Activation of Angiotensin-Converting Enzyme 2 Mitigates Doxorubicin Induced Nephrotoxicity via TLR4/NF-kB Down Regulation

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KEYWORDS
ACE2, Diminazene, Doxorubicin, NF-kB, Nephrotoxicity, TLR4.

ABSTRACT

The application of the anticancer antibiotic doxorubicin (Dox) has been limited due to the emergence of toxicities affecting vital organs, including the kidneys. The anti-trypanosomal drug diminazene aceturate (DIZE) was found to activate ACE2 and have multiple protective advantages. This study sought to elucidate the probable mechanisms and renoprotective benefits of DIZE in DOX-induced nephrotoxicity. Thirty male albino rats were divided into three groups: DOX, DOX+DIZE, and control groups, with ten rats in each category. After 8 weeks serum urea, serum creatinine, urine albumin, creatinine clearance, renal angiotensin II, renal angiotensin-converting enzyme 2, renal malondialdehyde, renal superoxide dismutase, renal tumor necrosis factor-alpha, renal interleukin-6, and renal gene expression of nuclear factor kappa B and Toll-like receptor 4 were assessed. Supplementary assessments of NF-kB and TLR4 immunoreactivity in the kidney were conducted. In the DOX group, serum levels of urea and creatinine, along with urinary albumin, renal MDA, renal TNF-α, renal IL-6, renal Ang II, and the renal gene expression and immune-reactivity of TLR4 and NF-κB, were significantly elevated compared to the control group; conversely, renal SOD, creatinine clearance, and renal ACE2 values in the DOX group were markedly diminished relative to the control. DIZE significantly improved the alterations caused by DOX-induced nephrotoxicity. DIZE affords renoprotection in DOX rats by downregulating the renal TLR4–NF-kB pathway and acting as an ACE2 activator, hence modifying the kidneys' anti-inflammatory and antioxidant properties.

Introduction

Doxorubicin (DOX), a broad-spectrum anthracycline antibiotic having anticancer characteristics, has been used successfully to treat a variety of malignancies. Unfortunately, toxicities to important organs, particularly the kidney, have limited Dox's use (Carvalho et al. 2009). Renal Dox-induced toxicity is primarily caused by the production of free radicals, which eventually leads to apoptosis activation (Ghibu et al., 2012). The biological effects of inflammation begin when proinflammatory cytokines are released as a result of free radical formation (Dinarello, 2006). Furthermore, because Dox accumulates preferentially in the kidney, it may cause nephrotoxicity due to its direct renal detrimental effect (Lee and Harris, 2011).

In response to external infections, the innate immune system's conserved family of pattern recognition receptors, termed toll-like receptors (TLRs), activates downstream inflammatory signaling pathways. The

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activity of nuclear factor kappa B (NF-κB) was associated with the activation of toll-like receptors 4 (TLR4) signaling, resulting in heightened production of pro-inflammatory cytokines and chemokines, resulting in localized inflammation and leukocyte accumulation. NF-κB acts as a downstream effector of the TLR4 signaling pathway (Zhu et al., 2018).

Doxorubicin activates the NLRP3 inflammasome in many cells by acting on TLR4. Activation of TLR4 leads to elevated NF-kB expression, which may translocate to the nucleus and promote the secretion of inflammatory cytokines (Ibrahim et al., 2021). TLR4 signaling contributes to Dox-induced toxicity, so blocking TLR4 may be a viable method to mitigate this toxicity (Sumneang et al., 2023).

The pathophysiology of DOX-induced toxicity is markedly affected by the renin-angiotensin system (RAS), and it has been shown that blocking the angiotensin converting enzyme (ACE)/AngII/AngII type 1 (AT1) receptor (AT1R) axis can mitigate DOX-induced toxicity (Ma et al., 2017). Angiotensin converting enzyme inhibitors and angiotensin receptor blockers have traditionally been associated with the inhibition of Ang II synthesis and AT1 receptor activation. However, especially at the tissue level, the degradation of AngII may also regulate Ang II concentrations (Ma et al., 2017).

Angiotensin-converting enzyme 2 (ACE2) mitigates the adverse effects of Ang II by converting it into Ang 1–7. The 1–7 peptide acts on Mas receptors to exert vaso-protective effects. The equilibrium between the advantageous ACE2/Ang 1–7 and the harmful ACE1/Ang II is crucial in tissue injury. Up-regulators of ACE2 that can restore the appropriate ACE/ACE2 equilibrium represent a potential therapeutic approach for renal damage, since the local balance between ACE and ACE2, along with Ang II and Ang 1-7, is crucial for regulating Ang II levels and its effects on kidney injury (Malek and Nematbakhsh, 2014). The anti-trypanosomal medicine Diminazene aceturate (DIZE) was shown to activate ACE2 and possess several advantageous features. Previous research (Malek and Nematbakhsh, 2014) has demonstrated that DIZE had reno-protective characteristics in rats with renal diseases.

The impact of DIZE on DOX-induced nephrotoxicity remains ambiguous. Moreover, the precise mechanisms through which DIZE confers reno-protective advantages remain ambiguous. The objective of this study was to investigate the potential protective effects of DIZE (an ACE2 activator) against DOX-induced nephrotoxicity and to explore the related mechanisms.

Material and Methods

Animals

All experiments, animal care, and usage were sanctioned by the Faculty of Medicine Ethics Committee at Menoufia University with IRB code 2/2025BIO13-1 and adhered to its regulations. For the present study, thirty male Wistar rats, each weighing between 160 and 190 g, were obtained from a local supply facility.

Rats were granted full access to standard feed and water. All efforts were undertaken to ensure animal welfare and mitigate stress and pain. Rats were randomly and uniformly divided into three experimental groups following acclimatization, with ten rats assigned to each group.

1. Rats in the control group received one milliliter of distilled water intraperitoneally (i.p.) and one milliliter

of distilled water (DIZE vehicle) subcutaneously (s.c.) once daily for three weeks.

2. DOX Group: A single intraperitoneal dose of 15 mg/kg DOX was given to the rats (Ajith et al., 2008; El-Sheikh et al., 2012). Doxorubicin was distributed in vials by HIKMA Specialized Pharmaceuticals in Egypt under the brand name "Adricin" Each vial contains DOX HCL 50 mg/25 ml. For three weeks, rats received one milliliter of distilled water s.c. once a day.
3. Doxorubicin/DIZE-treated (DOX+DIZE) group: the rats were given a single dose of DOX (15 mg/kg, i.p.) and DIZE (Sigma Aldrich, Steinheim, Germany), dissolved in distilled water at a dosage of 15 mg/kg/day, s.c for 3 weeks beginning from the first day of Dox treatment. (Al Suleimani et al., 2023; El-Domiaty et al., 2022).

Rats were placed in metabolic cages the day before the final day of therapy to collect urine that had been voided for the following 24 hours. After determining the volume of the urine samples, they were centrifuged for 10 minutes at 2000 revolutions per minute. The supernatant was then collected and kept at -20°C to determine urinary creatinine and urinary microalbuminuria levels.

Measurement of Microalbuminuria and creatinine clearance calculation

The microalbuminuria ELISA kit (Exocell Inc., Philadelphia, USA) was used to determine the amount of albumin in the urine. The creatinine clearance (mL/min) was calculated by multiplying the urine creatinine concentration (mg/dL) by the urine volume (mL/min) and divided into the plasma creatinine concentration (mg/dL), with values reported in milliliter per minute.

Sample Collection

At the termination of the treatment, the rats were sedated and blood was drawn from the vena cava. Urine was obtained from rats in metabolic cages one day before sacrifcation. The blood samples were kept at -20 °C until analysis. The rats were then sacrificed, and their kidneys were extracted and kept for subsequent biochemical analysis, real time PCR assay and histological analysis.

Biochemical Methods

Creatinine levels in plasma, urine, and urea were determined using colorimetric kits (Spectrum Diagnostics, Egypt). However, there are ELISA kits available for rats. The concentrations of renal TNF-α, IL-6, ACE2, and Ang II were measured using the following methods: (TNF-α: ERT2010 1, Assaypro LLC, Saint Charles, Missouri, USA, IL-6: ab100772, Abcam, Cambridge, UK, ACE2: Catalog No. MBS764117; MyBioSource, San Diego, CA, United States, Ang II: Catalog No. E-EL-R1430; Elabscience, Houston, TX, United States). Renal MDA and renal SOD were measured using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt) according to the manufacturer's recommendations.

Quantitative RT‑PCR (qRT‑PCR)

Rat kidney tissue was taken out, weighed, and either kept at -80 or used fresh for quantitative RT-PCR. Primers Express Software Version 3.0.1 (Applid Biosystems, USA) was utilized to generate gene-specific primers.

- The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC).
- The reverse primer was (GAGCAGTCATGTCCTTGGGT).
- The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT),
- The reverse was (GTCCTTGACCCACTGCAAGA).
- The forward primer for β-actin was (TGTTTG AGACCTTCAACACC),
- The reverse primer was (TAGGAGCCAGGGCAG TAATC).

The housekeeping control gene was β-actin. The source of all primers was Sigma-Aldrich (Chemie GmbH, Germany). Using an Applied Biosystems 7500 FAST 96-well PCR equipment (USA), RT-PCR tests were run in duplicate. Total RNA was extracted after TRI reagent (Sigma-Aldrich, UK) homogenized fresh or frozen rat renal tissue samples. Rat kidney tissue RNA was reverse-transcribed using a high-capacity RNA-to-cDNA kit (Applied Biosystems, CA, USA). The produced cDNA was then used to quantify the target gene's mRNA expression. Using β-actin as a reference, the comparative Ct ($2^{-\Delta Ct}$) approach was used to determine the relative amount of mRNA expression of the gene of interest.

Histopathological and Immuno-histochemical method

For histopathological studies by H&E, kidney tissue sections were fixed at 10% neutral buffered formalin. For immunohistochemical studies, the kidney paraffin sections (4 μm) were stained by TLR4 (Catalog No. GB11186; Servicebio, Wuhan, Hubei, China)), and NF-kB (Catalog No. ab32536, Abcam, Cambridge, United Kingdom).

Statistical analysis

Initially, the Kolmogorov-Smirnov test was used to verify that all of the data were normal. Tukey's post hoc tests were used in conjunction with the Analyses of Variances (ANOVA). Mean ± standard deviation (SD) was used to represent the results, and p values < 0.05 were deemed significant. The data was statistically analyzed using the Origin® program.

Results

The DOX group had significantly lower renal SOD, creatinine clearance, and renal ACE2 values compared to the control group. However, the DOX group had significantly higher serum levels of urea, creatinine, renal MDA, renal TNF-α, renal IL-6, renal Ang II, and renal gene expression of TLR4 and NF-κB. DOX+DIZE significantly increased renal SOD, creatinine clearance values, and renal ACE2 compared to DOX but decreased significantly compared to control. However, serum levels of urea, creatinine, urinary albumin, renal MDA, renal TNF-α, renal IL-6, renal Ang II, and renal gene expression of TLR4 and NF-κB were significantly decreased compared to DOX but significantly increased compared to control as shown in table 1.

Table (1): The serum urea, creatinine, creatinine clearance, urinary albumin, renal TNF-α, renal IL-6, renal MDA, renal SOD, renal ACE2, renal Ang II, TLR4 gene expression, and renal NF κB gene expression in all investigated groups.

	Control group mean ± SD	DOX group mean ± SD	DOX+ DIZE group mean ± SD
Serum Urea (mg/dl)	42.2±4.6	106.3±3.2 *	73.5±1.6 *#
Serum Creatinine (mg/dl)	0.52±.09	1.77±0.11 *	1.23±0.08 *#
Creatinine clearance (mL/min)	1.22±0.3	0.43±0.09 *	0.95±0.07 *#
urinary albumin (mg/day)	18.19±2.11	151.2±3.8 *	60.52±7.5 *#
Renal MDA (nmol/ g Tissue)	9.6 ±1.9	19± 0.3*	16.1± 1.26*#
Renal SOD (U/g. Tissue)	11 ±0.8	3.18±0.91*	6.39±0.67*#
Renal TNF-α (ng/ml)	20.6±0.37	43.2±1.71*	35.1±1.14*#
Renal IL-6 (pg/mL)	109±3.25	199±1.19*	169±3.1*#
Renal ACE2 (ng/mL)	4.81±0.12	0.81±0.09*	3.85±0.11*#
Renal AngII (Pg/mL)	30.5±1.41	114.9±3.27*	49.5±3.14*#
Renal TLR4 gene expression	1	2.9±0.08*	1.97±0.11*#
Renal NF-κB gene expression	1	3.2±0.08*	2.23±0.11*#

* Significant compared with control, # Significant compared with DOX, MDA: Malondialdehyde, SOD: superoxide dimutase, TNF-α: tumour necrosis factor alpha, IL-6: interleukin-6, ACE2: angiotensin converting enzyme 2, AngII: angiotensin II, TLR4: Toll like receptor 4, NF-κB: Nuclear factor K beta

Hematoxylin and Eosin staining:

Figure (1) illustrated sections from the control group that had the typical morphology of the renal glomerulus and renal tubules. The DOX group exhibited destruction of the renal tubules, glomeruli, and basement membrane. The DOX+DIZE demonstrated enhancement of renal glomeruli and dilation of renal tubules.

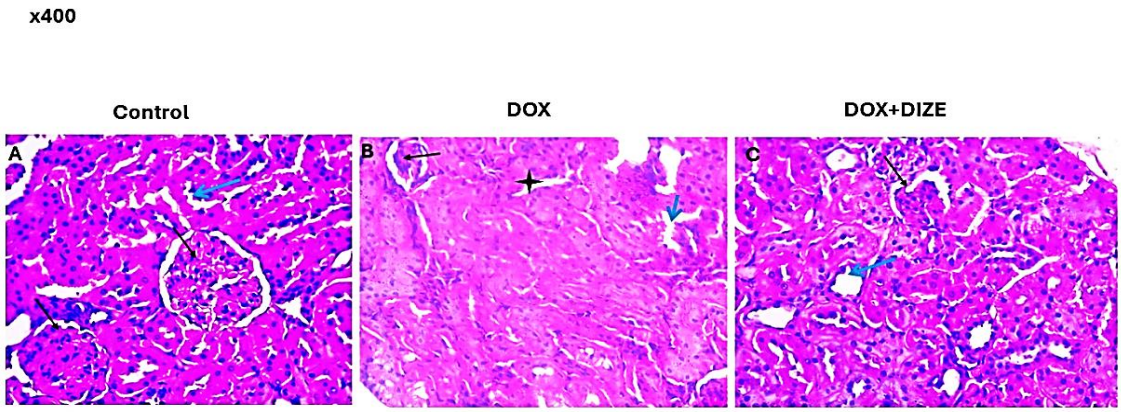

Fig. (1): H&E-stained renal sections in the examined groups (H&E ×400): (A) a photomicrograph of the control group, demonstrating the normal morphology of the kidney, including intact renal glomeruli (black arrows) and renal tubules (blue arrow). (B) a photomicrograph of the DOX group illustrating the damaged basement membrane (blue arrow), damaged urinary tubules (star), and disrupted glomeruli (black arrow). (C) a photomicrograph of the DOX+DIZE group, illustrating enhancement in the renal glomeruli (black arrow) and the tubules exhibiting dilatation (blue arrow).

Immunohistochemical results

Figure (2) A–D displayed that the percentage area of TLR4 was significantly elevated in the DOX group compared to the control group (58.05±0.02 vs. 9.02±0.15, $p<0.05$). Although this proportion remained elevated compared to the control group, it was significantly reduced in the DOX + DIZE group relative to the DOX group (17.4±0.33 vs. 58.05±0.02, $p<0.05$).

Figure (2) E–H indicated that the percentage area of NF-kB was significantly elevated in the DOX group compared to the control group (63±0.05 vs. 11.1±0.03, $p<0.05$). The proportion in the experimental group exceeded that of the control group; however, it was significantly reduced in the DOX+DIZE group compared to the DOX group (22.4±0.12 vs. 63±0.1, respectively, $p<0.05$).

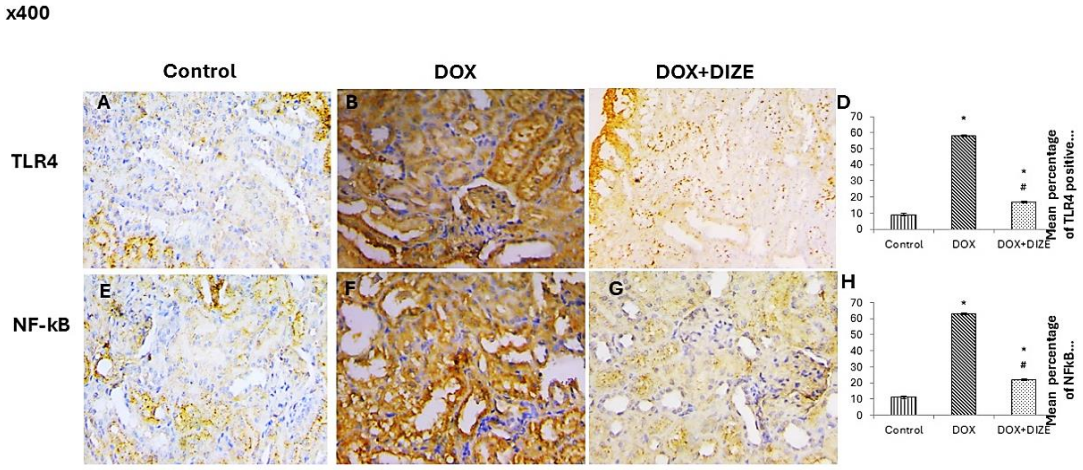

Fig. (2): Micrographs of the various experimental groups demonstrate a significant increase in TLR4 (A-D) and NF-kB (E-H) immunoreactivity in the DOX group, alongside a significant reduction in the DOX + DIZE group.

Discussion

Doxorubicin is a widely utilized chemotherapeutic agent for cancer treatment; nevertheless, its usage is mostly limited due to toxicities affecting the heart, kidneys, lungs, testicles, and hematological systems (Mohamed et al., 2011). DOX reduced renal function in the present study relative to the control group, corroborating previous findings (El-Sheikh et al., 2012). Marked elevations in blood urea and creatinine levels, alongside a reduction in creatinine clearance values relative to the control group, were indicative of DOX nephrotoxicity in our study. Histopathological modifications corroborated our findings. A reduction in glomerular filtration rate leading to elevated serum urea and creatinine levels is indicative of DOX's nephrotoxic effects. An elevation in glomerular filtration rate, a compromise in the structural integrity of renal cells, and kidney disease may result from these disturbances, potentially associated with the activation of oxidative stress and the inflammatory cascade (El-Sayed et al., 2017). Furthermore, DOX led to a marked reduction in renal SOD activity and an increase in MDA accumulation in kidney tissue, both of which intensified oxidative stress and reactive oxygen species (ROS) generation. These findings correspond with previous studies (El-Sayed et al., 2017).

Angiotensin II type 1 receptor (AT1R) antagonists, ACE inhibitors, and renin inhibitors are pharmacological agents that modulate the renin-angiotensin system (RAS)

and have shown encouraging outcomes in preserving renal architecture and functionality amid doxorubicin-induced renal injury (Liu et al., 2019). Moreover, these drugs regulate the unconventional, protective pathway of the renin-angiotensin system (RAS), which is facilitated by ACE2, Ang1–7, and the angiotensin II type 2 receptor (AT2R), while simultaneously mitigating the detrimental effects of traditional RAS activation (Ibrahim et al., 2009). Various animal models exhibiting renal impairment have demonstrated the therapeutic efficacy of DIZE being ACE2 activator (Silva de Almeida et al., 2020).

Diminazine is a prevalent antiparasitic medication that induces ACE2 as an off-target effect in a significant manner. The primary biological function of ACE2 is to counteract the pathophysiological effects of ACE by converting Ang II into Ang 1–7, which activates the Mas oncogene receptor (MasR) (Mizuiri and Ohashi, 2015). The ACE2/Ang 1–7/MasR axis has a variety of renoprotective effects. For instance, it reduces renal oxidative stress and inflammation, increases sodium excretion, and delays tissue fibrosis and remodeling (Simões e Silva et al., 2013).

The histopathological damage induced by DOX therapy was significantly improved, which is consistent with other research (Al Suleimani et al., 2023). Additionally, DIZE significantly improved kidney function parameters.

According to the study's results, DOX substantially increased Ang II levels and decreased ACE2 in comparison to the control group. Prominent Ang II action can be the consequence of either enhanced Ang II production or enhanced signaling from the Ang II receptors. Initially, doxorubicin increases the concentration of ang-II in plasma (Sobczuk et al., 2022). Ang-I levels were substantially increased by a single dosage of doxorubicin, which is believed to be a proxy for elevated renin activity (Rashikh et al., 2014). However, elevated ACE activity is associated with a different mechanism (Sobczuk et al., 2022).

In addition, DIZE substantially decreased Ang II and increased ACE2 activity in comparison to the control, a finding that is in accordance with other research (El-Domiaty et al., 2022). The ameliorative effect is attributed to the significant reduction in renal Ang II levels in DOX rodents that were supplemented with DIZE, as opposed to DOX animals. In this instance, renal Ang II decreased prior to the significantly increased renal ACE2. ACE2 is known to mitigate the adverse effects of ACE/Ang II by converting Ang II to Ang 1–7. DIZE can alleviate a variety of Ang II-mediated pathological diseases, such as oxidative stress, fibrosis, inflammation, and vasoconstriction, by acting as an ACE2 activator (Velkoska et al., 2016).

Angiotensin II induces the generation of ROS via modulating AT1R (Fujita, 2006). Furthermore, efferent arteriolar vaso-constriction induced by Ang II leads to glomerular hyperfiltration (Chalmers et al., 2006). Albuminuria results from the glomerular hyperfiltration that ensues, corroborating the findings of this study. Various intracellular mediators that induce inflammation and tissue damage are activated by intracellular reactive oxygen species mediated by Ang II (Zhang et al., 2007).

Our findings, corroborating previous research (Ayla et al., 2011), indicated that oxidative stress is a mechanism through which DOX exerts its nephrotoxic effects. Tissue damage results from disturbances in oxidant-antioxidant mechanisms, evidenced by lipid peroxidation and protein oxidation (Karaman et al., 2006). Histopathological alterations were presumed to correlate with the absorptive capacity of renal tubules,

leading to functional congestion of nephrons and subsequent renal dysfunction (Afsar et al., 2020).

Diminazine significantly mitigated the oxidative stress generated by DOX toxicity, consistent with other research (Malek and Nematbakhsh, 2014). The consequences of Ang1–7 upon ACE2 activation may elucidate the antioxidant properties of DIZE (Mizuiri and Ohashi, 2015). Recent research indicates that DIZE reduces ROS generation via downregulating NAPDH oxidase expression, independent of ACE2 activation (Rajapaksha et al., 2018). A prior study ascribed the antioxidant effect of DIZE to its capacity to reduce Ang II as an ACE2 activator (El-Domiaty et al., 2022).

The TLR4/NF-κB pathway appears to be crucial to the pathogenesis of numerous inflammatory disorders. The TLR4 pathway was activated, thereby initiating the NF-κB pathway and an NF –κB -dependent inflammatory response. This may exacerbate renal failure in both acute and chronic kidney conditions (Zhu et al., 2018).

Our results, aligning with prior studies, indicated that the DOX group exhibited elevated renal pro-inflammatory markers, alongside increased renal TLR4 and NF-kB gene expression and immune-reactivity, in comparison to the control (Tavakoli Dargani and Singla, 2019). This is also consistent with earlier research (El-Agamy et al., 2016), which associated the activation of NF-κβ expression with the substantial generation of ROS following DOX administration. This then prompts NF-κB to translocate to the nucleus, where it can initiate the expression of several inflammatory cytokine genes.

The expression of inflammatory cytokines may be directly produced by oxidative stress caused by DOX, and it was significantly increased after DOX administration (Wang et al., 2016). The inflammatory response induced by Ang II was suppressed by ACE2 (Zhong et al., 2011). Consistent with these findings, we observed that DIZE supplementation markedly downregulated pro-inflammatory cytokine expressions, while these expressions were dramatically increased in the DOX group. In a prior work, doxorubicin elicited the upregulation of TLR4 and NF-kB (Ibrahim et al., 2021).

This study also revealed that DIZE exerts a renal anti-inflammatory effect by dramatically reducing renal inflammatory cytokines, as reported by Chen et al. (2017). The recent discoveries elucidate DIZE's renal anti-inflammatory effects. Compared to the DOX group, DIZE supplementation significantly decreased the expression of TLR4 and NF-kB in rat renal tissue, as assessed by IHC and PCR. This aligns with prior studies (El-Domiaty et al., 2022). The suppression of the renal TLR4–NF-kB pathway accounts for DIZE's renal anti-inflammatory effect. DIZE's ability to suppress the renal TLR4–NF-kB pathway was associated with ACE2 activation, which hydrolyzed Ang II and mitigated its inflammatory effects on the kidneys. Ang 1–7, which are metabolites of ACE2 hydrolysis of Ang II, predominantly interact with Mas receptors to facilitate its protective effects (Chen et al., 2021).

Furthermore, in vitro studies indicate that the anti-inflammatory effects of diminazene are directly linked to the suppression of mitochondrial ROS generation (Al Suleimani et al., 2023).

Conclusion

Diminazine, as an ACE2 activator, facilitates renal anti-inflammatory and antioxidant properties in DOX rats by downregulating the renal TLR4–NF-kB pathway, hence providing a renoprotective effect. Consequently, DIZE may be

considered a prospective therapeutic option for the prevention of DOX-induced nephrotoxicity.

Conflict of interest

Authors declare that there is no conflict of interest

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