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SAFETY ASSESSMENT OF DOXYCYCLINE AFTER AN ORAL LONG-TERM ADMINISTRATION IN RATS

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ABSTRACT

Doxycycline has been widely studied for off-label use as neuroprotective and is capable to reduce oxidative stress in several neurodegenerative diseases. However, it is necessary to assess the safety of long-term exposure to doxycycline once neurodegenerative diseases generally require chronic treatments. Thus, we evaluated the safety of doxycycline in chronic use through hematological, biochemical, redox, and histological analysis. Male Wistar rats were divided into two groups: control (saline 0.9%) and experimental (10 mg/kg doxycycline/day), for 93 days, n=8 animals/group. After euthanasia, blood and liver were collected and analyzed. The hematimetric indices provided no significant differences between the control and experimental. Moreover, doxycycline did not interfere with the immune system, once the leukogram was similar between the two groups. Levels of urea and creatinine were also similar comparing the control and the experimental group. Doxycycline did not damage the livers'architecture, and aspartate and alanine aminotransferases presented typical activities. Regarding oxidative stress, the GSH-Px enzyme was the only parameter that presented an increase in the experimental group compared to the control. In conclusion, doxycycline proved to be a safe drug in long-term administration. The increase in the GSH-Px activity could be related to an increase in selenocysteine insertion induced by doxycycline, activating the enzyme.

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INTRODUCTION

Doxycycline is an antibacterial drug synthetically derived from oxytetracycline, a member of the tetracycline class of antibiotics (Kundu *et al*., 2015; Thillainayagam & Ramaiah, 2016). According to the Food and Drug Administration (FDA), doxycycline is used for the prevention or treatment of rickettsial infections, sexually transmitted infections, respiratory tract infections, bacterial infections, Lyme disease, ophthalmic infections, anthrax, acute intestinal amebiasis, diarrhea, and severe acne (FDA, 2018a), and in some specific cancers since doxycycline could inhibit cell proliferation and invasion, besides inducing apoptosis and blocking the gap phase (Kundu *et al*., 2015). Doxycycline has also been shown abilities against neurodegenerative diseases (Santa-Cecília *et al*., 2019; Dominguez-Mijide *et al*., 2021; Medina *et al*., 2021), Alzheimer disease by avoiding aggregation-prone proteins (Hicks *et al*., 2020; Chambraud *et al*., 2021; Kubota & Kirino, 2021), neuropathic pain (Jin *et al*., 2020), neuroinflammation (Balducci *et al*., 2018), Parkinson disease by avoiding inflammation (Forloni *et al*., 2021) or preserving glial cells, which are implicated in the function of dopamine neurons and regulate their survival and resistance to injury (Lazzarini *et al*., 2013), Huntington's disease, which involve excitotoxicity, mitochondrial damage, and inflammation, including microglia activation (Paldino *et al*., 2020), Creutzfeldt-Jakob disease, a degenerative brain disorder that leads to dementia and death (Pocchiari & Ladogana, 2015), in occurring diseases due to Hirano body formation in brain cells (Alzheimer's disease, Pick's disease, Guam amyotrophic lateral sclerosis and parkinsonism-dementia complex) (Pathak *et al*., 2021), transthyretin amyloidosis (Müller *et al*., 2020). Another hypothesized use of doxycycline is to prevent severe mental illness through microglia suppression during the intense synaptic pruning period of adolescence (Upmark *et al*., 2021). The neuroprotective role of doxycycline brings new perspectives for its repurposing as a disease-

modifying drug for synucleinopathies, aggregation of insoluble alpha-synuclein protein (González-Lizárraga *et al*., 2017), tauopathies, aggregation of the tau protein in the brain (Bortolanza *et al*., 2018), and many others targets of neurodegenerative diseases (Tribuiani *et al*., 2022). However, neurodegenerative diseases are generally chronic ones, and thus they need long treatments. In this way, this study aimed to evaluate the effects of doxycycline in rats, since demands for repurposing are growing, and consequences after oral long-termadministration on hematological and oxidative stress parameters, hepatic, and renal functions, are scarce in the literature.

MATERIAL AND METHODS

Experimental design: For this study, male rats Wistar (290-300 g) were purchased from the Central Animal Facility of the Instituto de Ciências Biomédicas, University of São Paulo, Brazil). They were housed in microenvironment isolation cages on a wood shavings bed at 22 ± 3 °C and 50 ± 5% humidity, on a 12 h light/dark cycle (lights on at 6 a.m.), with access to food (Presence®, Paulinia, Brazil) and water *ad libitum*. The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2012) and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny *et al*., 2010). The experimental protocols were approved by the institutional Committee for the Care and Use of Experimental Animals from the University of Sorocaba (protocol number 114/2017). Animals were randomly divided into control (n=8) and experimental (n=8) groups. Each group received via gavage 1 mL/day of saline 0.9% (control) or 1 mL/day of 10 mg/kg doxycycline diluted in saline 0.9% (experimental), for 93 days, and weighed weekly. Doxycycline hydrochloride was purchased from Sigma-Aldrich® (St. Louis, MO).

Blood collection and sample preparation: At the end of the study, animals were anaesthetized using a CO_2 chamber (W/Dump Door Med, Harvard Apparatus®) coupled to a CO_2 cylinder. Each animal in the chamber was submitted to CO_2 pressure (0.4 kgf/cm^2 for 45 seconds). Elevated concentrations of carbon dioxide induce hypercapnia (elevated levels of carbon dioxide in the blood) and decrease blood pH (Post, 1979), which reduces oxygen transport to the brain and results in anesthetization (Oberg *et al*., 2015). Immediately, animals were euthanized by exsanguination, and the maximum of blood was collected into a) heparinized polypropylene tubes or b) without anticoagulant, both previously identified according to each group.

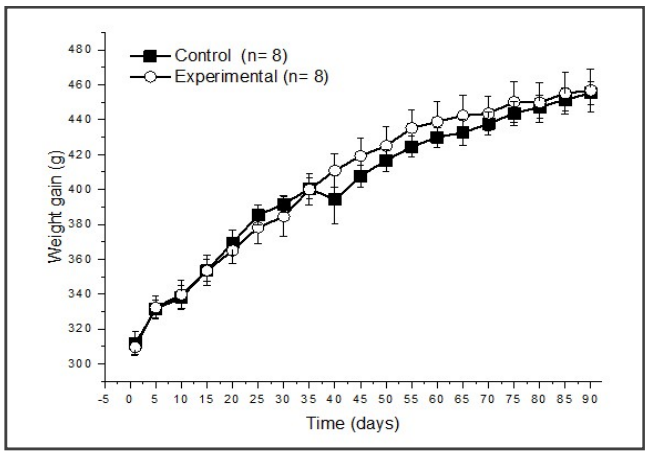

Figure 1. Weight gain (g) of rats exposed to 10 mg/doxycycline/daily for 93 days, via gavage. There was no statistical difference between the control and experimental groups

A part of heparinized blood was used for hematological and oxidative stress biomarkers, whereas another part was centrifuged for 15 minutes at 1,000 g for blood plasma separation, and then stored at −80°C. Blood without anticoagulant was also centrifuged for 15 minutes at 1,000 g for serum obtention, which was stored at −80°C for biochemical analyses. The liver was collected, weighed, fixed in 10% buffered formalin solution for 24 h at room temperature, further sectioned, and immersed in 70% ethanol until the routine of the histological procedures.

Hematological assessment: Total blood was immediately analyzed using Sysmex XS 1000i™ Hematology Analyzer (Roche, Basel, Switzerland), to obtain the count and levels of White Blood Cell (WBC), Lymphocytes, Monocytes, Eosinophils, Red Blood Cell (RBC), Hemoglobin, Hematocrit, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Platelets.

Biochemical analyses: To evaluate liver and kidney functionality, the hepatic injury was evaluated by aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and renal function was assessed by creatinine and urea. These biomarkers were analyzed using commercial kits, purchased from Roche Diagnóstica (São Paulo, Brazil) and the assay followed the recommendation of the manufacturer, using Cobas C111 analyzer (Roche®, São Paulo, Brazil).

Assessment of oxidative stress: Glutathione (GSH) was determined by sulfhydryl (SH) quantification using the Ellman method (Ellman, 1959). Briefly, 100 µL of Triton X-100 was added to 150 µL of blood followed by 100 µL of 10% trichloroacetic acid (TCA). After centrifugation (5,000 rpm, 10 min, 4 ^{o}C) the supernatant was diluted in 1 M phosphate buffer (pH 7.4) and an aliquot was subsequently mixed with 10 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to yield a yellow complex, the absorbance of which was read at 412 nm. The GSH concentration was expressed in mM. Glutathione peroxidase (GSH-Px) activity was assayed according to Paglia & Valentine (1967). Briefly, 10 mL of diluted blood was mixed with 390 mL of 100 mM potassium phosphate buffer, pH 7, containing 1 mM GSH, 0.1 U of GSH-reductase/mL, 0.15 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1.25 mM sodium azide and 100 µL of 0.4 mM hydrogen peroxide (H_2O_2). The GSH-Px activity was monitored as the decay in NADPH absorbance (greater activity = greater decay) for 2 min at 340 nm and was expressed in µmol NADPH min^{-1} g^{-1} of hemoglobin (Hb). Catalase (CAT) activity was assayed according to Aebi (1984). Briefly, 10 µL of blood was diluted in 590 µL of 50 mM potassium phosphate buffer, pH 7. A 20 µL aliquot was mixed with 70 µL of H_2O_2 to start the reaction and the enzymatic decomposition of H_2O_2 by CAT was monitored for 3 min at 240 nm. The reaction was run at 25 °C. Enzyme activity was expressed as k min^{-1} g^{-1} of Hb, where k is a rate constant. Lipid peroxidation was evaluated by quantifying thiobarbituric acid reactive substances (TBARS) according to Ohkawa *et al.* (1979). Briefly, 150 µL of plasma was mixed with 50 µL of 3 M NaOH and incubated at 60 $^{\circ}$C for 30 min. This step was followed by the addition of 250 µL of 6% H_3PO_4, 250 µL of 0.8% thiobarbituric acid (TBA), and 100 µL of 10% sodium dodecyl sulfate (SDS) followed by incubation at 80 °C for 60 min. The reaction between lipid peroxidation products and TBA results in a pink/rose-colored compound detected at 532 nm. The results were expressed as TBARS concentration in µmol mL^{-1} of plasma.

Histological analysis: The routine histological process was carried out using standard procedures for dehydration and embedding in paraffin, by Leica TP1020 Automatic Benchtop Tissue Processor (Leica Biosystem, São Paulo, SP, Brazil). Sections (4 µm thick) were mounted on slides, dewaxed, cleared in xylene, and hydrated in running tap water, after which they were double-stained with hematoxylin-eosin (H&E) and examined with a light microscope (Olympus CBA, Tokyo, Japan).

Statistical analysis: All numerical parameters were expressed as the mean ± SD. Statistical comparisons among the experimental groups were done using t-Student or one-way ANOVA followed by the Tukey-Kramer multiple comparisons test, with $p<0.05$ indicating significance in all cases. The normality of the data was assessed using the Shapiro-Wilk test before the statistical comparisons. All data

analyses were done using Origin© v.9.5 (OriginLab Corporation, Northampton, MA, USA) or Statistica v.8.0 (Dell, Round Rock, TX, USA).

RESULTS AND DISCUSSION

Clinically, all animals from the experimental group survived doxycycline exposure during long-term administration, looked like normal behavior and they had weight gain as the control group, showing they were under good conditions (Figure 1). The gain of weight is an important criterium to determine toxic effects in animals with weight reduction as consequence (Gerenutti *et al*., 1992; Gerenutti *et al*., 2008). In healthy human volunteers the use of doxycycline has been associated with weight gain, a finding that led to the proposal of doxycycline to treat malnourished children in developing countries and seen in patients submitted to long-term doxycycline and hydroxychloroquine treatment, due to modifications of gut microbiota at the phylum level (Angelakis *et al*., 2014).

Hematological parameters: Data for hematological parametersin the experimental group showed to be within the limits of normality compared to the control group (Table 1). Hematological indices are one of the most important parameters used for checking cellular morphological alterations, since compounds gain the systemic circulation to reach their target, and hematological changes are indirectly induced by aggressive external agents. The hematimetric indices provide an overview of the state of red blood cells, and no significant difference between the control and experimental groups was found, regarding the size, color, and size distribution of red blood cells. Therefore, we suggest doxycycline was a safe drug for red blood cells, in long-term administration. Leukogram is used to evaluate the count of defense cells and no differences were observed between the two groups; thus, doxycycline seems not to interfere with the immune system. Comparatively, neutropenia was reported in a young woman after doxycycline treatment (Foti *et al*., 2022). This finding is considered extremely rare, but it is a serious side effect. We can also observe there is no difference between the groups in terms of platelet count, one of the parameters for evaluating hemostasis. Thus, we can hypothesize this antibiotic does not increase the possibility of bleeding. A study in dogs with ehrlichiosis, an infection causing thrombocytopenia, treated with doxycycline 5 mg and 10 mg every 12 hours, showed that there was an increase in progressive platelet count (Rondelli *et al*., 2016).

Biochemical parameters

Renal function: Assessment of renal function is important to monitor the effects of doxycycline, under long-term administration.The tests conventionally used to screen kidney function after pharmacological treatment are creatinine and urea in serum.

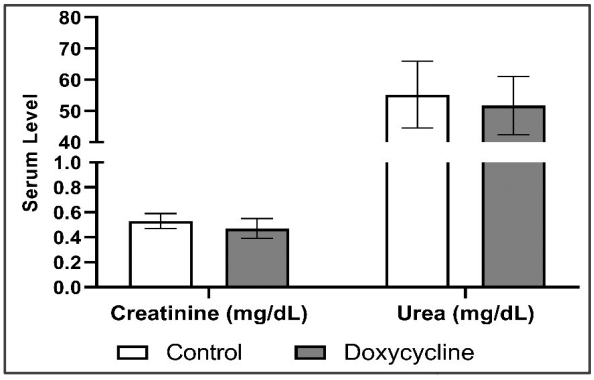

Figure 2. Renal function determined by creatinine and urea biomarkers (mean ± SD). p>0.05

Creatinine is the by-product of creatine phosphate and creatine in muscle, an endogenous marker for the assessment of glomerular function, creatinine is cleared from the blood entirely by the kidney. Decreased clearance by the kidney results in increased blood creatinine. Urea is a nitrogen-containing compound formed in the liver as the product of protein metabolism and the urea cycle, being eliminated via kidneys (about 85%) (Gounden *et al*., 2021). The renal function is presented in Figure 2. Levels of urea and creatinine were similar between the control and the experimental group, and within normality limits (46.7 ± 3.14; 0.41 ± 0.10 mg/dL, respectively to urea and creatinine) found elsewhere (Yoshida *et al*., 2020). The role of the liver comprises nutrient metabolism (including the control and maintenance of the blood glucose level), detoxification and excretion (hydrophobic metabolites and xenobiotics), synthesis of most plasma proteins, digestion through synthesis, biliary secretion, and conservation of bile acids that are essential both for optimum hydrolysis of dietary fat and for intestinal absorption of fatty acids and other lipids, including fat-soluble vitamins (Tennant, 1997). Here, we evaluated the hepatic function through transaminases, liver weight, and histology. The liver weight is a commonly applied endpoint in toxicology studies carried out routinely to assess liver mass (Cattley, 2013). Rodent liver weights vary by species and strain but typically fall in the 4–5 g range (2–3% of body weight) in rats (Rogers & Dintzis, 2018). The mean rat's body weight at the end of the long-term exposition to doxycycline was 455.2 ± 6.7 g (control) and 456.8 ± 12.1 g (experimental). Based on that information and applying the percentage of 2-3% for rats, the outcome would be 9.1-13.6 g (control) and 9.1 – 13.7 g (experimental). Considering the variation in each group (SD of 6.7 and 12.1, control and experimental, respectively) the mean values of livers in this study are within normality limits (Figure 3), which means doxycycline did not damage the livers when evaluated underweight gain of animals.

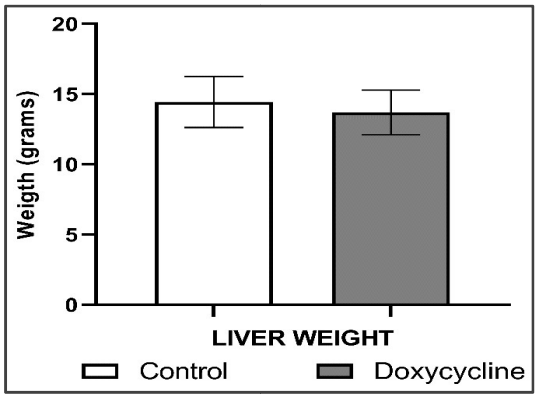

Figure 3. Weight mean ± SD (in grams) of livers for the control and doxycycline groups. p>0.05

Biochemically, the most common tests performed to evaluate injury hepatic are ALT and AST enzymes, which are normally released into serum, but when there is some injury, their activities increase and become useful diagnostically. Figure 4 shows the values of ALT and AST of both groups.

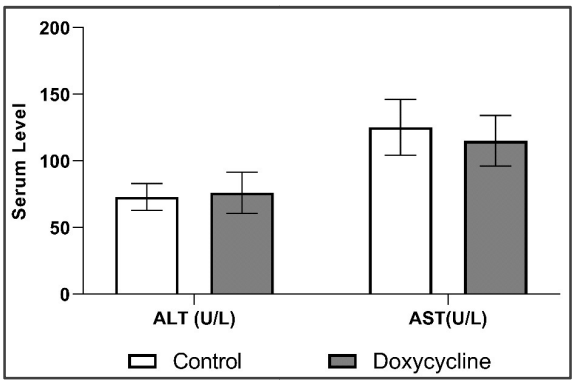

Figure 4. Hepatic function, determined by Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) enzyme, reported as Mean ± SD. p>0.05

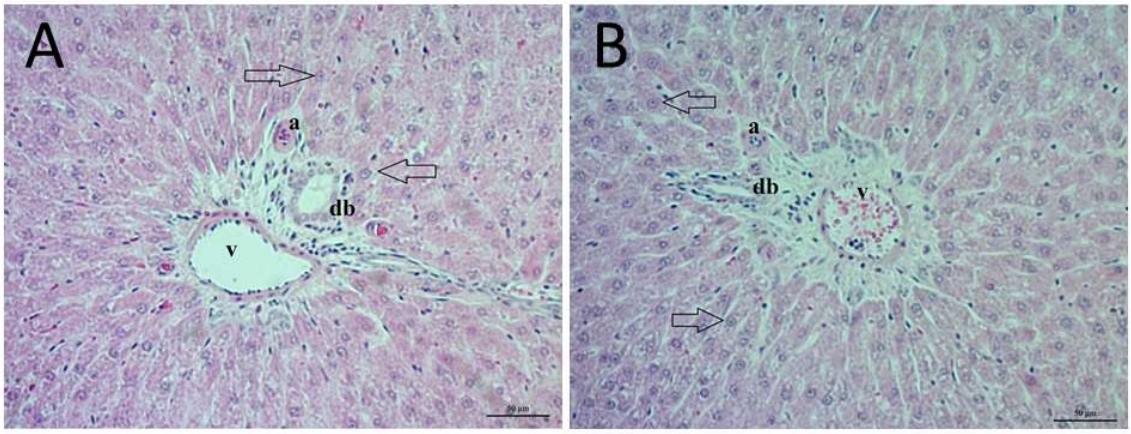

Figure 5. H&E photomicrography in the region of the hepatic portal system, branch of the central portal vein. A) Control group (x200) and B) Doxycycline group (x200). a = arterioles; v = vein; db= bile ductulus; black arrows = hepatocytes

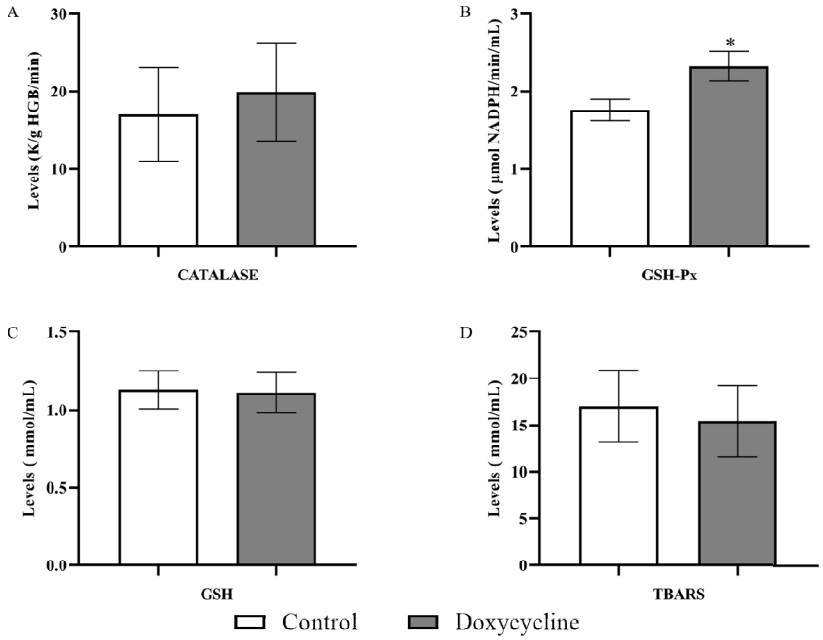

Figure 6. Redox status is assessed through A) CATALASE activity, B) GSH-Px activity, C) GSH levels and D) TBARS levels. *p< 0.05 compared to the control group

Table 1. Hematological parameters (Units of measurement are shown in parentheses)

Parameter	Control	Experimental
White Blood Cell (WBC, $10^3/\mu L$)	7.22 ± 0.92	5.54 ± 1.15
Red Blood Cell (RBC, $10^6/\mu L$)	8.11 ± 0.33	8.60 ± 2.2
Hemoglobin (g/dL)	14.58 ± 0.47	18.52 ± 10.55
Hematocrit (%)	46.97 ± 1.82	49.96 ± 5.70
Mean Corpuscular Volume (MCV) (fL)	57.9 ± 0.83	56.17 ± 14.75
Mean Corpuscular Hemoglobin (MCH) (pg)	17.98 ± 0.32	19.01 ± 0.58
Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dL)	31.06 ± 0.40	39.87 ± 0.59
Platelets ($10^3/\mu L$)	350.5 ± 127.4	311.5 ± 110.3

There was no statistical difference between the groups. AST values obtained from our control group are followed elsewhere (Tarig *et al*., 2017; Hasan *et al*., 2018), whereas ALT did not. Our ALT control group showed values higher than those found elsewhere (22 – 46 U/L) (Tarig *et al*., 2017; Hasan *et al*., 2018). In veterinary, it is accepted that in small animals with hepatocellular injury, the fractional increase in activity of ALT is 4-8 times higher than the corresponding increase in AST, which did not occur in our study. Besides, variations even interspecies are common (Tennant, 1997), and maybe is of major relevance to comparing our results, control and experimental, which show no hepatic damage in the experimental group.

We agree with the statement "it is possible to have quite modest but statistically significant increases in ALT that are not toxicologically relevant" (Cattley, 2013). In favor of regular results, representative images of the histological sections of livers from both groups are shown in Figure 5. A similar appearance in the liver architecture (control and experimental) excludes any damage induced by doxycycline. It appears that the use of doxycycline does not affect kidney function or lead to liver damage. A study with mice subjected to hemorrhage at 30 mmHg for 3 h and resuscitated with blood showed that doxycycline attenuates liver and kidney damage after shock (Kholmukhamedov *et al*., 2014).

Oxidative stress biomarkers: We used validated oxidative stress biomarkers to investigate possible toxicity induced by doxycycline after the long-term exposition, in rats (Figure 6). No oxidative changes were seen in any measured parameter (TBARS, CAT, GSH, and GSH-Px). TBARS are used for evaluating lipid peroxidation (Rael *et al*., 2004), whereas catalase is a heme-containing antioxidant enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen (Khare *et al*., 2019), and its increase in blood could indicate an elevated antioxidant status, which was not observed in this study. On the other hand, low doses of doxycycline were evidenced to neutralize the superoxide anion and other ROS species in human saphenous vein grafts (Saeed *et al*., 2019). GSH-Px is an enzyme that catalyzes the chemical reduction of hydrogen peroxide and lipid hydroperoxides by transforming GSH to GSSG (oxidized glutathione). It is known that the maintenance of redox homeostasis occurs at the expense of enhanced GSH consumption by increasing GSH-Px activity (Serviddio *et al*., 2002). Our findings showed a significant increase in the doxycycline group compared to the control (p=0,000009). Doxycycline interferes with the insertion of selenocysteine into selenoproteins, like GSH-Px. This increase in GSH-Px activity could be related to an increase in selenocysteine insertion, inducing activation of the enzyme. Limitations to the animal model are not significant since rats are scientifically accepted to develop a better understanding of the mechanisms of action of drugs due to a similar genome to humans (Howe *et al*., 2021), mainly when the study pointed out drug repurposing such as doxycycline. From the translational point of view, Weber *et al*. (2019) pointed out similarities between rats and humans and potential pitfalls to consider in trauma conditions, which is not applicable in this study of long-term exposure to oral doxycycline without toxic evidence.

CONCLUSION

Doxycycline showed to be safe at 10 mg/kg/day orally administered via gavage for more than 90 days, in rats, once no changes in hematological, renal, and hepatic biomarkers were observed. Regarding redox condition, in general, no alterations were reported, except for GSH-Px, in which doxycycline induced its activity.

Conflict of Interest Statement: The authors have no conflicts of interest related to this work.

Author Contributions: Conceptualization: NT, YOF; Methodology and Investigation: NT, ASBAJ, MAQJ, ELAC, RNC; Formal Analysis: YOF, DG. Writing - Original Draft: ASBAJ, ELAC; Writing - Review & Editing: EMAV, YOF, DG.

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