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Nicorandil ameliorates gentamicin-induced nephrotoxicity through Nrf2/HO-1, p38 MAPK/NF-κB p65/NO and miR-7/CHOP pathways

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Abstract: Gentamicin (GM) is a frequently prescribed aminoglycoside antibiotic. Nevertheless, its clinical use is restricted by its nephrotoxic properties. GM-mediated nephrotoxicity elicits chiefly from renal inflammation and oxidative stress. Nicorandil (NR), a synthetic ATP-dependent K channel activator and nitric NO donor, exerts vasodilator, anti-inflammatory, antioxidant, in addition to anti-apoptotic properties. This study was targeted to explore the protecting properties of NR against GM-mediated nephrotoxicity. A daily intraperitoneal dose of 100 mg/kg, of GM given for seven days, induced nephrotoxicity. NR (15 mg/kg) was administered orally every day for 21 days starting 14 days before challenging with GM. Nicorandil repressed the GM-mediated renal injury as verified by the amelioration of the histopathological changes and the considerable reduction in serum BUN, creatinine and KIM-1 levels, in addition to the decline in the relative kidney weight. Notably, NR caused a profound decline in oxidative stress which was verified by the boosted expression of Nrf2 and HO-1 in the renal tissue. Moreover, NR suppressed the inflammation induced by GM; it reduced renal IL-1β, TNF-α and p38 MAPK contents as well as NF-κB p65 expression. Furthermore, NR decreased renal levels of NO and increased eNOS expression. Besides, NR efficiently decreased the expression of Bax and caspase 3 while increased Bcl-2 in renal tissue. Lastly, NR remarkably upregulated the miR-7 and depressed the ER stress marker, CHOP. These findings show that treatment with NR could alleviate GM-mediated nephrotoxicity in rats, highlighting the roles of miR-7 and eNOS in modulating oxidative stress, inflammation in addition to ER stress.

Keywords: Nicorandil; Gentamicin; Nephrotoxicity; Endoplasmic reticulum; miR-7; eNOS; Rats.

1. INTRODUCTION

Gentamicin (GM) is a conventional aminoglycoside antibiotic retaining therapeutic effect against severe infections produced by Gram-negative organisms[1, 2] Accumulation of GM in the renal proximal tubular cells causes nephrotoxicity [3].

Gentamicin-induced nephrotoxicity principally involve elevated renal oxidative stress[4]. Oxidative stress recruits the inflammatory cells with subsequent release of the pro-inflammatory cytokines. In addition, it activates the p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor-κappa B (NF-κB) pathways, ultimately leading to renal inflammation[2]. Also, in response to the pro-inflammatory cytokines, 100- to 1000-fold nitric oxide (NO) is generated from the inducible NO synthase isoform (iNOS) more than the endothelial NO synthase isoform (eNOS). iNOS-generated NO has detrimental effects on different body organs[5]. Besides, increasing evidences support that oxidative stress and apoptosis are closely connected and are involved in pathophysiology of GM-induced renal injury[2, 6].

Moreover, the accumulation of GM in the endoplasmic reticulum (ER) can possibly prompt an ER stress. The ER stress motivates the unfolded protein response (UPR)[7]. Overload with UPR

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leading to apoptosis [8]. Additionally, ER stress triggers the expression of CCAAT-enhancer binding protein homologous protein (CHOP); a transcription factor that has a significant impact on apoptosis induction[9, 10]. Remarkably, the ER stress can be modified by microRNAs [11, 12] MicroRNAs are small non-coding RNA molecules that have been involved in almost all physiological as well as pathological processes [11].

Accordingly, a medication that can considerably disturb the pathogenic pathways of GM; increased oxidative stress, inflammation, ER stress and apoptosis, may be anticipated to provide significant defense against GM-provoked renal damage.

Nicorandil (NR), 2-[(pyridin-3-ylcarbonyl) amino] ethyl nitrate, exerts its vasodilatory effect by dual mechanisms: one is an ATP-dependent K channel opening and the other is releasing NO [13]. It is frequently used for treatment of heart failure and ischemic heart diseases, as well as for the inhibition of contrast-induced nephropathy [14, 15].

Previous studies have shown that NR offers significant protection against oxidative stress[16]. In addition, NR anti-inflammatory [17] and anti-apoptotic effects [18], were previously reported.

Nicorandil also was demonstrated to have beneficial effects in several models of experimental cardiac, pulmonary, hepatic as well as inflammatory bowel diseases [19-21]. Regarding the renal effect of NR, it was reported to protect podocytes from hyperglycemia-induced oxidative stress [13] and ameliorated renal injury induced by unilateral ureteral obstruction in rats [22]. Recently, NR was shown to prevent the deleterious effects of cyclosporine-A in the kidney via modulation of HIF-1α/VEGF/eNOS signaling [23]. However, the nephroprotective effect of NR against GM-induced renal toxicity has not been yet clarified.

Accordingly, this study was targeted to scrutinize the protecting effects of NR against GM-mediated renal injury in rats. We hypothesized that NR may improve the renal functions by amending the stressful oxidative, inflammatory, apoptotic as well as ER stress related signaling pathways.

2. METHODS

2.1. Drugs

GM, NR, pentobarbital and every additional used chemical were procured from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. GM was dissolved in saline. NR was suspended in 0.5 % carboxymethyl cellulose (CMC).

2.2. Kits & antibodies

All kits and antibodies used in the current study were used according to the manufacturers' guidelines, and their catalogue numbers and sources are provided in **Table 1**.

2.3. Experimental animals

The approval (No:292) was given to this study by The Ethics Committee of Faculty of Pharmacy, (Girls), Al-Azhar University, following the standard guidelines in the NIH Guide for the Principles of Laboratory Animal Care (Publications No. 85-23, revised 2011). Male Sprague Dawley rats, weighing (150 ± 20) g, were procured from the Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Rats were accommodated in sets of four/cage in the animal house of Faculty of Pharmacy (Girls), Al-Azhar University. Animals were kept at 25 ± 2 °C and humidity of 55% with a 12-hour light-darkness cycle. They had *ad libitum* access to water and standard diet (El-Nasr, Cairo, Egypt).

2.4. Experimental protocol

2.4.1. Experimental groups

After 7 days acclimatization period, 32 rats were randomly assigned into 4 groups (8 rats each) and treated as follows:

(1) Vehicle control (C) group was treated with 0.5 % CMC (2 mL/kg/day, orally) for 21 days and saline (2 mL/kg/day, intraperitoneally) from day 15 to day 21.

(2) Gentamicin (GM) group was treated with 0.5 % CMC (2 mL/kg/day, orally) for 21 days and GM (100 mg/kg/day, intraperitoneally) from day 15 to day 21[2, 24] **.**

(3) Gentamicin plus nicorandil (GM+NR) group was treated with NR (15 mg/kg/day, orally) for 21 days [5] and GM (100 mg/kg/day, intraperitoneally) one hour after NR treatment starting from day 15 to day 21.

(4) Nicorandil (NR) group was treated with NR (15 mg/kg/day, orally) for 21 days and saline (2 mL/kg/day, intraperitoneally) from day 15 to day 21.

2.4.2. Samples collection

On day 22, blood specimens were drawn through the retro-orbital venous plexus under anesthesia with 50 mg/kg pentobarbital [25]. Serum

was separated by centrifuging blood at 2500 rpm for 10 min at 4°C and used for biochemical assessment of BUN, creatinine and KIM-1. Then, rats were sacrificed by means of cervical dislocation and kidneys were directly excised, rinsed with ice-cold saline and weighed. All kidneys were cut into two equivalent parts. First part was kept at −80°C for western blotting, quantitative real-time polymerase chain reaction (QRT-PCR) or to prepare kidney homogenate; 1:10 w/v was prepared using ice-cold 0.1M phosphate buffer, pH 7.4. The supernatants collected after 20 min. of centrifugation (3000 g at 4 °C) were used for further biochemical investigation. The second part was placed in buffered formalin (10%) and utilized for histological analysis.

Table 1. Kits and antibodies used, catalogue number and source.

Kit	Catalog #	Source
Colorimetric assay		
Blood urea nitrogen (BUN)	UR 2110	Biodiagnostic Co., Cairo, Egypt
creatinine	CR 1251	
superoxide dismutase (SOD)	SD 2520	
total antioxidant capacity (TAC)	TA 2512	
malondialdehyde (MDA)	MD 2528	
NO	NO 2532	
Enzyme-linked immunosorbent assay (ELISA)		
Kidney injury molecule-1 (KIM-1)	MBS355395	MyBioSource, Southern California, San Diego, USA
interleukin-1 beta (IL-1β)	MBS825017	
p38 MAPK	MBS765087	
heme oxygenase-1 (HO-1)	MBS2508238	MyBioSource, Southern California, San Diego, USA
CHOP	MBS3808179	
Tumor necrosis factor-alpha (TNF-α)	CSB-E11987r	Cusabio Biotech Co., Wuhan, China
Bcl-2-associated X protein (Bax)	CSB-EL002573RA	
B-cell lymphoma 2 (Bcl-2)	CSB-E08854r	
mirVana miRNA Isolation Kit	AM1560	Thermo Fisher Scientific Inc., Waltham, MA, USA
TaqMan MicroRNA Reverse Transcription Kit	4366596	

Antibody	Catalog #	Source
nuclear factor E2-related factor 2 (Nrf2)	ab137550	Abcam, Cambridge, UK
NF-κB p65	3034	Cell Signaling Technology, USA
caspase-3	9662	
eNOS	32027S	
β-actin	A5316	Sigma-Aldrich; Merck KGaA, Darmstadt, Germany

2.5. Assessment of kidney functions

The serum levels of BUN and creatinine were colorimetrically assessed using specified kits according to the manufacturer's protocols using a Shimadzu UV-1601 UV visible spectrophotometer (Shimadzu, Kyoto, Japan). Also, KIM-1 serum level was evaluated using the relevant ELISA kit following the manufacturer's recommendations. The relative kidney weight was determined by calculating the percentage of kidney weight in relation to the total body weight (g/g) [2].

2.6. Assessment of oxidative stress biomarkers

Oxidative stress biomarkers; SOD, TAC and MDA were evaluated in renal tissue using relevant commercial colorimetric kits following manufacturer's guidelines (Biodiagnostic Co., Cairo, Egypt).

2.7. Enzyme linked immunosorbent assay

ELISA method was employed to measure level of inflammatory markers (TNF-α, IL-1β and p38 MAPK), apoptotic biomarkers (Bax and Bcl-2), HO-1 as well as CHOP in kidney tissues using

relevant ELISA kits according to the manufacturer's specifications.

2.8. Western immunoblotting

Kidney tissues were rinsed with PBS and then homogenized in ice-cold RIBA lysis buffer. Bradford Protein Assay Kit was utilized to determine the protein concentration. Equal quantities of protein from each sample were denatured using Laemmli sample buffer, resolved in SDS-PAGE (10%) and transferred to PVDF membrane (Millipore, USA). Membranes were blocked for 1 h in 3% BSA in TBS-Tween 20 buffer then, were incubated with one of the following primary antibodies: anti-Nrf2 (1:500), anti-NF-κB p65 (1:1000), anti-eNOS (1:1000) and anti-Caspase-3 (1:1000), at 4 °C overnight. Monoclonal antibody of β-actin (1:10000) was added and incubated at 4 °C for 1 h. The membranes were rinsed in TBS-Tween buffer to remove any excess antibodies then incubated with a suitable secondary antibody at room temperature for 1 h. Protein bands were visualized using the enhanced chemiluminescence (Ultra-Lum, Claremont, CA, USA) and quantified by densitometry using ImageJ analysis system.

2.9. Quantitative real-time polymerase chain reaction

QRT-PCR was performed to determine the relative expression of microRNA-7 (miR-7) in renal tissues. In brief, total RNA including miRNA was isolated from renal tissue sample using mirVana miRNA Isolation Kit and then, reverse transcribed to its cDNA using TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's recommendations. cDNA was quantified using a TaqMan-based RT-qPCR assay specific for miR-7 according to the manufacturer's protocol (Primers for miR-7 RT and PCR were present in the kits). All reactions were run in triplicate. The relative expression level of miR-7 was obtained using $2^{-\Delta\Delta CT}$ method. The internal control being used is SNORD68.

2.10. Histopathological examination

Kidney specimens from animals in different groups were dehydrated with graded ethanol (30-100 %). Tissue samples were embedded in paraffin using a hot air oven (56 ºC) for 24 h and then, were sliced into 4 µm sections, deparaffinized and stained with hematoxylin and eosin (H & E) for regular microscopic inspection [26].

2.11. Statistical analysis

Data were analysed by one way analysis of variance (ANOVA) tailed by Tukey's as post hoc test. Data were presented as mean and standard deviation. A $P < 0.05$ was regarded significant. GraphPad Prism (ISI®, USA) software (version 5) was applied to perform all statistical analysis and graphical sketching.

3. RESULTS

3.1. Effects of NR on GM-induced changes in the kidney functions in rats

Table 2 presents the serum BUN, creatinine and KIM-1 levels. Treatment of animals with GM for 7 days significantly increased serum BUN, creatinine and KIM-1 levels reaching 323.8 %, 738.9 % and 223.6 % respectively as compared with the control group. Rats that received NR besides GM showed a substantial decrease in serum BUN, creatinine and KIM-1 levels by 39.3 %, 64.2 % and 51.2 % respectively in comparison to the GM group. Additionally, the relative kidney weight was significantly increased by 51.5 % in the GM group with respect to the control group. Nicorandil significantly inhibited the increase in the relative kidney weight reaching 0.78 % in comparison to the GM group **(Table 2)**. Nicorandil therapy had no impact on its own on renal functions in comparison to control group. These outcomes showed that NR could efficiently correct GM-induced deteriorations in kidney functions.

3.2. Effects of NR on GM-induced renal oxidative stress in rats

In kidney tissues, the SOD activity and TAC were significantly reduced by GM treatment reaching 37.6 % and 42.5 %, respectively of the control values. This decline was reestablished by NR treatment as it significantly elevated these parameters by 160.7 % and 122.8 % respectively with respect to the GM group. In parallel, the renal tissue concentration of MDA was significantly elevated by 307.9 % in GM group relative to the control group, while its concentration was significantly attenuated by NR treatment reaching 48.3 % in comparison to the GM group. Nicorandil treatment without GM did not significantly alter the SOD activity, TAC or MDA compared to the control rats **(Table 3)**. These outcomes indicated that NR protected against GM-induced oxidative stress.

Table 2. Effects of NR on GM-induced changes in the kidney functions in rats.

Groups	Blood Urea Nitrogen (mg/dL)	Creatinine (mg/dL)	KIM-1 (pg/mL)	Relative kidney weight (%)
C	36.07 ± 8.51	0.18 ± 0.04	107.9 ± 4.89	0.33 ± 0.13
GM	116.80 ± 10.32 $^{@}$	1.51 ± 0.36 $^{@}$	349.2±31.40 $^{@}$	0.50 ± 0.04$^{@}$
GM+NR	70.92 ± 8.13 $^{@,\#}$	0.54 ± 0.14 $^{@,\#}$	170.3 ± 4.75 $^{@,\#}$	0.39 ± 0.29$^{\#}$
NR	40.70 ± 5.09 $^{\#,\$}$	0.20 ± 0.04 $^{\#,\$}$	104.4 ± 5.64 $^{\#,\$}$	0.30± 0.003$^{\#}$

NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, serum was separated and used to assess BUN, creatinine and KIM-1. In parallel, kidneys were excised and weighed to calculate the relative kidney weight. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ $p < 0.05$, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; BUN, blood urea nitrogen; C, control; GM, gentamicin; KIM-1, kidney injury molecule-1; NR, nicorandil.

Table 3. Effects of NR on GM-induced renal oxidative stress in rats.

Groups	SOD (U/mg protein)	TAC (mM/L)	MDA (nmol/mL)
C	43.52 ± 5.24	73.85 ± 14.42	46.78 ± 8.73
GM	16.37 ± 2.24 $^{@}$	31.38 ± 5.79 $^{@}$	190.8 ± 14.56 $^{@}$
GM+NR	42.68 ± 4.06 $^{\#}$	69.93 ± 4.45 $^{\#}$	92.08 ± 16.78 $^{@,\#}$
NR	46.28 ± 11.16 $^{\#}$	79.85 ± 6.54 $^{\#}$	45.73 ± 8.03 $^{\#,\$}$

NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess oxidative stress biomarkers. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ $p < 0.05$, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; GM, gentamicin; MDA, malondialdehyde; NR, nicorandil; SOD, superoxide dismutase; TAC, total anti-oxidant capacity.

3.3. Effects of NR on GM-induced changes in the renal Nrf2 protein expression and HO-1 content in rats

Figure 1 revealed that treatment of animals with GM significantly decreased the renal tissues Nrf2 protein expression as well as HO-1 content reaching 18.1 % and 21 % respectively of the control values. Oral NR treatment caused a substantial rise in both parameters to 373.7 % and 308.4 % respectively in comparison to the GM group. Nicorandil treatment without GM did not significantly alter Nrf2 protein expression or the HO-1 content in renal tissues compared with the control group. These outcomes indicated that NR protected against GM-induced oxidative stress via modulation of Nrf2/HO-1 pathway.

3.4. Effects of NR on GM-induced renal inflammation in rats

Figure 2 illustrated that administration of GM to rats markedly elevated the renal levels of TNF-α, IL-1β and p38 MAPK reaching 398.7 %, 324.4 % and 495.5 %, respectively as compared to normal control rats. Rats that received NR and GM exhibited a marked decrease in renal levels of TNF-α, IL-1β and p38 MAPK reaching 39.7 %, 49.3 % and 36.2 %, respectively as compared with the GM group. Moreover, western blotting revealed that the renal protein expression NF-κB p65 was significantly elevated in GM treated rats reaching 563 % compared to normal control rats. Oral NR administration significantly attenuated this elevation to 36.8 % of the GM group value. Again, NR treatment without GM did not significantly alter these parameters in renal tissues compared with the control animals. These results could imply that NR

treatment diminished the renal inflammation brought by GM.

3.5. Effects of NR on GM-induced changes in the renal NO content and eNOS protein expression in rats

Treatment with GM caused a considerable upsurge in the renal level of NO and a marked decline in eNOS protein expression reaching 312 % and 30.3 % respectively when compared to the control values. Following NR treatment, renal levels of NO significantly decreased and levels of eNOS protein expression markedly increased by 54.7 % and 186.7 % respectively in comparison with the GM group. Nicorandil treatment without GM did not significantly alter the renal levels of NO or eNOS protein expression in comparison to the control group **(Figure 3)**. These findings suggest that NR treatment attenuated GM-induced aberrations in NO and eNOS protein expression.

3.6. Effects of NR on GM-induced apoptosis in rats

Figure 4 revealed that injection of GM resulted in marked increase in Bax levels accompanied by a substantial decline in Bcl-2 levels in renal tissues reaching 349 % and 47.3 %, respectively in comparison to the control group. Upon treatment with NR, renal levels of Bax significantly declined and levels of Bcl-2 markedly increased with respect to the GM group by 56.2 % and 95.8 % respectively. On the other hand, expression caspase 3 was significantly increased by 463 % in the GM group in comparison to the control group, and NR treatment significantly attenuated this increase reaching 50.6 % in comparison to with the GM group. Nicorandil treatment alone did not significantly alter the renal levels of apoptotic markers in comparison to the control group. These outcomes confirmed that NR could depress GM-induced apoptosis.

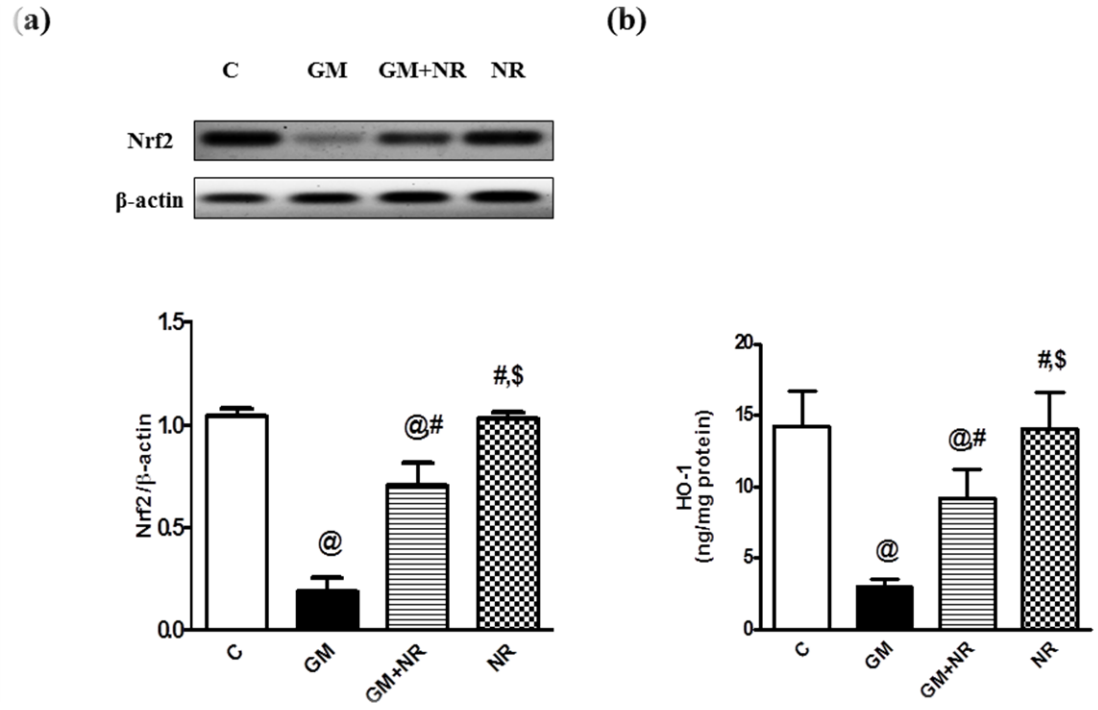

Figure 1. Effects of NR on GM-induced changes in the renal Nrf2 protein expression **(a)** and HO-1 content **(b)** in rats. NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess Nrf2 protein expression and HO-1 content. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ $p < 0.05$, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; GM, gentamicin; HO-1, heme oxygenase-1; NR, nicorandil; Nrf2, nuclear factor erythroid 2-related factor 2.

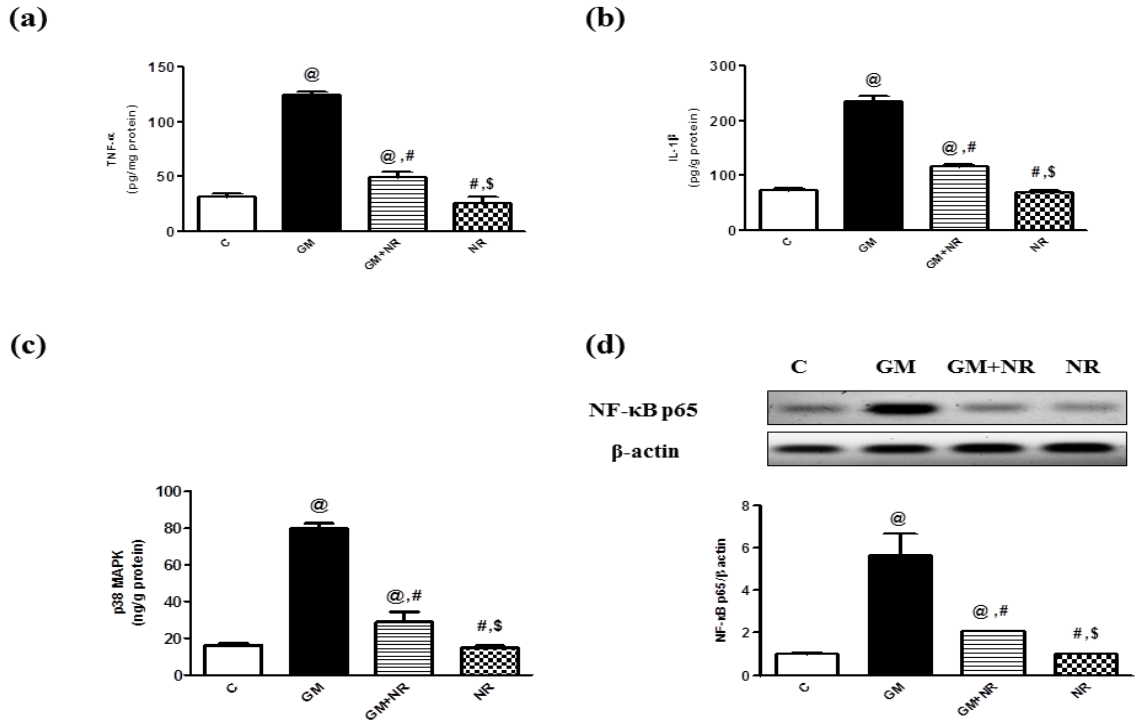

Figure 2. Effects of NR on GM-induced changes in renal levels of TNF-α **(a)**, IL-1β **(b)** and p38 MAPK **(c)** as well as NF-κB p65 protein expression **(d)** in rats.
NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess levels of TNF-α, IL-1β and p38 MAPK as well as NF-κB p65 protein expression. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ $p < 0.05$, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; GM, gentamicin; IL-1β, interlukin-1 beta; NF-κB p65, nuclear factor-κappa B p65; NR, nicorandil; p38 MAPK, p38 mitogen-activated protein kinase; TNF-α, tumor necrosis factor-alpha.

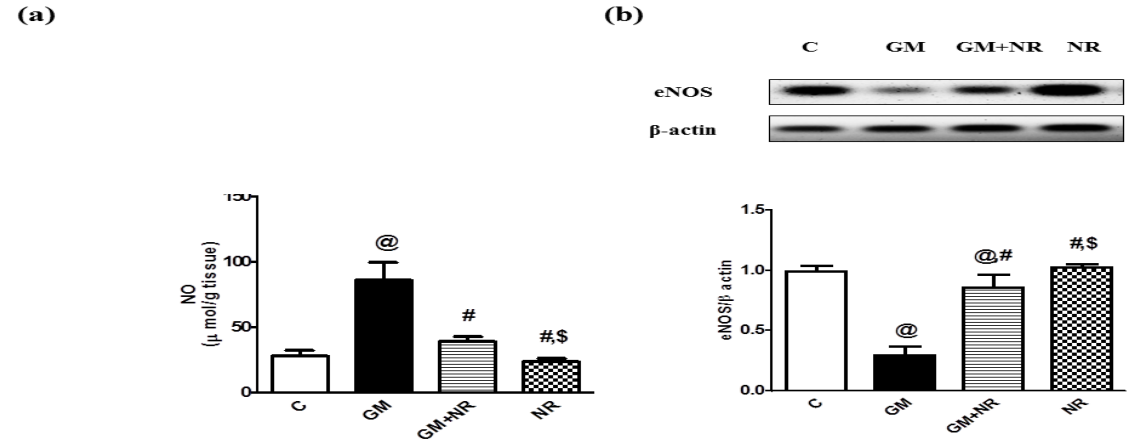

Figure 3. Effects of NR on GM-induced changes in the renal NO content **(a)** and eNOS protein expression **(b)** in rats.
NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess NO level and eNOS protein expression. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ $p < 0.05$, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; eNOS, endothelial nitric oxide synthase; GM, gentamicin; NR, nicorandil; NO, nitric oxide.

3.7. Effect of NR on GM-induced ER stress in rats

Table 4 illustrated that GM treatment produced a significant elevation in renal levels of CHOP, an ER stress marker, reaching 340.1 % in comparison to the control group. Nicorandil treatment lessened this increase to 49.4 % in comparison to the GM group. Nicorandil treatment

without GM did not alter the renal levels of CHOP with respect to the control group. These outcomes confirmed that NR could indeed lessen GM-induced ER stress.

Table 4. Effect of NR on GM-induced ER stress in rats.

Groups	CHOP (ng/mL)
C	19.23 ± 2.57
GM	65.40 ± 4.44 $^{@}$
GM+NR	32.28 ± 4.41 $^{@,\#}$
NR	15.10 ± 1.81 $^{\#,\$}$

NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess CHOP, an endoplasmic reticulum stress biomarker. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ p < 0.05, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; CHOP, CCAAT-enhancer binding protein homologous protein; GM, gentamicin; NR, nicorandil

3.8. Effect of NR on GM-induced changes in renal miR-7 gene expression in rats

Injection of GM caused a significant decline in the renal expression of miR-7 reaching 23.6 % in comparison to control group. Oral NR administration significantly elevated the renal miR-7 expression by 176 % as compared to GM group **(Table 5)**. These findings suggested that NR-induced protection against GM renal injury might be related to miR-7.

Table 5. Effect of NR on GM-induced changes in renal miR-7 gene expression in rats.

Groups	miR-7 (fold induction)
C	1.06 ± 0.04
GM	0.25 ± 0.02 $^{@}$
GM+NR	0.69 ± 0.08 $^{@,\#}$
NR	1.03 ± 0.04 $^{\#,\$}$

NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess miR-7 relative expression. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ p < 0.05, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; C, control; GM, gentamicin; miR-7, micro-RNA 7; NR, nicorandil.

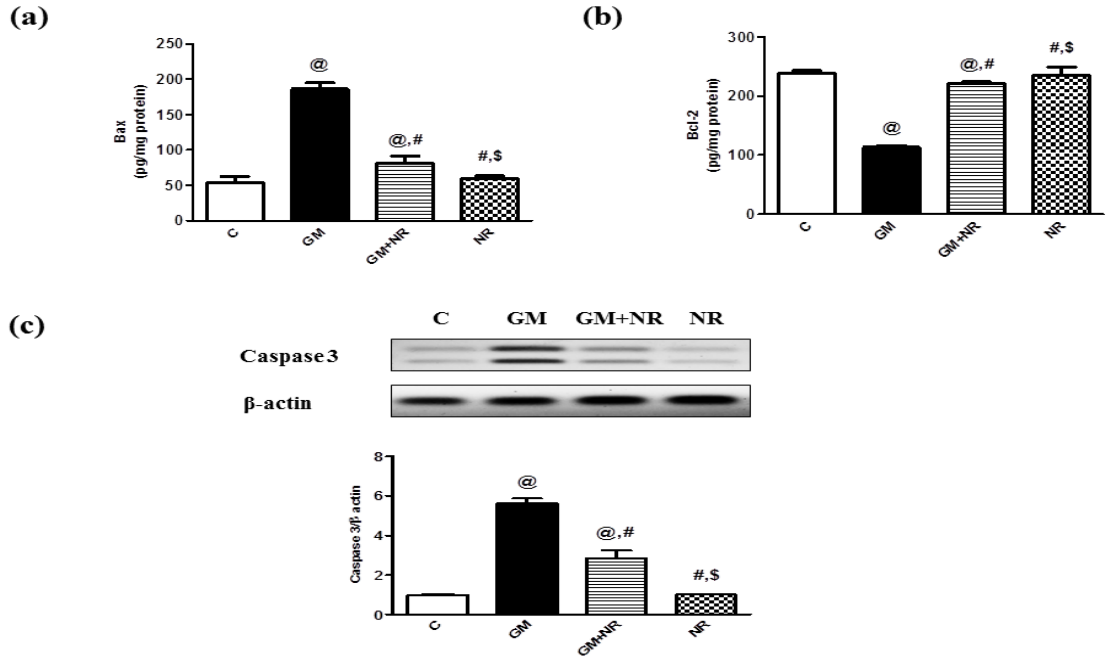

Figure 4. Effects of NR on GM-induced changes in renal levels of Bax **(a)** and Bcl-2 **(b)** as well as caspase 3 protein expression **(c)** in rats.

NR (15 mg/kg, orally) was daily administered for 21 days starting 14 days before GM (100 mg/kg/day) injection. On Day 22, kidney tissues were used to assess levels of Bax and Bcl-2 as well as caspase 3 protein expression. Values are presented as mean ± SD, with n = 6 animals/group. @, # or $ p < 0.05, denotes significant difference from C, GM or GM+NR group, respectively, using one way ANOVA followed by Tukey's as post-hoc test. ANOVA, analysis of variance; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; C, control; GM, gentamicin; NR, nicorandil.

3.9. Effects of NR on GM-induced changes in the renal histology in rats

The histopathological sections of kidneys are shown in **Figure 5**. Animals in vehicle control **(C)** group and nicorandil **(NR)** group showed normal histological architecture of the tubules ***(t)*** and glomeruli ***(g)*** at the cortical regions. Animals in gentamicin **(GM)** group showed evidence of diffused coagulative necrosis ***(co)*** of the lining tubular epithelium at the cortex. Additionally, peri-vascular oedema ***(o)*** and infiltration of inflammatory cells ***(m)*** were detected nearby the congested blood vessels ***(bv)***. In addition, there were degeneration ***(d)*** and desquamation of some tubules at the cortex. Gentamicin plus nicorandil **(GM+NR)** group showed only focal inflammatory cells infiltration ***(m)*** between the tubules at the cortex. The semi-quantitative histological score of the kidney sections is shown in **Table 6**.

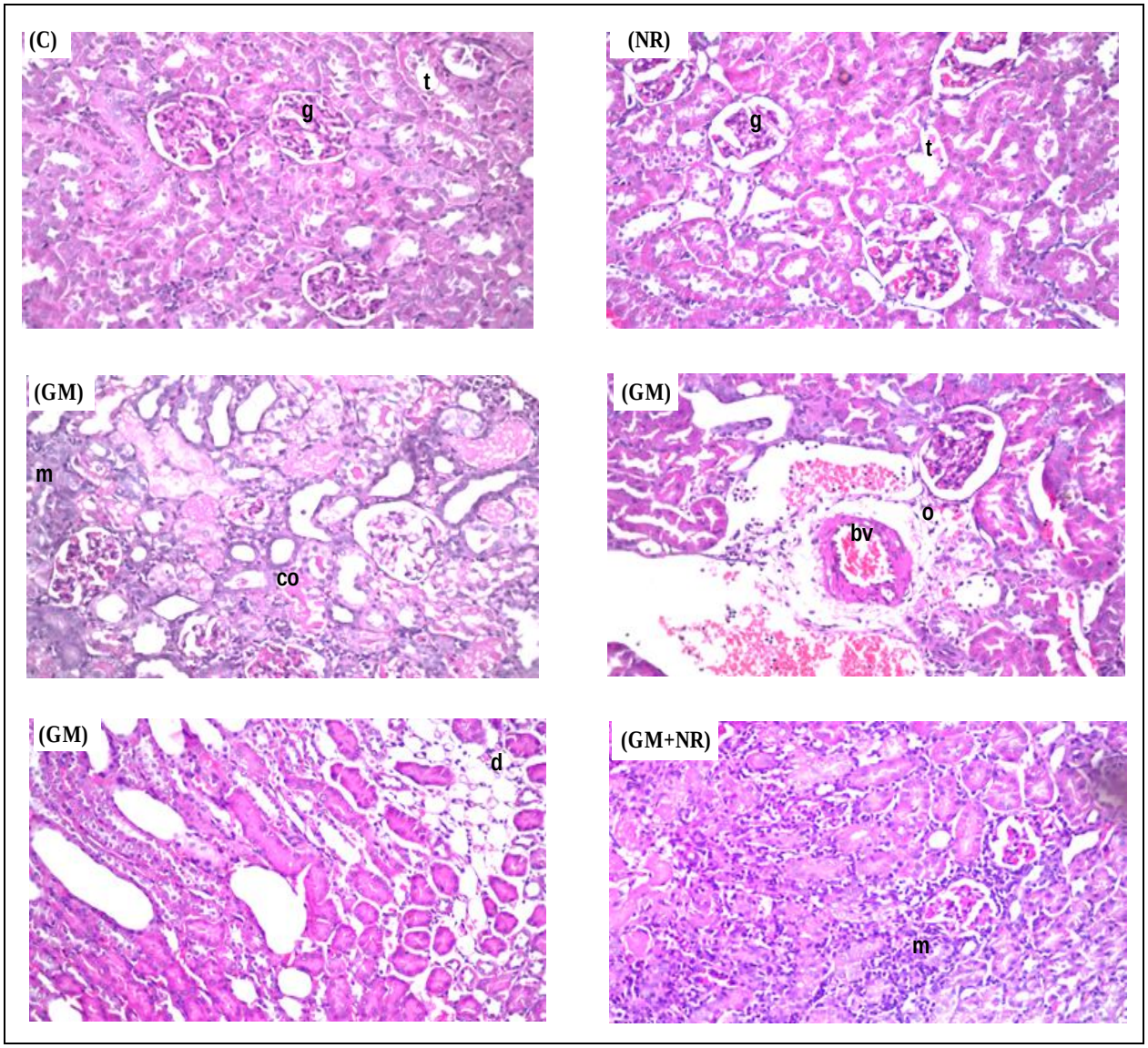

Figure 5. Effects of NR on GM-induced changes in the renal histology in rats.
H & E stained kidney sections from vehicle control **(C)** and nicorandil **(NR)** groups showed normal histological structure of the tubules ***(t)*** and glomeruli ***(g)*** at the cortex. Gentamicin **(GM)** group showed evidence of diffused coagulative necrosis ***(co)*** of the lining tubular epithelium at the cortical region. Additionally, peri-vascular oedema ***(o)*** and infiltration of inflammatory cells ***(m)*** were detected nearby the congested blood vessels ***(bv)***. In addition, there were degeneration ***(d)*** and desquamation of some tubules at the cortex. Gentamicin plus nicorandil **(GM+NR)** group showed only focal inflammatory cells infiltration ***(m)*** between the tubules at the cortex.

Table 6. Semi-quantitative histological score of the kidney sections taken from C (control), **GM** (gentamicin), **GM+NR** (gentamicin + nicorandil) and **NR** (nicorandil) groups.

Histopathological alterations	Groups			
	C	GM	GM+NR	NR
Tubular coagulative necrosis	–	+++	–	–
Congestion	–	++	–	–
Perivascular oedema & inflammatory cells infiltration	–	+++	–	–
Tubular degeneration	–	++	–	–
Focal inflammatory cells infiltration	–	++	++	–

4. DISCUSSION

The current study highlights the protective role of NR against GM-prompted nephrotoxicity potentially through targeting Nrf2 and NF-κB signaling, modulating eNOS and mitigating ER stress.

Gentamicin is the most clinically used aminoglycoside antibiotic. It has broad spectrum of activities against Gram-negative infectious bacteria. However, restrictions on its use are mainly due to nephrotoxic adverse effects [2, 27].

In the current work, GM injection caused obvious aberrations in both the structure as well as the function of renal tissues. The architectural abnormalities were verified by the histopathological findings; coagulative necrosis all over the lining tubular epithelium, perivascular edema as well as focal and perivascular inflammatory cells infiltration. These results were consistent with earlier researches[28, 29] .

On the other hand, the functional impairments were evinced by elevated serum BUN, creatinine and KIM-1 levels as well as the relative kidney weight. High levels of BUN and creatinine is a consequence of decrease in the glomerular filtration rate[30], an event indicative of kidney damage and renal tubular necrosis induced by GM [31]. After renal damage, the transmembrane glycoprotein KIM-1 is expressed more abundantly in the proximal tubular cells[32]. Its expression has been shown to be correlated with renal damage in various human renal diseases[33]. The increase in relative kidney weight in GM-treated animals may be attributed to edema of renal parenchyma, which is triggered by the renal inflammation induced by GM [2, 34, 35].

Nicorandil treatment significantly improved both structural and functional disruption. This nephron-protecting potential of NR could be enlightened by its vasodilatory effect that enhances the renal perfusion and excretion. Similar reports proposed that NR amends nephrotoxicity in different models of renal injury in rats [5, 18]. Therefore, NR may be beneficial in alleviating the kidney damage brought on by GM.

Accumulating evidences supported the involvement of oxidative stress response and production of reactive oxygen species (ROS) in the pathogenesis of GM nephrotoxicity. The culminated release of ROS along with impairment of renal antioxidant defense, exposed the renal cell membrane to attack of ROS, and accelerated lipid peroxidation in the renal tissue [36]. This chiefly lied behind the observed decrease in SOD activity, TAC and the elevated MDA level in the present study. These observations agreed with previous studies [2, 37, 38]. Consistent with these results, the GM-treated group showed a decrease in Nrf2 protein expression and HO-1 level in renal tissues [39]. Nrf2 is essential for reducing the oxidative stress response, through regulating antioxidant protein expression [40]. HO-1 is one of the enzymes that upregulated in response to Nrf2 stimulation and catalyzes the oxidative degradation of free dangerous heme into bilirubin [41].

Interestingly, oral NR administration mitigated the negative effects of GM-induced oxidative stress. Earlier, the capability of NR to decrease oxidative stress has been previously reported[42-45]

In the current study, treatment of animals with GM significantly amplified the renal levels of TNF-α, IL-1β, NF-κB p65 protein expression and its upstream associated kinase, p38 MAPK, favoring the role of GM-derived inflammation as a factor in development of nephrotoxicity.

GM-induced ROS formation is probably the key element in activation of NF-κB and p38 MAPK pathways and in the production of TNF-α and IL-1β [2]. NF-κB is a transcription factor that switched on in

response to oxidative stress and plays a vital role in launching the inflammatory cascades[46]. Moreover, GM nephrotoxicity participates in mesangial and vascular contraction and contributes to inflammation by the infiltration of various inflammatory cells and releasing different pro-inflammatory cytokines, as IL-1β and TNF-α, which trigger the NF-κB pathway and results in amplification of the inflammatory response[46].

In the current study, treatment of GM challenged rats with NR showed marked reduction in renal levels of TNF-α, IL-1β and p38 MAPK in addition to NF-κB p65 protein expression. In agreement with our results, NR alleviated acute lung injury induced by LPS by decreasing oxidative stress and repressing endothelial inflammation, an effect that might be caused by suppression of NF-κB and MAPK signaling pathways [42].

Although the basal release of NO is vital for renal functions, overproduction is largely associated with oxidative stress [47, 48]. Also, in response to the pro-inflammatory cytokines, 100 to 1000 fold NO is generated from the iNOS more than eNOS. The iNOS-generated NO has detrimental effects on different body organs [5]. In the current research, we detected elevated levels of NO accompanied by reduced expression of eNOS in renal tissues by GM treatment. These observations aligned with what was reported by **Abd-Elhamid** et al. and **Buffoli** et al. [48, 49]. In line, **Furusu et al**. have demonstrated the negative correlation between eNOS expression and the degree of renal injury in experimental animals [50].

In contrast, NR treatment reversed the impact of GM on NO production as well as eNOS expression [51]. Recently, NR has been reported to increase eNOS protein expression in bleomycin-induced pulmonary fibrosis [52], as well as nephrotoxic model of cyclosporine-A in rats [23]. Interestingly, it has been demonstrated that NR has the ability to enhance the expression eNOS in cardiac tissue via activation of an ATP-dependent K channel [44].

The present study presented apoptosis as one of the essential causes of GM-induced nephrotoxicity. This was evidenced by the marked increase in renal Bax levels and caspase-3 protein expression along with the significant decrease in Bcl-2, that ultimately leading to disruption of the Bax to Bcl-2 ratio. Bax acts as a pro-apoptotic protein, whereas Bcl-2 exerts its anti-apoptic effect via binding to the outer membrane of mitochondria and blocking cytochrome c activation [53]. These results are consistent with previous published reports[54, 55]. Conversely, NR treatment significantly reduced Bax and Caspase-3 and significantly increased the level of Bcl-2 hence renovating the Bax/Bcl-2 ratio in the NR treated animals as compared to the GM treated ones. These results are in line with earlier studies of **He** et al. and **Yu** et al. [42, 56]. Altogether, the antiapoptotic effects of NR might be related to its antioxidative properties as well as suppression of the p38MAPK-mediated activation of NF-κB [42].

Moreover, GM has been reported to bind with the eukaryotic 80S ribosome, activating an ER stress. ER stress activation is considered one of the fundamental pathways that supports the defense against different cellular stressful conditions [46, 57]. However, extremely activated ER could trigger apoptotic pathways and thereby result in cell death [10] .

Here, the significant elevation of CHOP, an ER stress biomarker, level in renal tissues revealed that GM treatment could induce an ER stress response and hence apoptosis[46]. This was already confirmed in our work by down regulation of the anti-apoptotic protein along with the up regulation of pro-apoptotic proteins. It is noteworthy that the increased CHOP is significantly attenuated by NR treatment. In accordance, NR had been demonstrated to diminish the expression of partaker proteins implicated in the ER stress response and apoptosis such as caspase 12, GRP78 and CHOP in injured astrocytes [17].

MicroRNAs are small, non-coding RNAs molecules involved in almost all physiological as well as pathological processes [11]. Emerging evidence has proposed that microRNAs can regulate an ER stress. For example, **Chitnis**, et al. investigated the association between miR-221 and CHOP-mediated apoptosis during ER stress [12]. On the other hand, **Dong** et al. showed that miR-7 was participated in oxygen-glucose deprivation-evoked ER stress in astrocytes. In this regard, the mRNA levels of Herpud2, an essential ER stress-related molecule, were apparently improved by miR-7 inhibitors and attenuated by using mimics of miR-7 [17].

Our study demonstrated the ability of GM to downregulate miR-7 and in turn miR-7-modulated ER stress thus enhancing the CHOP levels in renal tissues. Thus, our research suggested that miR-7 might be implicated in GM-induced ER stress. GM-

induced oxidative stress and ATP-depleting mechanisms contribute to the malfunction of ATP-dependent K channels. These channels are abundant in the ER and thus their malfunction is critically implicated in ER stress signaling pathway [58].

In the present study, NR, as an ATP-dependent K channels' opener, could prevent the decline in miR-7 elicited by GM and thus attenuating ER stress. These findings suggested that NR protected against GM-induced renal damage might be correlated with miR-7.

In line with our results, NR was stated to provide protection against ischemia/reperfusion injury in cardiac tissues and oxygen-glucose deprivation in astrocytes via attenuating ER response-induced apoptosis [17,59].

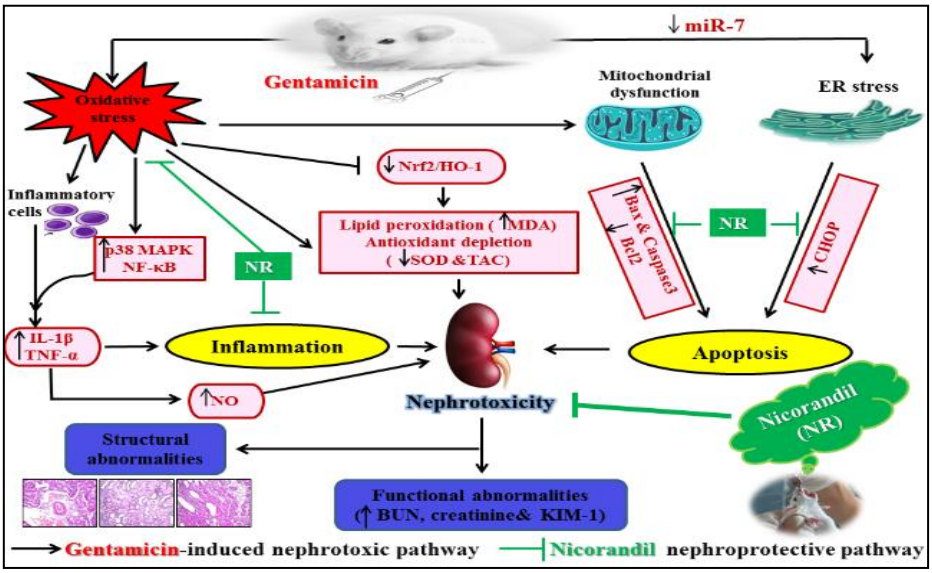

Figure 6. Graphical abstract of the modulatory nephroprotective effect of Nicorandil against Gentamicin-induced nephrotoxicity in rats.

5. CONCLUSIONS

In summary, this study affords evidence that NR can diminish GM-induced nephrotoxicity in rats by modulating oxidative and inflammatory stress responses as well as ER stress. Besides, we also highlighted the important roles of miR-7 and eNOS in nephroprotective effect exerted by NR.

Supplementary Materials:

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Conflicts of Interest: None of the authors has conflicts of interest to declare.

Ethical Statement: Everything in animals' techniques was done according to the Ethics Committee of the faculty of Pharmacy Al-Azhar University, Egypt (permit number: 203/2019). Unnecessary disturbance of animals, pressure and tough maneuver was avoided..

Author Contribution: Hebatalla I. Ahmed and Somaia A. Abdel-Sattar shared developing the research idea, designed the experiments, supervised the experiments performance, executed data analysis, wrote and revised the manuscript. Nashwa I. Abd El-Azeem performed the experiments, collected the data, carried out the graphical and statistical analysis and wrote the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

List of Abbreviations: ANOVA, analysis of variance; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; BUN, blood urea nitrogen; CHOP, CCAAT-enhancer binding protein homologous protein; CMC, carboxymethyl cellulose; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; GM, gentamicin; H & E, hematoxylin and eosin; HO-1, heme oxygenase-1; IL-1β, interlukin-1 beta; iNOS, inducible nitric oxide synthase; KIM-1, kidney injury molecule-1; MDA, malondialdehyde; miR-7, microRNA-7; NF-κB p65, nuclear factor-κappa B p65; NO, nitric oxide; NR, nicorandil; Nrf2, nuclear factor E2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase; QRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant

capacity; TNF-α, tumor necrosis factor-alpha; UPR, unfolded protein response.

Highlights: Nicorandil improves renal function in gentamicin-challenged rats; Nicorandil modulates gentamicin-induced oxidative and inflammatory stress responses; Nicorandil modulates Nrf2 and NF-κB transcriptional activities in gentamicin-challenged rats; Nicorandil ameliorates aberrations in eNOS expression induced by gentamicin; Nicorandil attenuated gentamicin-evoked endoplasmic reticulum stress.

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