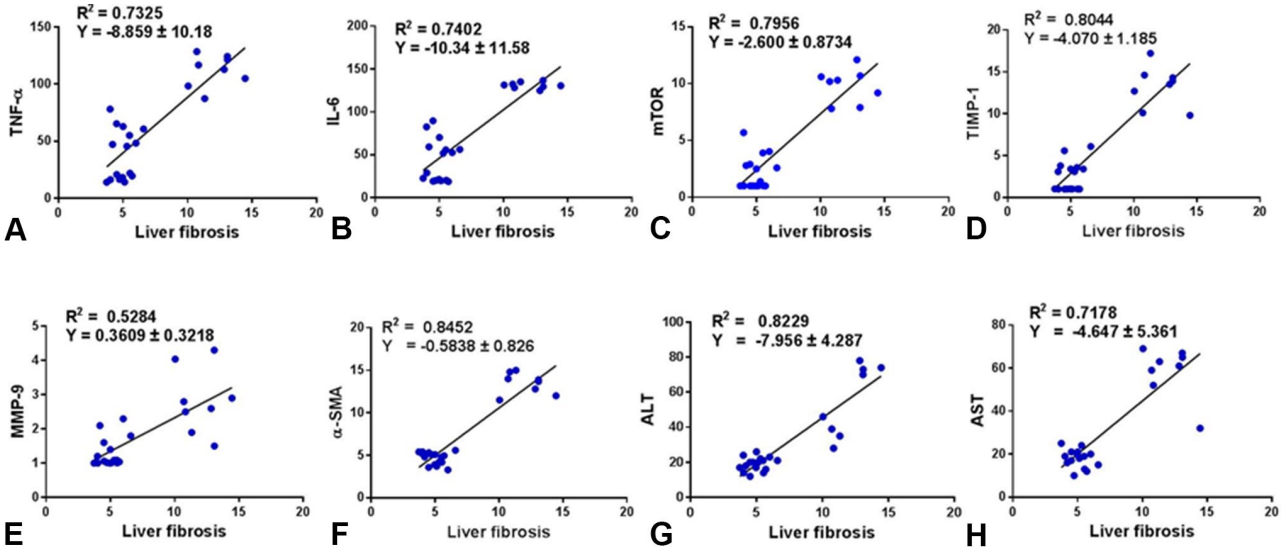

Fig. 5. Hepatic fibrosis score correlates with inflammation, mTOR, and biomarkers of fibrosis. The degree of hepatic fibrosis was assessed in all rats 2.5 months after the beginning of the experiment and a significant ($p<0.0001$) connection was noticed between hepatocyte damage versus TNF-α (a), IL-6 (b), mTOR (c), TIMP-1 (d), MMP-9 (e), α-SMA (f), ALT (g), and AST (h).

DISCUSSION

This report investigated the level of protection exerted by a combination of the pleotropic drug, metformin (Met) and the polyphenolic compound, resveratrol (Res) against chronic hepatic injury induced by the hepatotoxic chemical thioacetamide (TAA; C2H5NS) that has been used as a solvent in industry such as sulfide generator in the production of inorganic and organic compounds and pesticides. Various techniques like molecular, chemical, western blots, immunohistochemistry, and special stain for tissues were used to address the aim of the study; Met+Res can effectively protect against chronic liver injury caused by TAA in association with the inhibition of hepatic inflammation, mTOR, and biomarkers of fibrosis. The presented data here demonstrate that TAA intoxication caused a sharp increase in all parameters mentioned above, and Met+Res was able to effectively protect against the deleterious effects of TAA on liver tissue (Fig. 6). These results were therefore in agreement with our working hypothesis.

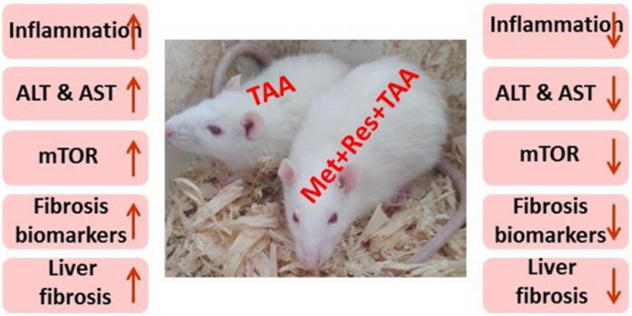

Fig. 6. Proposed model for hepatotoxicity induced by TAA and inhibited by Metformin (Met) plus resveratrol (Res). TAA: thioacetamide; mTOR: mammalian target of rapamycin.

Previous studies have shown partial effective inhibition of TAA- and CCl4-induced severe liver injury by metformin or resveratrol (Al-Hashem *et al*., 2019; Yu *et al*., 2019; Ebrahim *et al*., 2022). Indeed, (i) metformin partially protected the liver injury enzymes, inflammation, mTOR, TIMP-1, and α-SMA induced by TAA in rats (Al-Hashem *et al*., 2019); (ii) resveratrol also partially protected against inflammation, nitrosative stress, dyslipidemia, MMP-9, and hypoxia induced by TAA in liver and blood tissue (Ebrahim *et al*., 2022); and (iii) resveratrol slightly suppressed TNF-α levels in mice with hepatic fibrosis caused by CCl4 (Yu *et*

al., 2019). Whereas, our data presented here point to a more effective protection of the above mentioned parameters by Met+Res than either metformin or resveratrol alone, which justified the investigation. Indeed, previous reports point to the substantial inhibition of several pathological conditions by Met+Res such as hepatic steatosis via activating autophagy in association with the augmentation of cAMP/AMPK/SIRT1 cell signalling pathway (Afshari *et al*., 2023), inflammasomes-induced liver injury in diabetic rats (Rai *et al*., 2020), aging via mTOR and AMPK modulation (Sorrenti *et al*., 2022), and to treat diabetes supported by preclinical evidence that showed better effectiveness in protecting against diabetes (Dludla *et al*., 2020).

The liver is a recognized target organ for intoxication by TAA that led to hepatic fibrosis and cirrhosis after intraperitoneal injections of TAA for 12 weeks (Reif *et al*., 2004), which is in accordance with our data. However, they promoted MMP-9 as a collagenolytic enzyme, whereas our data and the work of Anavi *et al*. (2015) showed MMP-9 as a profibrogenic enzyme. Furthermore, our data that indicate the upregulation of TNF-α, TIMP-1, MMP-9, and α-SMA by TAA support published work showing (i) induction of liver fibrosis by TAA is associated with the activation of hepatic TNF-α, MMP-9, and TIMP-1 (Lin *et al*., 2017); and (ii) plasmid expressing the human antiinflammatory interleukin-10 (IL-10) decreased hepatic fibrosis in mice induced by CCl4 connected with the modulation of TIMP-1 and α-SMA (Chou *et al*., 2006).

Taken together, this study demonstrated in a rat model of chronic liver disease triggered by the toxic hepatitis chemical, thioacetamide the presence of an association between metformin plus resveratrol and the effective inhibition of hepatic tissue levels of inflammation, mTOR, fibrosis, as well as liver injury enzymes for duration of 10 weeks.

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RESUMEN: La respuesta del sistema inmunológico a estímulos dañinos conduce a la inflamación y los efectos adversos de la tioacetamida (TAA), una sustancia química tóxica para el hígado, están bien documentadas. Este artículo investigó el grado de protección proporcionado por el fármaco pleotrópico combinado metformina (Met), el polifenólico vegetal y el compuesto antiinflamatorio resveratrol (Res) en el tejido hepático expuesto a TAA, posiblemente a través de la inhibición de la citoquina inflamatoria, factor de necrosis tumoral α (TNF-α)/objetivo de la fibrosis hepática mediada por el eje de rapamicina (mTOR), así como mejora de la expresión de genes y proteínas profibróticas. Las ratas recibieron TAA (200 mg/kg mediante inyección intraperitoneal) durante 8 semanas a partir de la tercera semana (grupo experimental) o recibieron durante las dos primeras semanas del experimento dosis combinadas de metformina (200 mg/kg) y resveratrol (20 mg/kg) y continuaron recibiendo estos agentes y TAA hasta completar el experimento en la semana 10 (grupo tratado). Se observó un daño considerable al tejido hepático en las ratas experimentales, como lo revela el depósito de colágeno tisular en el área portal del hígado y un aumento sustancial ($p<0,0001$) en los niveles hepáticos del marcador inflamatorio, el factor de necrosis tumoral-α (TNF- α), así como los niveles sanguíneos de biomarcadores de lesión hepatocelular, alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST). TAA también aumentó los niveles en el tejido hepático de la molécula de señalización que promueve la fibrosis hepática (mTOR) y marcadores profibrogénicos; proteína actina del músculo liso alfa (α-SMA), inhibidor tisular de las metaloproteinasas-1 (TIMP-1) mRNA y matriz metaloproteinasa-9 (MMP-9) mRNA. Todos estos parámetros fueron protegidos ($p\leq0.0016$) por Met+Res. Además, se detectó una correlación significativa entre la puntuación de fibrosis hepática y la inflamación, las enzimas de lesión hepática, mTOR y los marcadores de profibrogénesis. Por lo tanto, estos hallazgos sugieren que Met+Res protege eficazmente el hígado contra el daño inducido por la tioacetamida en asociación con la regulación negativa del eje TNF-α/mTOR/fibrosis.

PALABRAS CLAVE: Tioacetamida; Inflamación; mTOR; Profibrosis; Fibrosis hepática; metformina; Resveratrol.

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