

Protective Effect of Alamandine on Doxorubicin-Induced Nephrotoxicity in Rats

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Research Article

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Abstract

Background: The objective of this study was to evaluate the protective effects of alamandine, a new member of the angiotensin family, against doxorubicin (DOX)-induced nephrotoxicity in rats.

Methods: Rats, intraperitoneally injected with DOX (3.750 mg/kg/week) to reach total cumulative dose of 15 mg/kg on day 35. Alamandine (50 μ g/kg/day) was administered to the rats via mini-osmotic pumps for 42 days. At the end of experiment, rats were placed in the metabolic cages for 24 h for measurement of water intake and urine output. After scarification, Serum and kidney tissues were collected, and biochemical, histopathological and immunohistochemical studies were carried out.

Results: Inflammatory cytokines (IL-1 β , IL-6), pro-fibrotic mediator (TGF- β), pro-inflammatory transcription factor (NF- κ B), renal MDA, creatinine clearance, BUN, and water intake were increased by DOX administration. On the other hand, renal SOD, renal GPx activity and urinary output were decreased in the DOX-treated group. Alamandine co-therapy decreased these effects, as confirmed by histopathology and immunohistochemical analysis

Conclusion: The results of this study suggest that alamandine has the potential in preventing the nephrotoxicity induced by DXR in rats.

Introduction

Doxorubicin (DOX) is an antibiotic anthracycline, first isolated in 1960, and has been used for 30 years for cancer treatment[1]. Doxorubicin has severe side effects such as cardiac and renal toxicity. DOX-mediated nephropathy is caused by the destruction of glomeruli and damages to the tubules[2]. The mechanism by which DOX induces nephropathy is complex. Several factors such as free radicals, lipid peroxidation, and decreased activity of antioxidant enzymes likely to be the primary mediators in the development of the nephrotic syndrome[3]. Inflammation also plays a significant role in kidney damage induced by DOX via the effect of topical cytokines and other cytotoxic factors[2]. DOX can also cause protein excretion in the urine by destroying the nephrons directly. DOX-induced nephropathy is a classic model of renal failure in the rats. Damage to the filtration barrier is the primary factor in protein excretion due to renal failure[2]. DOX is responsible for this filtration barrier damage[4].

Thus, a drug with anti-inflammatory and antioxidant effects can improve the side effects of DOX. Alamandine is a new member of the renin-angiotensin system family that plays an essential protective role in the cardiovascular system and kidney functions[5].

Alamandine, like Ang (1–7), is a heptapeptide, which differs only in one amino acid in the N-terminal region. Alamandine exerts its effects by binding to the MrgD receptor[6]. Animal models of cardiovascular disease and kidney disease have shown that Ang(1–7) and alamandine have anti-fibrotic, anti-thrombotic, and anti-inflammatory effects on kidneys and heart tissues[5]. In the animal model of sepsis

induction by polysaccharides in C57BL6/J mice, plasma and tissue levels of IL-1B, TNFa increased, and alamandine reduced these inflammatory cytokines and apoptosis in cardiac tissues[7].

Considering that many experimental and clinical reports have shown the protective role of Ang1-7 in renal hemodynamics and functions under different conditions[8] and since alamandine has similar effects to Ang (1-7) [9], We assumed that alamandine may have a protective effect on DOX-induced nephrotoxicity.

In this study, biomarkers of renal injury, such as BUN, creatinine, creatinine clearance, serum urea, albumin and inflammatory cytokines (IL-1 β , 1L-6), pro-inflammatory transcription factor, profibrotic mediator (TGF- β), renal antioxidant (SOD, GPx, MDA) were measured in DOX-treated rats and alamandine/DOX co-administrated rats. In addition, histological and immunohistochemistry analyzes were performed.

Materials And Methods

Materials:

This experiment was performed on 35 male Sprague–Dawley rats (180–220 g) obtained from the Experimental Animal Centre of Fasa University of Medical Sciences. All procedures have been carried out in accordance with relevant guidelines and regulations regarding the care and the use of animals for the experimental procedures and were approved by the Committee of Animal Care of the Fasa University of Medical Sciences (IR.FUMS.REC.1397.014) in compliance with the ARRIVE guidelines[10]. All Animals were acclimatized under the controlled standard conditions of 12 h light/12 h dark cycles, at temperature 20-22 ° C, standard pellet diets, and water ad libitum for one week before the start of experiments.

Alamandine, and DOX were obtained from Phoenix Pharmaceuticals Inc., CA, USA and Tocris Bioscience, respectively

Experimental Group Design

After one week of acclimatization to the cages, the rats were randomly divided into five groups:

1-Control group

2-Sham group that, received normal saline as a vehicle for 42 days via mini-osmotic pumps (model 2006; ALZET Osmotic Pumps, CA, USA) and was surgically placed subcutaneously between the scapulae. This group also received normal saline intraperitoneal (i.p) on day(s) 14, 21, 28 and 35.

3-DOX group: Received DOX (3.750 mg/kg) i.p on day (s) 14, 21, 28 and 35 to reach total cumulative dose (15 mg/kg).

4- Alamandine group: Received alamandine for 42 days by mini-osmotic pumps with an infusion rate of 0.15 μ l/h (50 μ g alamandine/kg/day).

5-DOX+Alamandine group: Received alamandine by mini-osmotic pumps for 42 days (50 µg alamandine/kg/day) and also DOX (3.750 mg/kg) i.p on day (s) 14, 21, 28 and 35 to reach total cumulative dose (15 mg/kg).

On 41th day, rats were housed in the individual metabolic cages. The 24-hour urine sample from each animal was collected for measuring the creatinine clearance, creatinine, albumin, and TGF-B levels. At the end of the experiment, the animals were euthanized with pentobarbital sodium (150 mg/kg, intraperitoneal, IP), the blood samples were taken, and the two kidneys were removed. The blood samples were centrifuged at 4000 rpm for ten minutes. Serums for evaluation of biochemical parameters were taken and maintained at -80 °C. Creatinine clearance as an estimate of the glomerular filtration rate (GFR) was determined based on the 24-hour urine samples obtained under the following formula and expressed as mL/min (1).: Creatinine clearance (ml/min) = mg creatinine/dl urine × ml urine 24 h /mg creatinine/dl serum × 1440

The right kidneys were dissected and washed with PBS (10 mM PO43-, 137 mM NaCl, and 2.7 mM KCl; pH = 7.4) and then dried on filter paper and weighed. Then were homogenized in the PBS and centrifuged at 10,000×g for 20 minutes at 4 ° C. The supernatant was stored at -20 ° C until the oxidative stress parameters, antioxidants, and TGF-β were further analyzed using Eliza kits.

Assessment of Water Intake and Urine Volume

It was achieved by using metabolic cages. Water intake was calculated by the difference between the last measured and the remaining volume over 24 hours. This measurement was considered as the daily water intake for every animal in each experimental group.

Both volumes for water intake and urine output were measured by measuring cylinders.

Assessment of inflammatory cytokines in serum:

IL-1β, IL-6 and NF-κB levels in the serum samples were determined using ELISA kits (Cloud-Clone Crop Technology co., Ltd., Wuhan, China), according to the manufacturer's protocol. All measurements were performed in duplicate.

Assessment of oxidative stress markers and TGF-β in kidney tissue and urine:

TGF-β levels in the urine and kidney tissue were determined using ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. All measurements were performed in duplicate. Samples of urine were collected in the metabolic cages and stored at 20°C. Before refrigeration, 10 mL of commercial protease inhibitor cocktail were added to the urine samples.

The markers of oxidative stress, malondialdehyde (MDA), superoxide dismutase (SOD), and Glutathione Peroxidase (GPx), were determined in kidney tissue homogenates using available kits according to the manufacturer's protocol

Immunohistochemistry (IHC)

IL-6, IL-1, P53, and NF-κB expressions were evaluated on paraffin-embedded tissues by a standard immunostaining assay. Briefly, Xylene and a graded alcohol series were used for deparaffinization and rehydration, respectively. Then the slides were incubated for 30 minutes in a blocking reagent containing 1.5% hydrogen peroxide in methanol. Antigen retrieval was performed on slides using microwave protocol and then incubated in serum for 30 minutes and immunostained with IL-6 (cat. no. sc-28343; Santa Cruz Biotechnology, Inc.), IL-1 (cat. no. sc-32294; Santa Cruz Biotechnology, Inc.), P53 (cat. no. sc-81168; Santa Cruz Biotechnology, Inc.) and NF-κB (cat. no. sc-48366; Santa Cruz Biotechnology, Inc.) primary antibodies, for one hour at room temperature. The slides were washed with PBS three times and then incubated with secondary antibodies for 30 minutes. The sections were stained using 3,30-diaminobenzidine (Dako liquid DAB color solution), and the slides were then counterstained with hematoxylin. An Olympus BX51 microscope was used to visualize the results (Olympus, Tokyo, Japan). Five different microscopic fields were selected randomly in each slide, and positive staining within each slide was measured by Image Pro Plus 6.1. Quantitative analysis was performed in a blinded manner.

Histopathological Studies

Left kidneys of male rats were harvested and fixed immediately in 10% buffered formalin phosphate (pH 7.4) for histological tests. The tissue samples were then dehydrated by passing through graded concentrations of alcohol, cleaned to remove alcohol by xylene, incorporated in paraffin, and allowed to harden. Subsequently, 5 µm sections of the paraffin blocks were prepared by microtome and remained floating in the water bath. Floating kidney sections were then mounted on microscopical slides, placed into the drying oven at 60 °C, stained with Harris' hematoxylin and 1% eosin, and histological examination was carried out with light microscopy.

Data and statistical analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Prism software v6 Inc., La Jolla, CA, USA). All data were expressed as mean ± standard deviation (SD). A one-way Analysis of Variance (ANOVA) was used to compare all groups, followed by Tukey post-hoc test.

Results

Effect of different treatments on water intake, urine output and renal toxicity markers:

As shown in Table 1, one week following doxorubicin injection, water intake and urine output checked. Rats in the DOX group developed an increase in water intake ($p = 0.003$) and a decrease in urine output ($P < 0.001$) when compared to the control group. Co-therapy with alamandine significantly decreased DOX effect. Alamandine alone has a diuretic effect compared to the control group.

Table 1
Effect of treatment with alamandine on water intake, urine output and renal toxicity markers in DOX-treated rats

| | Sham | Control | Ala | Dox | Dox + Ala |
|--|--------------|--------------|-----------------|------------------|-----------------|
| Water Intake (ml/kg/day) | 63.10 ± 8.68 | 62.69 ± 6.90 | 70.31 ± 6.68 | 81.11 ± 9.84** | 66.24 ± 10.21# |
| Urine output (ml/kg/day) | 27.29 ± 3.72 | 27.57 ± 3.55 | 34.03 ± 4.13### | 14.39 ± 4.34*** | 31.20 ± 4.90### |
| Serum Creatinine (mg/dl) | 0.71 ± 0.19 | 0.65 ± 0.16 | 0.67 ± 0.23 | 1.61 ± 0.31*** | 1.06 ± 0.38## |
| Serum BUN (mg/dl) | 22.81 ± 4.99 | 22.98 ± 5.21 | 19.22 ± 6.33 | 43.45 ± 13.44*** | 28.32 ± 9.23# |
| Serum Albumin (g/dl) | 4.06 ± 0.51 | 4.32 ± 0.58 | 3.96 ± 0.68 | 2.34 ± 0.78*** | 3.20 ± 0.81 |
| Urine Creatinine (mg/dl) | 76.12 ± 7.32 | 76.76 ± 6.98 | 81.24 ± 11.45 | 58.32 ± 13.82* | 68.98 ± 14.34 |
| Urine Albumin (mg/dl) | 1.03 ± 0.21 | 0.7 ± 0.19 | 1.07 ± 0.24 | 2.89 ± 0.36*** | 2.05 ± 0.39### |
| Creatinine Clearance (ml/min) | 0.57 ± 0.14 | 0.64 ± 0.17 | 0.90 ± 0.53 | 0.08 ± 0.3** | 0.40 ± 0.18 |
| Data are expressed as mean ± SD; n = 7 for each group. | | | | | |
| *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control; | | | | | |
| #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the DOX group. | | | | | |

One week after the last dose of DOX serum albumin was significantly decreased ($P < 0.001$) and urinary albumin increased ($P < 0.001$) compared to the control group. These changes in urinary albumin were significantly reduced by administration of alamandine (50mg/kg/day) ($P < 0.001$). Significant increases in serum BUN ($P < 0.001$) and serum creatinine ($P < 0.001$) concentrations were observed in rats treated with DOX alone, compared to the control group. However, these changes were effectively reversed by treatment of alamandine. The results also revealed that creatinine clearance levels in the DOX group significantly decreased ($p = 0.005$) compared to the control group. In the rat co-treated with alamandine, the creatinine clearance significantly increased compared to the DOX group.

Effect Of Different Treatments On Inflammatory Cytokines And Nf- κ b

DOX administration increased the serum levels of IL-1 β ($p = 0.04$), IL-6($p < 0.001$), NF- κ B ($p = 0.008$) compared to the control group, and alamandine co-therapy reduced these effects.

The level of TGF- β in the kidney and urine increased ($p < 0.001$) in the DOX-group compared to the control group and alamandine decreased this level (Table 2).

Table 2

Effect of treatment with alamandine on inflammatory cytokines and NF- κ B in DOX-treated rats

| | Sham | Control | Ala | Dox | Dox + Ala |
|---|---------------|---------------|---------------|------------------|--------------------|
| TGF- β in Urine (pg/mg creatinine) | 16.48 ± 5.69 | 17.20 ± 5.50 | 23.34 ± 8.44 | 79.89 ± 15.78*** | 56.22 ± 13.33***## |
| TGF- β in Kidney (pg/mg protein) | 142.7 ± 45.99 | 135.9 ± 22.56 | 179.1 ± 67.20 | 534.4 ± 99.90*** | 404.4 ± 77.23***## |
| Serum IL-1 β (pg/ml) | 363.4 ± 99.9 | 350.0 ± 86.8 | 342.2 ± 89.99 | 552.1 ± 109.3** | 453.9 ± 105.3 |
| Serum IL-6 (pg/ml) | 22.32 ± 5.53 | 20.10 ± 5.34 | 21.23 ± 6.56 | 47.46 ± 10.32*** | 34.98 ± 9.77# |
| Serum NF- κ B (ng/ml) | 6.52.1 | 6.31.9 | 6.41.9 | 10.22.2** | 7.32.1 |

Data are expressed as mean ± SD; n = 7 for each group.

P < 0.01, *P < 0.001 compared to the control;

#P < 0.05, ##P < 0.01 compared to the DOX group.

Effect Of Different Treatments On Renal Oxidative Stress Markers

DOX administration resulted in a marked increase in kidney MDA ($P < 0.001$), significant decrease in Kidney SOD ($P = 0.006$) and decrease in renal GPx Activity ($P = 0.002$) relative to controls and the co-treatment with alamandine reduced these effects (Table 3).

Table 3
Effect of treatment with alamandine renal oxidative stress markers in DOX-treated rats

| | Sham | Control | Ala | Dox | Dox + Ala |
|--|---------------|----------------|---------------|-----------------|------------------|
| Kidney SOD (U/g tissue) | 71.90 ± 10.62 | 74.42 ± 9.62 | 69.51 ± 11.96 | 48.52 ± 14.93** | 59.04 ± 16.09 |
| Kidney MDA (nmol/g tissue) | 4.44 ± 1.53 | 4.46 ± 0.94 | 5.52 ± 2.61 | 17.3 ± 5.31*** | 14.87 ± 5.36 |
| Kidney GPx Activity (u/mg protein) | 11.17 ± 2.81 | 11.72 ± 2.09 | 10.26 ± 2.51 | 4.91 ± 2.89*** | 6.61 ± 2.88** |
| Data are expressed as mean ± SD; n = 7 for each group. | | | | | |
| **P < 0.01, ***P < 0.001 compared to the control; | | | | | |

Immunohistological Study

All immunohistochemical sections for kidney tissue were seen in Fig. 1. The minimum expression of IL-6, IL-1, P53, and NF- κ b was seen in the control group. Moreover, the expression level of P53 was minimum and negligible in all groups. The intense staining of IL-6, IL-1, and NF- κ b was observed in DOX group($P < 0.01$).

However, the Alamandine + DOX treatment group showed significantly reduced expression of pro-inflammatory cytokines (IL-1, IL-6 and NF- κ b) compared with the DOX group. The evaluation of results in alamandine group revealed a close similarity to the control group, however, the expression level of IL-1 in this group was higher than those of the control group (Fig. 1).

Histopathological Study

All hematoxylin and eosin (H&E) stained kidney sections from different experimental groups were evaluated histologically (Fig. 2). The histopathological micrographs of a normal kidney have been showed in Fig. 1. The structure of the kidney was intact. Micrographs of the kidney section of alamandine group did not show any significant histopathological changes in harvested samples. The histopathological evaluation of the kidney in doxorubicin-treated animals showed severe proximal and distal tubular cell swelling (cell degeneration), tubular cell necrosis, and the classical architecture of kidney had been deteriorated (Fig. 2).

Discussion

DOX is used in the treatment of multiple solid tumors. But it has severe adverse effects on the kidneys[11]. The mechanisms by which DOX causes glomerular toxicity have not been fully clarified.

However, some previous reports have shown that reactive oxygen species and free radicals are the major contributors to DOX-induced nephrotoxicity[12, 13]. It is proposed that the conversion of DOX to its semiquinone form should play an essential role in its nephrotoxic actions[14]. Semiquinone is unstable under aerobic conditions and therefore reacts with molecular oxygen to form superoxide anion radicals[15]. As the number of primary free radicals increases, locally infiltrating neutrophils and active mesenchymal glomerular cells generate free radicals that cause kidney tissue damage[16]. DOX exert direct toxic damage to the glomerular base membrane, podocytes, glomerular endothelial cells and consequently induce tubular interstitial inflammation and fibrosis[17]. As a result, renal function is compromised following administration of doxorubicin, so that serum urea and creatinine concentrations increase, serum albumin, urea, and creatinine clearance decrease, and extreme proteinuria may be observed[18].

In the current research, we used a cumulative dose of doxorubicin injection and successfully induced an experimental nephrotic syndrome model. This model was characterized by albuminuria, hypoalbuminemia, increased BUN and creatinine serum levels (two significant indicators of renal function), decreased creatinine clearance (indicator of GFR), associated with increased oxidative stress and inflammatory factors. The toxic effects of DOX administration observed in the current study are consistent with previous studies in which renal function parameters, including serum urea and creatinine, increased[19, 20]. However, administration of almandine before, during and after doxorubicin injection was associated with improvement in renal function parameters through attenuated serum urea, creatinine, albumin, and creatinine clearance. This improvement in DOX-induced renal dysfunction has been confirmed based on histological and biochemical findings. DOX has been shown to increase oxidative stress in the kidneys, with evidence of increased lipid peroxidation and changes in the status of antioxidants[21]. Oxidative stress plays a crucial role in developing podocyte damage, glomerular sclerosis, and proteinuria[22]. Our data showed that almandine decreased ROS end products (MDA) and increased ROS-preventing enzymes (SOD and GPx) both in the kidneys and the circulation, suggesting that it could relieve renal oxidative stress in DOX-induced nephrotoxicity rats. The antioxidant property of almandine might partly explain its protective effects on albuminuria and glomerular basement membrane damage. MDA can be measured as an indicator of lipid peroxidation, and it is used indirectly to assess the degree of damage to the cell membrane[23]. SOD catalyzes the dismutation reaction of superoxide anion to hydrogen peroxide, which is then detoxified to oxygen and water by catalase or glutathione peroxidase. Such proteins are essential in the prevention of oxidative stress damage[24]. These results from this research are consistent with the previous study, which showed that almandin increased the expression of antioxidant proteins in ventricles exposed to I/R ventricle injury[25].

NF- kB as a transcription factor, plays a crucial role in regulating a wide variety of genes involved in the development of renal disease[26]. There is strong evidence for the pivotal role of NF- kB activation in the pathogenesis of DOX-induced renal inflammation[27]. NF- kB is responsible for inflammatory reactions via the mediation of TNF- α , IL-1 β , and IL-6 expressions[28]. In this study, serum and tissue elevations of TNF- α , IL-1 β , IL-6, and NFkB were observed in DOX-treated rats. In contrast, almandine treatment with DOX decreased NFkB, IL-1 β , TNF- α , and IL-6 compared with DOX-treated rats. These results suggest that

alamandine has anti-inflammatory effects and successfully reduced the injury of DOX in harvested tissues.

In this study, alamandine alone increased interleukin-1. Macrophages have MRD receptors. Possibly alamandine binds to its receptors on macrophages, causing an increase in the secretion of interleukin-1. However, this increase in interleukin production needs further investigation.

TGF- β was found to be a central mediator of renal fibrosis[29]. TGF- β_1 can be synthesized by a wide variety of cells, including macrophages, T and B fibroblast lymphocytes, and resident renal cells[30]. In present study, alamandine reduced renal TGF- β_1 levels. TGF- β_1 expression in the kidneys is considered to be the last common pathway leading to structural damage and fibrosis in a variety of glomerular diseases[31]. The urinary TGF- β_1 appears to be a marker of the severity of the glomerular damage. In our study urinary levels of TGF- β_1 were also increased and, alamandine co-therapy reduced this level.

P53 induction mediates cell apoptosis by activation of the caspase-3 protease family and apoptotic cell death[32]. However, the expression of P53 IHC in our data set has not changed in either the DOX or the alamandine groups, indicating that the reduced DOX side effects are independent of P53. It is possible that other pathways, including ROS, inflammatory cytokines, and NFkB, all work together to damage the kidneys. Given that elimination of intracellular H₂O₂ protects myocytes from DOX-induced apoptosis, probably by inhibiting NF- κ B activation[33], It is possible that alamandine may protect the kidneys by reducing superoxide and inhibiting the NF-B pathway.

The beneficial effects of alamandine were further illustrated by histological evaluation using H&E stain. Histopathologic examination showed marked pathological lesions characterized by severe proximal and distal tubular cell swelling (cell degeneration), tubular cell necrosis, and deterioration of the classical architecture of the kidney. Treatment with alamandine reduced pathological lesions. Surprisingly, the results of this study showed that alamandine alone increased urine volume. Accumulating evidence indicates that Ang1-7 can counterbalance the vascular and tubular action of AngII[34]. Ang 1–7 induces vasodilator, natriuretic, and diuretic effects through the mas receptor. It has been suggested that alamandine, a heptapeptide with an Ang1-7-like structure, exhibits actions similar to Ang1-7[5, 35–37]. The effects of alamandine on urine volume needs further investigation. Figure 3 shows an overview of the effects of alamandine on doxorubicin-induced nephrotoxicity.

In conclusion, our results suggest that alamandine could improve DOX-induced nephrotic syndrome in rats. The antioxidant and anti-inflammatory properties of alamandine probably contribute to its therapeutic effects in nephrotic syndrome. These findings provide significant evidence that alamandine could be considered as a potential therapeutic agent for the treatment of DOX-induced nephrotoxicity induced by DOX.

Declarations

Ethics approval:

The study was approved by the ethics committee of FUMS (IR.FUMS.REC.1397.014).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

There is no conflict of interest.

Funding

There is no funding source.

Authors' contributions

A.S.H. and K.J. conceived and planned the experiments. A.S.H., A.CH. and H.A. performed research. A.S.H. and K.J. analyzed data. A.S.H. and K.J. wrote the paper. All authors have read and approved the manuscript.

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Figures

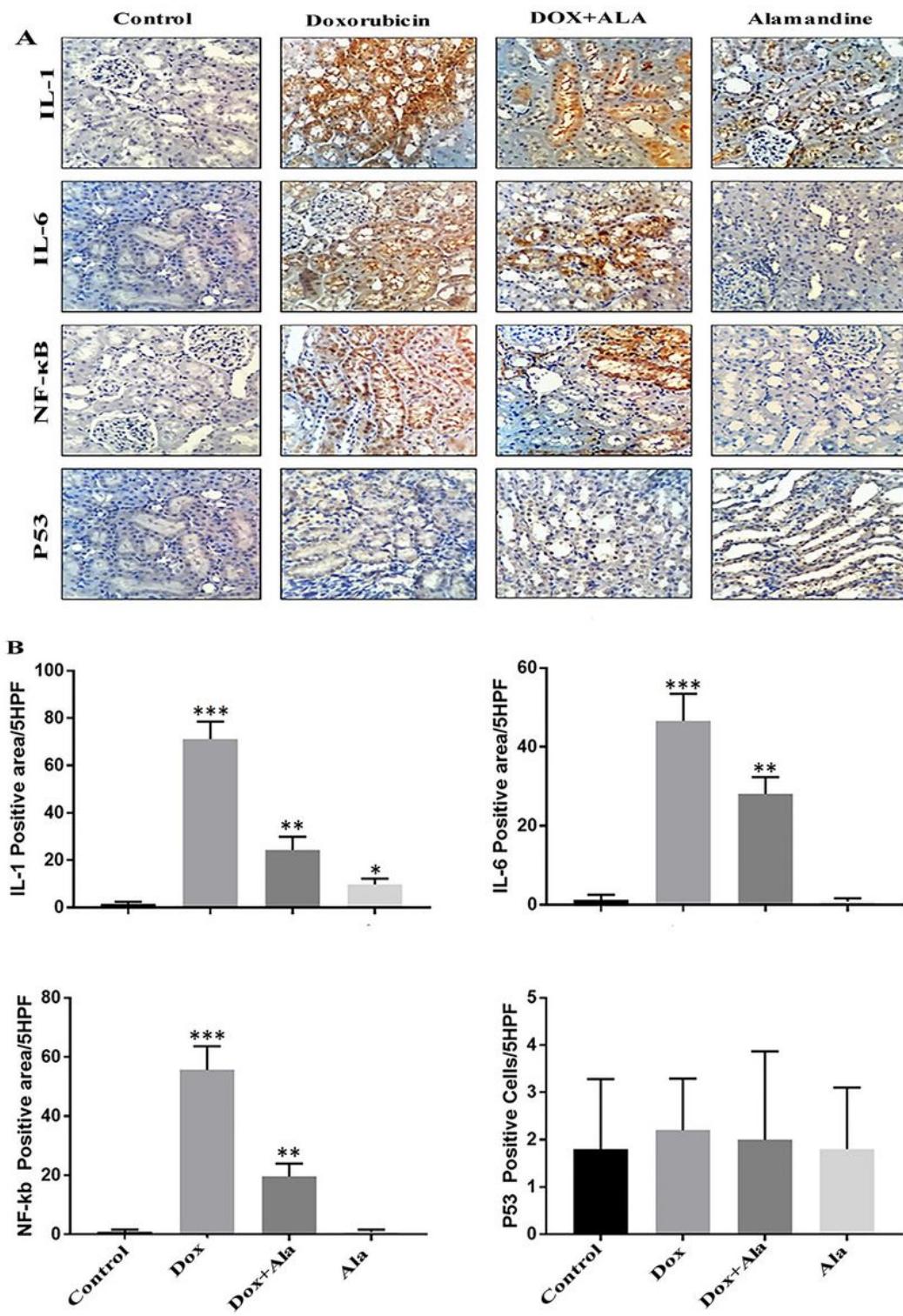


Figure 1

A: Immunohistochemistry of IL1, IL-6, P53, and NF-Kb in kidney. Magnification x400. B: Immunohistochemical analysis of the of IL1, IL-6, P53, and NF-Kb in kidney. *, **, ***: values indicate treatment group versus control group; ***P<0.001, **P < 0.01, *P < 0.05.

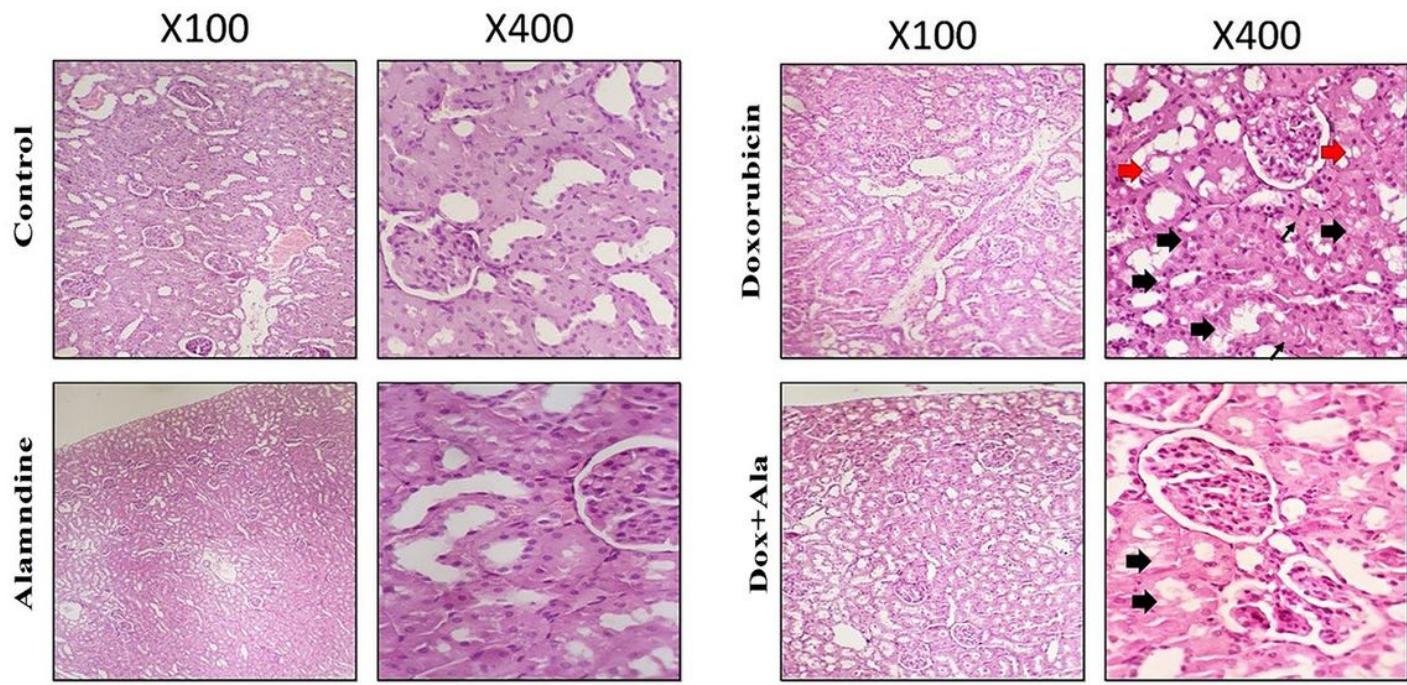


Figure 2

Histopathologic sections of kidney in different experimental groups, Thick black arrows: degeneration of tubular cells, thin arrows: cell necrosis, Red arrows: hyalin cast. H&E stain.

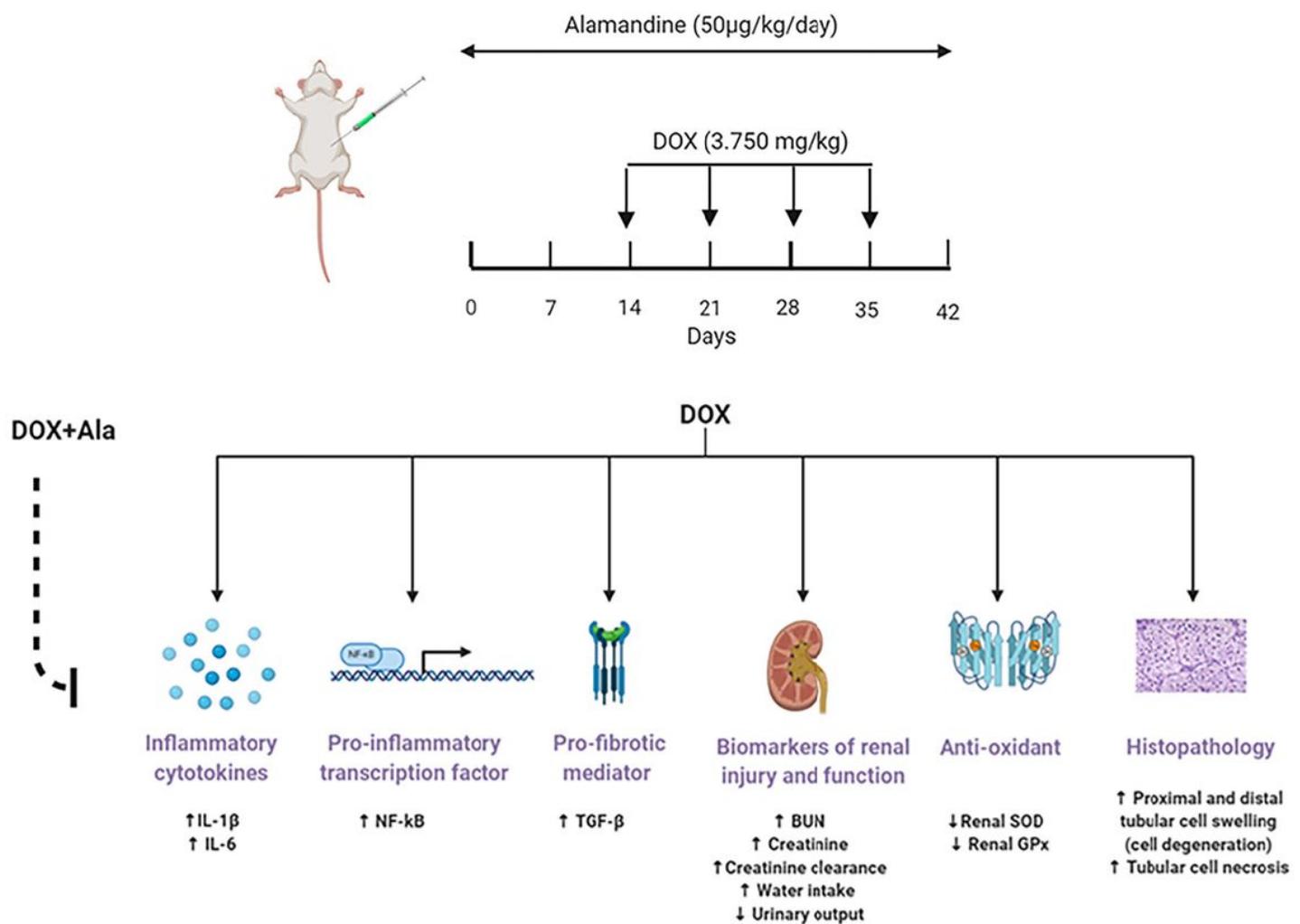


Figure 3

Graphic abstract showing the effect of alamandine on doxorubicin-induced nephrotoxicity.