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Research article

Anti-oxidant impact of Lisinopril and Enalapril against acute kidney injury induced by doxorubicin in male Wistar rats: involvement of kidney injury molecule-1

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ABSTRACT

Doxorubicin (DOX) is a standard anticancer agent exerting devastating effects as nephrotoxicity, hepatotoxicity and cardiotoxicity. The purpose of this study was to increase the clinical use of DOX through decreasing its detrimental effects via combination with ACE inhibitors to ameliorate the induced acute kidney injury (AKI). AKI was induced by a single injection of DOX (7.5 mg/kg; i.p.) as Group 1; control (vehicle), Group 2; DOX (7.5 mg/kg; i.p.) single dose, Group 3 and 4; Lisinopril (Lis, 20 mg/kg) and Enalapril (Enal, 40 mg/kg) orally administration for 15 consecutive days after DOX injection, respectively. Serum samples were used to measure creatinine and BUN, tissue samples were extracted to determine myeloperoxidase (MPO), malondialdehyde (MDA), total antioxidant capacity (TAC) and kidney injury molecule (KIM-1) using ELISA technique. Heme oxygenase (HO-1) RNA expression was quantified in tissue using real time polymerase chain reaction (PCR). Parts of the kidney tissue were kept in formalin for immunohistochemical demonstration of Cleaved Caspase-3 and NF-κβ immune staining and the other part was used for pathological examination. Oral treatment with Lis (20 mg/kg) and Enal (40 mg/kg) for 15 consecutive days reversed DOX effects as they reduced the serum creatinine and BUN, kidney levels of MPO and MDA, whereas the drugs increased tissue TAC. The administration of Lis and Enal with DOX also reduced KIM-1and HO-1 RNA expression. A significant decrease in cleaved caspase-3 and NF-κβ immunostainings in conjunction with pronounced amelioration in pathologies in the rat kidney were observed. We concluded that DOX adverse effects can be controlled by Lis and Enal.

1. Introduction

Acute kidney injury (AKI) represents a considerable burden in healthcare. Nephrotoxicity induced by various drugs is a major cause of AKI [1]. The effective anticancer agent doxorubicin (DOX) is one of these drugs that gained wide fame in curing different types of cancers. Unfortunately, DOX has been recorded to cause severe devastating adverse effects, e.g., cardiotoxicity, hepatotoxicity and nephrotoxicity [2].

DOX-induced nephrotoxicity occurred due to increased free radicals production, leading to membrane lipid peroxidation as well as deleterious effects on kidney tissue causing tubular degeneration [3]. The free radical production may be attributed to DOX's chemical constituent of the water-insoluble planar tetracycline which binds to the water-soluble sugar daunosamin. Furthermore, DOX itself can be transformed into a free radical that reacts with oxygen resulting in oxidative stress and ultimately cell death following the release of superoxide [4]. DOX has also been recorded to exert apoptotic effects by affecting mainly the mitochondrial pathway [5].

Nowadays, researchers are implementing wide investigation aiming to increase the clinical use of chemotherapeutic drugs via decreasing their detrimental adverse effects [6]. Hence, the use of various agents in combination with DOX is under investigation. Angiotensin-converting enzyme inhibitors (ACEIs) are widely prescribed for patients suffering from hypertension and congestive heart failure. It has also been reported that scavenging of both free radicals and oxidants was attributed to the presence of sulfhydryl group in ACE inhibitors. Whereas, lipid peroxidation was reduced by both sulfhydryl- and non-sulfhydryl containing ACE inhibitors [7].

This study will examine the role of two non-sulfhydryl ACE inhibitors; *viz.*, Lisinopril (Lis) and Enalapril (Enal) in the protection against DOX-

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induced AKI in rats by determining their effect on oxidative stress referring to regulation of KIM-1 and HO-1 RNA expression. The study also will go in-depth to test the immunohistochemical expression of Cleaved caspase-3 as well as NF-KB and measure the quantitative scores of both parameters.

2. Material and methods

2.1. Drugs

Doxorubcin vials (Adricin®, Hikma pharmaceuticals, Egypt), lisinopril tablets (zestril®, AstraZeneca, UK) and enalapril tablets (Enalapril®, October Pharma S.A.E., Egypt) were used in the current study. All other chemicals used in the experiment were of the highest available grade.

2.2. Animals

Forty male Wistar rats (250–350 g) purchased from the animal breeding unit at the National Research Centre -Dokki- Giza – Egypt. Animals were housed in cages with water and food ad-libitum, and the animal room was kept at constant temperature of 20 ± 1 °C on a 12-hour light/12-hour dark cycle. Adequate measures were taken to minimize pain or discomfort of the animals. Experimental protocol was approved by the Ethics and Animal Care Committee of the National Research Centre (NRC-MREC), and following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.3. Experimental protocol

Forty male wistar rats were divided randomly into four groups (n = 10). Group 1 (Control -ve) was given saline for 15 consecutive days. Groups 2, 3 and 4 were injected with single dose of DOX (7.5 mg/kg; i.p.) to induce AKI [8]. Group 2 (DOX group) was administered DOX only. Group 3 (DOX + Lis) and group 4 (DOX + Enal) administered DOX followed by Lis (20 mg/kg; p.o.) and Enal (40 mg/kg; p.o.) for 15 consecutive days. 48 hours after the end of the experiment, blood samples were collected from the retro-orbital plexus under mild ketamine anesthesia from the overnight-fasted animals into sampling tubes. Serum was used for measurement of biochemical parameters (creatinine, blood urea nitrogen (BUN)) using specific diagnostic kits (Biodiagnostic, Egypt). After blood sampling, animals were sacrificed by cervical dislocation under anesthesia and kidneys were extracted, part of each kidney was kept at -80°c and used for measurement of myeloperoxidase (MPO), malondialdehyde (MDA), total antioxidant capacity (TAC) and kidney injury molecule (KIM-1) using ELISA kits. Heme oxygenase (HO-1) RNA expression was quantified in kidney tissue using real time polymerase chain reaction (PCR). The other part of the kidney was kept in formalin for histopathological and immunohistochemical studies. The experimental protocol is presented in Figure 1.

2.4. Preparation of tissue samples

Kidneys were homogenized (MPW-120 homogenizer, Med instruments, Poland) in PBS to obtain 20% homogenate that was stored overnight at –80 °C. The homogenates were centrifuged for 5 min at 5000 x g using a cooling centrifuge (Sigma and laborzentrifugen, 2k15,

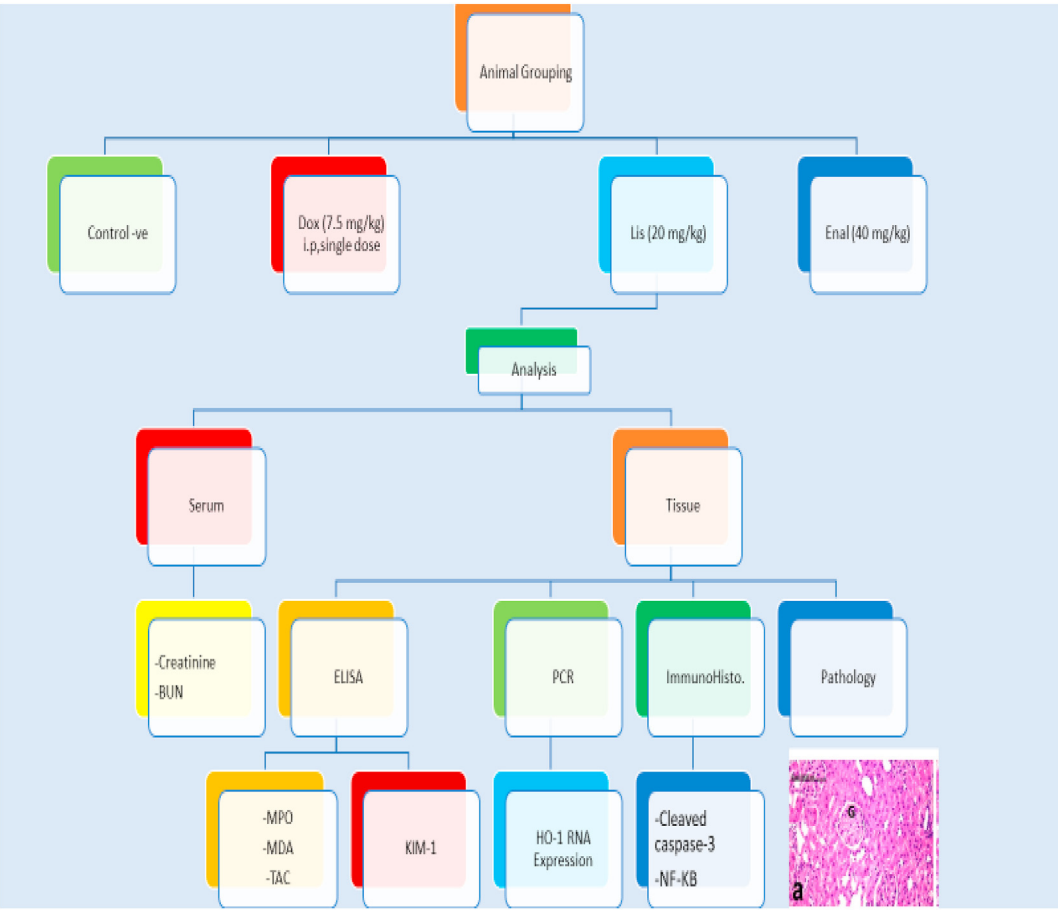


Figure 1. Flow chart for the experimental protocol.

Germany). The supernatant was removed immediately and assayed for oxidative stress biomarkers: myeloperoxidase (MPO), malondialdehyde (MDA) and total antioxidant capacity (TAC) according to the following methods [9, 10] and [11] respectively using specific ELISA kits according to manufacturer's instructions. Kidney injury molecule-1 (KIM-1) concentration was measured using specific ELISA kits according to manufacturer's instructions (Cloud-Clone Corp-USA). All results are calculated per 1mg of total protein.

2.5. Real-time polymerase chain reaction (PCR) quantification of heme oxygenase-1 (HO-) RNA expression

Heme oxygenase 1 (HO-1) RNA was extracted from tissues using TRIzol RNA Mini kit Cat No. 15596-026 (Invitrogen, Carlsbad, CA). RNA extracted from tissue homogenate was eluted in 40 ul of nuclease-free distilled water and stored at 20 °C until analysis and quantified using standard laboratory protocol as recommended. Quantification of HO-1 PCR was carried out by using HO-1 PCR Fluorescence Quantitative Diagnostic Kit Cat No. (M R 246187) with Masrecycler realpex (Eppendorf, Westbury, N.Y.)

- HO-1:
  Forward 5-ATGGCCTCCCTGTACCACATC-3
  Reverse 5-TGTTGCGCTCAATCTCCTCCT-3
- Housekeeping control gene is –β actin
- RNA was transcribed using the SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad,CA)

2.6. Histopathological examination

Directly after sacrifice of animals, parts of the kidneys from normal and treated rats were removed and fixed in 10% neutral formalin. The tissues were then routinely processed and embedded in paraffin blocks. Finally, the tissues were cut into 5 μm thick sections and stained with H&E. Pathological scoring of kidney damage was semi-quantitatively assessed in ten random low power fields (10X); according to the percentage of tissue showing damage; as described by Altınkaynak et al., (2018) [12]. Grading system scaled from 0 to 3 was used; in which 0 = normal histological structure, 1 = kidney damage in less than 25% of tissue, 2 = kidney damage in 25%–50% of tissue and 3 = kidney damage in ˃50% of tissue. The pathological parameters used in this evaluation were vacuolar degeneration of kidney tubules, glomerular congestion, glomerular atrophy and interstitial inflammatory cell infiltration.

2.7. Immunohistochemical analysis

Immunohistochemical procedures for the demonstration of Cleaved Caspase-3 and NF-κβ immune staining were performed according to the method of Khalil et al, (2019) [13]. Briefly, the paraffin-embedded kidney sections were dewaxed and rehydrated. The sections were then incubated with rabbit monoclonal anti-Caspase-3 (ab 184787, abcam) and rabbit monoclonal anti NF-κβ (ab 32360, abcam) antibodies. Diaminobenzidine (DAB) was used to demonstrate the immune reaction. The cells with cytoplasmic and/or nuclear staining were considered positive for cleaved caspase-3, but cells with nuclear staining were considered positive for NF-κβ. For assessment of the immune reactivity, a semi-quantitative grading system scaled from 0 to 3 was used relying on the percentage of positive cells in ten high power fields (40X) as previously described [14] in which 0 = no staining; ≥1 to ˂ 2 = positive staining in <30% of cells per HPF; ≥2 to ˂3 = positive staining in 30–70% of cells per HPF and≥3 = positive staining in >70% of cells per HPF.

2.8. Statistical analysis

Data represented as mean ± SE. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by *Tukey-Kramer* test for multiple comparisons.

On the other hand, Caspase3 and NF-κβ immunohistochemical staining assessment was analyzed by performing Kruskal-Wallis non-parametric ANOVA test followed by Mann-Whitney *U* test. In all cases, a *P* value of <0.05 was assumed to denote statistical significance.

3. Results

3.1. Measuring kidney biochemical parameters

Kidney biochemical parameters (Creatinine and **BUN**) were determined in the sera and the obtained findings in Table 1 showed a significant increase (*$p < 0.05$) in creatinine and BUN concentrations in DOX induced group (1.12 ± 0.03 and 42.78 ± 0.9 mg/dl respectively) when compared to the normal control group (0.67 ± 0.01 and 27.56 ± 0.4 mg/dl respectively) showing an increase in creatinine and BUN concentration to 167.36% and 155.22% respectively in DOX induced group as compared to control -ve group. Oral treatment of DOX induced rats with Lis (20 mg/kg) caused a significant reduction ($^{@}p < 0.05$) in creatinine and BUN level (0.68 ± 0.04 and 30.02 ± 0.6 mg/dl) respectively as compared to DOX induced group restoring creatinine and BUN concentrations into normal levels. Moreover, a significant decrease ($^{@}p < 0.05$) in creatinine and BUN level was observed in the Enal (40 mg/kg) treated group (0.83 ± 0.03 and 37.59 ± 1.5 mg/dl) respectively when compared to the DOX induced group showing a decrease in creatinine and BUN concentration to 74.6% and 87.8% respectively as compared to DOX induced group. These results indicate that Lis and Enal might have a regenerative effect on kidney function by reducing creatinine and BUN biomarkers in the DOX induced rat model.

3.2. Oxidative stress biomarkers

Oxidative stress biomarkers (MPO, MDA and TAC) were measured in the kidney tissue homogenate. The obtained data in Table 2 showed a significant increase (*$p < 0.05$) in MPO and MDA and a significant decrease (*$p < 0.05$) in TAC levels in DOX induced group (4.07 ± 0.18 ng/mg total protein, 10.9 ± 0.3 nmol/mg total protein and 1.44 ± 0.08 mM/mg total protein) respectively when compared to the normal control

Table 1. Effects of lisinopril and enalapril on biochemical parameters in a doxorubicin-induced rat model of acute kidney injury.

Groups	Creatinine (mg/dl)	Blood Urea Nitrogen (BUN) (mg/dl)
Control -ve	0.67 ± 0.01	27.56 ± 0.4
Dox (7.5 mg/kg)	1.12 ± 0.03*	42.78 ± 0.9*
Lis (20 mg/kg)	0.68 ± 0.04@	30.02 ± 0.6@
Enal (40 mg/kg)	0.83 ± 0.03*@	37.59 ± 1.5*@

Data were represented as (mean ± SE, n = 10/group). Statistics were done using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests for confirmation. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril.
*$p < 0.05$ represents a significant difference from the control negative group.
@$p < 0.05$ represents a significant difference from the DOX group.

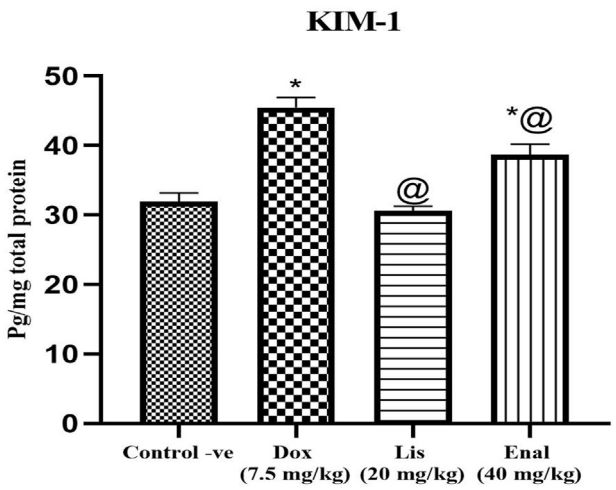


Figure 2. Effect of Lis and Enal on KIM-1 in DOX induced rats. All data are presented as mean ± SE. (n = 10), a *P* value of <0.05 was assumed to denote statistical significance. *$P < 0.05$ Compared to the normal control -ve, $^{@}P < 0.05$ *vs* Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril; KIM-1, Kidney injury molecule.

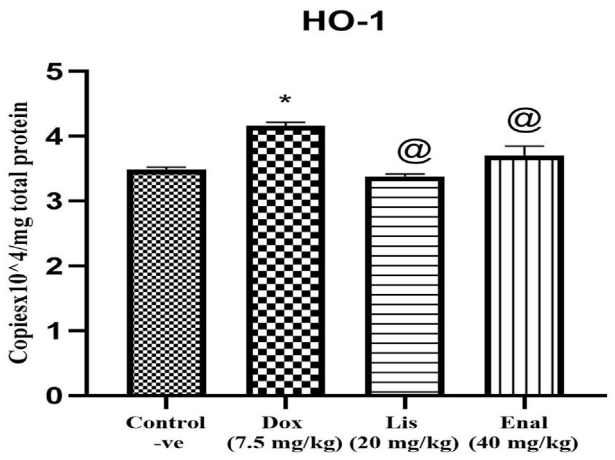


Figure 3. Effect of Lis and Enal on Heme oxygenase-1 (HO-) RNA expression in DOX induced rats. All data are presented as mean ± SE (n = 10), a *P* value of <0.05 was assumed to denote statistical significance. *$P < 0.05$ Compared to the normal control -ve, $^{@}P < 0.05$ *vs* Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril; KIM-1, Kidney injury molecule.

group (2.92 ± 0.13 ng/mg total protein, 7.6 ± 0.17 nmol/mg total protein and 1.89 ± 0.02 mM/mg total protein) respectively showing an increase in MPO and MDA concentration to 139.6% and 143.4% and a decrease in TAC levels to 76.35% respectively in DOX induced group as compared to control -ve group. Oral treatment of DOX induced rats with Lis (20 mg/kg) caused a significant reduction ($^{@}p < 0.05$) in MPO and MDA level (2.59 ± 0.07 ng/mg total protein and 7.44 ± 0.26 nmol/mg total protein) as well as a significant increase ($^{@}p < 0.05$) in TAC (1.86 ± 0.07 mM/mg total protein) respectively as compared to DOX induced group. Same results were obtained in Dox induced group treated with Enal (40 mg/kg) where a significant decrease ($^{@}p < 0.05$) in MPO and MDA level was observed (3.27 ± 0.24 ng/mg total protein and 8.23 ± 0.17 nmol/mg total protein) as well as a significant increase ($^{@}p < 0.05$) in TAC (1.78 ± 0.1 mM/mg total protein) respectively as compared to DOX induced group. These results indicate that treatment of Dox induced groups with Lis (20 mg/kg) and Enal (40 mg/kg) restored MPO, MDA and TAC into normal levels determining a prominent antioxidant activity of both ACE inhibitors.

3.3. Assessment of kidney injury molecule-1 (KIM-1)

Kidney injury molecule-1 (KIM-1), also known as hepatitis A virus cellular receptor-1 and T-cell immunoglobulin mucin-1, is a transmembrane glycoprotein highly up-regulated in proximal tubular cells following kidney injury. Therefore we investigated the effect of Lis (20 mg/kg) and Enal (40 mg/kg) on KIM-1 in tissue homogenate and the obtained results in Figure 1 showed a significant increase ($^{*}p < 0.05$) in KIM-1 level in DOX induced group (45.49 ± 1.4 Pg/mg total protein) when compared to the normal control group (31.93 ± 1.27 Pg/mg total protein) showing an increase in KIM-1 level to 142.44% in DOX induced group as compared to control -ve group. Oral treatment of DOX induced rats with Lis (20 mg/kg) showed a significant reduction ($^{@}p < 0.05$) in KIM-1 level (30.6 ± 0.64 Pg/mg total protein) as compared to DOX induced group restoring KIM-1 into normal levels. Moreover, a significant decrease ($^{@}p < 0.05$) in KIM-1 level was observed in the Enal (40 mg/kg) treated group (38.69 ± 1.52 Pg/mg total protein) respectively when compared to the DOX induced group showing a decrease in KIM-1 level to 85.04% in Enal treated group as compared to DOX induced group. These results indicate that Lis and Enal might have a regenerative effect on kidney tissue by reducing KIM-1 level in the DOX induced rat model. Data is presented at Figure 2.

3.4. Quantification of heme oxygenase-1 (HO-1) RNA expression

Heme oxygenase (HO) is the rate-limiting enzyme in the degradation of heme. It converts heme to biliverdin via a reaction that produces carbon monoxide and liberates iron. HO-1 is strongly induced by oxidative stress. Heme oxygenase-1 (HO-1) RNA expression was assessed in the tissue homogenate and the obtained results in Figure 2 showed a significant increase ($^{*}p < 0.05$) in HO-1 RNA expression in DOX induced group (4.16 ± 0.05 Copies x10^4/mg total protein) when compared to the normal control group (3.48 ± 0.03 Copies x10^4/mg total protein) showing an increase in HO-1 RNA expression to 119.4% in DOX induced group as compared to control -ve group. Oral treatment of DOX induced rats with Lis (20 mg/kg) and Enal (40 mg/kg) showed a significant reduction ($^{@}p < 0.05$) in HO-1 RNA expression (3.38 ± 0.04 and 3.71 ± 0.14 Copies x10^4/mg total protein) as compared to DOX induced group restoring HO-1RNA expression into normal levels. These results indicate that Lis and Enal might have a promising protective effect on acute

Table 2. Effects of lisinopril and enalapril on oxidative stress biomarkers in a doxorubicin-induced rat model of acute kidney injury.

Groups	MPO (ng/mg total protein)	MDA (nmole/mg total protein)	TAC (mM/mg total protein)
Control -ve	2.92 ± 0.13	7.6 ± 0.17	1.89 ± 0.02
Dox (7.5 mg/kg)	4.07 ± 0.18*	10.9 ± 0.3*	1.44 ± 0.08*
Lis (20 mg/kg)	2.59 ± 0.07$^{@}$	7.44 ± 0.26$^{@}$	1.86 ± 0.07$^{@}$
Enal (40 mg/kg)	3.27 ± 0.24$^{@}$	8.23 ± 0.17$^{@}$	1.78 ± 0.1$^{@}$

Data were represented as (mean ± SE, n = 10/group). Statistics were done using one-way analysis of variance (ANOVA) followed by *Tukey's* multiple comparison post hoc tests for confirmation. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril; MPO, Meyloperoxidase; MDA, Malondialdehyde; TAC, Total Antioxidant Capacity.
$^{*}p < 0.05$ represents a significant difference from the control negative group.
$^{@}p < 0.05$ represents a significant difference from the DOX group.

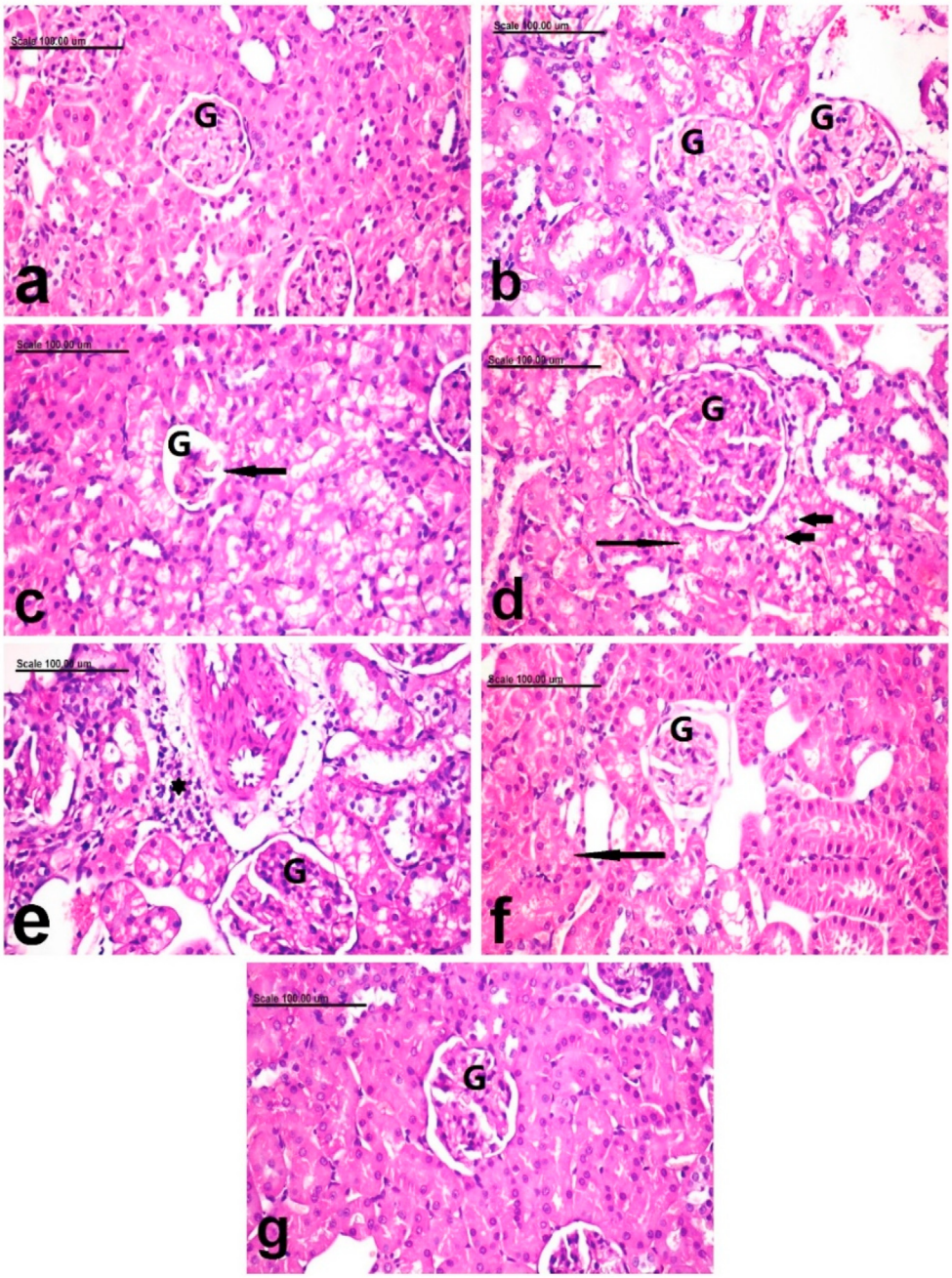


Figure 4. Photomicrograph of kidney tissues from, (a) normal rats showing normal kidney tubules and glomeruli (G), (b,c,d,e) DOX-treated rats showing congestion of glomerular tuft (G) (b), glomerular atrophy with shrinkage of glomerular tuft (arrow) (c), vacuolar degeneration of kidney tubular epithelium with intracytoplasmic aggregation of hyaline droplets (long thin arrow) and pyknosis of their nuclei (short thick arrows) (d), and mild focal interstitial infiltration with mononuclear cells (astrix) (e), (f) Lis group showing mild granular degeneration of some kidney tubular epithelial cells (arrow), and (g) Enal group showing normal glomeruli (G) and tubules (Stain: H&E, scale bar = 100μm).

Table 3. Illustrates the mean pathologic score of kidney damage recorded in the kidneys of normal and different treated groups.

Groups	Mean pathologic score of kidney damage (Mean ± SE)
Control -ve	0.10 ± 0.10
Dox (7.5 mg/kg)	2.70* ± 0.15
Lis (20 mg/kg)	0.60@±0.30
Enal (40 mg/kg)	0.50@±0.22

All data represent the mean ± SE (n = 10).
*P < 0.05 Indicate the significant differences compared to the control -ve group.
@P < 0.05 vs DOX group Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril.

kidney injury by reducing HO-1 RNA expression in the DOX induced rat model. Data is shown at Figure 3.

3.5. Histopathological findings

The histopathologic scoring of kidney damage assessed in normal and different treated groups was illustrated in Figure 4h. Normal kidney tubules and glomeruli were demonstrated in the kidneys of normal rats; with no evidence of kidney damage (Figure 4a). In contrast, pronounced kidney damage was demonstrated in the kidneys of DOX group. Variable glomerular lesions ranging from glomerular congestion (Figure 4b) to glomerular atrophy with shrinkage of glomerular tuft (Figure 4c) were demonstrated in this group. In addition, nearly most kidney tubules revealed extensive vacuolar degeneration of their epithelial lining with intracytoplasmic aggregation of hyaline droplets and pyknosis of their nuclei which appeared small and intensely basophilic (Figure 4d). Mild

Table 4. Effect of lisinopril and enalapril on kidney cleaved caspase-3 and NF-κβ expression recorded in doxorubicin-induced rat model of acute kidney injury.

Groups	Cleaved caspase-3 expression (% of positive cells/HPF) (Mean ± SE)	NF-κβ expression (% of positive cells/HPF) (Mean ± SE)
Control -ve	0.10 ± 0.10	0.10 ± 0.10
Dox (7.5 mg/kg)	2.90* ± 0.10	2.60* ± 0.16
Lis (20 mg/kg)	1.70*@±0.26	1.40*@±0.16
Enal (40 mg/kg)	1.30*@±0.21	1.10*@±0.10

All data are represented as mean ± SE. A semi-quantitative scale graded from 0 to 3 was used in which 0, no staining; ≥1 to < 2, positive staining in <30% of cells per HPF; ≥2 to <3, positive staining in 30–70% of cells per HPF; or ≥3, positive staining in >70% of cells per HPF. Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril.

*$P < 0.05$ compared to the normal control -ve, @$P < 0.05$ *vs* Dox group.

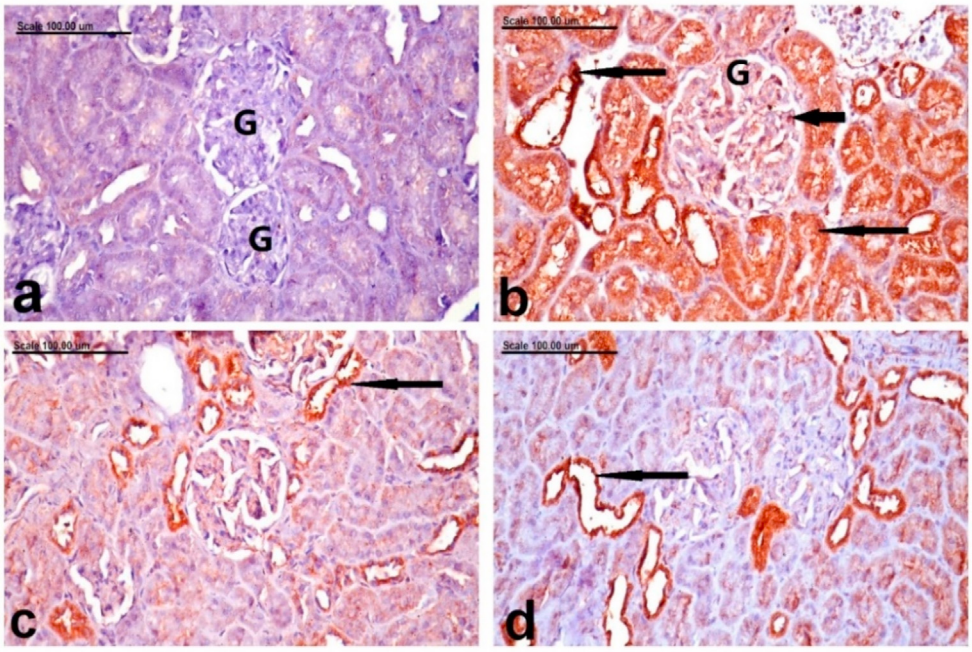
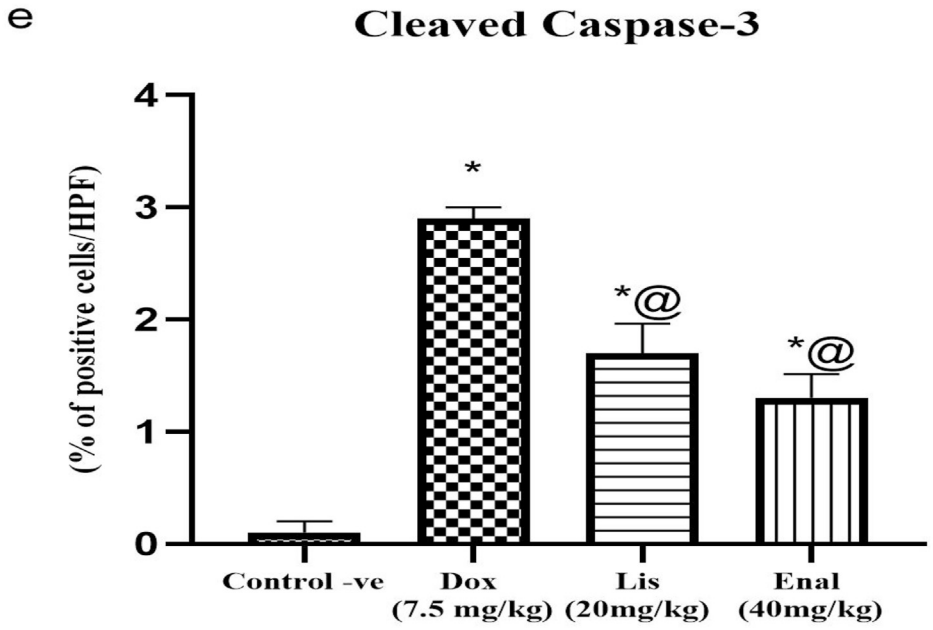


Figure 5. Representative images of cleaved caspase-3 in the, (a) normal rats showing no cleaved caspase-3 immune staining, (b) DOX-treated rats showing significant increase of immune positive cells with intense cytoplasmic and/or nuclear staining of cells of glomerular tuft (short thick arrow) and epithelial lining kidney tubules (long thin arrows), (c) Lis group showing reduction of percentage of caspase-3 positively stained cells (arrow), (d) Enal group showing decreased percentage of positively stained cells with intense cytoplasmic and/or nuclear staining (arrow) and (e) quantification of its expression level as assessed by immunohistochemical staining (cleaved caspase-3 immunohistochemical staining, scale bar = 100μm). Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril.



focal interstitial infiltrations with mononuclear cells were also observed (Figure 4e). Regression of the histopathological lesions, with only mild granular degeneration of some kidney tubular epithelial cells, was recorded in Lis group (Figure 4f). Pronounced amelioration with normal glomeruli and tubules is demonstrated in Enal group (Figure 4g). The mean pathological score of kidney damage is recorded in Table 3.

3.6. Immunohistochemical findings

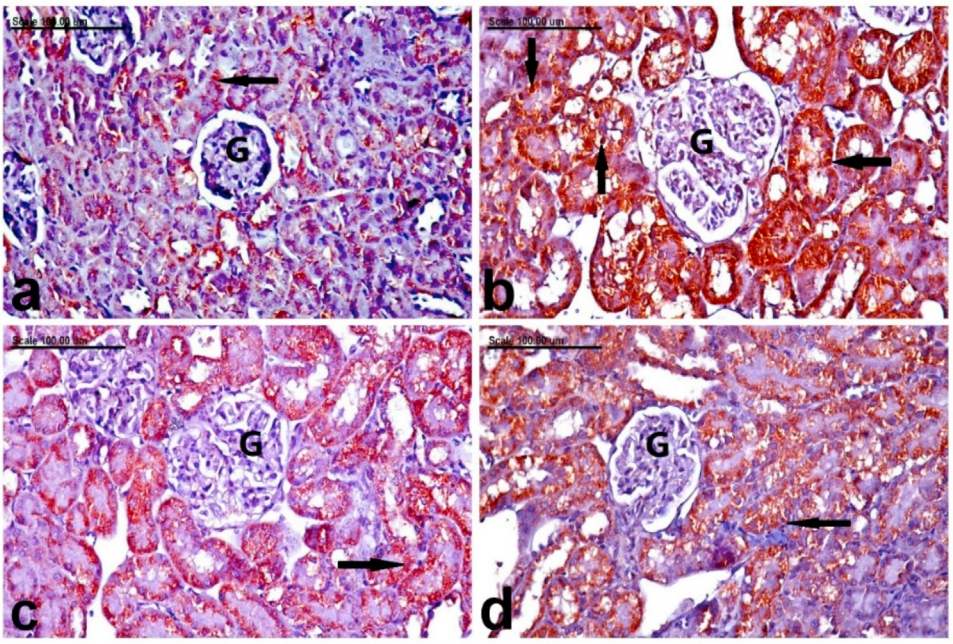
The results of cleaved caspase-3 and NF-κβ expression recorded in the kidney tissue of normal and treated rats are illustrated in Table 4.

3.6.1. Cleaved caspase-3 expression

Caspase-3 is one of the caspase enzymes having a fundamental role in the implementation of apoptosis. It is mainly located in damaged tubules and in some glomerular and interstitial cells. No cleaved caspase-3 immune staining was demonstrated in the kidneys of normal rats (Figure 5a). On the contrary, significant increase of cleaved caspase-3 expression with significant increase of immune positive cells was recorded in the kidneys of DOX group. The immune reactivity was characteristically demonstrated in the cells of glomerular tuft and epithelial lining kidney tubules with intense cytoplasmic and/or nuclear staining (Figure 5b). On the other side, significant decrease of caspase-3 expression with reduced percentage of positively stained cells was recorded in the kidneys of Lis and Enal groups with non-significant difference between them (Figure 5c & d, respectively). Quantification of cleaved caspase-3 expression levels is presented in Figure 5e.

3.6.2. NF-κβ expression

The nuclear factor (NF)-κB is a key regulator of pro-inflammatory and pro-apoptotic gene transcription that is highly up-regulated as a result of oxidative stress. Kidneys of normal rats showed normal cytoplasmic



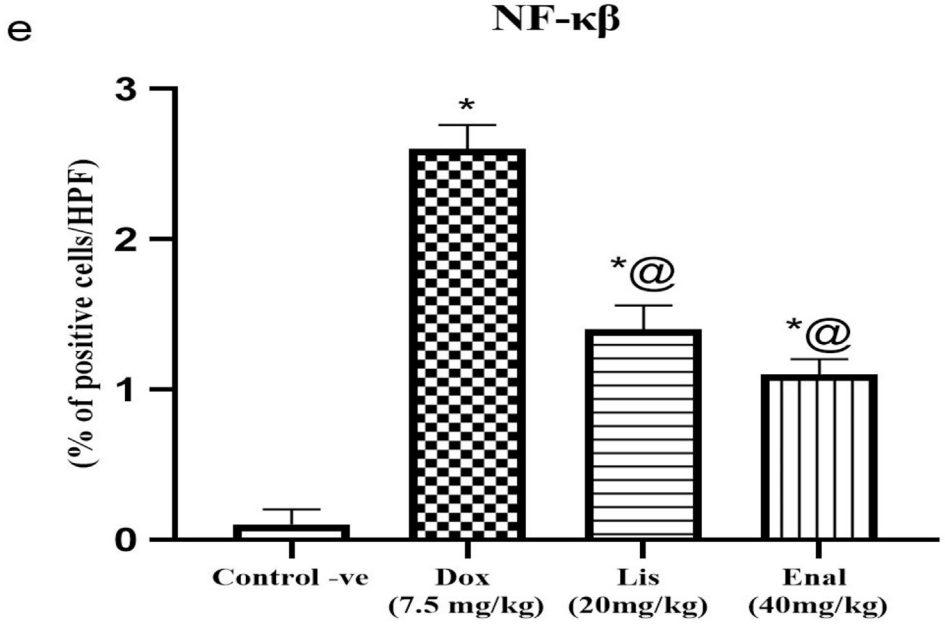


Figure 6. Representative images of NF-κβ in the (a) normal rats showing normal cytoplasmic staining of kidney tubules with no evidence of nuclear staining (arrow), (b) DOX-treated rats showing significant increase of positively stained cells with intense nuclear staining (arrows), (c) Lis group showing kidney tubules showing few cells with nuclear staining (arrow), (d) Enal group showing normal cytoplasmic staining (arrow) with sparse cells with nuclear staining and (e) quantification of its expression level as assessed by immunohistochemical staining. (NF-κβ immunohistochemical staining, scale bar = 100μm). Dox group. Dox, Doxorubicin; Lis, Lisinopril; Enal, Enalapril.

staining of kidney tubules with no evidence of nuclear staining (Figure 6a). In contrast, kidneys of Dox group showed significant increase of NF-κβ expression with increase percentage of positively stained cells which shows intense nuclear staining (Figure 6b). Significant decrease of percentage of NF-κβ positively stained cells were recorded in Lis and Enal groups, in which most of kidney tubules showed normal cytoplasmic staining. Only sparse cells showed nuclear staining (Figure 6 c & d, respectively). Quantification of NF-κβ expression levels is presented in Figure 6e.

4. Discussion

Doxorubicin (Adriamycin) is a secondary metabolite, isolated from *Streptomyces peucetius* in 1960s, targeting topoisomerase II enzyme. DOX is used as an efficient standard drug for the treatment of cancer either alone or in combination with other chemotherapeutic drugs to treat different types of malignancies [15]. DOX treatment is accompanied by deleterious toxic effects on various body organs e.g., bone marrow, heart, kidneys and liver. Therefore; in order to increase the clinical use of chemotherapeutic drugs; researchers have to prevent or at least decrease its side effects to the minimum bearable level. Any drug that can reduce the toxicity of DOX will be useful in such clinical conditions [16].

The precise mechanism of DOX-induced AKI is not yet fully understood, but it is assumed to occur due to free radical formation and membrane lipid peroxidation [17]. DOX also has a direct destructive effect on the kidney tissue because it favorably accumulates in the kidney [18].

AKI induced by DOX (7.5 mg/kg) was determined through assessment of different parameters in both sera and kidney tissues. Serum biochemical analysis showed a significant increase in creatinine and BUN as compared to control -ve group. Previously, the increase in BUN and creatinine in response to DOX was attributed to glomerular toxicity [19] and elevated glomerular mean transcapillary hydraulic pressure due to activation of the renin-angiotensin system (RAS) [20]. Oral administration of antihypertensive ACE inhibitors *viz* Lis and Enal to AKI induced rats caused a significant decrease in kidney function parameters as compared to DOX treated group. This effect could be due to intrarenal efferent vasodilation, resulting in filtration pressure reduction and improvement of the impaired kidney function. Our result was in line was those recorder by Navis et al., (1996) [21].

Dox also appeared to cause impairment in the natural antioxidant defense mechanism of the body showing a significant increase in MPO and MDA contents. On the other hand, DOX caused a significant decrease in TAC concentration when compared to the control -ve group. Previous studies attributed these findings to the ring structure of anthracycline of DOX that increases the enzymatic and non-enzymatic single-electron redox cycle release of ROS from molecular oxygen [22]. Various researches showed that free radicals induced by DOX treatment cause depletion of the antioxidant defense system and thus increase the oxidation process of both lipids and proteins in tissues of DOX-treated rats [23]. Oral administration of Lis (20 mg/kg) and Enal (40 mg/kg) in DOX induced AKI in rats caused an impressive improvement of the antioxidant defense system that was impaired as a response to DOX treatment. Although Lis and Enal are non-sulfhydryl ACE inhibitors, they exhibited a defensive role against free radical damage. In a previous study, authors reported that non-sulfhydryl ACE inhibitors inhibited free radical-induced injury mainly by activation of prostacyclin synthesis. Both Lis and Enal could also ameliorate programmed cellular death through the up-regulation of constitutive eNOS via an increase of bradykinin [24]. Another study reported that lipid peroxidation was reduced by both sulfhydryl- and non-sulfhydryl containing ACE inhibitors [7].

KIM-1 is a type 1 membrane protein that is found at insignificant levels in normal rat kidneys; whereas it is tremendously increased and localized in proximal tubular cells in acute kidney injury induced by DOX [25]. Our result agreed with this study where the group treated with DOX showed a significant increase in KIM-1 level as compared to control -ve group. Administration of Lis (20 mg/kg) and Enal (40 mg/kg) showed significant reduction in KIM-1 as compared to DOX induced group. Our result was adjacent with those of Li et al., (2019) [26] who determined that the non-sulfhydryl ACE inhibitor; fosinopril (Fos) can significantly inhibit the expression of KIM-1, an early marker of kidney injury in rats exerting good renoprotective effects. A comparative study was done between Fos and Lis to control induced memory deficit, they found that both drugs showed good results but as Fos is more lipophilic due to the presence of a bicyclic ring, it exhibited better efficacy due to better ability to penetrate the brain [27]. On the other hand, we found that both Lis and Enal showed a good amelioration of induced KIM-1 concentration similar to that caused by Fos in case of DOX induced AKI [26]. The decrease of KIM-1 was attributed to the blockade of RAS exerted by ACE inhibitors [28].

HO-1 is a phase II enzyme expressed by HO-1 RNA. It was proved to respond to oxidative stress and cellular damage. The over-expression of HO-1 induces lipid peroxidation-dependent cell death and ferroptosis; a newly recognized iron leading to cell death [29]. The harmful effects of HO-1 were reported in different diseases, including AKI and neurodegeneration [30]. HO-1 is expressed in very low levels in approximately all cells. HO-1 RNA expression is up-regulated in the injured cells triggering HO induction, particularly in the kidney [31]. On the other hand, studies showed that HO-1 is known to metabolize heme into biliverdin/bilirubin; which are potent antioxidants, suggesting a cytoprotective effect against different stress-related impairment. We can declare that HO-1 may have both a dark and a bright side [32]. Whether the HO-1 is up-regulated in response to oxidative stress for a cytoprotective or for a cell destructing role, our current study showed that treatment with DOX up-regulated HO-1 RNA expression, this finding may be due to induction of free radicals' release in response to DOX. Administration of Lis (20 mg/kg) and Enal (40 mg/kg) showed significant reduction in HO-1 RNA expression as compared to DOX induced group. As mentioned, Lis and Enal had the ability to improve the antioxidant defense system impaired as a response to DOX treatment, though ameliorating HO-1 RNA expression induced by DOX.

Our pathological results also showed pronounced kidney damage as demonstrated in the kidneys of DOX group in the form of variable glomerular lesions and extensive tubular vacuolar degeneration. Same results were obtained in a recent study [33]. Oral administration of Lis (20 mg/kg) showed regression of the histopathological lesions and a pronounced amelioration with normal glomeruli and tubules was demonstrated with Enal (40 mg/kg). These results could be attributed to the protective effect of both Lis and Enal represented by their antioxidant, antihypertensive and antiapoptotic effects. Their efficacy and protective effect against early kidney damaged was previously reported [34, 35].

In the current immunohistochemical study, a significant increase in cleaved caspase-3 and NF-κβ expression with increase percentage of positively stained cells that showed intense nuclear staining in group injected with DOX when compared with control negative group that showed no cleaved caspase-3 immune staining and normal cytoplasmic staining of kidney tubules with no evidence of NF-κβ nuclear staining. Same results for increase cleaved caspase-3 were obtained in a previous study [36] which stated that one of the apoptotic pathway targeted by the DOX was the mitochondrial pathway that resulted in the release of mitochondrial Cytochrome *c*, which in turn interacts with Apaf-1, caspase-9, and dATP, forming a complex called the apoptosome. This apoptosome induces the key cell death protease; caspase-3. The significant increase in NF-κβ expression was also in accordance with previous findings [37] which attributed the activation of NF-κβ expression to the prominent release of ROS in response to DOX treatment and in turn leads to the translocation of NF-κβ into the nucleus where it can activate various inflammatory cytokine genes. Administration of Lis (20 mg/kg) and Enal (40 mg/kg) significantly reduced caspase-3 expression and NF-κβ with reduced percentage of positively stained cells was recorded in the kidneys. All of the above findings suggest that both Lis and Enal has a

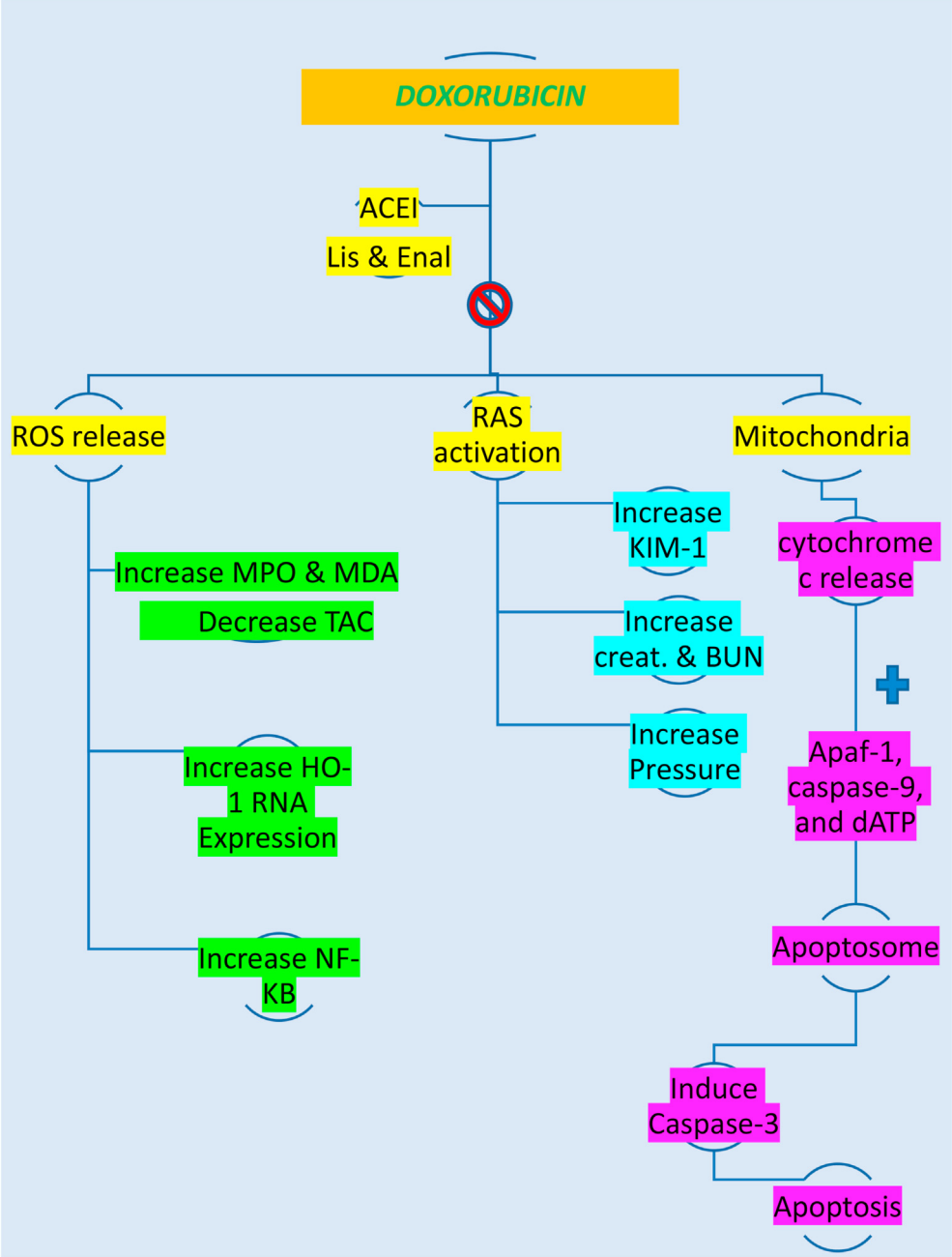


Figure 7. Flow Chart for induction Of AKI in response to Dox and the reversal effect of ACEI (Lis & Enal). ROS, Reactive Oxygen Species; RAS, Renin Angiotensin System.

prominent renoprotective effect through their antioxidant, antihypertensive and antiapoptotic properties. The antioxidant effect of Lis and Enal reduced the ROS release and lipid peroxidation [7] and thus reduced the induction of NF-κβ that was previously reported to increase as a response to induced ROS post DOX administration. The anti-apoptotic effect attributed to up-regulation of constitutive eNOS via an increase of bradykinin [24] could be the reason the significant decrease in cleaved caspase-3; a marker for apoptosis. The underlying mechanisms are illustrated in Figure 7.

5. Conclusion

Lis (20 mg/kg) and Enal (40 mg/kg) succeeded to improve kidney function and ameliorate the oxidative stress and apoptotic effects. Lis and Enal also improved the up-regulation of HO-1 RNA expression as a consequence of AKI induced by DOX treatment. Likewise, the pathological studies showed a prominent regenerative effect of kidney tissue by treatment of Lis and Enal along with DOX. From all these results, we can recommend addition of both Lis and Enal to DOX in cancer treatment regimen to control the detrimental AKI induced by DOX.

Declarations

Author contribution statement

G. F. Asaad: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Hassan: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

R. E. Mostafa: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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