



## Evaluation of the Diuretics Effects in the Inflammatory and Redox Responses in a Doxorubicin-Induced Ns Model.

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### ABSTRACT

Nephrotic syndrome (NS) is a renal condition characterized by proteinuria, edema formation, hypoalbuminemia, and dyslipidemia. Evidence indicates that the immune response plays a fundamental role in disease evolution and maintenance. Although diuretics are used in the NS treatment, it is not known whether they have any effect on immune and redox responses. Therefore, the present study aimed to evaluate the furosemide effects in the inflammatory and redox responses in a doxorubicin-induced NS model. Eighteen male adult Wistar rats were divided into 3 groups: Control (n = 6) - received intravenous injection of saline solution; DOXO (n = 6) - received intravenous injection of doxorubicin (7.5 mg/kg); DOXO-F (n = 6) - received intravenous injection of doxorubicin (7.5 mg/kg) and were later treated with furosemide by gavage (5.0 mg/kg). At the end of 36 days of treatment were evaluated: urine protein concentration, blood leukocyte count, kidney histology, cytokine levels (TNF- $\alpha$ , INF- $\gamma$ , and TGF- $\beta$ ), antioxidant levels (FRAP), and enzyme activity (CAT and SOD), besides markers of oxidative stress (TBARS and protein carbonyl) in renal tissue. Data were analyzed with ANOVA and Tukey test when necessary ( $p < 0.05$ ). At the dosage used in this study, Furosemide promoted an increase in global blood leukocytes and reduced lymphocyte blood count. It was also observed that furosemide reduced TNF- $\alpha$  and increased TGF- $\beta$  levels in renal tissue. In addition, furosemide increased the levels of oxidative stress markers (TBARS and protein carbonyls) and the activity of antioxidant enzymes (SOD and CAT). Thus, furosemide showed anti-inflammatory effects in rats with nephropathy, by reducing TNF $\alpha$  levels and increasing antioxidant activity in kidney tissue.

**Keywords:** Nephrotic Syndrome. Furosemide. Animal model. Cytokines. Redox status

### I. INTRODUCTION

Nephrotic syndrome (NS) is one of the commonest renal conditions in the world, caused by primary kidney diseases or induced by secondary causes, such as diabetes mellitus, lupus erythematosus, bacterial infections or medications. Clinically, NS is characterized by intense proteinuria, edema formation, hypoalbuminemia and dyslipidemia. These conditions develop from the increased permeability of the renal glomerular membrane, leading mainly to urinary elimination of massive amounts of proteins and others complications (Wang and Greenbaum, 2019).

Although the NS mechanism is not fully understood, evidence indicates that the immune system plays a fundamental role in the disease evolution. For example, immune response may cause the disruption of the glomerular filtration barrier, which would be a major factor in the clinical manifestations of the primary or secondary NS (Daehn and Duffield, 2021). Also, inflammatory infiltrates of macrophages and T lymphocytes were observed in the tubulointerstitial zone in NS early stages (Araya et al., 2009). In addition, other studies show that cytokines and reactive oxygen species can alter the permeability of the glomerular capillary wall (Al-Eisa et al., 2017; Colucci et al., 2018; Ece et al., 2004). Aggravatingly, proteinuria provides the recruitment of more macrophages, prolonging the inflammatory response in the interstitium (Abreu et al., 2017; WANG et al., 2001).

Regarding NS treatment, in patients with greater renal impairment, loop diuretics are indicated to contain the disease evolution. The oral treatment with furosemide reduces edema formation and controls blood pressure through its natriuresis and diuresis mechanisms (Fallahzadeh et al., 2017; Kallash and Mahan, 2021; Mahmoodpoor et al., 2020; Varghese and Majumdar, 2021). Additionally, furosemide also showed anti-inflammatory and antioxidant effects when using other experimental models (Arumugam



et al., 2014; Bryniarski et al., 2021; dos Santos et al., 2018), suggesting that this drug could also have an effect on NS inflammatory and oxidant responses.

In this context, it remains to be explored whether furosemide has any action on inflammatory and redox state markers in NS. Therefore, the aim of this study was to evaluate the furosemide effects in the inflammatory and redox responses in a doxorubicin-induced NS model.

## II. MATERIALS AND METHODS

### 1. Ethical statement

All experimental procedures were approved by the Ethics Committee on the Use of Animals of Federal University of Jequitinhonha and Mucuri Valleys - UFVJM (protocol number 001/2019) and occurred according to the ethical principles of animal use (CONCEA, 2016). Eighteen male Wistar rats were obtained from Experimental Physical Training Laboratory/UFVJM and kept under controlled environmental conditions, with free access to food (Nuvilab<sup>®</sup> CR-1) and potable water.

### 2. Experimental design

On the first day of the experiment (D0), 18 male Wistar rats (six-week-old) were randomly placed in three groups: Control (n = 6) - received intravenous injection of saline solution (isotonic and pyrogen-free sodium chloride solution 0.9%); DOXO (n = 6) and DOXO-F (n = 6) - received intravenous injection of doxorubicin hydrochloride (7.5 mg/kg of body weight) (Pereira et al., 2015a). The furosemide administration (5.0 mg/kg of body weight) occurred by gavage only for the DOXO-F group, from the seventh day (D7) onwards (D36). The Control and DOXO groups received potable water under the same conditions. The treatments were performed daily until the day of euthanasia (D36).

### 3. Protein evaluation

Protein levels were measured using 24-hour urine samples, collected weekly in metabolic cages (Insight<sup>®</sup>, Ribeirão Preto, São Paulo, Brazil) on D0 (before doxorubicin injection), D7, D14, D21, D28 and D35 (after doxorubicin injection). First, the urine samples (24 hour) were diluted in the proportion of 100 microliters of the sample for each 9.9 mL of deionized water (Gomes et al., 2017). Proteinuria analyses were carried out by colorimetric Bradford assay (Bradford, 1976).

### 4. Euthanasia and sample collection

On D36, the animals were anesthetized (xylazine 8 mg/kg; ketamine 60 mg/kg) and euthanized by exsanguination process. The blood was collected to perform blood leukocyte count. The left kidney was removed and sectioned for histological analysis, in addition to cytokines and redox state evaluations.

### 5. Blood Leukocyte Count

For global leukocyte count, 10 $\mu$ L of the blood sample from each animal was diluted in 190 $\mu$ L of Turkey's solution (red cell lyser) and count in a Neubauer hemocytometric chamber (Neubauer Improved<sup>®</sup>, Marienfeld – Germany) under light microscopy (Olympus Optical Co., Ltd. Microscope – BX41 TF – Japan). Differential white blood cell counts were performed in blood smears using an optical microscope (Olympus-BX41 TF, Japan) after May-Grunwald-Giemsa staining (Pereira et al., 2015a).

### 6. Histological analysis

The left kidney was segmented into coronal sections, fixed and stained with hematoxylin and eosin (HE) and Masson's Trichrome (MT) for the analysis of morphological changes and kidney fibrosis, according to Pereira et al., 2015a. Histological images were analyzed using light microscopy (Microscope Olympus Optical CO. Ltd – BX41 TF-Japan) and scanned by a camera (NIKON, Eclipse E220) attached to the microscope. The ImageJ<sup>®</sup> (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) software was used for the analysis.

### 7. Cytokines analyses

For cytokines analyses, renal samples were homogenized in the extraction solution (0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 kIU aprotinin) prepared in PBS. The homogenates were centrifuged at 10,000  $\times$  g for 10 min at 4 °C. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interferon- $\gamma$  (INF- $\gamma$ ) and Transforming growth factor- $\beta$  (TGF- $\beta$ ) levels were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems<sup>®</sup>) and as previously described (Rocha-Gomes et al., 2022). TGF- $\beta$ /TNF- $\alpha$  ratio was calculated and classified as an anti-inflammatory marker (Babania et al., 2021).



## 8. Redox state

For tissue preparation, the kidney was homogenized in cold PBS (50mM, pH 7.0), and centrifuged at 750 g for 10 min at 4 °C, as previously described (Rocha-Gomes et al., 2021).

The total antioxidant capacity was evaluated using the ferric reducing antioxidant power (FRAP) method, monitored at 550nm, with FeSO<sub>4</sub> used as standard (Benzie and Strain, 1996; Rocha-Gomes et al., 2021). For the activity of the antioxidant enzyme superoxide dismutase (SOD), the pyrogallol oxidation method was used. The oxidation process was measured at 420nm for 250s at intervals of 10s and 37°C (MARKLUND and MARKLUND, 1974; Rocha-Gomes et al., 2021). Catalase (CAT) activity was assessed by metabolizing hydrogen peroxide. The readings were performed in a microplate reader every 15 seconds for 1 minute at 25°C (Nelson and Kiesow, 1972; Rocha-Gomes et al., 2021).

The lipid peroxidation evaluation was performed using the thiobarbituric acid reactive substances (TBARS) method with malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) as the standard, being monitored at 532nm using (Ohkawa et al., 1979; Rocha-Gomes et al., 2021). Protein carbonyls were evaluated in pellets from the homogenates and were determined using the 2,4-dinitrophenylhydrazine (DNPH) method,

monitored at 370 nm (Levine et al., 1990; Rocha-Gomes et al., 2021).

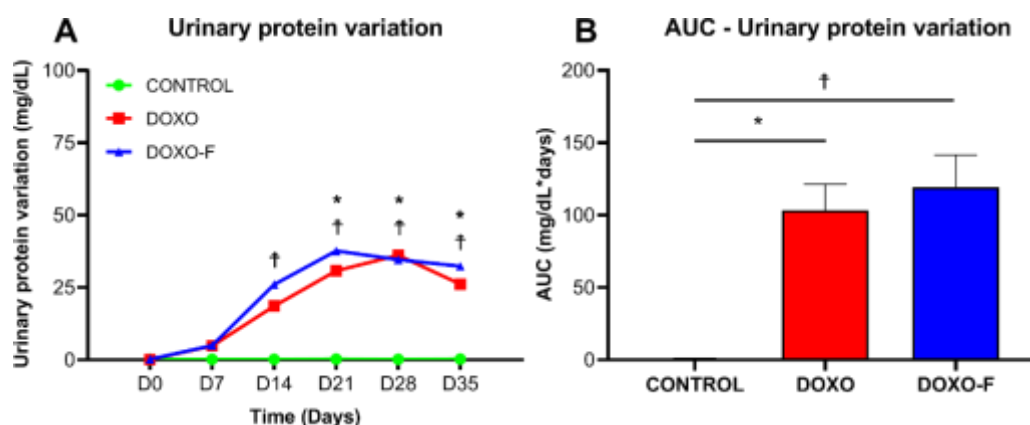
Protein content was quantified using BSA (1 mg/mL) as the standard (Bradford, 1976). The results of the redox state were corrected for the amount of protein in the samples. All redox analyses were performed in triplicate, using a plate reader (UV/visible U-200 L Spectrophotometer).

## 9. Statistical analyses

Analyses were performed using the GraphPad Prism 8.0 (GraphPad, La Jolla, California, USA). The results were expressed as mean and standard deviation (SD), with a significance level of 95% ( $p < 0,05$ ). According to normality, the data were analyzed using the one-way or two-way analysis of variance (ANOVA), with Tukey post hoc test or Kruskal Wallis test. Area under the curve (AUC) were calculated from the baseline using the trapezoidal method in the GraphPad Prism 8.0.

## III. RESULTS

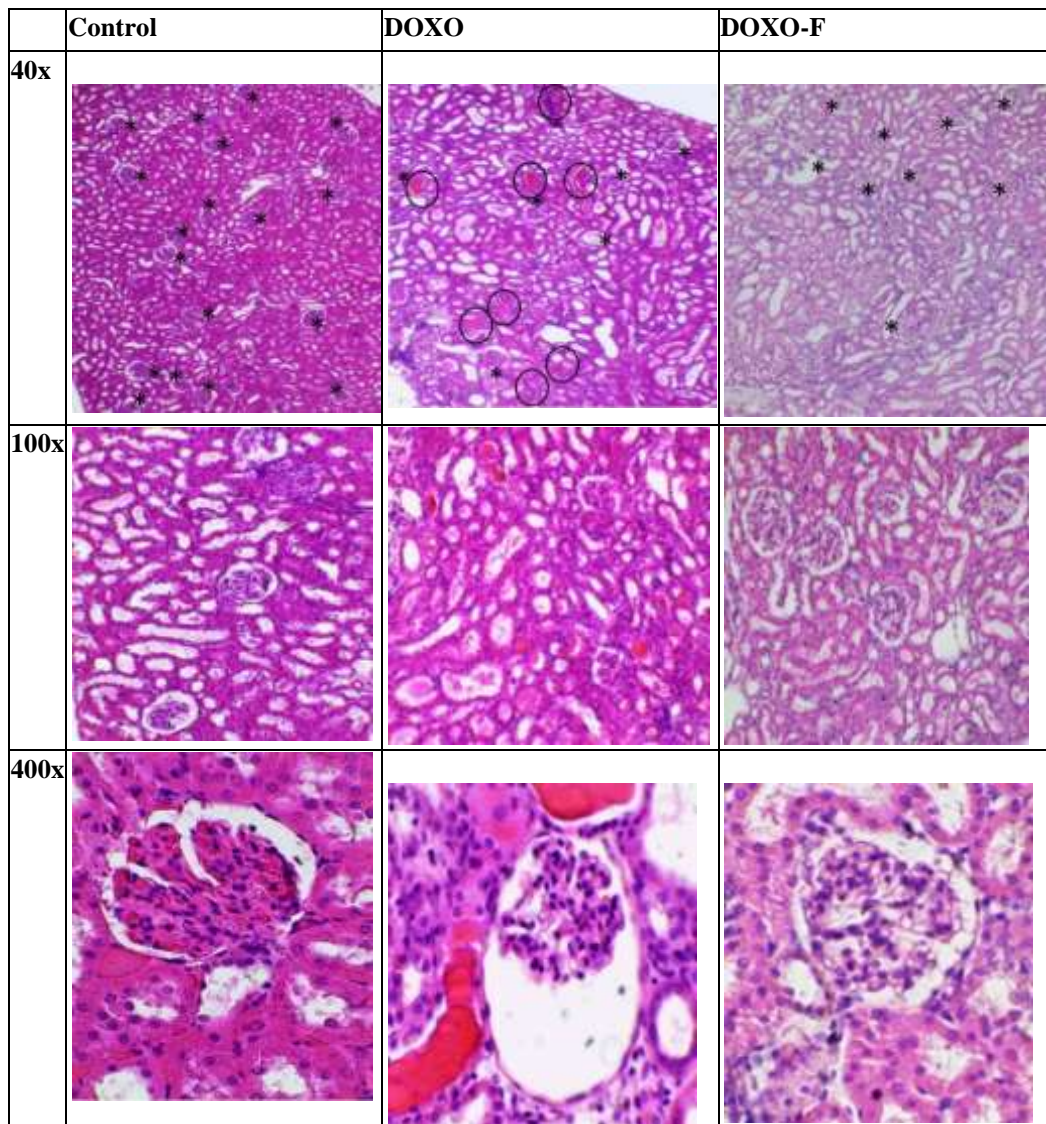
Significant differences were observed in the protein levels of the DOXO group from D21 and in the DOXO-F group from D14 both compared to the Control group (Figure 1A). AUC analysis showed that the DOXO and DOXO-F groups had higher proteinuria compared to the Control group (Figure 1B).



**Figure 1.** Urinary protein variation (A) and its respective area under the curve (B) from rats with doxorubicin-induced NS. Data are presented as mean  $\pm$  SD. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). Data presented as mean  $\pm$  SD. Urinary protein variation was analyzed by two-way ANOVA and area under the curve (AUC) by one-way ANOVA, both with Tukey post-test.  $n = 6$ ; \* $p < 0.05$  DOXO vs. Control groups; †  $p < 0.05$  DOXO-F vs Control groups.

Histological evaluation by HE staining showed a reduction in the number of glomeruli, increased hyalinization of the renal parenchyma and interstitial cellularity, indicative of an

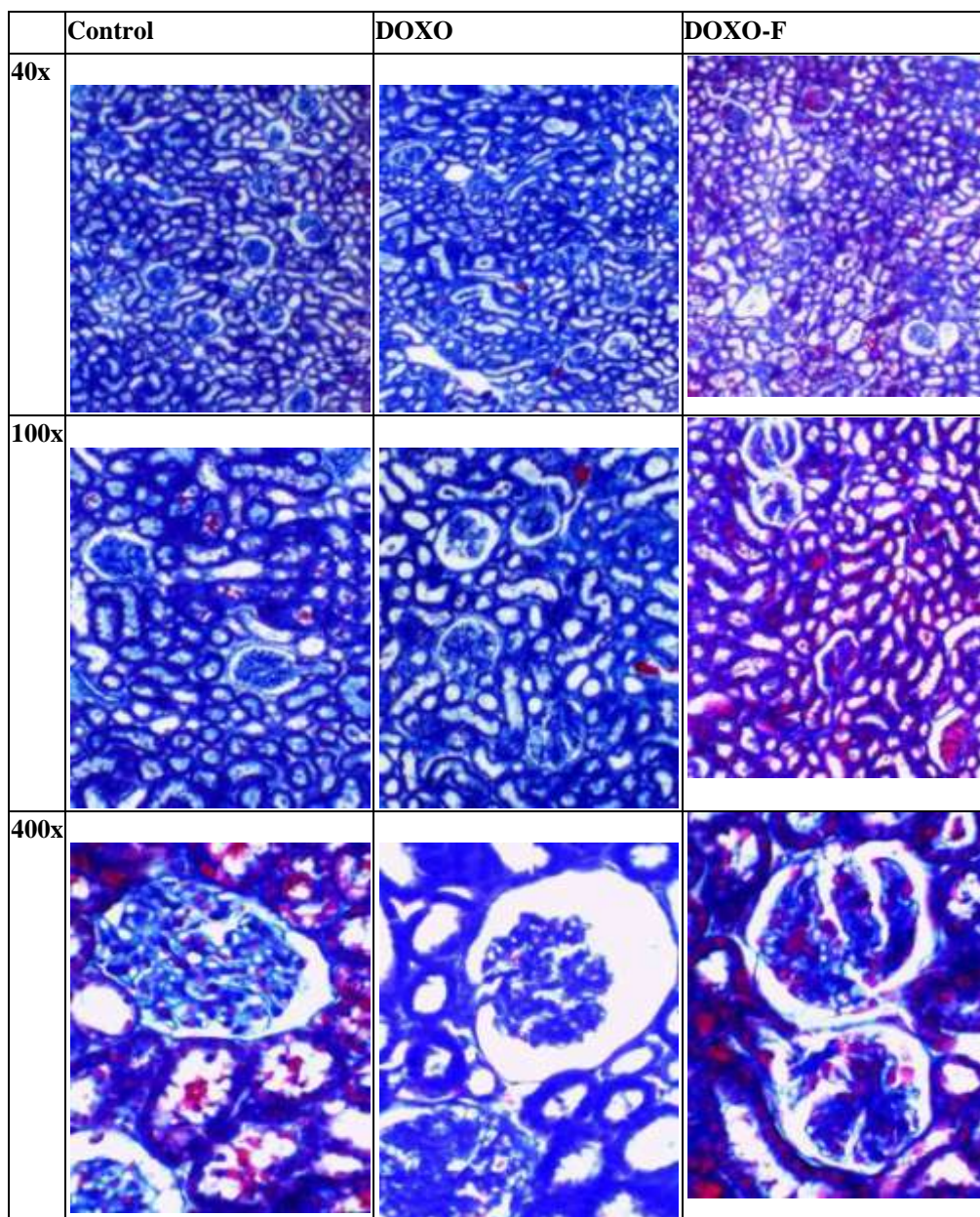
inflammatory infiltrate in DOXO group compared to Control. In the DOXO-F group, more glomeruli count and mild atrophy were observed compared to the DOXO group (Figure 2).



**Figure 2.** Renal histology from rats with doxorubicin-induced NS. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). The circled areas represent hyaline formations, while the \* shows the presence of glomeruli. The photomicrographs are presented in HE staining at 40x, 100x and 400x.

MT staining of kidney sections revealed an accumulation of collagen fibers among glomerular capillaries in the Bowman's capsule of the glomeruli in the DOXO group. The DOXO-F

group was more similar to the control group and showed a slight deposition of collagen fibers in the glomeruli (Figure 3).

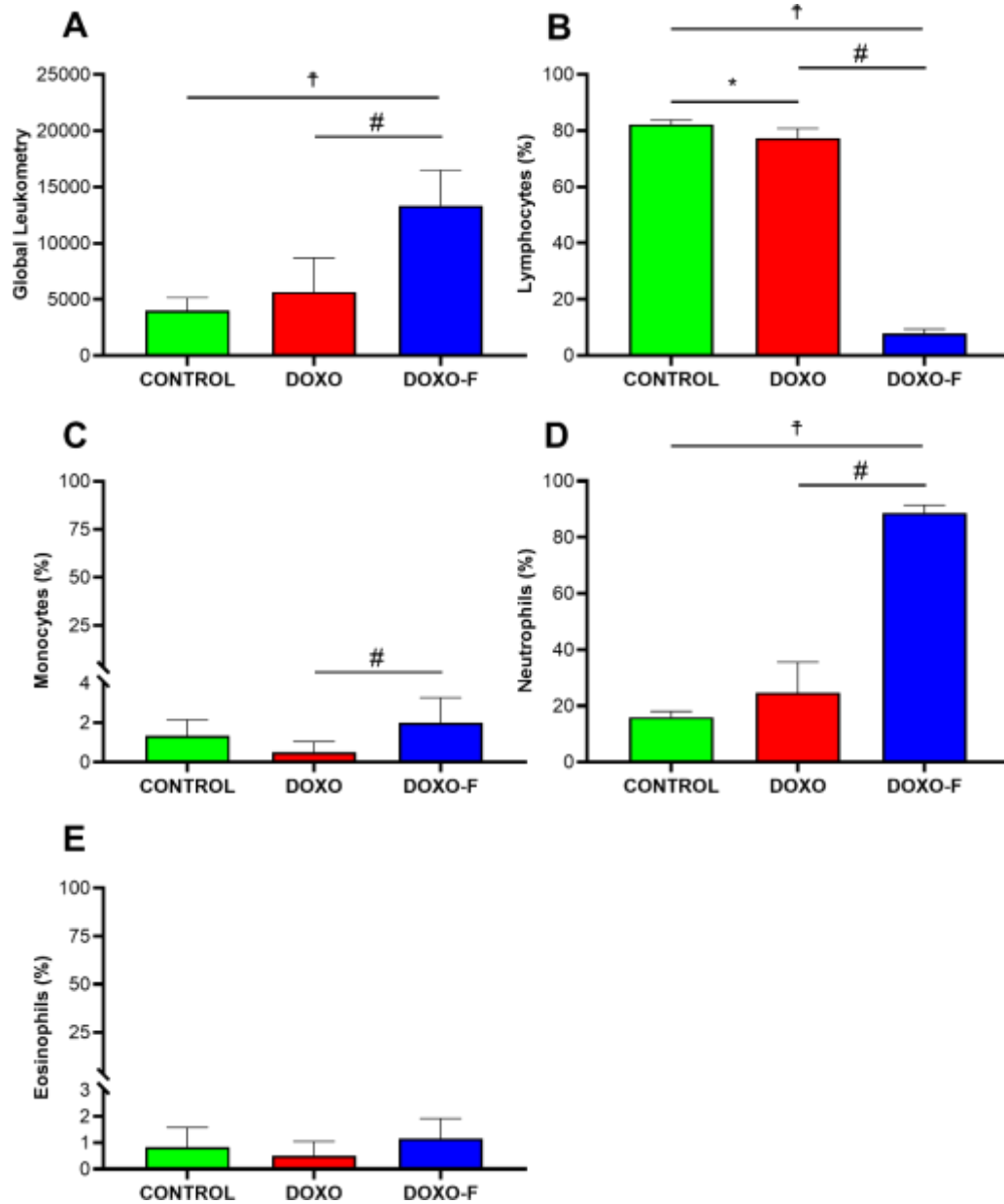


**Figure 3.** Renal histology from rats with doxorubicin-induced NS. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). The photomicrographs are presented in MT staining at 40x, 100x and 400x.

The DOXO-F group had higher values in the global leucocyte count when compared to the DOXO and CONTROL groups (Figure 4). Regarding the percentage of lymphocytes, the DOXO and DOXO-F groups presented lower values compared to the CONTROL group (Figure 4). It was also observed that the DOXO-F group

had a lower percentage of lymphocytes than the DOXO group. Furthermore, the DOXO-F group presented a higher percentage of monocytes compared to the DOXO group (Figure 4).

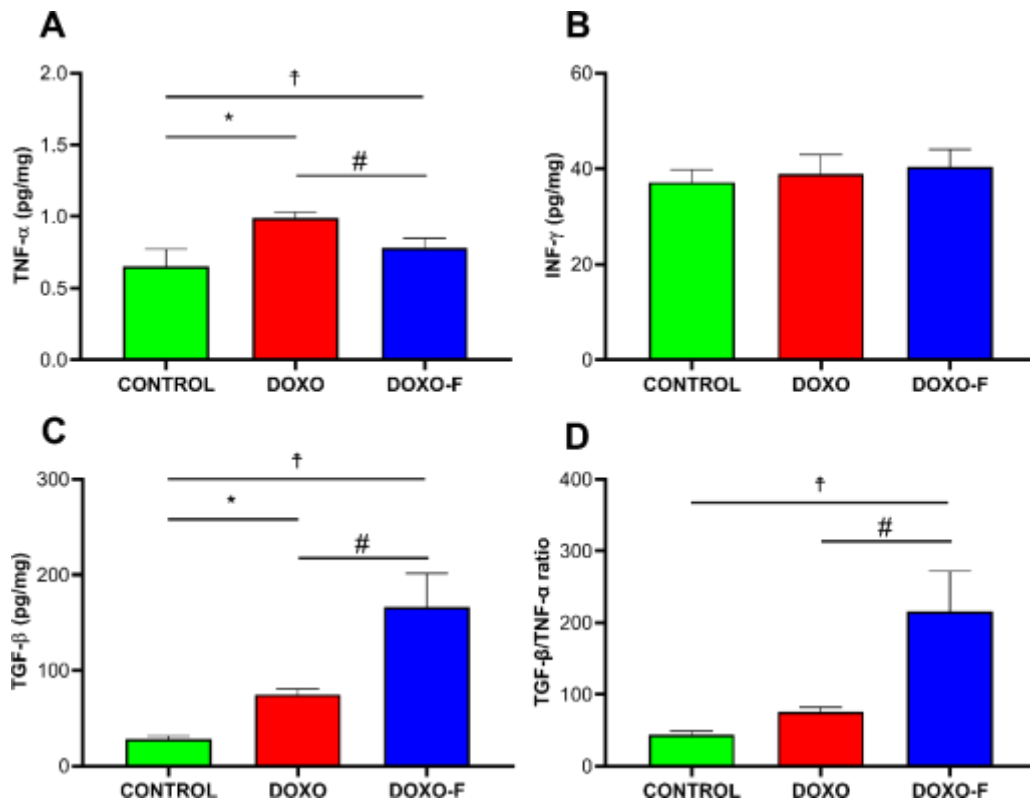
Regarding the neutrophils number, higher amounts of these cells were observed in the DOXO-F group (Figure 4).



**Figure 4.** Global leukocyte count (A); percentage of lymphocytes (B), monocytes (C), eosinophils (D) and neutrophils (E) in the blood of rats with doxorubicin-induced NS. Data are presented as mean  $\pm$  SD. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). Data presented as mean  $\pm$  SD. Data were analyzed by one-way ANOVA with Tukey post-test. n = 6; \*p < 0.05 DOXO vs. Control groups; † p < 0.05 DOXO-F vs Control groups; # p < 0.05 DOXO vs DOXO-F groups.

Significant increase in the renal concentration of TNF- $\alpha$  were showed in DOXO and DOXO-F groups when compared to the CONTROL group. In addition, the DOXO group

had higher concentration of this cytokine compared to the DOXO-F group (Figure 5).

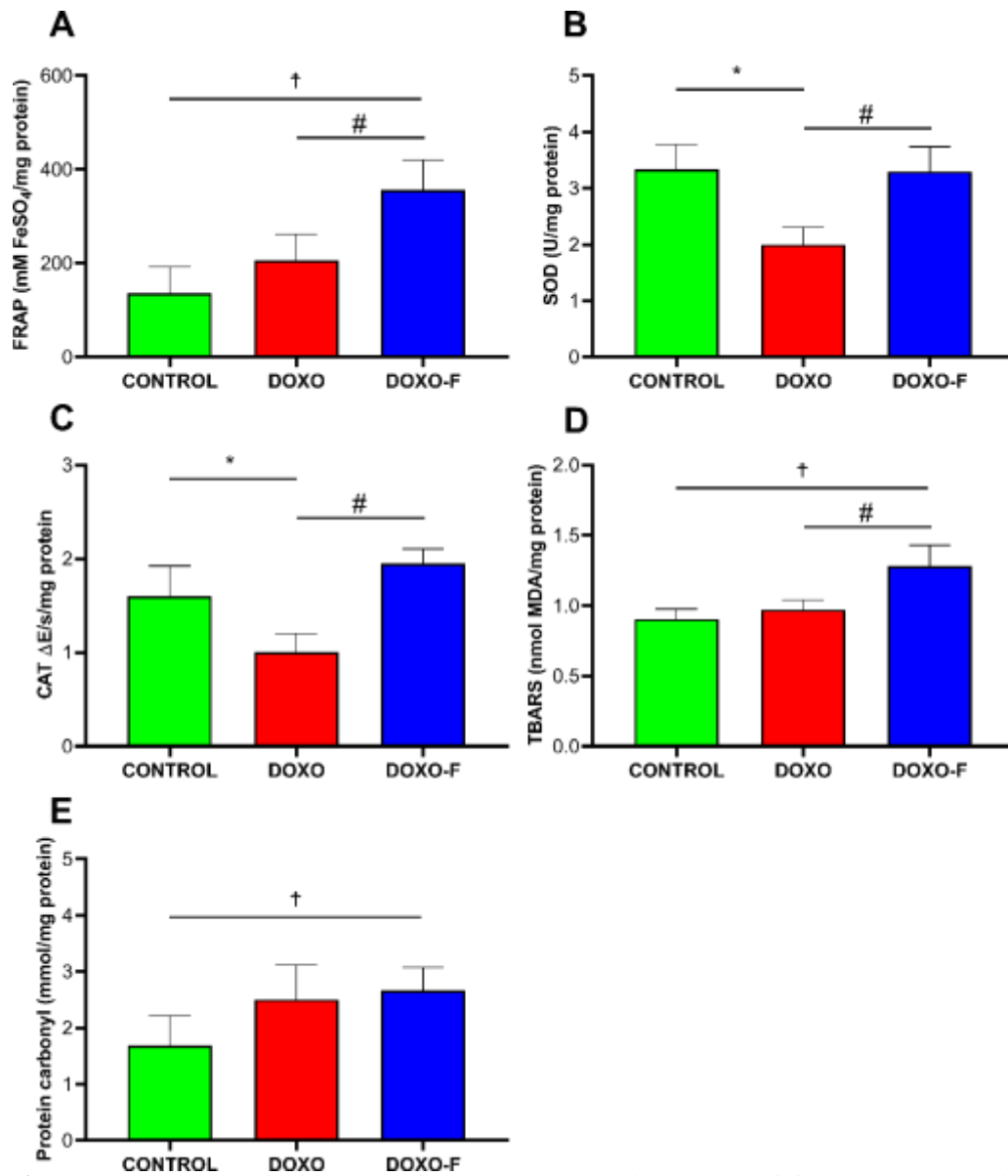


**Figure 5.** Renal levels of TNF- $\alpha$  (A), INF- $\gamma$  (B), TGF- $\beta$  (C) and TGF- $\beta$ /TNF- $\alpha$  ratio from rats with doxorubicin-induced NS. Data are presented as mean  $\pm$  SD. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). Data presented as mean  $\pm$  SD. Data were analyzed by one-way ANOVA with Tukey post-test. n = 6; \*p < 0.05 DOXO vs. Control groups; † p < 0.05 DOXO-F vs Control groups; # p < 0.05 DOXO vs DOXO-F groups.

The renal concentration of TGF- $\beta$  was significantly higher in the DOXO and DOXO-F groups compared to the CONTROL group. There was also increase in TGF- $\beta$  in DOXO-F group compared to the DOXO group. Furthermore, DOXO-F group showed a higher TGF- $\beta$ /TNF- $\alpha$  ratio compared to DOXO and CONTROL groups.

No difference was showed for the INF- $\gamma$  renal levels (Figure 5).

Regarding FRAP levels, a higher antioxidant capacity was showed in the DOXO-F group compared to the DOXO and CONTROL groups. In addition, the activities of SOD and CAT enzymes were higher in DOXO-F and CONTROL groups compared to the DOXO group (Figure 6).



**Figure 6.** Renal FRAP total antioxidant capacity (A); SOD (B) and CAT (C) activity; TBARS (D) and protein carbonyl (E) concentrations from rats with doxorubicin-induced NS. Data are presented as mean  $\pm$  SD. Control – Negative Control Group (no nephropathy and no treatment). DOXO – Positive Control Group (with nephropathy and no treatment). DOXO-F – Test Group (with nephropathy and treatment with oral Furosemide). Data presented as mean  $\pm$  SD. Data were analyzed by one-way ANOVA with Tukey post-test.  $n = 6$ ; \* $p < 0.05$  DOXO vs. Control groups; †  $p < 0.05$  DOXO-F vs. Control groups; #  $p < 0.05$  DOXO vs. DOXO-F groups.

DOXO-F group showed an increase in TBARS levels compared to the CONTROL and DOXO groups. Also, there was an increase in the expression of the protein carbonyl in the DOXO-F group compared to the CONTROL group (Figure 6).

#### IV. DISCUSSION

The use of diuretics has been recognized as one of the main treatments for primary or

secondary NS. However, the role of the diuretics in NS inflammatory and redox responses has not yet been determined. Here, it was shown that the doxorubicin-induced NS model (DOXO and DOXO-F groups) led to proteinuria and high glomerular damage, both characteristic conditions in NS. Additionally, the DOXO group showed increased levels of inflammatory cytokines and low activity of antioxidant enzymes in renal tissue. In contrast, furosemide (DOXO-F group) reduced the damage caused by doxorubicin, promoted an anti-





inflammatory response and increased activity of antioxidant enzymes in the kidney.

The presence of proteinuria is considered an expressive marker of kidney damage and is part of the NS diagnosis (Kopp et al., 2020). Proteinuria occurs due to increased protein filtration by glomeruli and a reduction in the rate of tubular reabsorption, causing podocyte and tubular lesions, interstitial changes, edema formation, fibrosis and inflammatory cell infiltrates (Ogawa-Akiyama et al., 2020; Tapia and Bashir, 2021). Corroborating other studies, the groups that received the doxorubicin injection (DOXO and DOXO-F) showed an increase proteins concentration in the urine (Elsherbini and Ebrahim, 2020; Pereira et al., 2015b), confirming the effectiveness of the model in inducing NS.

The use of doxorubicin is also linked to glomerular atrophy and increased capillary permeability, factors that are associated with the proteinuria (Xiang et al., 2021). This condition can be observed in the histological analysis of the present study, in which the DOXO and DOXO-F groups showed a reduction in the glomeruli number in relation to the CONTROL group. However, it is important to highlight that the furosemide administration attenuated glomeruli loss in relation to the DOXO group, which may suggest an improvement in the NS pathophysiological evolution.

The increase in the leukocytes migration in animals with nephropathy may be related to an exacerbated immune response, also described using other experimental nephropathy models (Kelly et al., 2009; Kitamoto et al., 2009). Therefore, the lower levels of blood lymphocytes presented by the DOXO-F group are suggestive of the migration of these cells to the renal tissue, since lymphocytes are one of the several cells responsible for the TGF- $\beta$  production (Lv et al., 2018; Stolfi et al., 2020).

Several studies have identified the role of inflammation in the development and NS evolution (Colucci et al., 2018; Daehn and Duffield, 2021; Pereira et al., 2015a). In the present study, the DOXO and DOXO-F groups showed an increase in the levels of renal TNF- $\alpha$ . This is one of the main cytokines involved in the inflammatory processes, being released by activated mast cells and macrophages, also stimulating other cells of the immune system (Mehaffey and Majid, 2017). Hence, it is possible to assume that the elevation of TNF- $\alpha$  is linked to the doxorubicin injection and NS development, with consequent inflammatory response and tissue damage.

Interestingly, the DOXO-F group had lower levels of renal TNF- $\alpha$  compared to the DOXO group. Some studies had already shown anti-inflammatory action of furosemide in vitro using human peripheral blood mononuclear cells, reporting reduction of cytokines, such as IL-6, IL-8 and TNF- $\alpha$  (Bryniarski et al., 2021;

Yuengsrigul et al., 1999). In addition, the DOXO-F group had higher concentrations of renal TGF- $\beta$  and a greater TGF- $\beta$ /TNF- $\alpha$  ratio compared to the other groups. TGF- $\beta$  has an important role in the regulation of processes such as tissue repair and apoptosis, besides acting in the control of immune system homeostasis and inflammation (Teicher, 2021). To the best of our knowledge, this is the first study to shown that the furosemide application can reduce renal levels of a pro-inflammatory cytokine (TNF- $\alpha$ ) and increase the concentration of one involved in tissue repair (TGF- $\beta$ ). These data suggest an anti-inflammatory role in renal tissue promoted by furosemide, with a consequent attempt to return to homeostasis.

The increase in reactive oxygen and nitrogen species can lead to oxidative stress, causing damage to proteins and lipids, raising the levels of TBARS and carbonyl derivatives in proteins. In contrast, to contain tissue oxidative stress advance, there is an increase in the production of antioxidant enzymes to maintain the redox state homeostasis (Bakunina et al., 2015). In the present study, the DOXO-F group showed an increase in the expression of TBARS and carbonyl protein, but also an elevation in the activity of SOD and CAT enzymes, as well as in the FRAP levels. The renal oxidative stress has been shown in other studies that used doxorubicin or different models of kidney injury (Boonsanit et al., 2006; Khajevand-Khazaei et al., 2019). Furthermore, patients with NS have a strong correlation between oxidative stress and disease progression (Daenen et al., 2019). However, the increased activity of antioxidant enzymes in the DOXO-F group suggests that furosemide may triggered a response to contain oxidative damage. These results reinforce those observed in cytokine levels, indicating an anti-inflammatory and antioxidant action of furosemide.

Some limitations were noted in this study. For a better understanding of the anti-inflammatory action of furosemide on renal tissue, other inflammatory markers, as well as evaluation at different timepoints are necessary. In addition, the direct dosage of reactive species, evaluation of Nrf2 (nuclear erythroid factor 2-related factor 2) and the markers of its pathway expression, could be key elements to further understand the interaction



of the redox state with the NS evolution. Finally, the need for future studies with other doses of furosemide and longer exposure time is evident.

The present study showed that the use of furosemide can attenuate the NS progression, possibly through an anti-inflammatory and antioxidant action. These findings are important to increase the discussion around NS therapy, since in some cases corticosteroids and immunosuppressants are used, which have serious long-term adverse effects (Chin et al., 2021; Zhao and Liu, 2020). Hence, the demonstration of the anti-inflammatory properties of furosemide may be useful for discussing new clinical approaches.

## V. CONCLUSION

Furosemide reduced TNF $\alpha$  levels and increased antioxidant activity in the renal tissue of rats with doxorubicin-induced nephropathy, thus demonstrating a beneficial effect in attenuating the inflammatory response in an experimental model of nephropathy.

## ETHICS APPROVAL

This study was previously approved by the Animal Ethics Committee of Federal University of Jequitinhonha and Mucury Valleys (UFVJM) regarding the Guiding Principles in the Care and Use of Animals, with an approved protocol number of 001/19.

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