Metformin Inhibits ROS/TNF-α Axis-Mediated Chronic Kidney Disease Induced by TAA Independent of Leukocyte Infiltration in Association with the Inhibition of Kidney Injury Biomarkers

La Metformina Inhibe la Enfermedad Renal Crónica Mediada por el Eje ROS/TNF-α Inducida por TAA Independientemente de la Infiltración de Leucocitos en Asociación con la Inhibición de Biomarcadores de Lesión Renal

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SUMMARY: The toxic effects of thioacetamide (TAA) and carbon tetrachloride on the human body are well recognized. In this study, we examined whether TAA intoxication can induce kidney leukocyte infiltration (measured as leukocyte common antigen CD45) associated with the augmentation of the reactive oxygen species (ROS)/tumor necrosis factor-alpha (TNF-α) axis, as well as biomarkers of kidney injury with and without metformin treatment. Rats were either injected with TAA (200 mg/kg; twice a week for 8 weeks) before being sacrificed after 10 weeks (experimental group) or were pre-treated with metformin (200 mg/kg) daily for two weeks prior to TAA injections and continued receiving both agents until the end of the experiment, at week 10 (protective group). Using basic histology staining, immunohistochemistry methods, and blood chemistry analysis, we observed profound kidney tissue injury such as glomerular and tubular damage in the experimental group, which were substantially ameliorated by metformin. Metformin also significantly ($p<0.05$) inhibited TAA-induced ROS, TNF-α, urea, and creatinine in harvested kidney homogenates and blood samples. In addition, a significant ($p<0.0001$) positive correlation between kidney tubular epithelial cell injury and the serum levels of biomarkers of oxidative stress, inflammation, and kidney injury was observed. However, TAA caused no significant ($p>0.05$) increase in kidney expression of CD45 positive immunostaining cells. In conclusion, we found that TAA induces kidney injury in association with the augmentation of ROS/TNF-α axis, independent of leukocyte infiltration, which is protected by metformin.

KEY WORDS: Thioacetamide; Kidney injury; ROS; Inflammation; CD45; Metformin.

INTRODUCTION

Industrial toxicants such as thioacetamide (TAA) and carbon tetrachloride that induce liver and kidney damage are used in medical research to generate animal models that mimic human diseases, enabling the dissection of major signalling pathways involved in the pathophysiology of the disease, as well as evaluating potential protective agents (Mochizuki *et al.*, 2009; Al-Hashem *et al.*, 2019). TAA is a severe hepatotoxic compound that causes liver fibrosis, cirrhosis, and liver cancer (De Minicis *et al.*, 2013). TAA also induces kidney damage via ROS activation and inhibition of the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway, which plays a significant role in cell metabolism, growth, and proliferation (Ghosh *et al.*, 2016). DNA damage in kidney cells measured as a sharp

elevation of 8-hydroxy-2-deoxyguanosine (8-OHdG) was reported in rats treated with TAA (Zargar *et al.*, 2019).

Infiltration of leukocytes into the renal system in response to insults is well-documented. For example, (i) kidney leukocyte infiltration is highly expressed in a mouse model of chronic nephritis induced by lupus, which was correlated with the severity of the disease (Adalid-Peralta *et al.*, 2008); (ii) kidney infiltration of macrophages, monocytes, and helper T cells were observed post ischemia-reperfusion renal injury (Ysebaert *et al.*, 2003); and (iii) circulating leukocytes contribute to the development and progression of nephropathy in patients with type 2 diabetes (Chung *et al.*, 2005). In addition, inflammatory cytokines such as TNF-α and IL-1

are highly expressed in renal tissue in kidney diseases such as diabetic nephropathy (Donate-Correa *et al.*, 2021) and end-stage kidney failure (Ortega & Fornoni, 2010).

The antidiabetic drug, metformin ameliorates several types of liver and kidney diseases such as nonalcoholic fatty liver disease (Matafome *et al.*, 2011), protects primary rat hepatocytes against oxidative stress-induced apoptosis (Conde de la Rosa *et al.*, 2015), kidney injury secondary to diabetes (Dawood *et al.*, 2022), and nephrotoxicity induced by the antibiotic gentamicin (Morales *et al.*, 2010). However, the potential infiltration of leukocytes into kideny tissue upon TAA intoxication has not been invesigated before in animal models. Therefore, this study examined whether TAA injection into rats can induce kidney leukocyte infiltration associated with the activation of the ROS/ TNF-α axis with and without metformin treatment.

MATERIAL AND METHOD

Animals. Male albino rats weighing 180-200 g were used in the experiment. The study followed 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 was approved by the Ethical Committee at King Khalid University. The rats were housed at a controlled room temperature with 12-hour light/dark cycles and had free access to food and water.

Experimental design. After acclimatization, the rats were separated into three groups (n=8 per group). The control group (Control) of rats were not 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 (Met+TAA) of rats was given Met (200 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 culled by cervical dislocation, and kidney tissue specimens were harvested.

Measurements of MDA, TNF-α, hsCRP, Urea, and Creatinine. ELISA kits for blood determination of malondialdehyde (MDA) from Cyaman Chemical, Ann Arbor, MI, USA; TNF-α (Abcam, Cambridge, UK), and hsCRP (Assaypro, Saint Charles, MO, USA) were used to measure MDA, TNF-α, and hsCRP according to the manufacturer's instructions. Blood urea and creatinine were measured using colorimetric methods according to the manufacturer's instructions (BioAssay System, USA).

Histological examination. Harvested kidney specimens were fixed overnight in 10 % formalin and then dehydrated with ascending grades of alcohol. Paraffin blocks were prepared by the standard method, and 5-μm thick sections were deparaffinized and rehydrated. Kidney sections were then stained with hematoxylin and eosin (H&E) staining, as well as Sirus red staining to assess kidney collagen deposition as previously reported (Dawood *et al.*, 2022).

Morphometry. The degree of kidney tubular injury was analysed in 10 non-overlapping fields for each group and given a semi-quantitative score of 0-3 (0 = no injury, 1 = mild injury, 2 = moderate injury, and 3 = severe injury).

Immunohistochemistry of CD45. Immunohistochemical staining was performed using anti-CD45 (Abcam, Cambridge, UK) as a marker for leukocyte infiltration. Antigen retrieval was conducted, followed by the application of the primary antibody overnight in a humidity chamber and the secondary antibody for 30 minutes. Sections were co-stained with Meyer hematoxylin.

Statistical analysis. Data were analyzed using SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. To detect a significant relationship between two different parameters, Pearson correlation was performed. A significance level of $p \leq 0.05$ was used.

RESULTS

Induction of hepato-renal injury in rats by TAA. TAA is a known hepato-nephrotoxic agent (Schyman *et al.*, 2018). Therefore, to investigate the aim of this study, we first sought to demonstrate in our animal model the induction of liver and kidney injury by TAA. Injection of rats with TAA (200 mg/kg body weight) twice a week for 8 weeks caused the development of hepatonephrotoxicity as demonstrated by a sharp increase in the blood levels of the hepatic injury biomarker, ALT (Fig. 1A) and the kidney injury biomarker, creatinine (Fig. 1B). In addition, a representative Sirus red-stained image of kidney sections prepared from the TAA group (Fig. 1D) shows intense thick collagen deposition in the interstitium (star) and in the basement membranes (arrow). Whereas, another image of kidney section at a similar magnification prepared from the control rats (Fig. 1C) reveals a weak collagen deposition in the basement membrane of the renal corpuscle and the convoluted tubules (arrowhead).
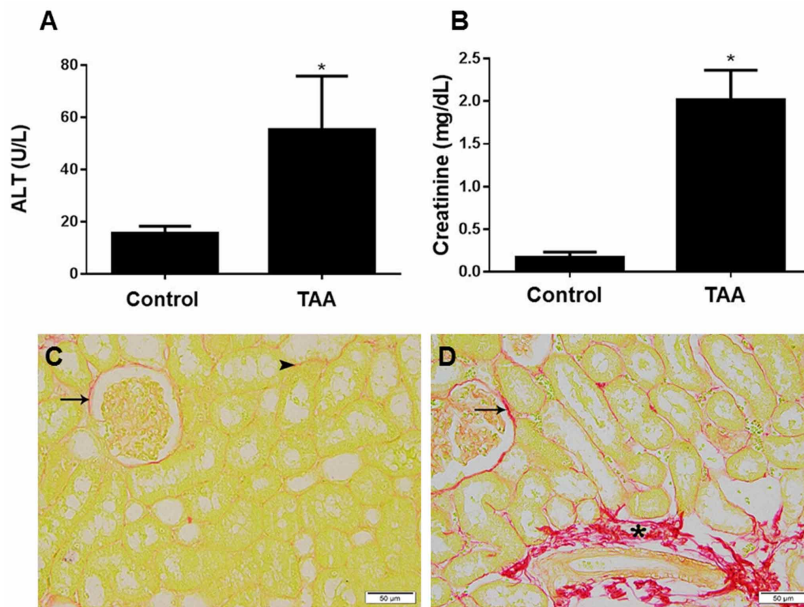
Fig. 1. TAA induces hepato-renal intoxication in rats. Blood levels of ALT (A) and creatinine (B) were assessed after 10 weeks in the control and experimental (TAA) rats' groups. Presented p values are significant. *p<0.0001 versus control. (C and D) Sirus red stained images (x200) of kidney sections from the control (C) and TAA (D) groups are depicted. ALT: alanine aminotransferase; TAA: thioacetamide.

TAA-induced ROS, TNF-α, and biomarkers of kidney injury are inhibited by metformin. ROS induction is a hallmark of renal injury (Dallak *et al.*, 2022). Therefore, we evaluated ROS levels (measured as MDA), TNF-α, hsCRP, urea, and creatinine in all rats with and without the incorporation of metformin. TAA increased the blood levels of MDA (Fig. 2A), TNF-α (Fig. 2B), hsCRP (Fig. 2C), urea (Fig. 2D), and creatinine (Fig. 2E), which was significantly (p<0.0001) inhibited by metformin treatment (Met+TAA) to levels comparable to control in (A) and (B). Whereas, the treatment with metformin showed only partial inhibition (p≤ 0.0073) comparable to control in (C) and (D). However, TAA intoxication had no effect on the animals' body weight (Fig. 2F).
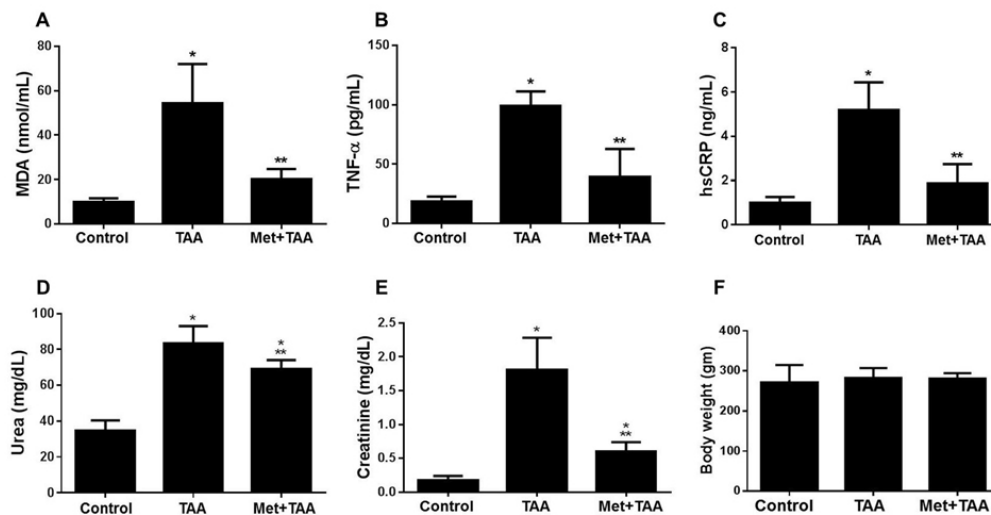
Kidney leukocyte infiltration is not induced in chronic renal disease caused by TAA in rats. Conflicting data on the induction of blood leukocytes upon TAA intoxication were reported (Enciso *et al.*, 2022; Al-Attar, 2022). Therefore, we sought to determine whether leukocyte infiltration into kidney tissues upon TAA intoxication is observed in the presence and absence of metformin treatment. The biomarker of leukocytes (CD45) was assessed in kidney sections prepared from all rats' group using immunohistochemistry. There was no difference in the intensity of CD45 +ve immunostaining between the control (Fig. 3A), the model group (TAA) (Fig. 3B), and the treated group (Met+TAA) (Fig. 3C). Quantitative analysis of CD45 immunostaining area percent revealed no significant (p>0.05) difference between all rats (data not shown).

Basic histology staining (H&E) of kidney sections revealed a normal tissue architecture from the control group (Fig. 3D). Whereas, a substantial damage to the renal corpuscles (the glomerulus and the Bowman's capsule) as well as the renal tubules displayed in tissue sections obtained from the model group (Fig. 3E). The treatment group (Figs. 3F and 3G) showed a significant (p<0.0001) protection to the kidney architecture. However, metformin provided partial protection.

Fig. 2. Metformin (Met) inhibits TAA-induced MDA, TNF-α, hsCRP, urea, and creatinine. Blood levels of hsCRP (A), TNF-a (B), MDA (C), urea (D), and creatinine (E) as well as body weight (F) were assessed after 10 weeks in all rats. Presented p values are all significant; *p≤ 0.0073 versus control, **p<0.0001 versus TAA. MDA: malondialdehyde; TNF-α: tumor necrosis factor-alpha; hsCRP: high sensitive C-reactive protein; TAA: thioacetamide.
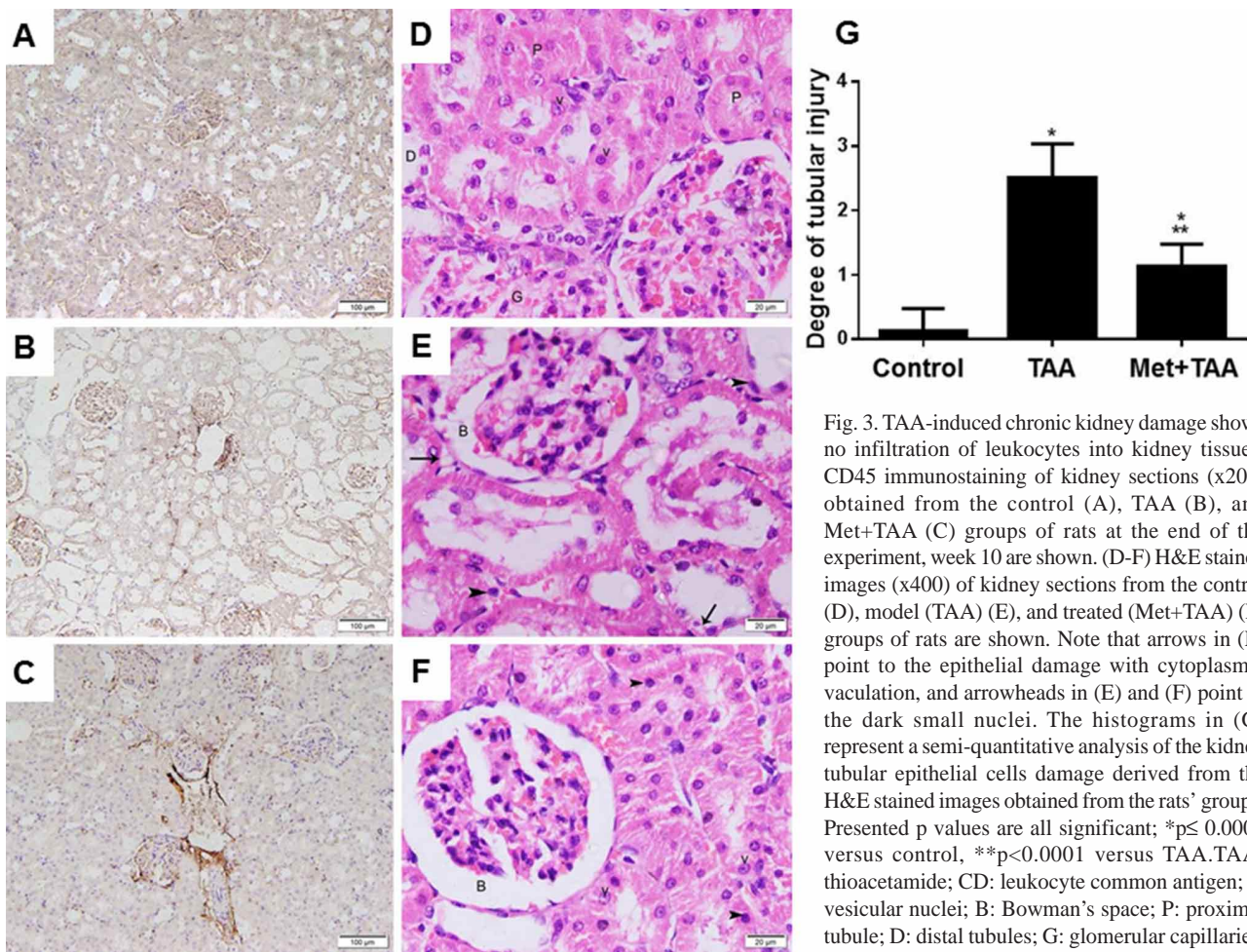
Fig. 3. TAA-induced chronic kidney damage shows no infiltration of leukocytes into kidney tissues. CD45 immunostaining of kidney sections (x200) obtained from the control (A), TAA (B), and Met+TAA (C) groups of rats at the end of the experiment, week 10 are shown. (D-F) H&E stained images (x400) of kidney sections from the control (D), model (TAA) (E), and treated (Met+TAA) (F) groups of rats are shown. Note that arrows in (E) point to the epithelial damage with cytoplasmic vaculation, and arrowheads in (E) and (F) point to the dark small nuclei. The histograms in (G) represent a semi-quantitative analysis of the kidney tubular epithelial cells damage derived from the H&E stained images obtained from the rats' groups. Presented p values are all significant; *$p \leq 0.0003$ versus control, **$p < 0.0001$ versus TAA.TAA: thioacetamide; CD: leukocyte common antigen; v: vesicular nuclei; B: Bowman's space; P: proximal tubule; D: distal tubules; G: glomerular capillaries.
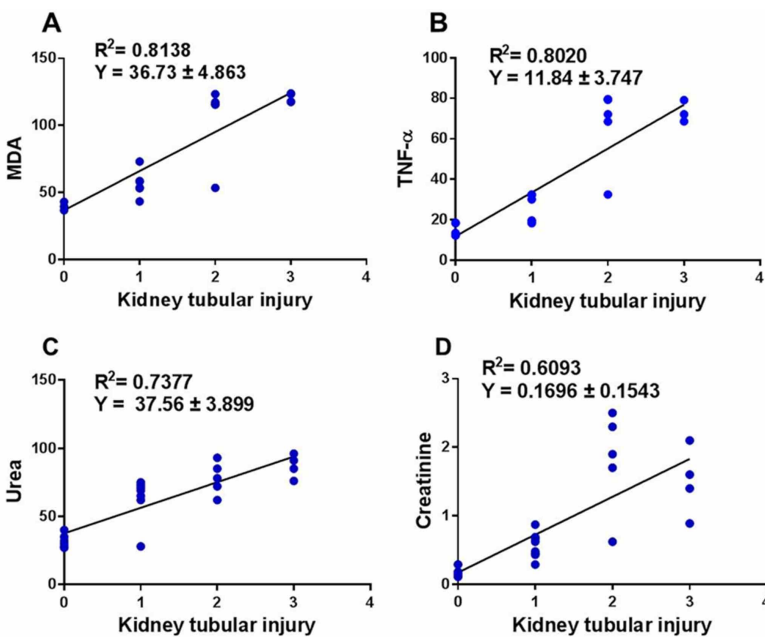
Correlation between kidney tubular injury score and kidney injury biomarkers. The correlation between the score of tubule injury derived from H&E staining kidney tissue and ROS, TNF-α, as well as biomarkers of chronic kidney disease (urea and creatinine) was assessed. This correlation links kidney TAA intoxication with these parameters associated with kidney injury. It also supports the diverse effects of metformin. A significant ($p < 0.0001$) positive correlation between the degree of tubular injury and MDA ($r = 0.902$) (Fig. 4A), TNF-α ($r = 0.896$) (Fig. 4B), urea ($r = 0.859$) (Fig. 4C), and creatinine($r = 0.781$) (Fig. 4D) was observed.

Fig. 4. Correlation between the scoring of kidney tubular damage and biomarkers of oxidative stress, inflammation, and kidney injury. Degree of renal tubular damage was evaluated in all rats at the end of the experiment to enable drawing a link between renal tubule injury and MDA (A), TNF-α (B), urea (C), and creatinine (D). MDA: malondialdehyde; TNF-α: tumor necrosis factor-alpha.

DISCUSSION

In this study, we investigated whether TAA-induced nephrotoxicity is associated with kidney infiltration of leukocytes in a rat model of chronic kidney disease, as well as the augmentation of the ROS/TNF-α axis and degree of renal tubule damage in the presence and absence of metformin. Our data showed that TAA intoxication increased both, the ROS/TNF-α axis and tubular injury, but not CD45. However, metformin significantly inhibited all these parameters, indicating that metformin is an appropriate medicine in chronic kidney disease. These conclusions are supported by the data indicating that TAA induced kidney injury, oxidative stress, TNF-α, hs-CRP, urea, and creatinine, but not leukocyte infiltration (CD45) into kidney tissue, which were substantially inhibited by metformin (Figs. 1-3). Additionally, our data showed a positive correlation between tubule injury scoring and all the above-mentioned parameters (Fig. 4), which supports our conclusion, mentioned above and is summarized in Fig. 5. Therefore, our data support our working hypothesis.

Upregulation of ROS and TNF-α tissue expression is demonstrated in many renal diseases such as chronic kidney disease secondary to diabetes (Navarro-González & Mora-Fernández, 2008; Dabla, 2010), acute kidney injury caused by acetaminophen toxicity in rats (Dallak *et al.*, 2022), as well as TAA-induced kidney ROS (Ghosh *et al.*, 2016) and TNF-α (Ghanim *et al.*, 2022). In addition, an association between TNF-α–induced liver injury and ROS was reported (Schwabe & Brenner, 2006). Therefore, these reports are in agreement with our findings shown in Figs. 2 and 3. Moreover, our data that point to the inhibition of ROS and TNF-α by metformin induced due to TAA intoxication in rats are in agreement with previous work on metformin inhibition of TAA-induced liver injury and fibrosis, as well as TNF-α (Al-Hashem *et al.*, 2019) and ROS (Al-Hashem *et al.*, 2018).

Kidney CD45+ve immunostaining was reported in diabetic mice (Zheng & Epstein, 2021; Fu *et al.*, 2022), which is not in agreement with our data displaying negative CD45 immunostaining in kidney tissue treated with TAA (Fig. 3). However, our data are in concordance with previous reports showing (i) no effects of TAA (10 and 30 mg/kg administered orally for 28 days) on blood leukocyte count in rats (Lim *et al.*, 2022); and (ii) no significant difference in total blood leukocytes, lymphocytes, and body weight in rats treated for 24 weeks with 200 mg/kg TAA injected intraperitoneally (Enciso *et al.*, 2022).

In summary, the presented data in this study demonstrate that in a rat model of TAA-induced chronic kidney disease, the induction of ROS and inflammation without kidney infiltration of leukocytes seemed to be inhibited for a period of 10 weeks with metformin in rats. Additionally, a link between renal tubule injury and the above parameters is observed, with metformin showing beneficial renal pleiotropic effects. Efforts are underway to assess the self-renewed kidney resident macrophages that can promote inflammation and fibrosis, as well as expressing high and low levels of F4/80 and CD68 markers, respectively (Puranik *et al.*, 2018).

ACKNOWLEDGMENTS

Fig. 5. Proposed model for chronic kidney disease induced by TAA which appear to be inhibited by metformin. TAA: thioacetamide; Met: metformin; ROS: reactive oxygen species; CD: leukocyte common antigen.

AL-HASHEM, F. La metformina inhibe la enfermedad renal crónica mediada por el eje ROS/TNF-α inducida por TAA independientemente de la infiltración de leucocitos en asociación con la inhibición de biomarcadores de lesión renal. *Int. J. Morphol., 41(4)*:1191-1197, 2023.

RESUMEN: Son bien conocidosos los efectos tóxicos de la tioacetamida (TAA) y el tetracloruro de carbono en el cuerpo humano. En este estudio, examinamos si la intoxicación por TAA puede inducir la infiltración de leucocitos renales (medida como antígeno leucocitario común CD45) asociada con el aumento de las especies reactivas de oxígeno (ROS)/factor de necrosis tumoral-alfa (TNF-α), así como biomarcadores de daño renal con y sin tratamiento con metformina. A las ratas se les inyectó TAA (200 mg/kg; dos veces por semana durante 8 semanas) antes de sacrificarlas a las 10 semanas (grupo experimental) o se les pretrató con metformina (200 mg/kg) diariamente durante dos semanas antes de las inyecciones de TAA y continuaron recibiendo ambos agentes hasta el final del experimento, en la semana 10 (grupo protector). Usando tinción histológica básica, métodos de inmunohistoquímica y análisis químico de la sangre, observamos una lesión profunda del tejido renal, como daño glomerular y tubular en el grupo experimental, que mejoraron sustancialmente con la metformina. La metformina también inhibió significativamente (p<0,05) las ROS, TNF-α, urea y creatinina inducidas por TAA en muestras de sangre y homogeneizados de riñón recolectados. Además, se observó una correlación positiva significativa (p<0,0001) entre la lesión de las células epiteliales tubulares renales y los niveles séricos de biomarcadores de estrés oxidativo, inflamación y lesión renal. Sin embargo, TAA no provocó un aumento significativo (p>0,05) en la expresión renal de células de inmunotinción positivas para CD45. En conclusión, encontramos que el TAA induce la lesión renal en asociación con el aumento del eje ROS/TNF-α, independientemente de la infiltración de leucocitos, que está protegida por metformina.

PALABRAS CLAVE: Tioacetamida; Lesión renal; ROS; Inflamación; CD45; Metformina.

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