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Avanafil has anti-inflammatory and anti apoptotic effect through amelioration of TLR4, MAPK P38, NF-κB, TNF-alpha and IL-1β signaling pathway.

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ABSTRACT:

Doxorubicin (DOX) nephrotoxicity is a fatal case that deprives us from the therapeutic benefits of DOX against cancer. This study aimed at showing the potential protective effects of avanafil; a novel phosphodiesterase enzyme inhibitor on doxorubicin induced nephrotoxicity in rats. Nephrotoxicity was induced by administration of DOX (2.6 mg/kg, *i.p.* twice per week for 4 weeks). Avanafil was administered in a daily dose of (10 mg/kg, *p.o.*) for 4 weeks. DOX nephrotoxicity was evident by the significant increase in the kidney function biomarkers (creatinine, urea, albumin and total protein), oxidative stress markers (glutathione reduced (GSH), lipid peroxides (MDA), Nrf-2, catalase and superoxide dismutase SOD), inflammatory markers (TLR4, MAPK P38, NF-κB, TNF-alpha and IL-1β) and the apoptotic biomarkers (BAX and Bcl-2). All these parameter were significantly attenuated by avanafil treatment. Moreover, avanafil prevented the histopathological alterations induced by doxorubicin. This study proved a novel significant nephroprotective effect of avanafil

KEYWORDS:, Doxorubicin, Nephrotoxicity, Inflammation, Apoptosis.

1. INTRODUCTION

Doxorubicin (DOX), belonging to the anthracycline class of antibiotics, is commonly used to treat several types of cancer including leukemia, lymphoma and haematopoietic tumors as well as solid tumors such as breast, pulmonary, uterine, ovarian and cervical and breast cancer **(Zidan et al., 2018, Sun et al., 2016, Shi et al., 2018)**. However, cancer chemotherapy usually demolishes the normal physiological homoeostasis and affects multiple organs during the course of treatment. Despite its extensive clinical utilization in the fight against a variety of human malignancies, treatment with the conventional DOX is limited because of its multi-organ toxicities

including renal damage and nephrotoxicity **(Wang et al., 2000, Hertzan Levy et al., 2000)**, cardiac, pulmonary, testicular and hematological toxicities **(Khames et al., 2019)**.

Nephrotoxicity is a hindering factor during DOX usage, as it induces renal inflammation and oxidative stress (Benzer et al., 2018), renal fibrosis **(Cardoso et al., 2018)**, hyperuricemia **(Khames et al., 2017b)**, besides inflammation and apoptosis. Considering the nephrotoxicity parameters that were estimated in all the previous work it was shown to be limited only to oxidative stress and hyperuricemia markers **(Khames et al., 2017b, Khan et al., 2018)**. However, this study will discuss DOX nephrotoxicity from another point of view.

Toll like receptor 4 (TLR4) is a leading pathway for inflammation. It is a part of innate immunity activated by several ligands including bacterial lipopolysaccharides, poisons and drugs (Kuzmich et al., 2017). Its activation results in activating mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NFκB) signaling and tumor necrosis factor alpha (TNF-alpha) inducing severe inflammatory response **(Zhu et al., 2015, Kuzmich et al., 2017, Molteni et al., 2016)**. A previous study confirmed the role of TLR4 in renal diseases and tubular damage but the mechanism isn't completely understood (R Nair, 2015). Therefore, this study will assess the role of TLR4 activation in doxorubicin-induced nephrotoxicity.

Avanafil, a new second generation phosphodiestrase 5 inhibitor (PDE5I), is commonly used for the treatment of erectile dysfunction and it showed the least adverse effects between the members of PDE5I family **(Corona et al., 2018)**.

Avanafil was previously reported to protect against obesity-induced oxidative stress and inflammation **(Moon et al., 2018)** as well as bone remodeling and oxidative stress in osteoporosis. In addition, avanafil

protected cardiac cells against ischemia reperfusion **(Korkmaz-Icöz et al., 2018)** and against contrast induced nephropathy **(Afsar et al., 2015)**.

There is a necessity to develop adjuvant therapy to be used in conjunction with DOX chemotherapy to improve the efficacy of the treatment and to reduce the associated undesirable side effects. Therefore, we hypothesized that avanafil might prevent the kidney injury caused by doxorubicin and in this study we will try to investigate the possible effects and mechanisms of action of avanafil on renal injury caused by doxorubicin through TLR4 pathway.

2. Material and methods

2.1. Animals

Adult male Sprague-Dawley rats (200g ±30) were obtained from the breeding colony of the animal house of the National Organization for Drug Control and Research (NODCAR, Giza, Egypt). Animals had free access to food and water. They were maintained at 22–24˚C and 40–60% relative humidity and diurnal light cycles in animal holding rooms. Animals were adapted two weeks in their place before the start of the experiments. Experimental procedures were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Research Ethical Committee of Faculty of Pharmacy, Beni-Suef University (Beni-Suef, Egypt) to comply with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, 1996). animals were carefully handled in such a manner that they do not suffer from unnecessary pain. Animals were treated in a friendly manner without squeezing or pressure, providing a good care towards the health and wellbeing of animals

2.2. **Reagents and chemicals**

Doxorubicin was provided as Adriablastina vial (10 mg/5 ml doxorubicin

hydrochloride), which was purchased from Pharmacia Italia S.P.A. Italy. Avanafil was purchased from sigma-aldrich, USA. All the other chemicals were of the highest purity and analytical grade.

2.3. **Induction of nephrotoxicity**

Rats were injected with a dose of doxorubicin (15 mg/kg, twice weekly i.p., for four weeks)

2.4. Experimental design

Rats were randomly divided into four groups (n=12-14 per each), A control (CONT) group received saline. The second group received doxorubicin injection (as mentioned above). The third group received avanafil (10 mg/kg/day, p.o, for four weeks).The fourth group received doxorubicin and avanafil for four weeks, avanafil was given one hour before doxorubicin injection. At the end of the experiment blood samples were collected from retro-orbital sinus plexus and sera were separated by centrifugation at 1000g for 10 min and stored at −80°C for serological measurements of the renal function tests. Afterwards, animals were sacrificed by decapitation under anesthesia, both kidneys of each animal were dissected out, and portions of them were fixed in 10% formalin solution for histopathological examination while others portions were homogenized in 50 mM phosphate buffer (pH 7.4), and stored at -80°C till estimation of biochemical parameters and western blot examinations.

Biochemical analysis

2.5. Assessment of renal functions

Colorimetric assay kits for the determination of renal functions biomarkers blood urea nitrogen (BUN), serum creatinine, serum albumin and

total protein levels using (Biomed-diagnostics, Cairo, Egypt). Rat Cystatin-C (Cys-C) kit for measurement of renal cystatin in addition to Rat Kidney injury molecule (KIM-1) ELISA Kit for measurement of renal KIM-1 were obtained from Mybiosource (San Diego, CA**,** USA). All procedures were performed according to the manufacturers' instructions.

2.6. Assessment of inflammatory markers in renal tissues

Protein levels of TLR-4, NF-κB, Nrf-2, and p38-MAPK were assessed using Western blot technique using TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE) which was provided by Bio-Rad Laboratories, TNC, USA Catalog. NO. 161-0181.

The results were expressed as arbitrary units after normalization for β-actin protein expression

2.7. Assessment of TNF-α and IL-1 β in renal tissues

Rat TNF-α and IL-1β were measured by the ELISA kits obtained from RayBiotech Inc. (Parkway, LaneSuite Norcross, GA).

2.8. Measurement of renal oxidative stress biomarkers

ELISA kits purchased from my biosource (San Diego, CA, USA) were used for measurement of malondialdehyde (MDA) and glutathione reduced (GSH), myeloperoxidase (MPO), catalase (CAT), and superoxide dismutase (SOD) on the basis of the manufacturer's instructions.

2.9. Measurement of protein expression of Bax/Bcl2

B-cell lymphoma 2 (Bcl-2) protein, Bcl-2-Associated-X (Bax)-protein and Bax/Bcl-2 ratio were determined using western blot technique, Protein levels of Bax/Bcl2 in renal tissues of different treatment groups (1–4) were assessed using western blot technique. Proteins were extracted by trizol reagent, and protein concentrations were estimated by Bradford assay. The

primary antibodies used were raised in rabbit anti-Bcl-2 antibody (13-8800 Thermo Fisher Scientific), anti-Bax antibody (MA5-14003 Thermo Fisher Scientific).

2.10. Renal histopathological examination

Autopsy samples were taken from the kidney of rats in different groups and fixed in 10% formol saline for twenty four hours. Washing was done with tap water, then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin & eosin stain for routine examination through the light electric microscope **(Suvarna et al., 2018)**.

2.11. Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed using a prism computer program (GraphPad software Inc. V5, San Diego, CA, USA). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparison Test as a post hoc test. Probability values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of avanafil on biochemical renal function markers.

Normal control values for the serum levels of urea, creatinine, total protein and albumin were 33.17 ± 1.9 mg/dl, 0.18 ± 0.013 mg/dl, 5.59 ± 0.15 mg/dl and 3.5 ± 0.25 mg/dl, respectively. Normal control values for the renal contents of KIM-1 and cystatin C were 2.202 ± 0.09 pg/g tissues. and 0.6617 ± 0.05 pg/g tissues. Doxorubicin significantly increased serum levels of urea nitrogen and creatinine associated with a significant decline in total protein and albumin levels as compared to the normal control group. Additionally, doxorubicin significantly increased renal contents of KIM-1 and cystatin C.

However, co-treatment with avanafil significantly suppressed and reversed the increase of urea nitrogen, creatinine, KIM-1 and cystatin C levels and counteracted the reduction of total protein and albumin, respectively, as compared to doxorubicin group (Table 1).

3.2. **Effect of avanafil on oxidative stress markers.**

Normal control values for the renal contents of MDA were 5.350 ± 0.41 nmol/g tissue and the renal activities of GSH , SOD, CAT, Nrf-2 and MPO were nmol/g. tissue, 71.15 ± 2.90 mg/g tissue, 5.89 ± 0.11 mg/g tissue, 120.0 ± 1.09 mg/g tissue,1.03± 0.02 mg/g tissue and 39.25 ± 2.39 mg/g tissue respectively.

Doxorubicin treatment caused a significant increase in the renal MDA content and MPO activity associated with a marked decrease in renal GSH content as well as a significant decrease in Nrf-2 ,SOD and CAT activities, respectively, as compared to the normal control group.

Treatment with avanafil clearly attenuated the renal MDA content and MPO activity and nearly restored renal endogenous GSH content and Nrf-2,

SOD and CAT activities to normal control values as compared to doxorubicin group (Fig. 1A-F).

3.3. Effect of avanafil on inflammatory mediators.

Normal control values for TLR4, P38 MAPK, NF-κB, IL.1 β and TNF-α were 1.00 ± 0.01, 1.005 ± 0.002, 1.007 ± 0.003, 14.93±1.30 and 24.88 ± 0.86 pg/g tissue, respectively.

Doxorubicin treatment caused a marked increase in renal content of TLR4, P38 MAPK, NF-κB, IL.1 β and TNF-α as compared to the normal control group.

Co-treatment with avanafil clearly suppressed renal content of TLR4, P38 MAPK, NF-κB, IL.1 β and TNF-α as compared to doxorubicin group (Fig. 2A-E).

3.4. Effect of avanafil on apoptotic markers.

Normal control values for the pro-apoptotic protein BCL2 Associated X protein (Bax), and anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) were 1.012 ± 0.01 and 1.018 ± 0.016.

Doxorubicin increased apoptotic markers significantly as compared to the normal control group.

As shown in Fig. (3A-C) after co-administration of avanafil the level of Bax was significantly decreased while Bcl-2 level was significantly increased in the renal tissue resulting in inhibition of apoptosis as compared with doxorubicin group

3.5. Effect of avanafil on renal histopathology (Fig 4).

1- Kidney sections from control and avanafil only treated groups showed no histological appearance of kidney structures (Fig 3A-B). BUT, Doxorubicin treated group showed focal inflammatory cells

infiltration with few fibroblastic cells proliferation were detected in between the tubules and glomeruli at the cortex The endothelial cells lining the tufts of the glomeruli showed vacuolization (Fig.4). The cortical stromal blood vessels showed congestion (Fig.4) as well as perivascular oedema (Fig.4). The corticomedullary portion showed focal fibrosis (Fig.5) and focal hemorrhages (Fig.6) in between the tubules. There were swelling in the lining tubular epithelium with obliteration in the tubular lumen (Fig.7) while other tubules at the corticomedullary portion had vacuolar degeneration in the lining epithelium. Interestingly, avanafil co-treated group showed only mild focal inflammatory cells infiltration.

Tabe 1. Effect of avanafil on serum level of urea, creatinine, total protein, albumin, Kidney injury molecule and cystatin in doxorubicin-induced nephrotoxicity in rats.

Group	Serum urea (mg/dl)	Serum creatinine (mg/dl)	Serum total protein g/dl	Serum albumin g/dl	Kidney injury molecule pg/mg tissue	Serum cystatin pg/mg tissue
Control saline	33.17 ± 1.9	0.18 ± 0.01	5.59 ± 0.15	3.48 ± 0.25	2.2 ± 0.09	0.66 ± 0.05
Doxorubicin	83.83 ± 1.35[a]	1.64 ± 0.009[a]	3.775 ± 0.05[a]	1.5 ± 0.04[a]	12.17 ± 0.35[a]	2.86 ± 0.06 [a]

Avanafil	29.33 ± 1.99[b]	0.16 ± 0.02[b]	5.97 ± 0.43[b]	2.9 ± 0.2[b]	1.73 ± 0.16[b]	0.73 ± 0.03[b]
avanafil + Doxorubicin	40.83 ± 3.9[ab]	0.46 ± 0.03[ab]	5.05 ± 0.16[ab]	3.56 ± 0.30[ab]	3.2 ± 0.10 [ab]	0.77 ± 0.06[ab]

Statistical analysis was carried out by one way ANOVA followed by Tukey-Kramer Multiple Comparison Test. Each value represents the mean ± standard error of 8 rats (S.E.).

a Significantly different from normal control group value at $p < 0.05$.

b Significantly different from doxorubicin group value at $p < 0$.

Table 2: Effect of avanafil on histopathlgical alterations in doxorubicin-induced nephrotoxicity in rats.

Group / Histopathlgical alteration	Control saline	Doxorubicin	Doxorubicin + avanafil	Avanafil
Tubular degeneration	–	++	–	–
Fcal inflammatory cell infiltration	–	++	–	–
Focal fibrosis	–	++	–	–
Vaculisation of glomerular endothelium	–	+++	–	–
Cngestion in blood vessels	–	++	–	–
Perivascular edema	–	++	–	–
Focal fibrosis	–	++	–	–
haemorrhage Focal	–	++	–	–
Degeneration in the tubules	–	++	+	–

Statistical analysis was carried out by one way ANOVA followed by Tukey- Multiple Comparison Test. Each value represents the standard deviation of 8 rats (S.E.).

a Significantly different from normal control group value at $p < 0.05$.

b Significantly different from doxorubicin group value at $p < 0$.

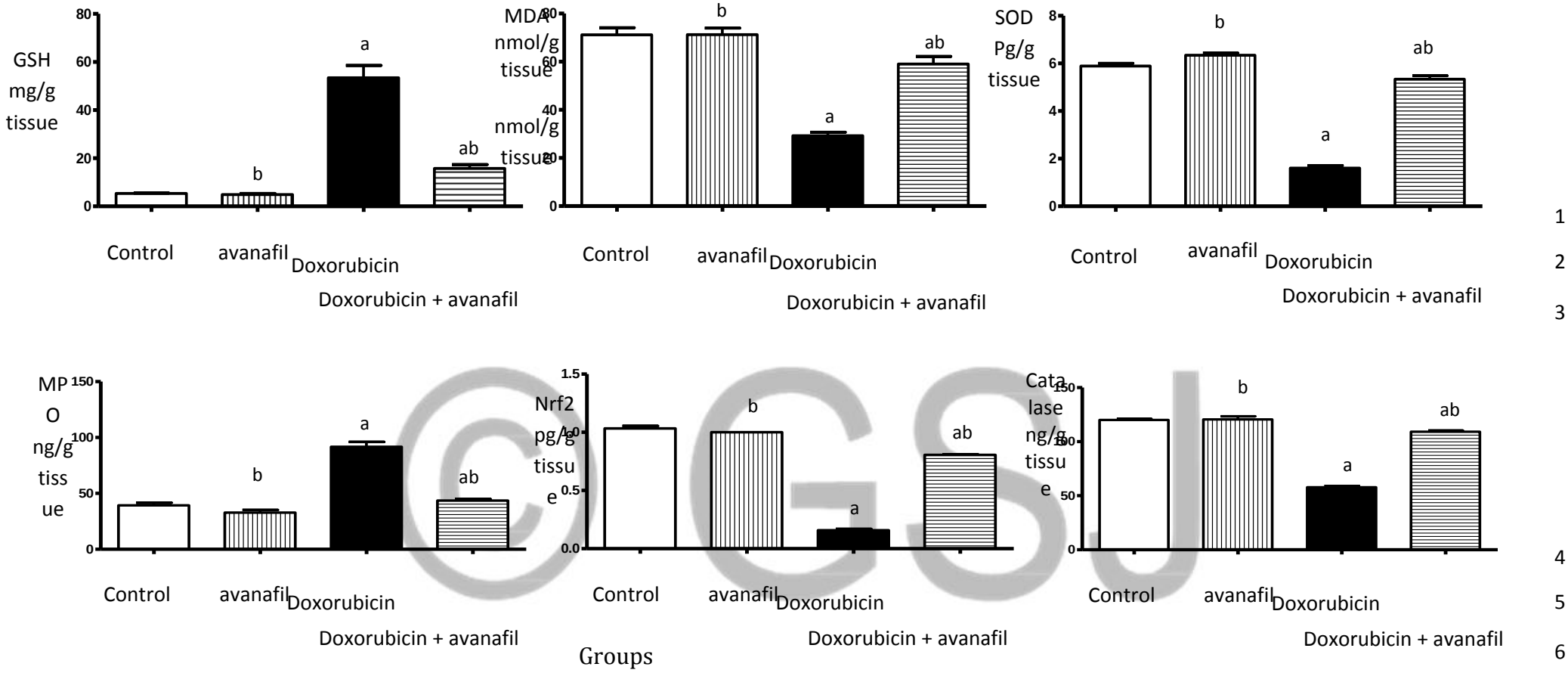

Figure 1: Effect of Avanafil on renal content of GSH, MDA, SOD, MPO, Nrf2 and catalase in doxorubicin-induced nephrotoxicity in rats.

Each value represents the mean of 8-10 rats ± standard error of the mean (SE.).

Statistical analysis was carried out by one way ANOVA followed by Tukey- Kramer Multiple Comparison Test.

[a] Significantly different from normal control group value at $p < 0.05$.
[b] Significantly different from doxorubicin group value at $p < 0.0$.

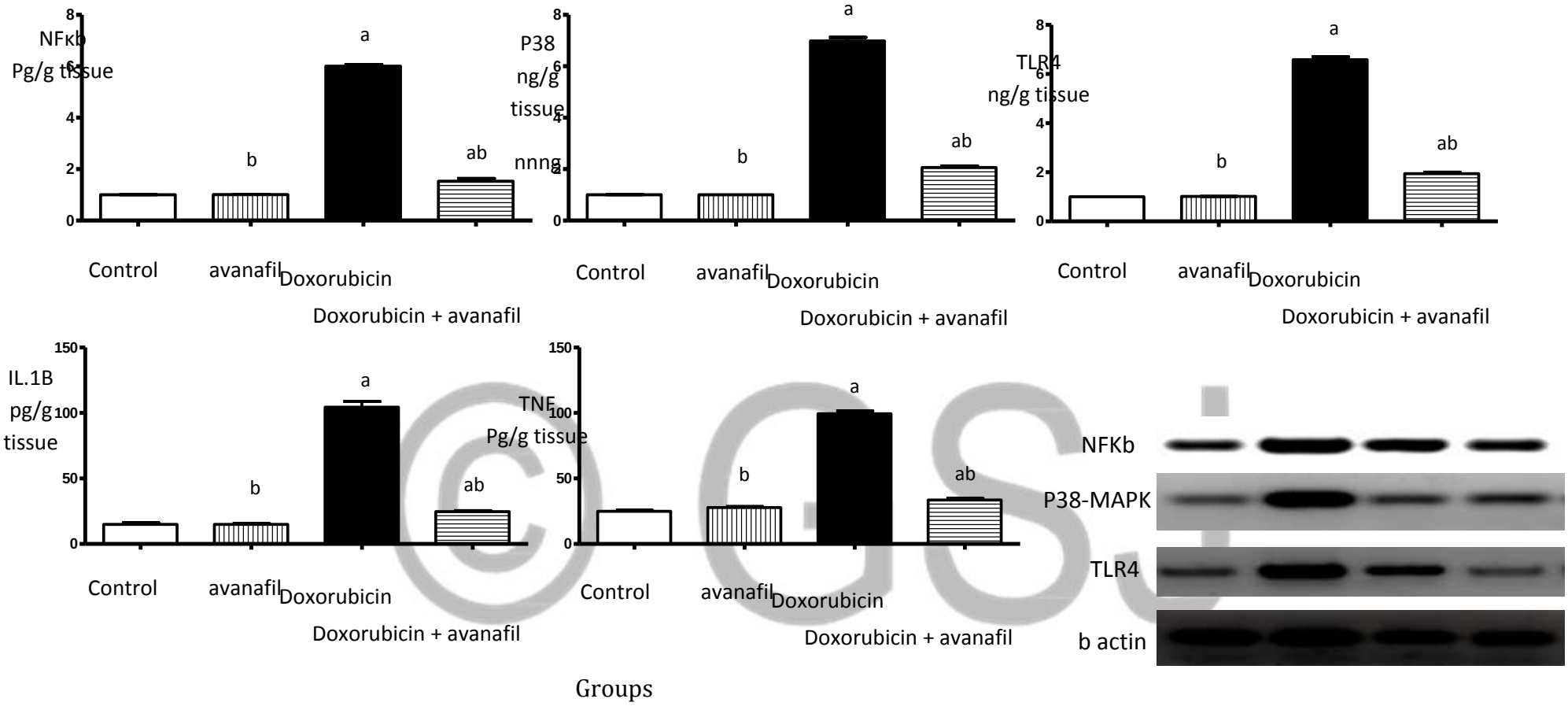

Figure 2: Effect of Avanafil on renal content of NFKb, P38 MAPK, TLR4, IL-1B and TNF in doxorubicin-induced nephrotoxicity in rats.

Each value represents the mean of 8-10 rats ± standard error of the mean (SE.).
Statistical analysis was carried out by one way ANOVA followed by Tukey- Kramer Multiple Comparison Test.
[a] Significantly different from normal control group value at $p < 0.05$.
[b] Significantly different from doxorubicin group value at $p < 0.0$.

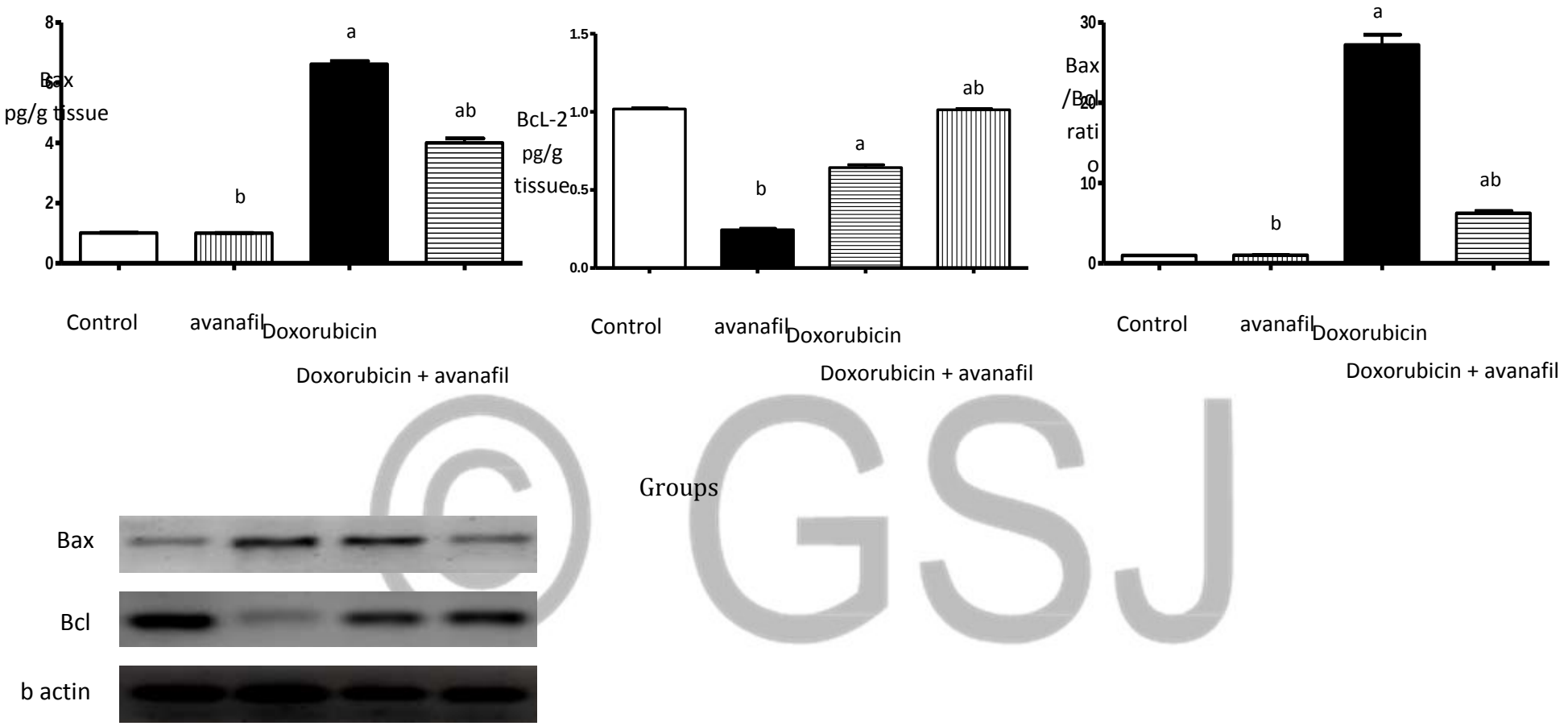

Figure 3: Effect of Avanafil on renal content of Bax and Bcl in doxorubicin-induced nephrotoxicity in rats.

Each value represents the mean of 8-10 rats ± standard error of the mean (SE.).
Statistical analysis was carried out by one way ANOVA followed by Tukey- Kramer Multiple Comparison Test.
[a] Significantly different from normal control group value at $p < 0.05$.
[b] Significantly different from doxorubicin group value at $p < 0.0$.

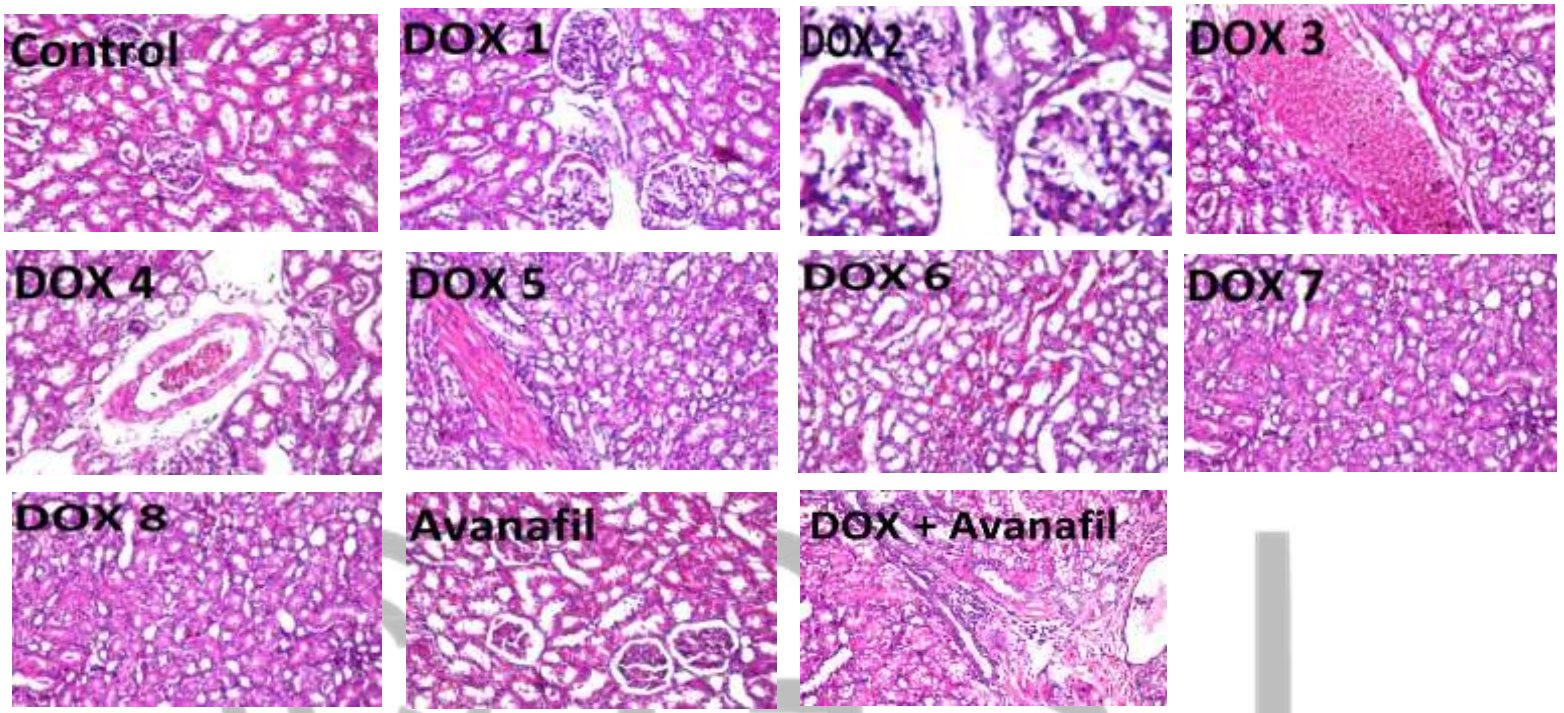

Figure 4: Effect of Avanafil on kidney sections from different groups of doxorubicin-induced nephrotoxicity in rats stained with hematoxylin/eosin (H/E) and examined under a microscope.

Doxorubicin treated rats showed degenerative changes; focal inflammatory cells infiltration (DOX 1), The endothelial cells lining the tufts of the glomeruli showed vacuolization (DOX 2), The cortical stromal blood vessels showed congestion (DOX 3) , as well as perivascular oedema (DOX 4), The corticomedullary portion showed focal fibrosis (DOX 5), and focal haemorrhages in between the tubules (DOX 6), There were swelling in the lining tubular epithelium with obliteration in the tubular lumen (DOX 7), while other tubules at the corticomedullary portion had vacuolar degeneration in the lining epithelium (DOX 8), There was no histopathological alteration as recorded in (avanafil) , There was no histopathological alteration in the glomeruli and tubules at the cortex (Fig.25), while the corticomedullary portion showed focal inflammatory cells infiltration with fibroblastic cells proliferation in between the degenerated tubules (DOX +avanafil).

Discussion

The serious adverse effects of doxorubicin make its indication is severely limited. These undesirable side effects include cardiac, renal, hepatic, and testicular toxicites.

The pathway of DOX-induced nephrotoxicity is sequential in which one step leads to another step, where DOX can induce oxidative stress **(Khan et al., 2018)**, that aggravates immune function toward ROS produced by DOX metabolites leading to the next step which is inflammation and apoptosis**(Khames et al., 2020). (Khames et al., 2017a, Khames et al., 2019)** reported that, hyperuricemic effect of DOX results in deterioration of renal function.

In this study, administration of DOX induced a significant elevation in serum levels of urea, creatinine, KIM-1and cystatin C, but DOX administration reduced the beneficial proteins as interpreted by the significant decline in serum level of albumin and total protein. These results agreed with

This increase in the nephrotoxicity biomarkers is due to structural and functional deterioration as a result of DOX oxidative metabolites that accumulate in nephrons

Dox administration resulted in a significant increase in MDA content and MPO activity associated with a significant decrease in GSH content in renal tissues as well as SOD and catalase activities and a significant increase in Nrf-2 level, these findings agree with the studies of …….(write their names) **(Dai et al., 2018, Renu and Gopalakrishnan, 2019)**. The increase in MDA content is a logic result of the resultant oxidative stress from ROS that react with proteins and lipid **(Lee and Harris, 2011)**.

Doxorubicin significantly increased the inflammatory mediators; TLR4, P38 MAPK, NF-κB, IL-1β, and TNF-α in renal tissues. These results

agreed with is due to oxidative reactions**(Yao et al., 2017)**, since ROS produced by DOX metabolites, after depletion of endogenous anti-oxidant enzymes, induce renal tissue injury leading to inflammatory cascade**(Shi et al., 2018)**

Doxorubicin induced apoptosis in renal tissue as evidenced by heightened the expression of Bax l and decreasing Bcl-2 level. This can be attributed to DOX-induced oxidative stress and inflammatory response , This agreed with **)Tury et al., 2018, Khan et al., 2018(**

Data of the current study showed that, avanafil pretreatment prevented DOX-induced nephrotoxicity as concluded from the decreased serum levels of urea, creatinine, KIM-1 and cystatin-C. These results agreed with

Moreover, avanafil restored the normal values of GSH, SOD and catalase and suppressed the elevated MDA content and MPO activity in the renal tissue ,this agreed with**)Della Camera et al., 2018, Corona et al., 2016(** The renoprotective effect of avanafil against oxidative stress is due to its chemical structure, where avanafil is rich in nitrogen and oxygen atoms that can neutralize free radicals by giving electrons.

The anti-inflammatory effect of avanafil was proved through normalizing the renal levels of TLR4, P38 MAPK, NF-κB, IL-1β and TNF-α. These findings agreed with the results of **(Korkmaz-Icöz et al., 2018)**. The anti-inflammatory effect of avanafil depends on its antioxidant effect and its protective effect against lipid peroxidation.Also, this study showed that avanafil significantly decreased Bax level and significantly increased Bcl-2 level in renal tissues. This strong antiapoptotic effect is thought to be mediated through the anti-oxidant structure of avanafil and its free radicals scavenging ability**)**.**Huyut et al., 2018, Korkmaz-Icöz et al., 2018(**

The aforementioned biochemical results were coupled with the histopathological improvements shown in doxorubicin+avanafil group which

include improvement the glomerular and proximal tubular integrities.to confirm nephroprotective effect.

Conclusion:

Avanafil is a promising nephroprotective agent against DOX nephrotoxicity. This is indicated via modulation of the **TLR4/P38 MAPK/NFκ-B inflammatory signaling pathway** besides inhibiting oxidative stress and apoptosis.

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