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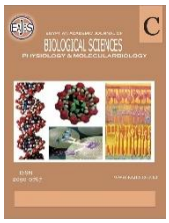

Immunomodulation by Simvastatin: A Key Mechanism in Attenuating Methotrexate-Induced Kidney Injury Through NF-κB and Oxidative Stress

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ABSTRACT

Methotrexate (MTX) is a widely used chemotherapeutic and immunosuppressive agent known to induce renal toxicity through mechanisms involving oxidative stress, inflammation, and apoptosis. This study aimed to evaluate the renoprotective effects of SIM against MTX-induced nephrotoxicity in rats. Thirty-two male Sprague-Dawley rats were randomly divided into four groups: control, SIM, MTX, and MTX+SIM. MTX administration significantly elevated serum Cr and urea levels increased renal MDA, and suppressed antioxidant enzymes, including SOD, CAT, and GPx. It also upregulated inflammatory markers such as NF-κB, TNF-α, IL-6, and IL-1β, and triggered apoptosis via increased expression of p53, cytochrome c, Bax, and caspase-3. Co-treatment with SIM significantly mitigated these pathological changes, restoring renal function, enhancing antioxidant defenses, suppressing inflammation, and downregulating apoptotic proteins. These findings highlight the multi-targeted therapeutic potential of SIM in mitigating MTX-induced renal injury and preserving kidney integrity.

INTRODUCTION

Despite their frequent use in managing malignancies, chemotherapeutic drugs are potentially hazardous, causing harmful side effects in various organs that necessitate careful management (Chen *et al.,* 2009; Minami *et al.,* 2010). A key example is Methotrexate (MTX), a potent antimetabolite and antifolate medication. It works by disrupting cellular metabolism and inhibiting the synthesis of purines and pyrimidines, thereby preventing cellular growth. Due to this mechanism, MTX is valuable as both an immunosuppressive agent and a chemotherapeutic drug. It is often prescribed in combination with other agents to treat a wide range of cancers, including acute lymphoblastic leukemia, osteosarcoma, breast cancer, brain tumours, lymphomas, and various carcinomas (Crews *et al.,* 2004; Khan *et al.,* 2012; Wu *et al.,* 2017; Chen *et al.,* 2020; Koźmiński *et al.,* 2020).

The antifolate mechanism of Methotrexate (MTX) involves interference with thymidine and purine biosynthesis, which is essential for DNA replication, repair, and cellular proliferation. Since the renal system is responsible for eliminating 90% of the drug, nephrotoxicity represents one of the most significant side effects of MTX therapy (Sales & Foresto, 2020; Jalili *et al.,* 2020). High-dose regimens, in particular, may induce acute renal failure in 2–12% of patients, primarily through the precipitation of the drug in the renal tubules a pathology termed crystalline nephropathy (Sales & Foresto, 2020).

Further toxicities associated with MTX treatment encompass mucositis, hepatitis, and gastrointestinal complications (Ramsey *et al.,* 2018).

MTX-induced renal damage is driven by the intratubular accumulation of both the drug and its 7-hydroxy metabolite, which establishes a vicious cycle that exacerbates toxicity (Hamed *et al.,* 2022). The pathogenic process is multifactorial, involving oxidative injury, inflammatory infiltration, and apoptotic cell death (Arab *et al.,* 2018; Wasfey *et al.,* 2023). At a molecular level, studies have demonstrated that this is mediated by the activation of NADPH oxidase, resulting in massive ROS generation (Abraham *et al.,* 2010), coupled with the disruption of the cytoprotective Nrf2 signaling pathway (Hussein *et al.,* 2020). The resulting state of severe oxidative stress then activates pro-inflammatory cascades, such as those involving TNF-α, leading to further renal tissue injury (Aladaileh *et al.,* 2019).

Beyond their primary role in preventing hypercholesterolemia and cardiovascular disease (Kapur & Musunuru, 2008), statins exhibit beneficial pleiotropic effects. These lipid-lowering agents have demonstrated the ability to mitigate OS and inflammation in DM and its complications (Al-Rasheed *et al.,* 2018; Al-Rasheed *et al.,* 2017). Evidence suggests these positive outcomes stem not only from cholesterol regulation but also from cholesterol-independent properties, including plaque stabilization, and direct antioxidant and anti-inflammatory actions (Verdoodt *et al.,* 2018). Consequently, statins have been shown to inhibit inflammation and OS in experimental models of kidney injury, such as renal ischemia/reperfusion and gentamicin nephrotoxicity (Ozbek *et al.,* 2009; Teshima *et al.,* 2010). The objective of this work was to evaluate the potential renoprotective effects of simvastatin (SIM) in the context of methotrexate (MTX) nephrotoxicity, with a specific focus on mechanisms underlying its amelioration of oxidative stress and inflammatory pathways.

MATERIALS AND METHODS

Experimental Animals:

The study utilized 32 adult male Sprague-Dawley rats (180–200 g), which were housed in groups of four under controlled conditions (24 ± 2°C, 55 ± 5% humidity, 12-hour light/dark cycles) with unrestricted access to food and water. Following a one-week acclimatization period, the rats were randomly allocated into four equal groups of eight. The protocol received prior approval from the Medical Research Ethics Committee at Umm Al-Qura University (Approval code: HAPO-02-K-012-2025-10-2940).

Experimental Treatment Protocols:

Male rats (n = 8 per group) were randomly allocated into four groups for a 10-day experiment: a control group receiving saline; a SIM group orally administered simvastatin (40 mg/kg/day) for 10 days (Nežić *et al.,* 2020); an MTX group injected with a single dose of methotrexate (20 mg/kg, i.p.) on day 4; and an MTX+SIM group receiving both treatments at the aforementioned doses and schedule. The MTX dosage was selected based on previous studies (Armagan *et al.,* 2015; Hafez *et al.,* 2015). On day 10, rats were euthanized via cervical dislocation under diethyl ether anesthesia. Both kidneys were collected; the right kidney was fixed for histological examination, while the left kidney was homogenized for biochemical analysis. Blood samples were centrifuged, and the obtained sera were stored at −80°C until further use.

Assessment of Renal Function and Oxidative Stress Parameters:

Serum levels of urea and Cr, markers of renal function, were measured using commercial kits (Bio-Diagnostic, Egypt; Urea: UR2110, Creatinine: CR1250) and reported in mg/dl.

To assess oxidative stress, kidney tissue homogenates were prepared. Briefly, frozen renal cortex samples were thawed and homogenized in a 10-fold volume (w/v) of ice-cold Tris-HCl buffer (pH 7.4) containing a 1% protease inhibitor cocktail. The

homogenization was carried out at 4000 rpm, and the resulting supernatant was collected following centrifugation for subsequent analysis. The supernatant was then used to spectrophotometrically determine lipid peroxidation and antioxidant enzyme activities. The extent of lipid peroxidation was assessed by quantifying MDA levels according to the method of Wong *et al.* (1987), with results expressed in nanomoles per gram of tissue (nmol/g). The activities of key antioxidant enzymes were analyzed as follows: SOD by the technique of Sun *et al.* (1988) in units per gram of tissue (U/g), CAT by the method of Aebi (1984) in micromoles per milligram of tissue (μmol/mg), GPx according to Koracevic *et al.* (2001) in micrograms per milligram of tissue (μg/mg)

ELISA Analysis:

The concentrations of inflammatory cytokines (NF-κB p65, TNF-α, IL-6, IL-1β; Cat# E-EL-R0674, E-EL-R2856, E-EL-R0015, MBS2023030) and apoptosis-related proteins (p53, Cytochrome-C, Bax, caspase-3; Cat# ERA47RB, MBS165286, MBS2512405, MBS261814) were quantified in kidney homogenates using specific ELISA kits according to the manufacturers' protocols.

Histological Analysis:

For histopathological analysis, the renal tissues were fixed in formalin and then processed through a series of steps including dehydration, clearing with xylene, and paraffin embedding. The embedded tissues were then sliced into thin sections (4–6 μm) and stained with hematoxylin and eosin (H&E) for examination (Bancroft & Gamble, 2008).

Statistical Analysis:

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in GraphPad Prism software (version 8.0.2). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Protective Effect of Simvastatin on MTX-Induced Renal Impairment and Histological Alterations:

A single intraperitoneal administration of methotrexate (MTX) on the fourth day of the study significantly elevated serum levels of renal function markers creatinine and urea ($p < 0.05$; Fig. 1A, B). This biochemical impairment was corroborated by histological examination of the renal cortex, which revealed marked tubular degeneration, dilation, and cast formation (Fig. 1E). In contrast, control rats exhibited normal renal function levels and preserved histological architecture, including intact glomeruli and tubules (Fig. 1C, D). Notably, coadministration of MTX with simvastatin (SIM) resulted in a significant improvement in renal function parameters and a marked reduction in tubular degeneration (Fig. 1F). These findings demonstrate the renoprotective effect of SIM, as evidenced by both biochemical and histological evaluations.

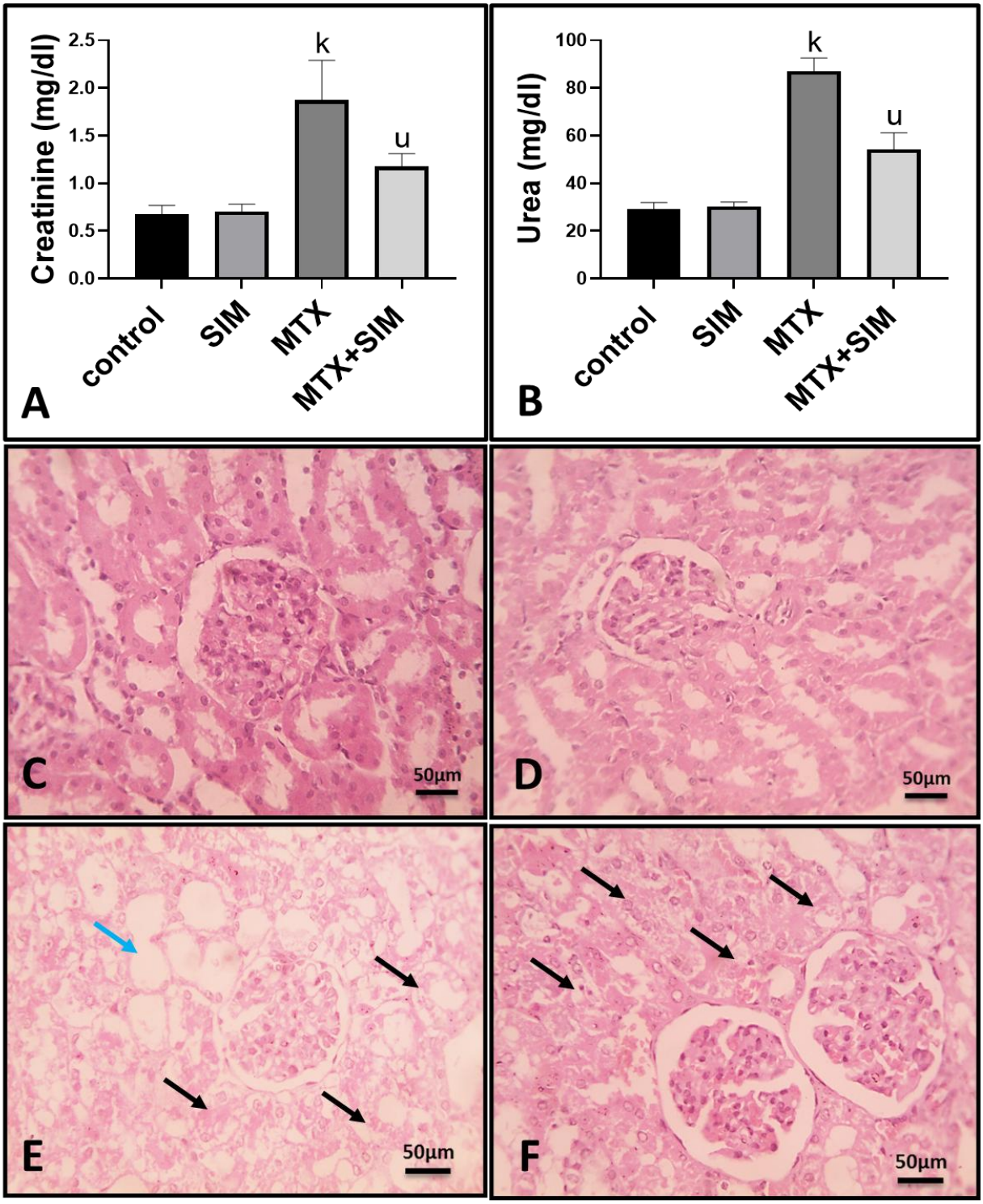

Fig.1: (A, B) Serum level of creatinine and urea in different groups. (C, D) Microscopic pictures of HE stained kidneys of control rats and rats received SIM showing normal cortex. (E) Rats received MTX showing severe tubular degeneration (black arrows), tubular dilation & atrophy (blue arrows). (F) kidneys of rats received MTX+SIM showing decreased tubular degeneration (black arrows) in cortex. Magnifications X200 bar50. $^{k}p< 0.05$ indicates significancy to control animals, $^{u}p< 0.05$ documented significancy to MTX group.

Oxidative Stress Modulation by Simvastatin in MTX-Induced Nephrotoxicity:

As shown in Figure 2, administration of methotrexate (MTX) induced significant oxidative stress in renal tissues, evidenced by elevated levels of lipid peroxidation marker malondialdehyde (MDA) and a marked reduction in antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) compared to normal rats. Conversely, the MTX+SIM group exhibited a notable reversal of these changes, with decreased MDA levels and restoration of antioxidant enzyme activities. These findings underscore the potent antioxidant properties of SIM in mitigating MTX-induced nephrotoxicity.

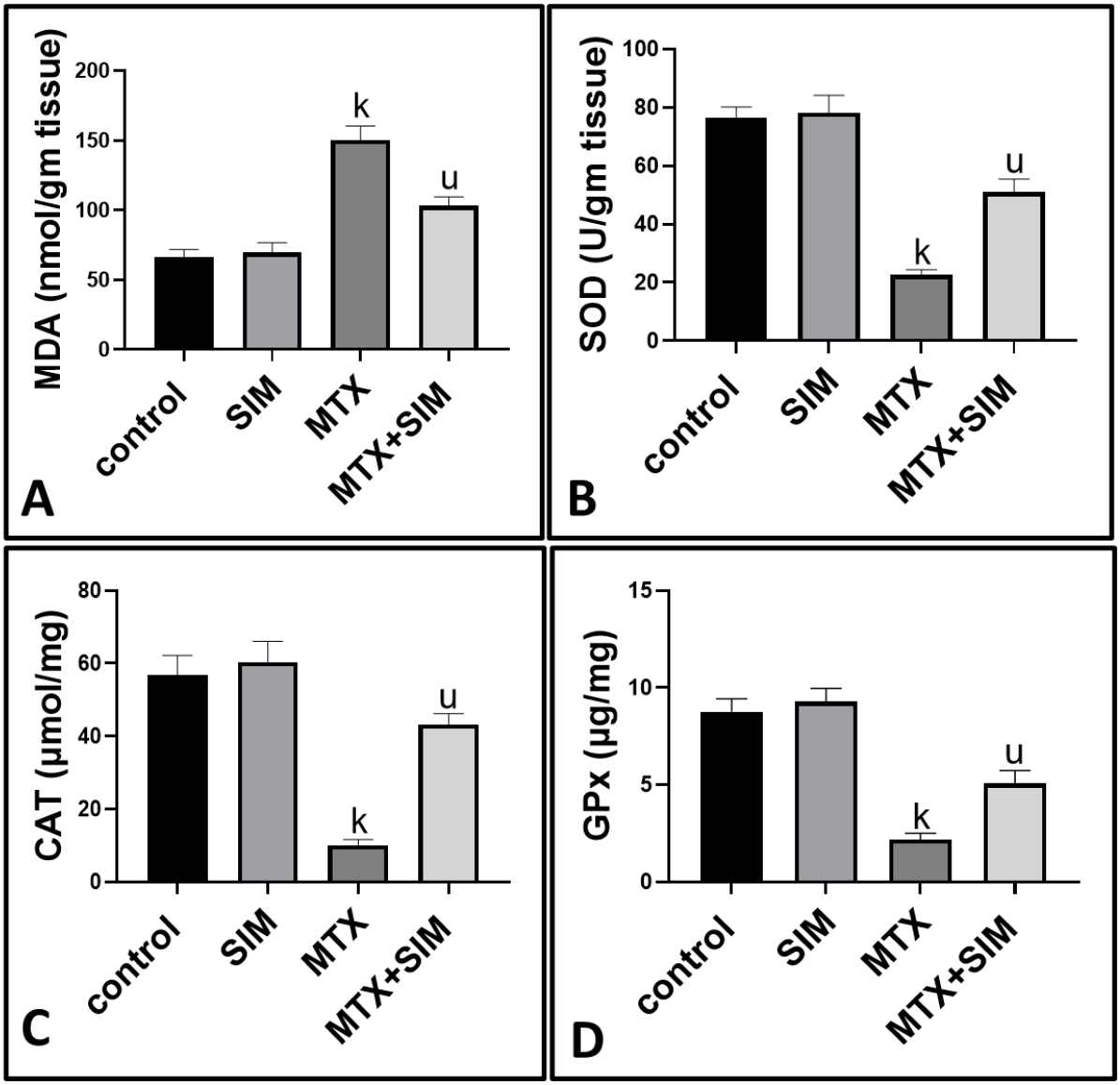

Fig. 2. Renal Homogenate level of (A) MDA, (B) SOD, (C) CAT, (D) GPx in different groups. $^{k}p< 0.05$ vs normal rats, $^{u}p< 0.05$ vs MTX treated rats.

Anti-Inflammatory Effect of Simvastatin Against MTX-Induced Renal Inflammation:

ELISA analysis revealed a significant elevation in the inflammatory transcription factor NF-κB and its associated cytokines, TNF-α, IL-6, and IL-1β, in the MTX-treated group compared to the control rats (Fig. 3A–

D). In contrast, coadministration of simvastatin (SIM) with MTX resulted in a marked reduction in these inflammatory markers relative to the MTX group. These findings strongly support the anti-inflammatory potential of SIM in mitigating MTX-induced renal inflammation.

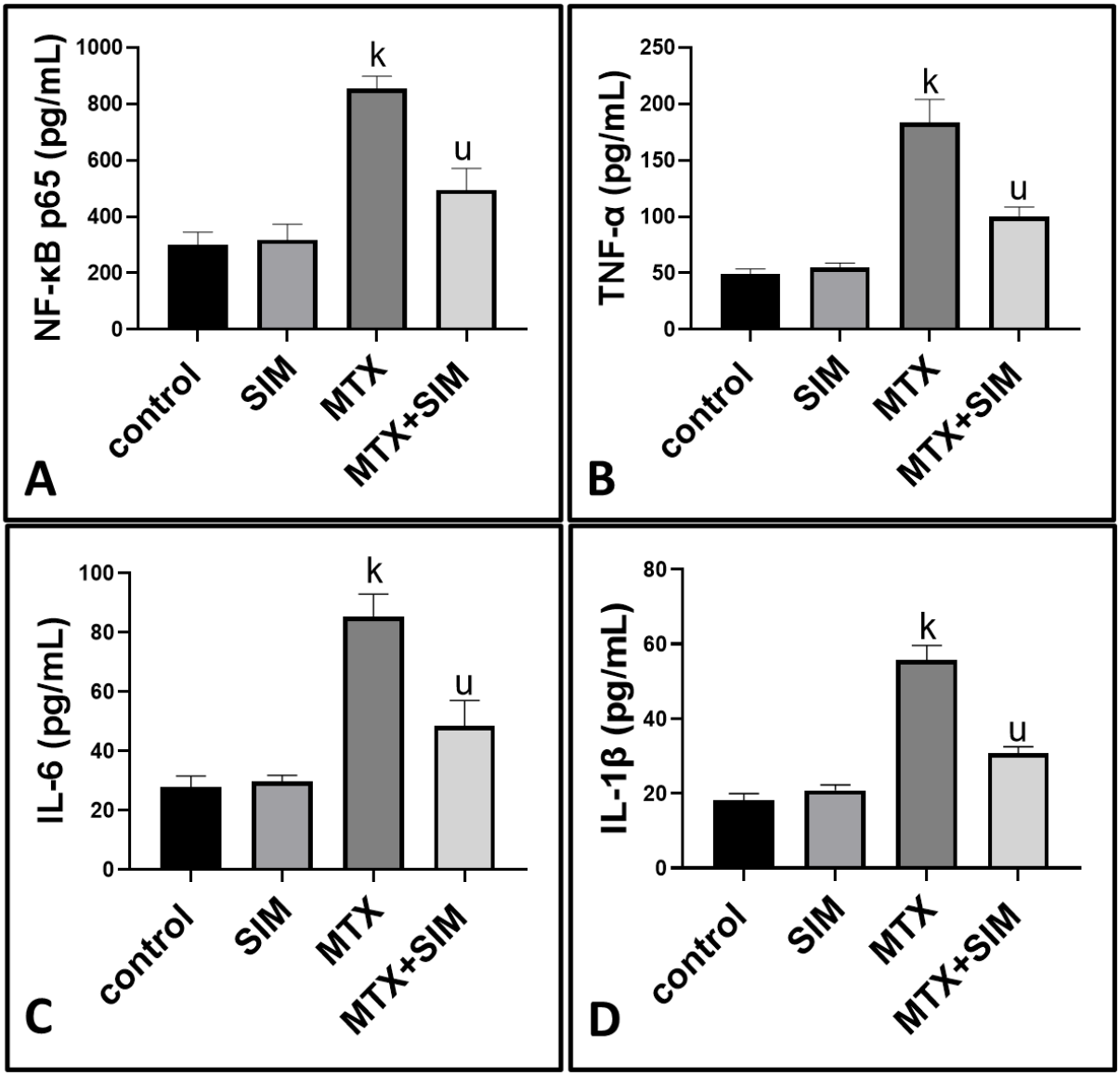

Fig. 3. Protein level of (A-D) NF-κB, TNF-α, IL-6, IL-1β in different experimental groups. $^{k}p< 0.05$ means significance to normal group, $^{u}p< 0.05$ means significance to MTX group.

Antiapoptotic Effect of Simvastatin in MTX-Induced Renal Tubular Apoptosis:

Methotrexate (MTX) administration led to a significant upregulation of apoptotic markers, including p53, cytochrome-C, Bax, and caspase-3, compared to the normal control group. Interestingly, co-treatment with simvastatin (SIM) markedly attenuated the expression of these apoptotic proteins (Fig. 4A–D). These results highlight the anti-apoptotic efficacy of SIM in protecting against MTX-induced renal tubular cell apoptosis.

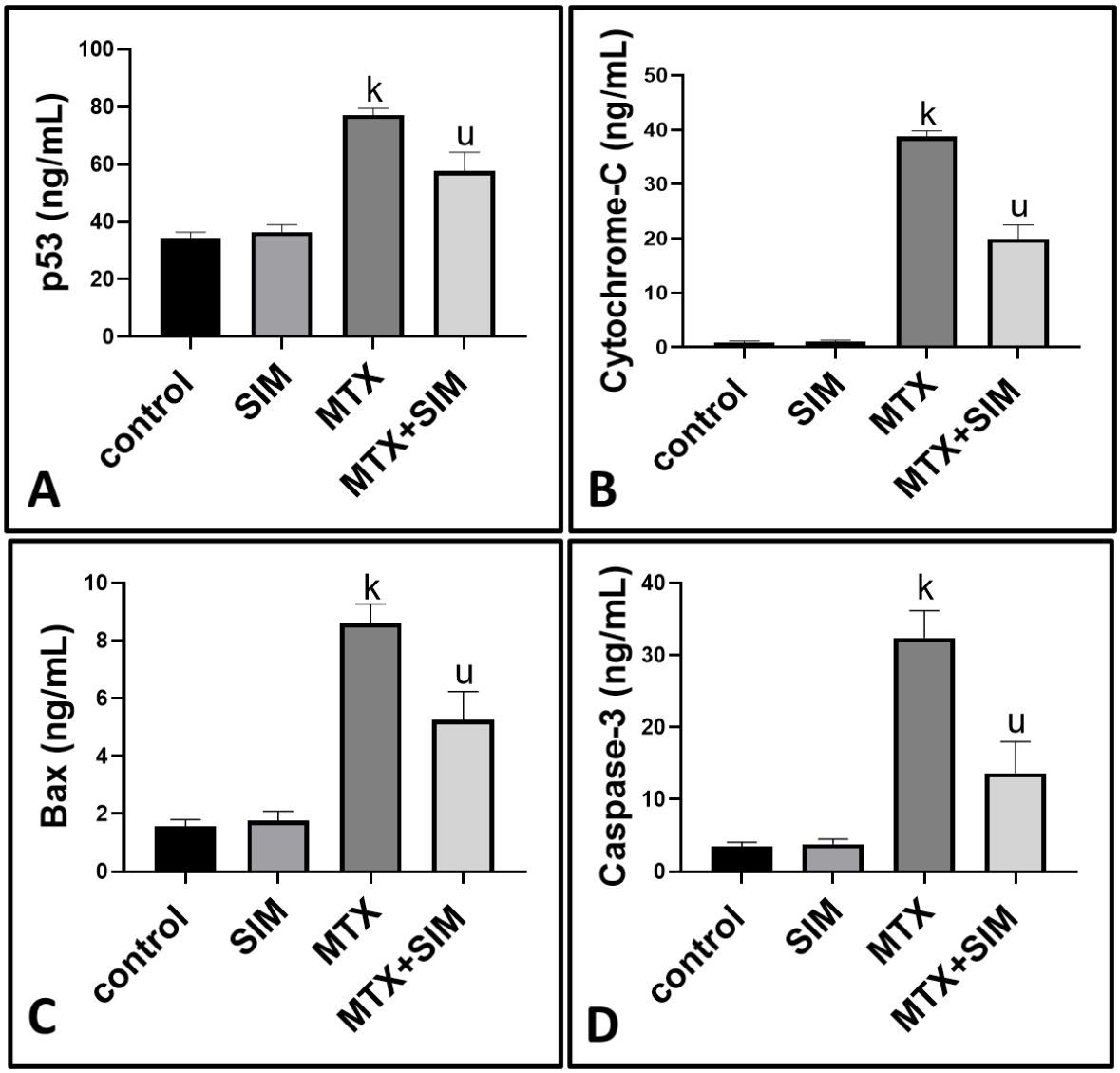

Fig. 4. ELISA analysis of apoptotic markers in renal tissues across experimental groups: (A) p53, (B) cytochrome-C, (C) Bax, and (D) caspase-3. Data are presented as mean ± SD. $^{k}p < 0.05$ indicates significance compared to the control group; $^{u}p < 0.05$ indicates significance compared to the MTX-treated group.

DISCUSSION

Although Methotrexate (MTX) is a widely used immunosuppressive and anticancer drug (Pannu, 2019; Howard *et al.,* 2016; Weinblatt, 2018), its benefits are often limited by serious side effects and toxicity (Jalili *et al.,* 2020), particularly kidney damage (nephrotoxicity). This renal injury, which targets the tubules, is driven by inflammation and oxidative stress (Pannu, 2019; Abd El-Twab *et al.,* 2016; Mahmoud *et al.,* 2018). Consequently, finding agents that can protect against this nephrotoxicity is a critical medical goal.

Urea and creatinine are primary markers of renal function and play a critical role in diagnosing renal injury and assessing kidney health (Türk *et al.,* 2022). These biomarkers are standard indices of glomerular function (Amini *et al.,* 2019), and elevated levels in the bloodstream typically indicate impaired kidney function (Salazar, 2014). In our study, the MTX-treated group exhibited a significant deterioration in renal function compared to the control group, consistent with findings from previous research (El-Saed *et al.,* 2025; Mahmoud *et al.,* 2025). Conversely, co-treatment with simvastatin

(SIM) and methotrexate (MTX) resulted in normalization of renal function, accompanied by notable improvements in renal morphology and a reduction in tubular degeneration. These findings align with those reported by Hasan *et al.* (2024), who demonstrated the protective effects of simvastatin in a diabetic nephropathy model, showing reductions in urinary microalbuminuria, serum BUN, and creatinine levels, along with histological improvements. Similarly, the study by Işeri *et al.* (2007) corroborated the nephroprotective role of simvastatin in cisplatin-induced nephrotoxicity.

Accumulating data underscores the detrimental role of reactive oxygen species (ROS) in kidney health, where they contribute to nephron damage and disrupt the architecture and function of renal tubules and glomeruli (Devrim *et al.,* 2005). Methotrexate (MTX), in particular, has been identified as a potent inducer of ROS generation (Al-Otaibi *et al.,* 2012). Our findings, consistent with previous reports, revealed that MTX administration significantly elevated renal tissue levels of MDA, a marker of lipid peroxidation, while concurrently reducing antioxidant enzyme levels. However, combined treatment with SIM and MTX resulted in a marked decrease in lipid peroxidation and a significant elevation in antioxidant enzymes such as GPx and SOD, compared to the MTX-only group. These results are supported by the work of Goodarzi *et al.* (2017), who demonstrated the antioxidant effect of simvastatin against chromium-induced renal damage through suppression of MDA and elevation of GSH. Similarly, (Al-Otaibi *et al.,* 2012) reported a lack of protective effect of simvastatin against contrast-induced oxidative stress. The antioxidant properties of simvastatin can be attributed to its multi-faceted mechanisms: (1) Reduction of lipid peroxidation simvastatin significantly lowers MDA levels, indicating reduced oxidative damage to renal tissues Mhaidat *et al.,* 2016; (2) Enhancement of antioxidant enzymes it boosts the activity of key enzymes such as GST, SOD, and catalase, which help neutralize ROS (Al-Otaibi *et al.,* 2012); (3) Improved nitric oxide bioavailability simvastatin enhances endothelial function by increasing nitric oxide levels, thereby reducing oxidative stress and improving renal perfusion (Mhaidat *et al.,* 2016); and (4) Upregulation of the Nrf2/HO-1 signaling pathway, which plays a critical role in cellular antioxidant defense (Zhang *et al.,* 2017).

Oxidative stress serves as the primary trigger for MTX-induced inflammation, leading to the activation of NF-κB, a central regulator of the redox response (Ahmad *et al.,* 2021). This activation initiates the transcription of inflammatory markers such as TNF-α, whose upregulation intensifies the inflammatory cascade by promoting immune cell infiltration and inducing cell death, thereby exacerbating renal pathology Fahmy *et al.* (2025). In our study, rats treated with MTX showed a marked increase in NF-κB expression and inflammatory cytokines compared to the control group. These findings align with previous work by Fahmy *et al.* (2025), who highlighted the role of inflammation in MTX-induced nephron-toxicity in a rat model. Interestingly, co-treatment with SIM and MTX demonstrated anti-inflammatory effects by reversing the elevated protein levels of inflammatory indicators. This observation is supported by Hasan *et al.* (2024), who reported that SIM exerted anti-inflammatory effects in a diabetic nephropathy model through inhibition of NF-κB and associated cytokines. The anti-inflammatory mechanism of SIM is attributed to its ability to prevent NF-κB activation by stabilizing its inhibitor, IκBα, thereby blocking its phosphorylation and degradation. This inhibition prevents the translocation of the NF-κB p65 subunit into the nucleus, effectively suppressing the transcription of inflammatory genes (Lee *et al.,* 2007).

The overproduction of ROS and pro-inflammatory cytokines is a key trigger for the mitochondrial apoptotic pathway (Heidari *et al.,* 2018). Our study demonstrated that MTX induces renal apoptosis by increasing

the protein levels of cytochrome-c, p53, Bax, and caspase-3, indicating activation of the intrinsic apoptotic pathway. In contrast, the SIM+MTX treatment group showed a notable reduction in renal apoptosis, as evidenced by decreased levels of apoptotic markers. These findings are consistent with previous work by Hasan *et al.* (2024), who reported that SIM mitigates apoptosis in a diabetic nephropathy model by downregulating the mRNA and protein expression of caspase-3 and upregulating the gene expression of Bcl-2. Additionally, Mahmood *et al.* (2008) documented the anti-apoptotic properties of SIM in a traumatic brain injury model, highlighting its ability to suppress caspase-3 expression.

CONCLUSIONS

Simvastatin demonstrated a multifaceted protective role against methotrexate-induced kidney damage. It improved renal performance, preserved tissue architecture, and counteracted oxidative imbalance. Additionally, simvastatin suppressed inflammatory mediators and reduced cellular death signals, confirming its therapeutic potential in maintaining renal integrity under toxic stress.

List of Abbreviations:

NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells
MTX – Methotrexate
SIM – Simvastatin
AKI – Acute Kidney Injury
ROS – Reactive Oxygen Species
OS– Oxidative stress
DM – diabetes mellitus
GSH – Glutathione
MDA – Malondialdehyde
SOD – Superoxide Dismutase
CAT – Catalase
Cr – Creatinine
BUN – blood urea nitrogen
ELISA – Enzyme-Linked Immunosorbent Assay
IL-1β – Interleukin-1 beta
IL-6 – Interleukin-6
TNF-α – Tumor Necrosis Factor-alpha
H&E – Hematoxylin and Eosin
GST – glutathione S-transferase

Declarations:

Ethical Approval and Consent to Participate: The protocol received prior approval from the Medical Research Ethics Committee at Umm Al-Qura University (Approval code: HAPO-02-K-012-2025-10-2940).

Competing Interests: The author declares no conflicts of interest.

Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

Authors' Contributions: Main Author.

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