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Combined use of systemic quercetin, glutamine and alpha-tocopherol attenuates myocardial fibrosis in diabetic rats

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ABSTRACT

This study aimed to analyze the effects of the quercetin (100 mg/kg), 1% glutamine and 1% α-tocopherol antioxidants in the myocardium of rats with streptozotocin-induced diabetes mellitus. Twenty male rats were

Abbreviations: SOD-1, superoxide dismutase 1; GPx-1, glutathione peroxidase-1; IL-1-β, interleukin-1β; IL-10, interleukin 10; TGF-β, transforming growth factor beta; FGF-2, fibroblast growth factor 2; DM, diabetes mellitus; DC, diabetic cardiomyopathy; ROS, reactive oxygen species; RNS, reactive nitrogen species; MPO, myeloperoxidase; MDA, malonaldehyde; GSH, glutathione; ATP, adenosine triphosphate; STZ, streptozotocin; N, not supplemented normoglycemic animals (control); D, not supplemented diabetic animals; NT, normoglycemic animals treated with quercetin, L-glutamine and α-tocopherol; DT, diabetic animals treated with quercetin, L-glutamine and α-tocopherol; COBEA, Brazilian College of Animal Experimentation; FW, Final body weight; CW, cardiac weight; CW/FW, weight/final body weight; EDTA, ethylenediaminetetraacetic acid; HTAB, cetyl trimethyl ammonium bromide; DTNB, 5.5'-Dithiobis 2-nitrobenzoic acid; NF-kβ, Nuclear factor kappa B.

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subdivided into four groups (n = 5): N (normoglycemic); D (diabetic); NT (normoglycemic treated with antioxidants); and DT (diabetic treated with antioxidants) treated for 60 days. Clinical parameters, oxidative stress markers, inflammatory cytokines, myocardial collagen fibers and immunoexpression of superoxide dismutase 1 (SOD-1), glutathione peroxidase-1 (GPx-1), interleukin-1β (IL-1-β), transforming growth factor-beta (TGF-β), and fibroblast growth factor-2 (FGF-2) were evaluated. Results showed reduced body weight, hyperphagia, polydipsia and hyperglycemic state in groups D and DT. The levels of glutathione (GSH) were higher in NT and DT compared to N ($p < 0.01$) and D ($p < 0.001$) groups, respectively. Greater GSH levels were found in DT when compared to N animals ($p < 0.001$). In DT, there was an increase in IL-10 in relation to N, D and NT ($p < 0.05$), while GPx-1 expression was similar to N and lower compared to D ($p < 0.001$). TGF-β expression in DT was greater than N ($p < 0.001$) group, whereas FGF-2 in DT was higher than in the other groups ($p < 0.001$). A significant reduction in collagen fibers (type I) was found in DT compared to D ($p < 0.05$). The associated administration of quercetin, glutamine and α-tocopherol increased the levels of circulating interleukin-10 (IL-10) and GSH, and reduced the number of type I collagen fibers. Combined use of systemic quercetin, glutamine and alpha-tocopherol attenuates myocardial fibrosis in diabetic rats.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease that affects the production of insulin by pancreatic β-cells, causing hyperglycemia, which can lead to several chronic complications [1]. In the circulatory system, DM can result in increase of vascular endothelial cells proliferation, which may disrupt the blood flow in the capillaries and, consequently, promote cardiac ischemia [2]. Many diabetic patients are also affected by diabetic cardiomyopathy (DC), which is considered to be the leading cause of death among these individuals [3–5].

The development of DC is induced by complex metabolic abnormalities, such as increased levels of oxidative stress and inflammation, that can lead to structural and functional effects [6–11], including myocardial hypertrophy [12,13], necrosis and focal fibrosis [14–16], heart failure and arrhythmias [17,18].

Oxidative stress is a pathophysiological condition characterized by excessive production of reactive oxygen and nitrogen species (ROS and RNS, respectively). In DM, oxidative stress is accompanied by an imbalance of the endogenous redox system [19–21]. Hyperglycemia may be involved in the pathogenesis of heart failure because it causes mitochondria dysfunction in the myocardium [22], which triggers redox imbalance and compromise the mechanical and electrical functions of the diabetic heart [23], and it also stimulates extracellular matrix remodeling, leading to myocardial stiffening [24]. In addition, excessive ROS in the myocardium activates pro-inflammatory pathways even after normalization of blood glucose [25].

Several experimental studies have evaluated the oxidative stress related to DM both at the systemic level, through the analysis of myeloperoxidase (MPO), malonaldehyde (MDA) and glutathione (GSH) levels, and at the tissue level, by means of superoxide dismutase (SOD) and glutathione peroxidase (GPx) expression [26–30]. In addition, oxidative stress resulting from DM also can induce changes in pro-inflammatory [7,16,30] and anti-inflammatory enzymes [31].

Aiming to minimize cell damage caused by hyperglycemia-induced oxidative stress, different antioxidant agents have been used, including quercetin, glutamine and α-tocopherol (vitamin E). Quercetin is a flavonoid present in the human diet with an antioxidant and anti-inflammatory activity, with a protective effect on the heart when administered before ischemia [32–34]. It prevents cell damage, apoptosis [35] and ventricular hypertrophy, decreases myocardial damage and infarct size [36] and shows an antihypertensive effect [37,38].

Glutamine is the most concentrated amino acid in the body, with crucial functions in the maintenance of tissues and organs [39]. Under normal physiological conditions, glutamine is synthesized by the body, but under pathological conditions, the synthesis is diminished, leading to changes in energy metabolism [40]. This amino acid can protect the heart from damage caused by ischemia and reperfusion, through reduction of cardiomyocyte apoptosis [41], improving energy metabolism and stimulating the production of adenosine triphosphate (ATP) and GSH in cardiomyocytes [42].

The α-tocopherol is a natural fat-soluble antioxidant, widely distributed in tissues and plasma [43,44]. It has the ability to reduce blood glucose [45], to prevent the propagation of ROS-induced reactions in biological membranes and to decrease the speed of chain reactions in lipid peroxidation [46]. In the heart, α-tocopherol has been shown to prevent myocardial infarction [47], cardiomyopathy and heart failure resulting from DM [48]. However, the effects of α-tocopherol on blood glucose levels, as well as its cardioprotective effect, are still unclear [44,49].

Supplementation with multiple antioxidants (ascorbic acid, α-tocopherol, β-carotene, N-acetyl cysteine and selenium) for treatment of diabetic cardiomyopathy attenuated cardiac dysfunction in diabetic rats [50]. In the current study, we have evaluated the effect of the combined therapy with quercetin, L-glutamine and α-tocopherol on oxidative stress and on systemic and myocardial inflammation of rats with diabetes induced by streptozotocin (STZ).

2. Materials and methods

2.1. Animals

Twenty male Wistar rats (*Rattus norvegicus*) from the Central Animal Facility of the State University of Maringá were used. The animals were kept under controlled environmental conditions of temperature (22 + 2 °C) and lighting (12 h light/12 h darkness cycle), housed in polypropylene boxes with a drinking trough and feeder, and received water and standard rodent chow (Nuvilab, Colombo, PR, Brazil) ad libitum. The rats were randomly distributed into four groups (n = 5), as follows: N – not supplemented normoglycemic animals (control), D – not supplemented diabetic animals, NT – normoglycemic animals treated with quercetin, L-glutamine and α-tocopherol, DT – diabetic animals treated with quercetin, L-glutamine and α-tocopherol. All procedures are in accordance with ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by CEUA/UFRN (Protocol no. 020/2016). The results of this study are deposited in the repository of the Federal University of Rio Grande do Norte:
https://repositorio.ufrn.br/jspui/bitstream/123456789/25212/1/EfeitoTratamentoConjunto_Purifica%C3%A7%C3%A3oo_2018.pdf.

2.2. Induction of diabetes mellitus

For the induction of DM, the animals in groups D and DT were fasted for 14 h, and after this period, they received an intravenously (penile vein) single dose of diluted STZ (35 mg/kg body weight; Sigma, St Louis, MO, USA) diluted in citrate buffer pH 4.5 (10 nM), under inhalation anesthesia with 3% isoflurane. To simulate the same stress caused for the induction of DM, the animals in groups N and NT were subjected to the same procedures described above, but they received a citrate buffer solution pH 4.5 (10 nM) intravenously (penile vein) without the

diabetogenic agent.

Diabetes was induced by streptozotocin (35 mg/kg). After the 72-hour induction period, the capillary blood glucose of the animals was evaluated using an Accucheck® portable glucometer, all animals with glucose above 250 mg/dL were considered diabetic and able to participate in the study group (D or DT). Blood glucose was measured once a week in the animals of groups D and DT to monitor the maintenance of diabetes and all animals remained diabetic until the end of the experiment. We have added this information in the new version of the manuscript. At the end of the experiment, the animals were weighed and euthanized with an intraperitoneal injection of sodium thiopental (100 mg/kg of body weight; Abbott Laboratories, Chicago, IL, USA), and the blood collected by cardiac puncture was used to determine the final blood glucose (FG). For both analyzes, the glucose oxidase method was used [51].

2.3. Experimental design

The average body weight of the animals was 313.0 g at the beginning of the experiments. The weight of the animals was verified at the end of the 60-day experimental period. The amount of water (mL) and feed (g) ingested was quantified seven times throughout the experiment and the average of the seven measurements was calculated to determine the water intake (WI) and feed intake (FI) per animal.

Animals in groups NT and DT were treated with quercetin (100 mg/kg of body weight; Acros Organics, Fair Lawn, NJ, USA), 1% L-glutamine (1% of body weight; Fagron do Brasil Farmacêutica Ltda, São Paulo) and 1% α-tocopherol (250 mg/day; α-tocopherol; Zhejiang NHU, China). Quercetin and L-glutamine were diluted in water, while α-tocopherol was diluted in mineral oil. For each animal, the treatment with each of the substances was performed by daily gavage, which was divided into three stages with a difference of one hour between them: 1) gavage with quercetin; 2) gavage with L-glutamine; and 3) gavage with α-tocopherol.

The animals in groups N and D went through the same gavage process as in groups NT and DT, but they received only water. Treatments started on the day of diabetes induction, with daily treatment for 60 consecutive days.

2.4. Collection and processing of the materials

At the end of the experimental period, the animals were weighed and euthanized with an intraperitoneal injection of sodium thiopental (100 mg/kg of body weight; Abbott Laboratories, Chicago, IL, USA). Blood was collected by cardiac puncture for the evaluation of blood glucose and cytokines levels, as well as MPO, MDA and GSH activity as described below. Then, a thoracotomy was performed to collect the heart of the animals.

The hearts were washed in phosphate-buffered saline (PBS) pH 7.4, weighed (CW – cardiac weight), and then fixed in a 10% buffered formaldehyde solution for 48 h. After this period, the ventricles were separated from the atria, and the former were destined for routine histological procedures with dehydration in a growing series of alcohols (80%, 90%, absolute I, II and III), clearing with xylene (three baths) and paraffin embedding. Slides were made with 3 μm-thick sections of the sample and used for: 1) picrosirius red histochemical technique for collagen and cardiac fibrosis analysis; 2) immunohistochemical techniques to determine SOD-1, glutathione peroxidase-1 (GPx-1), interleukin-1β (IL-1β), transforming growth factor-beta (TGF-β) and fibroblast growth factor-2 (FGF-2) expression. Final body weight (FW) and cardiac weight (CW) were used to determine the heart weight/final body weight (CW/FW) coefficient, an indicator of cardiac fibrosis [52].

After euthanasia liver and kidney samples were excised quickly and washed with cold isotonic saline. Each segment was weighed and cut longitudinally. Three sections of liver and kidney were analyzed. The specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Sections of 5 μm thickness were obtained for haematoxylin – eosin staining (H&E) and examined by light microscopy (40x, Olympus BX50, Morphology Department / UFRN). Histological sections were stained using hematoxylin and eosin (Easypath) and examined under light microscopy (Nikon Eclipse 2000 equipped with Nikon DS-Fi2; Nikon Corporation, Tokyo, Japan). Liver pathology (Portal area and centrilobular vein**)** was scored as follows: steatosis (the percentage of liver cells containing fat): $< 25\% = 1$, $< 50\% = 2$, $< 75\% = 3$, $> 75\% = 4$; inflammation and necrosis: 1 absence or 2 presence [53]. For the kidneys, the following characteristics and scores were attributed: Hyperemia (1: absence 2: moderate 3: intense), hemorrhage (1: absence 2: moderate 3: intense), lymphoid infiltrate (1:absence 2: moderate 3: intense), fibrosis (1:absence 2: moderate 3: intense), glomerular change: (1: absence or 2: presence), foci of tubular necrosis (1:absence or 2: presence). Pathology was scored in a blinded manner by one of the authors and by an outside expert in rodent liver/kidney pathology.

2.5. Dosage of myeloperoxidase (MPO) and oxidative stress markers

After blood collection by cardiac puncture, the serum of 5 animals/group was separated and stored in the freezer at − 80 °C, until its use in the following experiments.

For determination of MPO, the samples were weighed to calculate the amount of HTAB (cetyl trimethyl ammonium bromide buffer) for each sample. The volume of HTAB 20 × the weight of the sample was used, and these were then kept with HTAB in the proportion of 50 mg-1 mL on a cold surface. The samples were homogenized with polytron, and the homogenate was submitted to the action of the sonicator for 5 min. Then, the homogenate went through two freezing cycles (two hours each), then it was centrifuged at 5000 rpm at 4 °C for 20 min and frozen for 24 h. In the next day, the supernatant was collected. The reading solution was prepared (27 mL of distilled water, 3 mL of potassium phosphate buffer and 5 mg of O-dianisidine) and kept protected from light. Then, the preparation of 15 μL of 1% hydrogen peroxide was carried out. A volume of 200 μL of the reading solution was added to 7 μL of the sample, and these were placed in two rows of the reading plate. In the third row, only 200 μL of HTAB was placed for later color comparison between the samples. Elisa Polaris® microplate reader at 450 nm was used to perform two readings with an interval of one minute between them [53].

In the MDA assay, serum was homogenized in 200 μL of Trizma and then the eppendorfs with the samples were completed with Trizma to avoid extravasation. The samples were centrifuged at 11,000 rpm for 10 min at 4 °C. Next, 300 μL of the supernatant was transferred to eppendofs to which the chromogenic reagent (1-methyl-2-phenylindole 10.3 mM in acetonitrile) was added at the proportion of 750 μL of the reagent for each 100 mg or 100 μL of sample. Afterwards, the samples were incubated in 225 μL of 37% HCL, in a water bath at 45 °C for 40 min. The material was again centrifuged (11,000 rpm, for 5 min at 4 °C) and then 300 μL supernatant was transferred to each well of the 96-well plate. The last row of the plate was filled with Trizma for later color comparison. A microplate reader (Elisa Polaris®) was used to read the absorbances each sample, measured at 586 nm or immediately below [53].

For the glutathione (GSH) assay, 1 mL of 0.02 M ethylenediaminetetraacetic acid (EDTA) (1 mL of EDTA for every 100 μL of sample) was added to 100 μL of each serum sample. Then, they were homogenized with polytron. To 400 μL of the sample homogenate, 320 μL of distilled H2O and 80 μL of 50% trichloroacetic acid (TCA) were added. The material was centrifuged at 3000 rpm for 15 min at 4 °C, and 100 μL of the supernatant was transferred to a 96-well plate containing 200 μL of 0.4 M Tris (4.84 g of TRIS + 10 mL of EDTA 0, 2 M + qsp 100 mL distilled water) and 25 μL 0.01 M DTNB (5.5'-Dithiobis 2-nitrobenzoic acid (Sigma-Aldrich)) (chromogenic reagent). The last row of the plate was filled with Tris buffer for color comparison. Reading was performed by a microplate reader (Elisa Polaris®) at 412 nm and the results were described as units of GSH per milligram of tissue (U/mg)

[53].

2.6. Inflammatory cytokine levels

Systemic inflammation was examined by blood levels of the IL-1β, TNF-α and IL-10 cytokines from the stored (− 80 °C) serum samples of all animal groups. The samples were homogenized and processed to determine levels of IL-1β (detection range: 62.5–4000 pg/mL; sensitivity or lower limit of detection [LLD]: 12.5 ng/mL of IL-1β mouse recombinant), TNF-α (detection range: 62.5–4000 pg/mL; sensitivity or LLD: 50 ng/mL recombinant mouse TNF-α) and IL-10 (detection range: 62.5–4000 pg/mL: sensitivity or LLD: 12.5 ng/mL of recombinant mouse IL-10) by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) [53].

Subsequently, 96-well plates were incubated for 12 h at 4 °C with anti-IL-1, anti-TNF-α and anti-IL-10 mouse antibody (4 μg/mL, DuoSet ELISA Development kit, R&D Systems Catalog, DY501 and DY510, respectively). After sensitization of the plates, the samples were added in duplicate, and the standard curve was added in various dilutions, followed by incubation for 2 h at 4ºC. The plates were then washed three times with PBS/Tween-20 buffer solution (0.05% SIGMA) and incubated with and incubated with diluted biotinylated anti-IL-1, anti-IL-10 and anti-TNF-α monoclonal antibody (1:1000 μL with 1% bovine serum albumin/Tween) at room temperature for 1 h. Next, the plates were washed, and 50 μL of the diluted HRP-streptavidin complex (1:5000 μL) was added. After 15 min, the o-phenylenediamine color reagent (OPD, 50 μL) was added and the plates were incubated in the absence of light at 37 °C for 15 min. The enzymatic reaction was stopped by adding H_2SO_4 (1 M), and the optical density was measured at 490 nm in a spectrophotometer for IL-1β, TNF-α and IL-10. Cytokine concentrations contained in the samples were calculated from a standard curve with 11 points, obtained by serial dilution, with initial concentrations of 4000 pg/mL for and IL-1β, TNF-α and IL-10. Results were expressed as pg/mL of supernatant/mg of serum [53].

2.7. Analysis of cardiac fibrosis

Analysis of cardiac fibrosis was performed by evaluating collagen deposition and volume density (V_V), as described below, in 3 μm-thick samples stained by the histochemical technique of Picrosirius red (collagenous fibers type I and III) without polarization [52,54]. The samples were collected with an interval of 15 μm between them, with a total of 15 sections per animal. In each section, nine images were captured using the 40 × objective, three from each region (right ventricle, interventricular septum and left ventricle), totaling 45 images per animal.

2.8. Immunohistochemistry

Three-μm thick tissue sections were prepared on silanized glass slides and destined for immunohistochemical techniques to determine the expression of SOD-1, GPx-1, IL-1β, TGF-β, and FGF-2 (n = 3) in the myocardium of the rats. The slides were subjected to two baths in xylene for 10 min each for deparaffinization, then rehydrated in decreasing series with alcohol (absolute I, II and III, 95% alcohol and 80% alcohol). Next, the specimens were pretreated with PBS buffer pH 7.4 for antigen retrieval, and with 6 μL of proteinase K for 15 min, and then washed in PBS. Endogenous peroxidase blockade was performed with 3% hydrogen peroxide (Synth, Diadema-SP, Brazil) for 10 min. The slides were washed twice in distilled water, and the endogenous peroxidase blocking step was repeated, and washed again in distilled water. To prevent non-specific staining, the slides were treated with block protein from the DAKO X0909 kit (Dako North America, Inc., Carpinteria-CA, USA) for 10 min and then washed in distilled water twice, and twice in PBS. The samples were incubated with the primary antibodies SOD-1 (SC 11404, Santa Cruz Biotechnology, Interprise, Brazil; 1:800), GPx-1 (SC 22145, Santa Cruz Biotechnology, Interprise, Brazil; 1:200), IL-1β (SC 7884, Santa Cruz Biotechnology, Interprise, Brazil; 1:800), TGF-β (SC 146, Santa Cruz Biotechnology, Interprise, Brazil; 1:400) and FGF-2 (SC 1360, Santa Cruz Biotechnology, Interprise, Brazil; 1: 800), and kept in a humid chamber at 4 °C for a period of 18 h. The material was washed with PBS buffer and incubated with secondary antibody conjugated with streptabComplex/HRP Duet, Mouse/Rabbit (K0492 DAKO, Dako North America, Carpinteria, CA, USA) at room temperature. Two washes were carried out with PBS buffer and the DAKO Liquid DAB + substrate Chromogen System kit (K3468 DAKO, Dako North America, Carpinteria, CA, USA) was applied for 10 min. The material was washed in distilled water and stained with Harris Hematoxylin (MHS16-Sigma, Sigma, St. Louis, MO, USA) for 15 min and then washed in running water for 10 min, dehydrated in 95% alcohol and in absolute alcohol (three times) for 10 s each, and in xylene (three times) for 10 s. Then, the slides were mounted with a cover slip and Permount mounting medium [53].

2.9. Image capture and volume density (V_V) analysis

The intensity of SOD-1, GPx-1, IL-1β, TGF-β, and FGF-2 and collagen were evaluated by stereological analysis of volume density (*Vv*) as detailed below. Forty-five images of the myocardium (right ventricle, interventricular septum and left ventricle) per animal were captured on a 40x objective, with a Nikon Digital DXM 1200 camera (Melville, NY, USA) coupled to an Olympus BX50 microscope (Center Valley, PA, USA), and Motic Image Plus 2 image capture software (Motic Group Co., Shanghai, China), at LAICI Laboratory, UFRN Department of Morphology, to assess volume density (*Vv*) [55] of collagen fibers and the expression of SOD-1, GPX-1, IL-1β, TGF-β and FGF-2. The analysis was made with assistance of a point test system (composed of 475 points) in the ImagePro Plus 7 software (Media Cybernetics Inc., Rockville, MD, USA). For that, the following formula was used: $Vv = Pint/-Pref$, where Pint is the total number of points that touched the cells of interest and Pref is the total number of points in the test system.

2.10. Statistical analysis

All results were expressed as mean ± standard error of the mean. Analysis of variance (ANOVA) followed by Tukey's test (GraphPad Prism 6 Software, La Jolla, CA, USA) was used. The significance level adopted for all tests was 5%.

3. Results

3.1. Physiological parameters of the animals

Results regarding body and heart weight, water and feed consumption, and plasma glucose of the animals are shown in Table 1. Biometric data revealed that the animals of the D and DT groups presented a lower body weight than those from the N group ($p < 0.001$). No differences in the body weight were observed between DT and D groups, as well as between NT and N ($p > 0.05$). Heart weight was lower in D and DT animals compared to N group ($p < 0.001$). The DT and D groups showed similar heart weight ($p > 0.05$), while the NT animals presented a higher heart weight compared to the N animals ($p < 0.05$). The heart weight/body weight ratio was higher in D ($p < 0.01$) and NT ($p < 0.05$) groups when compared to N animals. No statistically significant difference was found between DT group and the animals in the D and N groups.

Mean water intake was higher in D, NT and DT groups compared to the animals in the N group ($p < 0.001$). An increase in water intake was observed in DT compared to D group ($p < 0.001$). Feed consumption was higher in D and DT groups than in N ($p < 0.001$). Also, DT animals presented an increase in the feed intake compared to those in D group ($p < 0.05$), whereas no difference was identified between NT and N groups ($p > 0.05$).

Initial and final glucose levels were higher in D and DT groups

Table 1
Biometric parameters, food consumption and glycemic levels of the animals in the groups N, D, NT and DT.

Groups	FW (g)	HW (g)	HW/FW (g)	WI (mL)	FI (g)	IG	FG
N	428.6 ± 4.47[a]	1.57 ± 0.037[a]	0.00366 ± 0.00008[a]	8.23 ± 0.29[a]	5.29 ± 0.11[a]	139.2 ± 3.61[a]	140,0 ± 3,08[a]
D	286.4 ± 12.58[b]	1.22 ± 0.036[b]	0.00422 ± 0.00010[b]	35.21 ± 0.66[b]	10.89 ± 0.30[b]	428.2 ± 2.60[b]	422,2 ± 1,93[b]
NT	413.6 ± 6.15[a]	1.73 ± 0.037[c]	0.00418 ± 0.00014[b]	12.43 ± 0.51[c]	6.23 ± 0.13[a]	144.8 ± 1.39[a]	147,4 ± 3,70[a]
DT	313.0 ± 4.18[b]	1.26 ± 0.004[b]	0.00404 ± 0.00005[ab]	47.64 ± 0.65[d]	12.07 ± 0.38[c]	468.4 ± 3.47[c]	465,2 ± 3,87[c]

Mean ± standard deviation for the final body weight – FW (g), heart weight – HW (g), heart weight/body weight ratio – HW/FW (g), water intake/animal – WI (mL), feed intake/animal – FI (g), initial blood glucose – IG and final blood glucose – FG (mg/dL) of the animals in the groups N, D, NT and DT. Different letters in the same column indicate statistically significant difference by Tukey's test ($p < 0.05$), n = 5/group.
Source: https://repositorio.ufrn.br/jspui/bitstream/123456789/25212/1/EfeitoTratamentoConjunto_Purifica%C3%A7%C3%A3oo_2018.pdf.

compared to N and NT ($p < 0.001$). In DT, there was an increase in IG and FG compared to D ($p < 0.001$), while NT showed similar values to N group ($p > 0.05$).

3.2. Oxidative stress and systemic inflammation

Comparison of MPO activity between D and N groups showed no statistically significant difference ($p > 0.05$). The animals from DT group

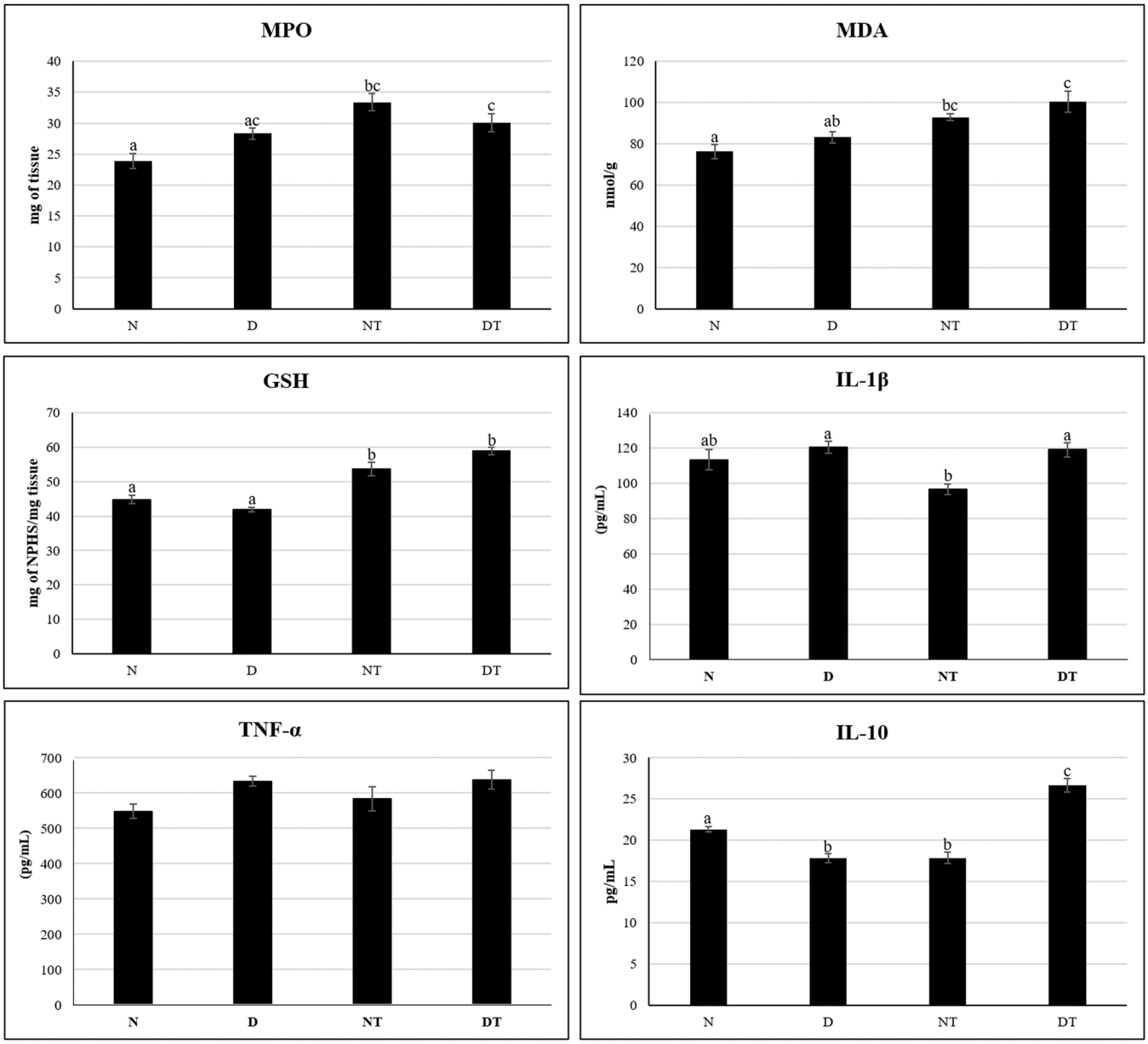

Fig. 1. Oxidative stress and systemic inflammation. Effect of combined antioxidant treatment on blood levels of MPO (U/mg tissue), MDA (nmol/g), GSH (NPHS mg/mg tissue), IL-1β (pg/mL), TNF-α (pg/mL) and IL-10 (pg/mL) in the groups N, D, NT and DT. Results are expressed as mean ± standard error (n = 5). Different letters indicate a statistically significant difference by Tukey's test ($p < 0.05$).
Source: https://repositorio.ufrn.br/jspui/bitstream/123456789/25212/1/EfeitoTratamentoConjunto_Purifica%C3%A7%C3%A3oo_2018.pdf.

presented similar MPO levels compared to those in D group ($p > 0.05$), but a higher activity than N animals ($p < 0.05$). A higher MPO activity was found in NT group compared to N ($p < 0.001$) (Fig. 1A).

Comparing D and N groups, no differences were observed in MDA values ($p > 0.05$). DT group revealed higher MDA compared to D ($p < 0.05$), and to N groups ($p < 0.001$). The level of MDA in the NT group was higher than N group ($p < 0.05$) (Fig. 1B).

The levels of GSH were higher in NT and DT compared to N ($p < 0.01$) and D ($p < 0.001$) groups, respectively. Greater GSH levels were found in DT when compared to N animals ($p < 0.001$) (Fig. 1C).

Regarding the cytokines associated with inflammation, no statistically significant differences were detected in IL-1β levels between groups N, D and DT groups (Fig. 1D). Likewise, no differences were observed in the levels of TNF-α between the experimental groups ($p > 0.05$) (Fig. 1E). IL-10 concentrations were lower in D and NT compared to N group ($p < 0.01$). In DT, there was an increase in IL-10 levels in comparison to N and D groups ($p < 0.001$) (Fig. 1F).

3.3. Oxidative stress and inflammation in the myocardium

The expression of SOD-1 was higher in D than in N group ($p < 0.001$). SOD-1 expression in DT was similar to D ($p > 0.05$), but greater than N group ($p < 0.001$). No differences were observed in the expression of SOD-1 when comparing NT and N groups ($p > 0.05$) (Fig. 2A–E). As for the results of GPx-1 expression, it was higher in D compared to N group ($p < 0.001$). In DT, there was a lower GPx-1 expression than D group ($p < 0.001$), while NT presented a higher expression than N ($p < 0.001$) (Fig. 2F–J).

Expression of IL-1β in the myocardium was higher in D compared to N animals ($p < 0.001$). In DT group, no significant difference in IL-1β expression was found when compared to D, but there was a higher expression compared to N group ($p < 0.001$). There were no differences in IL-1β immunoexpression between NT and N groups ($p > 0.05$) (Fig. 2K–O).

A higher expression of TGF-β was observed in D compared to N group ($p < 0.001$). DT animals exhibited an increased TGF-β expression compared to those in the N group ($p < 0.001$), but no differences were found when DT was compared to D ($p > 0.05$). NT and N showed a similar TGF-β expression ($p > 0.05$) (Fig. 3A–E). There was no significant difference in FGF-2 expression between N, D and NT groups ($p > 0.05$). In DT, FGF-2 expression was higher than in the other experimental groups ($p < 0.001$) (Fig. 3F–J).

3.4. Quantification of collagen fibers in the myocardium

The amount of type I collagen fibers in the myocardium was greater in D than in N ($p < 0.001$). DT group exhibited a decrease in collagen type I when compared to D ($p < 0.05$), and an increase compared to N ($p < 0.001$). The amount of collagen was significantly greater in NT than N group ($p < 0.001$) (Fig. 3L–K).

3.5. Histopathological analysis (liver and kidney)

3.5.1. Liver

Histopathological data on the liver of animals showed that there were no statistically significant difference among groups, with a score of 1 (Fig. 4). The liver pathology (Portal area and centrilobular vein) showed steatosis with the percentage of liver cells containing fat:

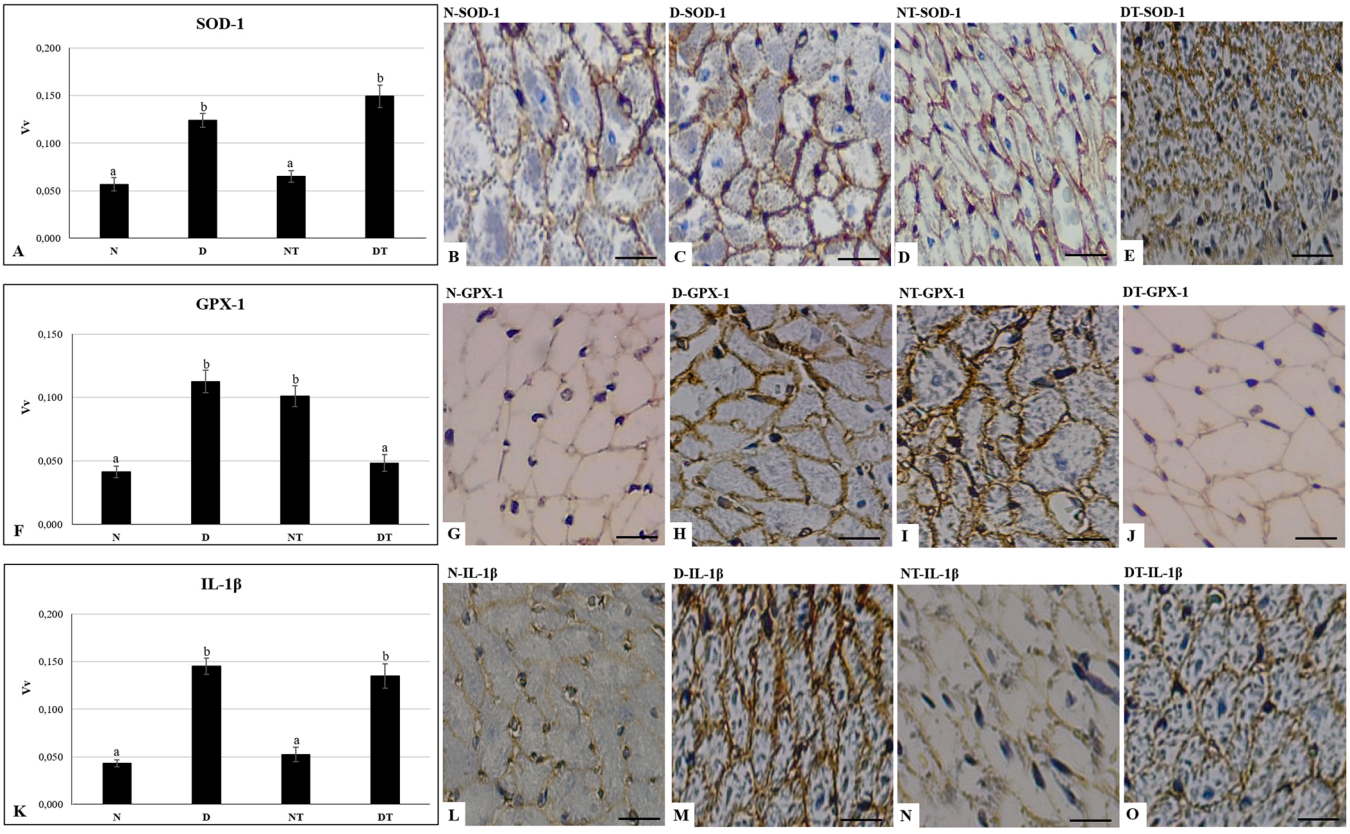

Fig. 2. Oxidative stress and myocardial inflammation. Effect of the combined treatment of antioxidants on the expression of SOD-1, GPx-1 and IL-1β in the myocardium (A, F and K). Representative photomicrograph of SOD-1 (B–E), GPx-1 (G–J) and IL-1β (L-O) expression in the myocardium of the animals from N, D, NT and DT groups, respectively. Data are expressed as mean ± standard error (n = 5). Different letters indicate a statistically significant difference by Tukey's test ($p < 0.05$). Scale bar: 100 μm.
Source: https://repositorio.ufrn.br/jspui/bitstream/123456789/25212/1/EfeitoTratamentoConjunto_Purifica%C3%A7%C3%A3oo_2018.pdf.

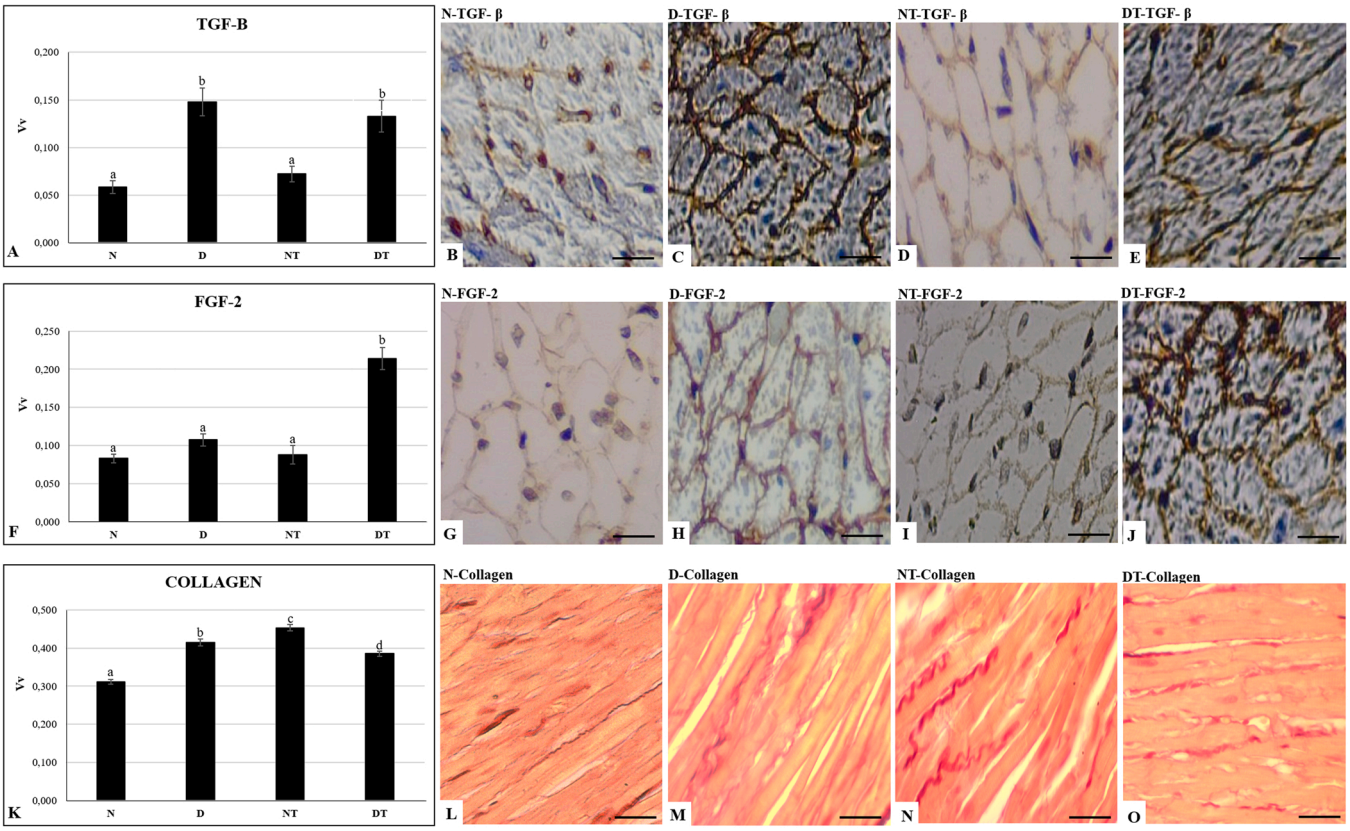

Fig. 3. Cell proliferation and myocardial fibrosis. Effect of the combined antioxidants treatment on the expression of TGF-β, FGF-2 and quantification of collagen fibers in the myocardium of rats (A, F and K). Representative photomicrograph of the expression of TGF-β (B-E), FGF-2 (G-J) and collagen fibers (picrosirius red staining) (L-O) in the myocardium of the animals from groups N, D, NT and DT, respectively. Data are expressed as mean ± standard error (n = 5). Different letters indicate a statistically significant difference by Tukey's test ($p < 0.05$). Scale bar: 100 μm.
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< 25%; Absence of inflammation and necrosis, p > 0.05, Fig. 4.

3.5.2. *Kidney*

The histopathological analysis in N group showed normal glomerulus. and normal convoluted tubules. NT showed glomerulus with vascular congestion. D showed podocyte death, tubular atrophy and inflammatory infiltrate. DT showed inflammatory infiltrate and tubular atrophy, Fig. 5. The statistical analysis showed the following results for the features: Hyperemia: N comparate with NT, D and DT, p < 0.001; Lymphoid infiltrate: N comparate with D, p < 0.01; DT comparate with D p < 0.01; DT comparate with D, p < 0.05; Glomerular change: N comparate with NT, D and DT, p < 0.001; Foci of tubular necrois: N comparate with NT, D and DT, p < 0.001, Fig. 5.

4. Discussion

In this study, a reduction in body weight was observed in the animals of D and DT groups, as well as an increase in the consumption of water (polydipsia) and feed (polyphagy), and the animals of D group even showed a loss of body mass compared to the beginning of the experiment. In DM, the reduction in body weight of may occur due to the cellular inability to metabolize glucose, generating a compensation process characterized by oxidation of adipose and muscular tissue to supply the organism needs, which has also been shown in other studies with diabetic animals [6,15,56,57], including with the administration of vitamin E and L-glutamine [45,58,59]. However, isolated supplementation with quercetin in diabetic animals is associated to an increase in body weight, probably due to the restoration of homeostasis of the glycolytic pathway, causing a decrease in water and food intake [60].

The reduction in cardiac weight of D and DT rats observed in this study was also evidenced in other studies using quercetin [16] and, when applying the CW/FW coefficient, the results indicated an increase in diabetic animals without treatment, which was also previously evidenced in diabetic rats [8,48]. Collectively, these findings suggest a cardiac hypertrophy [52,53], which can lead to arrhythmia and heart failure [44].

Regarding glycemic levels, the present results demonstrated DM induction after one week of STZ administration (GI) and maintenance of hyperglycemia in D and DT animals, in accordance with distinct diabetes research with animal models [6,15,30,56]. Different substances have been tested to control blood glucose, such as L-glutamine [58,59], vitamin E [45,47] and quercetin [6,16,32,34]. However, in this study, the combined treatment with these three antioxidants was not beneficial in the DT group, as it promoted an increase in glycemic levels beyond those observed in the animals of the D group.

There is evidence that supplementation with quercetin (10 mg/kg of body weight/day) decreases plasma glucose levels in diabetic animals [62], but in studies with obese diabetic rats, no change was observed [34]. On the other hand, high doses of L-glutamine (500–100 mg/kg of body weight) reduced blood glucose in diabetic rats [58]. Supplementation with vitamin E also diminished blood glucose in diabetic rats [45,47]; however, the results were dose-dependent, which can relate to our findings that showed no positive effect on this parameter after combined treatment with different antioxidants [26].

Hyperglycemia increases the risk of cardiomyopathies [15,56], probably by increasing cardiac oxidative stress and inflammation, even though individuals with DC are often asymptomatic until reaching advanced stages of the disease [3].

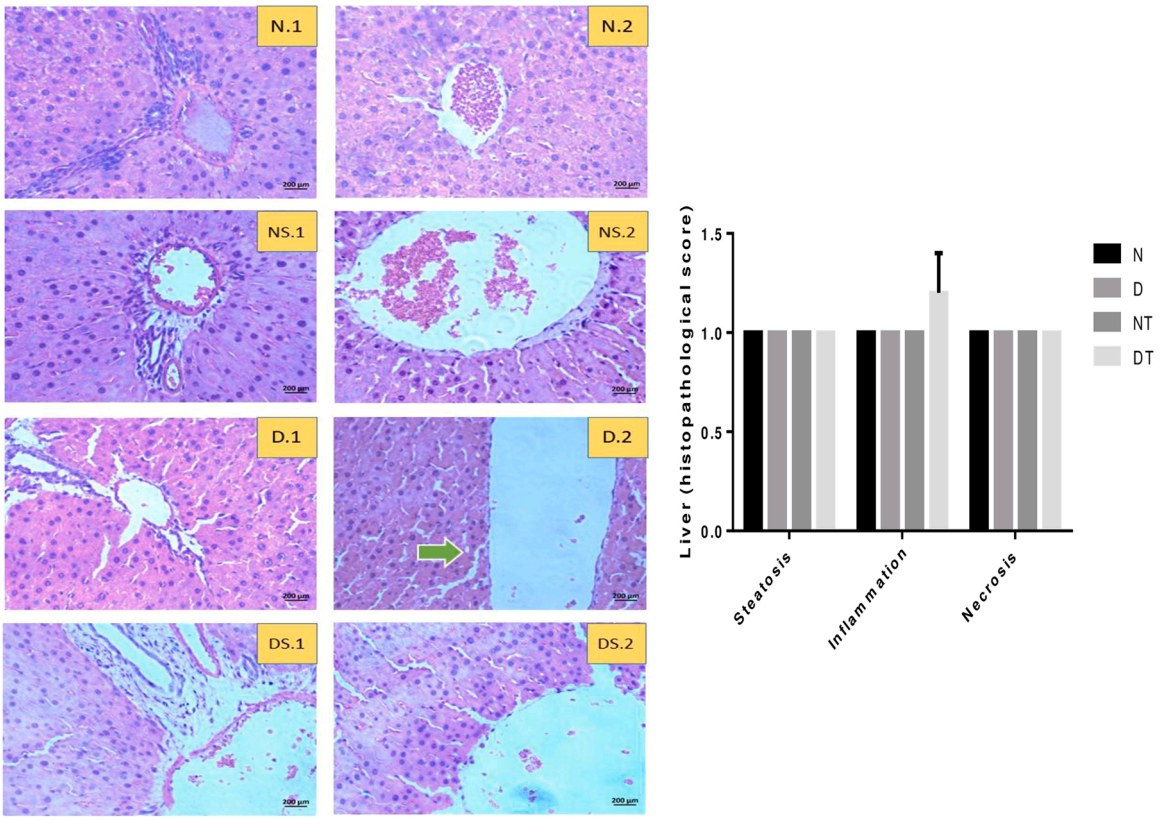

Fig. 4. Score and histopathological aspects of the liver. N1, NS1, D1, DS1: portal area; N2,NS2, D2, DS2: **centrilobular vein** (N1, N2) N – not supplemented normoglycemic animals (control); (NS1; NS2) NT – normoglycemic animals treated with quercetin: (D1; D2) D – not supplemented diabetic animals; (DS1;DS2) DT – diabetic animals treated with quercetin, L-glutamine and α-tocopherol.

The findings of our study regarding the systemic and tissue oxidative stress revealed an increase in the blood levels of MPO and MDA in the groups treated with antioxidants, as well as the levels of GSH. In the myocardium, there was an increase in SOD-1 expression in diabetic animals with and without treatment and, in relation to GPX-1, an increase was identified in the D group, and a reduction was found in DT animals. These data indicate that, at the systemic level, there is an increase in oxidative stress induced by DM in association with the antioxidant treatment, as well as an attempt of the body to reverse this oxidative stress state, through greater activation of the glutathione redox pathway, while at tissue level, the treatment promoted an increase in the antioxidant pathway by greater expression of SOD-1 and reduction of GPX-1, a finding similar to those observed in studies with diabetic animals treated with quercetin [16].

Increased fatty acid oxidation is also directly related to cardiac malfunction. In this regard, the analysis of MPO and MDA is helpful in the diagnosis of heart disease [27–29]. Different studies have shown an increase in MDA and a decrease in GSH in diabetic animals [26,30,63]. However, treatment with antioxidant agents such as Ginkgo biloba extract associated to rutin and quercetin, and with L-glutamine has resulted in a reduction in MDA and an increase in GSH levels [58].

Increased cardiac oxidative stress in STZ-DM rats may lead to the characteristic myocardial changes of DC [63]. Reduction in SOD levels has been observed in animals with DM [26,30], however, treatment with L-glutamine [58] or quercetin [34] stimulated increased SOD expression in rat hearts diabetics, suggesting that these antioxidants can decrease the levels of reactive oxygen species, reversing the inhibition of SOD induced by diabetes.

The relationship between oxidative stress and inflammation has been widely described. In this study, systemic inflammation was evaluated by the dosage of pro-inflammatory cytokines IL-1β and TNF-α, with differences between D, DT and N groups. However, the dosage of IL-10, an anti-inflammatory cytokine [26,52], was lower in D and higher in DT compared to N, indicating less activation of the anti-inflammatory pathway in diabetic animals to the detriment of a higher anti-inflammatory activity in DT animals. An increase in pro-inflammatory cytokines such as IL-1β and TNF-α is usually identified in DM [5,7,16,30,64], while a reduction in the anti-inflammatory cytokine IL-10 is frequently observed in DM patients [5,26], which corroborates our findings. In addition, the combined treatment with antioxidants, resulted in an increase in the anti-inflammatory pathway, an important finding of our study, since IL-10 inhibits the action of other pro-inflammatory cytokines [31].

At the tissue level, the increase in the cytokine IL-1β expression in animals from groups D and DT in the current study, indicates a local inflammatory process, which was not reversed by the combined treatment with antioxidants, although we have observed an effect on the activation of antioxidant pathways. Increased pro-inflammatory cytokines IL-1β and TNF-α has also been observed in the myocardium of diabetic rats in previous studies [6–8,16,30]. Studies with diabetic rats have shown increased oxidative stress in the myocardium due to lower SOD expression and higher MDA level, and tissue inflammation characterized by increased expression of pro-inflammatory cytokines (IL-6 and TNF-α), with gingerol supplementation showing an anti-inflammatory activity by reducing cytokines and MDA levels [11, 30].

In infarcted myocardium, inflammation leads to immune system cells infiltration to digest the damaged tissue and to stimulate tissue repair by fibroblast proliferation and healing, but tissue changes will depend on the degree and time of inflammation, as well as other factors such as stress oxidative [17].

Oxidative stress and inflammation play a role on cardiac hypertrophy, which occurs as a compensatory process and, in the long term, may result in fibrosis and heart failure, which are related to the expression of

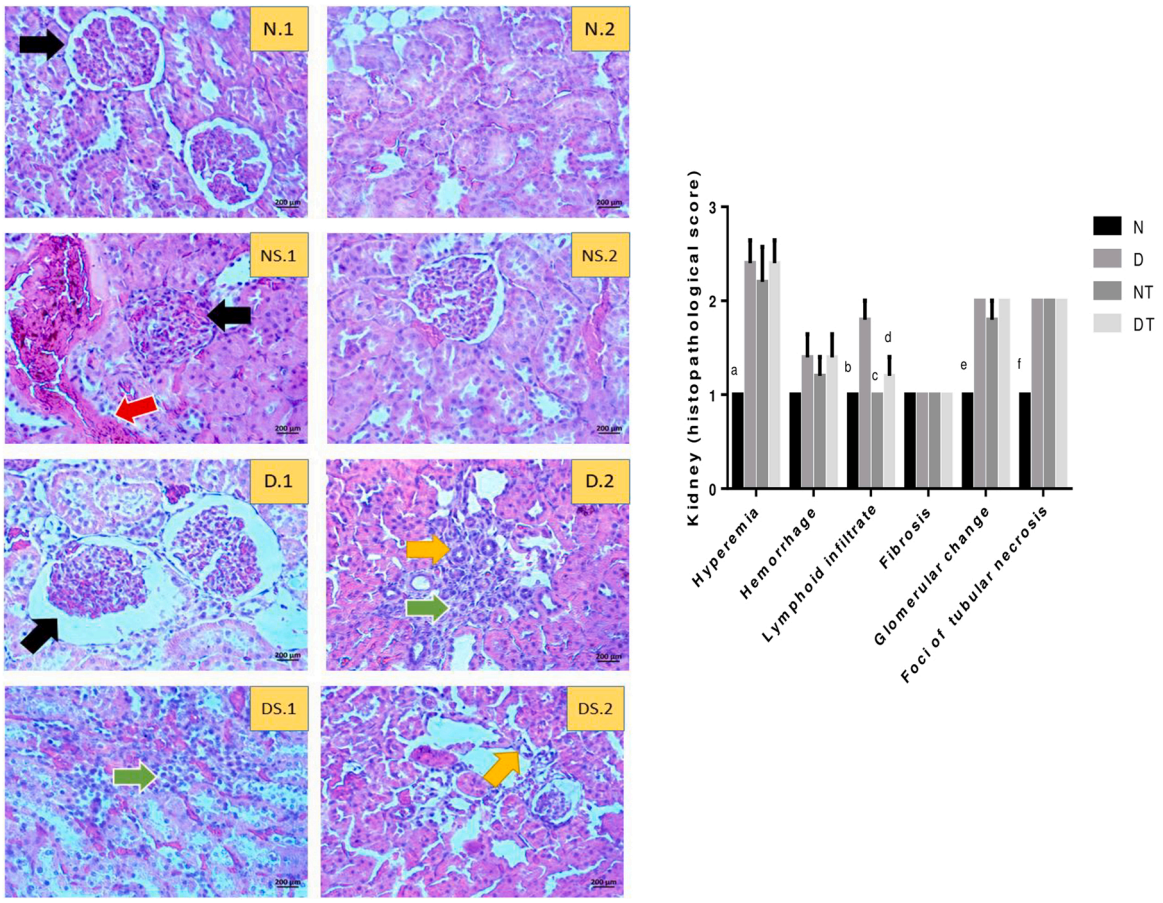

Fig. 5. Score and histopathological aspects of the kidney. N1, NS1, D1, DS1: portal area; N2,NS2, D2, DS2: **centrilobular vein** (N1, N2) N – not supplemented normoglycemic animals (control); (NS1; NS2) NT – normoglycemic animals treated with quercetin: (D1; D2) D – not supplemented diabetic animals; (DS1;DS2) DT – diabetic animals treated with quercetin, L-glutamine and α-tocopherol. N.1 – black arrow indicates normal glomerulus. N.2 – normal convoluted tubules. NS.1 – black arrow indicates glomerulus with vascular congestion; red arrow indicates congested vessel. D.1 – black arrow indicates podocyte death. D.2 – yellow arrow indicates tubular atrophy; green arrow indicates inflammatory infiltrate. DS.1 - yellow arrow indicates inflammatory infiltrate. DS.2 – yellow arrow indicates tubular atrophy. Hyperemia (a): N comparate with NT, D and DT, $p < 0.001$; Lymphoid infiltrate (b) N comparate with D, $p < 0.01$; (c) DT comparate with D $p < 0.01$; (d) DT comparate with D, $p < 0.05$; (e) Glomerular change: N comparate with NT, D and DT, $p < 0.001$; (f) Foci of tubular necrois: N comparate with NT, D and DT, $p < 0.001$.

TGF-β1, a key mediator in collagen synthesis [15].

Cardiac remodeling by increase in collagen deposition in the heart of rats with STZ-induced diabetes promoted morphofunctional changes, such as cardiac stiffening and a decrease in the systolic and diastolic strain rate [24,53,65].

In the myocardium, two main types of collagen fibers are found, type I and type III, and TGF-β is a pro-fibrotic cytokine that stimulates the synthesis of type I collagen [52,54]. In this study, there was an increase in the expression of TGF-β1 and in the amount of collagen fibers in D animals, while in DT group, an increase in TGF-β1 and FGF-2, and a reduction in the amount of collagen fibers was observed. Production and deposition of collagen in the extracellular matrix of cardiomyocytes is a normal physiological mechanism, but in pathological processes such as DM it is increased, characterizing cardiac fibrosis [63,65]. In a study with post-ischemic fibrosis, treatment with flavonoids improved ventricular remodeling and function, decreasing the expression of TGF-β1 and the amount of collagen [66], while the administration of curcumin decreased the synthesis of type I and type III collagen and TGF- expression in the myocardium of diabetic rats [53].

Treatment of diabetic cardiomyopathy with multiple antioxidants in diabetic rats reduced the oxidative stress, lipid peroxidation levels, nuclear factor kappa B (NF-kβ), and cytokines TNF-α, IFN-c, TGF-β and IL-10, which significantly diminished cardiac dysfunctions [50].

Another important agent in cardiac remodeling is FGF-2 which, in addition to its widely known angiogenic action, controls cardiogenic differentiation by regulating the cell differentiation process [67,68]. FGF signaling in cardiomyocytes is critical for the maintenance of cardiac homeostasis. After myocardial infarction, low molecular weight FGF-2 (Lo-FGF-2) would have cardioprotective action promoting angiogenesis, whereas high molecular weight FGF-2 (Hi-FGF-2) would lead to myocardial hypertrophy and to reduction of its contractile function [69,70].

5. Conclusions

Our findings indicate that the associated administration and systemic use of antioxidants did not change the indexes indicative of DM (weight reduction, hyperphagia, polydipsia and glycemia). However, it induced the activation of redox regulatory pathways (with an increase in GSH levels), increased levels of circulating IL-10 in the blood, as well as increased expression of FGF-2 and decreased deposition of collagen fibers in the myocardium.

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CRediT authorship contribution statement

Natan Reyges Castro da Purificação: Conceptualization, Methodology, Writing – review & editing, Investigation. **Vinícius Barreto Garcia:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Flávia Cristina Vieira Frez:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Camila Caviquioli Sehaber:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Kaio Ramon de Aguiar Lima:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Marília Fabiana de Oliveira Lima:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Roseane De Carvalho Vasconcelos:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation. **Aurigena Antunes de Araujo:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation. **Raimundo Fernandes de Araújo Júnior:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation. **Silvia Lacchini, Flávia de Oliveira:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Juliana Vanessa Colombo Martins Perles:** Methodology, Software, Validation, Writing – review & editing, Investigation. **Jacqueline Nelisis Zanoni:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation. **Maria Luiza Diniz de Sousa Lopes:** Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation. **Naianne Kelly Clebis:** Conceptualization, Methodology, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Investigation.

Data Availability

The results of this study are deposited in the repository of the Federal University of Rio Grande do Norte: https://repositorio.ufrn.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

"The datasets generated during and/or analyzed during the current study are available in the Biblioteca Central Zila Mamede/UFRN repository, https://repositorio.ufrn.br/handle/123456789/25212".

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. All procedures are in accordance with ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by CEUA/UFRN (Protocol no. 020/2016).

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Further reading

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