Evaluation of Cardiovascular Effects of Carvacrol in a D-(+)-Galactose-Induced Aging Model

ABSTRACT

Aim: To evaluate the cardiovascular effect of carvacrol treatment in a D(+)galactose accelerated aging model, investigating effects on vascular reactivity, oxidative stress, and systolic blood pressure (SBP).
Methodology: Eight-week-old male Wistar rats (Rattus norvegicus) were used for oral treatment for eight weeks. Organ baths were used for vascular reactivity studies (FEN, ACh, and NPS), fluorescence microscopy to detect reactive oxygen species (ROS, using DHE probe), and Tail-Cuff for systolic blood pressure (SBP) measurements. Non-linear regression was used to create the concentration-response curves. Emax denotes the tissue's maximum response.
Results: The aged rats showed a significant increase in fluorescence intensity by the DHE probe compared to the CTL group (CTL=100 ± 3.6%, n=5 and Dgal=167.7 ± 7.9%, n=5, respectively). However, the levels of ROS in the carvacrol-treated groups were significantly attenuated in the Dgal+C50 (138.8 ± 4.5%, n=5) and Dgal+C100 (130.0 ± 5.5%, n=5) groups. The animals of the Dgal group presented hypertension through the significant increase in SBP compared to the CTL group (CTL=135.9 ± 3.9 mmHg, n=6, Dgal=170.9 ± 2.0 mmHg, n=9, respectively). The increased SBP of Dgal rats could be reversed by treatment with carvacrol (Dgal+C50=137.9 ± 2.7 mmHg, n=5, and Dgal+C100=124.6 ± 8.2 mmHg, n=5, respectively. On the other hand, carvacrol was unable to restore the ACh-induced vasorelaxation effect found in CTL (Emax=100.0 ± 3.9%), Dgal (Emax=84.9 ± 4.4%), Dgal+C50 (Emax=84.9 ± 4.4%) and Dgal+C100 (Emax=82.1 ± 6.2 %).
Conclusion: Carvacrol shows protective antioxidant effects capable of reducing SBP in aged animals, being an important tool in promoting healthy aging.

Keywords: carvacrol, aging, d-galactose-induced aging model, oxidative stress, antioxidant, systolic blood pressure,

1. INTRODUCTION

The aging process, characterized by the gradual decline of cellular, molecular, and tissue functions, is regarded as the leading risk factor for the development of age-related diseases, such as cardiovascular diseases (CVDs), which are the leading cause of morbidity and mortality worldwide [1, 2]. Data indicate that by 2050, approximately a quarter of the world's population will comprise the elderly. However, despite the population's increased life expectancy, individuals do not necessarily experience an improvement in their quality of life [3, 4].

Cardiovascular aging is a dynamic process caused by several mechanisms, including progressive function and structure change, resulting in compromised cardiovascular homeostasis [5]. These changes are associated with increased synthesis and release of Reactive Oxygen Species (ROS) [4]. Briefly, with oxidative stress, vessels and the heart become stiffer, and endothelial dysfunction as one ages, a factor that predisposes to the onset of CVDs [6]. Due to the high prevalence of CVDs in aging, understanding the causes and associated mechanisms is important [7, 8].

It has been shown in the literature that rats given D-(+)-galactose for eight weeks to induce aging developed oxidative stress, vascular remodeling, changes in cardiac anatomy, and senescent cell accumulation, similar to naturally aged rats. Thus, this accelerated aging model is a reliable experimental aging model at the cardiovascular system level and can be widely used [9, 10, 11]. The exploration of biomarkers and the search for new therapeutic targets, especially those with antioxidant activity that can act to slow or reverse the aging process, has aroused much interest [12]. Natural products have been a constant inspiration for medication research and development. Carvacrol, a natural compound of the monoterpene class, is the constituent of the essential oil produced by numerous aromatic plants and spices, such as black cumin (Nigella sativa L.), marjoram (Origanum majorana L.), oregano (Origanum vulgare L.) and thyme (Thymus

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vulgaris L.) [13,14]. Aside from its antioxidant properties, carvacrol also has antimicrobial, bactericidal, antifungal, anticancer, and immunomodulatory properties [15].

This study aimed to evaluate the effect of carvacrol on cardiovascular changes in aging, investigating its impact on blood pressure, vascular reactivity, and oxidative stress in rats with accelerated aging induced by D-(+)-galactose.

2. MATERIAL AND METHODS

2.1 Animals

Eight- and nine-week-old male Wistar rats (Rattus novergicus) from the Animal Production Unit (UPA) of the Institute for Research in Drugs and Medicines (IPeFarM) of the Federal University of Paraíba (UFPB) were used. The animals were kept under appropriate environmental conditions, temperature (22 ± 1˚C), a 12-hour light-dark cycle (6-18 hours), with free access to water and food (Nuvilab CR-1, Quimtia1).

2.2 Chemical substances

The following substances were used: acetylcholine hydrochloride (ACh), L(-)phenylephrine hydrochloride (Phe), cremofor®, D-(+)-galactose, dihydroethidium (DHE), sodium nitroprusside (SNP). All were obtained from Sigma-Aldrich Brasil Ltda (São Paulo-SP, Brazil). The substances were solubilized in water, kept at 0 to 4 °C, and only removed at each experiment. In addition, Carvacrol (5-isopropyl-2-methyl phenol) was purchased from Sigma-Aldrich Brasil Ltda and solubilized in a mixture of cremofor® and physiological saline (NaCl 0.9%).

2.3 Study design

The animals were randomly assigned to four experimental groups: the control group (CTL), which received saline vehicle solution (NaCl 0.9%) intraperitoneally (i.p.), and the D-(+)-galactose group (Dgal), which received D-(+)-galactose 250 mg/Kg i. p. and the D-(+)-galactose + carvacrol 50 mg/Kg (Dgal+C50) and D-(+)-galactose + carvacrol 100 mg/Kg (Dgal+C100) groups, which received D-(+)-galactose 250 mg/Kg i.p. and carvacrol 50 mg/Kg or 100 mg/Kg intragastrically (i.g.), respectively. All groups underwent eight weeks of treatment with daily administration. The CTL and Dgal groups were given a saline solution that had been solubilized in the same proportion as carvacrol in cremofor®. At the end of the treatments, the physical characteristics of the animals in each group were examined.

2.4 Systolic blood pressure monitoring

The SBP of the rats was measured weekly using the tail-cuff method, as previously described [16] (Panlab, Harvard Apparatus, Spain). To measure the blood pressure, the rats were kept in a heated acrylic container (28-30ºC) for 10 minutes prior to the measurement to make the caudal artery pulsation more readily detectable. At least three successive measurements were recorded in the data acquisition system (LabChart® software, version 7.1; ADInstruments, Colorado Springs, CO) to obtain the mean SBP.

2.5 Vascular reactivity

The animals were anesthetized with ketamine (75mg/kg) and xylazine (10 mg/kg) and sacrificed by exsanguination. After euthanasia, the superior mesenteric artery was immediately removed and maintained cold (4°C) in a Tyrode's solution containing the following composition (mM): NaCl 138.16, KCl 4.0, MgCl2 1.05, NaH2PO4 0.42, CaCl2 2, NaHCO3 10.0; Glucose 5.6 [17]. The rings were then suspended vertically in isolated organ baths (Panlab Multi-Chamber Organ Baths, ADInstruments, Australia) by two stainless steel metallic rods and immediately submerged in 10 mL of 37°C Tyrode's solution with a carbogenic mixture (95% O2 and 5% CO2), maintained at pH 7.4, and under a stabilizing tension of 0.75 g, for 60 minutes. Voltage changes were measured using isometric transducers (MLT020, ADInstruments, Australia) and recorded in a PowerLab® data acquisition system (ML870/P, LabChart version 7.0, ADInstruments, Australia).

The contractility of the mesenteric rings was tested in the presence of an increasing and cumulative addition of Phe (1 nM– 30 nM). Furthermore, the mesenteric ring relaxing responses of the treated groups were evaluated by increasing, and cumulative addition of ACh (0,1 nM—30 M) and SNP (1 pM—30 M) in Phe (1 M) induced contractions.

2.6 Evaluation of superoxide anion production

Reactive oxygen species (ROS) generation in the rat superior mesenteric artery was detected with the fluorescent dye DHE, as previously described [18]. The rat mesenteric arteries were isolated, embedded into the Tissue Tek Compound (OCT) embedding medium, and frozen in liquid nitrogen. Subsequently, sections of rat arteries (10 µm) were incubated with 5 µM DHE at 37 ºC for 30 minutes in a humid chamber and protected from light. The fluorescence intensity emitted by DHE was used to measure the superoxide anion production in the different groups. The digital images were captured using a fluorescence microscope (NIKON Eclipse Ti-E, NIKON, Japan) for further analysis.

2.7 Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Non-linear regression was used to create the concentration-response curves. Emax denotes the tissue's maximum response. For statistical analysis, one-way ANOVA was used, followed by the Bonferroni post-hoc test. The differences between the means were considered significant when $P < 0.05$. The data were analyzed and plotted in the statistical software GraphPad Prism 7.0®. The maximum relaxation corresponded to the maximum effect (Emax) for the highest concentration used.

3. RESULTS AND DISCUSSION

The main finding of this study reveals that treatment for eight weeks with the monoterpene carvacrol was able to prevent vascular oxidative stress and attenuate the increase in systolic blood pressure involved in the D-(+)-galactose-induced model.

D-(+)-Galactose-induced accelerated aging mimics natural aging and has been widely used in antiaging pharmacological studies [19]. Natural products have gained prominence in the search for pharmacological agents for antiaging activity, especially those with antioxidant activity [20, 21]. In this context, due to the recent prominence of carvacrol as an antioxidant of natural origin, the present study sought to evaluate its action on components of the cardiovascular system of aging rats.

The animals studied looked different at the end of each treatment. For example, the rats in the CTL, Dgal+C50, and Dgal+C100 groups had smooth, healthy-looking, and shiny hair with uniform colors, whereas the animals in the Dgal group had curly, coarse, and opaque hair with darker regions and severe hair loss.

In the aging process, the heart and blood vessels show a gradual imbalance of homeostasis and important changes in functional responses and morphology, leading to tissue adaptations [22]. To evaluate the effect of carvacrol on alterations in the vascular response induced by D-(+)-galactose, concentration-response curves were constructed for contracting and relaxing agents.

Figure 1 depicts the vascular reactivity results. Increasing and cumulative addition of Phenylephrine (Phe, 10^{-9} - $3x10^{-4}$ M) promoted concentration-dependent contraction in arteries of all four experimental groups. Increasing and cumulative addition of Phe promoted concentration-dependent contraction in arteries of all four experimental groups. The Dgal group (91.2 ± 9.03%, n=9) showed no significant increase in contractile response by Phe when compared to the CTL group (100.5 ± 12.2%, n=11), suggesting that the D-(+)-galactose-induced accelerated aging model does not develop hypercontractility of the rat mesenteric artery, corroborating with Guevara-Balcazar et al. (2017) [23], who identified similar results. On the other hand, in animals treated with carvacrol at a dose of 50 mg/kg, a significant increase in the contractile response to Phe was observed (Dgal+C50, 120.4 ± 10.4 %, n=9). However, the contractile response to Phe in the Dgal+C100 group (82.8 ± 10.11%, n = 7) was not different from that of both CTL and Dgal groups. Thus, the highest contractile response observed in the Dgal+C50 group appears to be an isolated dose-specific effect. Further studies are needed to investigate the underlying mechanisms implicated in the contractile effect induced by Phe in rats treated with carvacrol at a dose of 50 mg/kg.

In terms of vascular structure and functionality, the endothelium is a highly active monolayer that modulates vascular tone, thromboresistance, cell adhesion, and smooth muscle cell proliferation, among other functions, as well as the production of various vasoprotective molecules, such as NO. The endothelium has an important role in vascular function by acting directly in the balance of oxygen supply to tissues, remodeling vascular structures, and regulating the tone and diameter of the vessel [24, 25]. Thus, in the present study, we evaluated the NO-mediated relaxant response by the action of ACh at the vascular endothelium level.

In the vascular reactivity studies, when evaluating the relaxant response, it was found that the Dgal group significantly decreased the percentage of ACh-induced relaxation when compared to the CTL group. Furthermore, the vasorelaxant effect induced by acetylcholine (ACh, 10^{-10} - $3x10^{-4}$ M) in the rings precontracted with Phe (Figure 1B) was attenuated in the Dgal group (84.9 ± 4.4%, n=8) compared to control animals CTL (100.0 ± 3.9%, n=5). Therefore, it is suggested that the group submitted to the aging model induced by D-(+)-galactose presented endothelial dysfunction. This may occur due to increased superoxide anion generation and, as a result, decreased NO bioavailability, compromising ACh-

dependent vasodilation in the vessel [26, 27]. Our results corroborate those obtained by Dai et al. (2018) [28] that show an impairment of ACh-induced relaxation in aortas of rats aged by D-(+)-galactose (at a dose of 150 mg/kg), thus showing endothelial dysfunction. The carvacrol-treated groups showed similar effects to the Dgal animal group, being Dgal+C50 (84.9 ± 4.4%) and Dgal+C100 (82.1 ± 6.2 %, n=7), indicating that carvacrol treatment could not reverse the endothelial dysfunction promoted by D-galactose in rats.

In addition to evaluating endothelium-dependent relaxation, it is important to investigate whether there is any impairment in pathways directly involved in vessel relaxation. Therefore, we used the inorganic compound SNP, an NO donor in biological systems, through enzymatic and non-enzymatic mechanisms [29].

Cumulative SNP curves (10^{-12} - $3x10^{-6}$ M) in rings precontracted with Phe had no difference between the different groups (Figure 1C): CTL (100.0 ± 4.5%, n=6), Dgal (107.7 ± 3.8%, n=5), Dgal+C50 (92.2 ± 4.7%, n=5) and Dgal+C100 (113.2 ± 8.7, n=5), suggesting that the accelerated aging model is not associated with changes in the nitric oxide pathway in vascular smooth muscle. These findings back up the findings of Rezende et al. (2021) [30], who investigated the effect of SNP on rat corpora cavernosa strips after treatment with carvacrol at 50 and 100 mg/kg and showed the same responses.

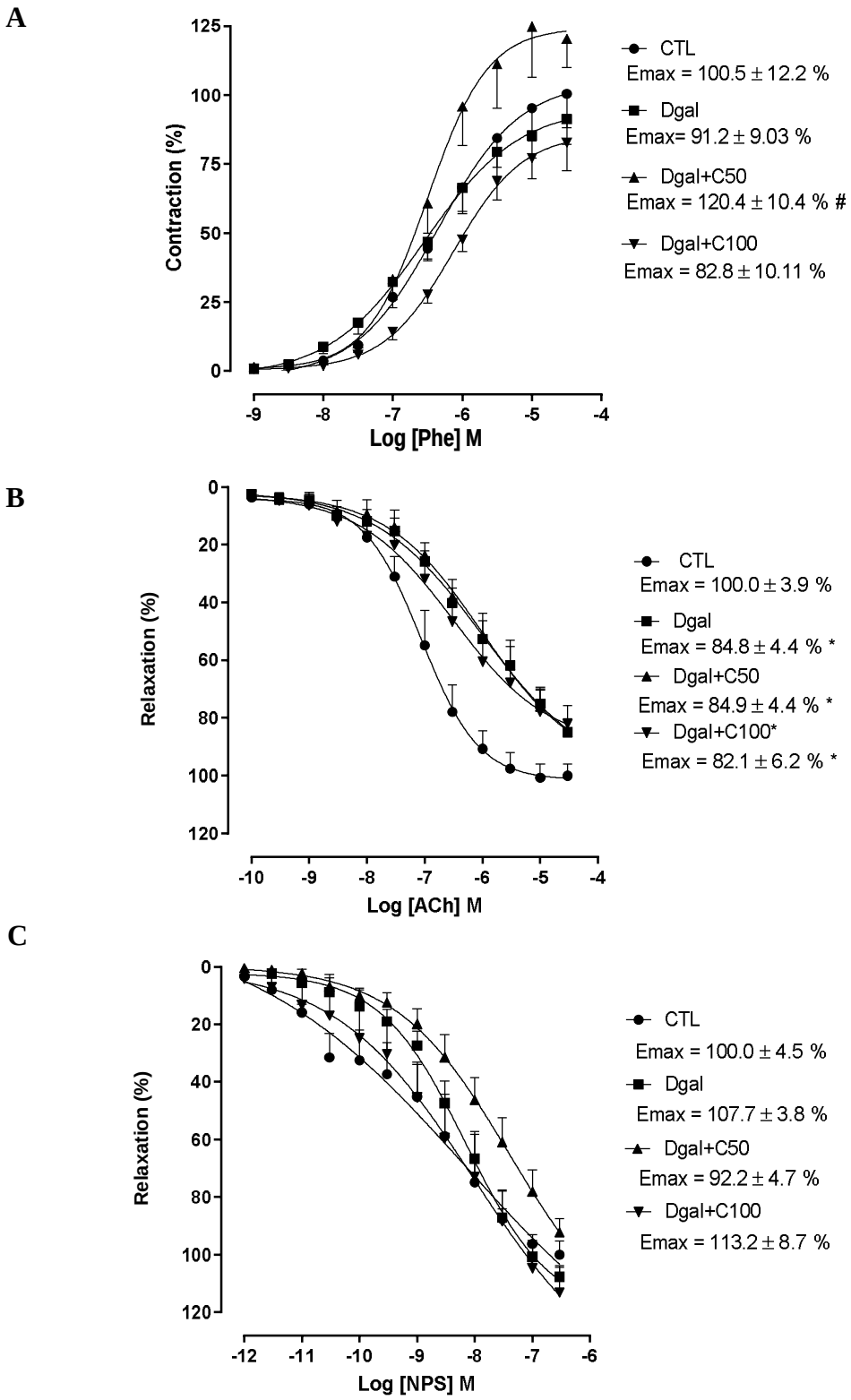
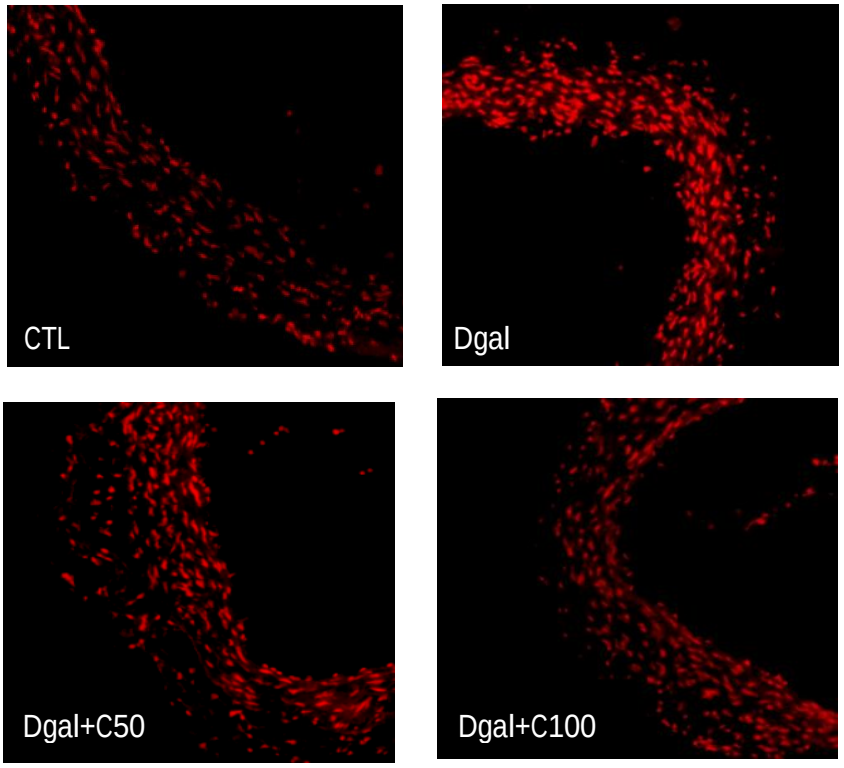


Figure 1 - Study of vascular reactivity in mesenteric artery of aged rats. A) Concentration-response curves for Phe (10^{-9} - $3x10^{-4}$ M). Groups: CTL (control, n = 11), Dgal (D-(+)-galactose 250 mg/kg, n = 9), Dgal+C50 (D-(+)-galactose 250 mg/Kg + carvacrol 50 mg/Kg, n = 9); Dgal+C100 (D-(+)-galactose + carvacrol 100 mg/Kg, n = 7). B) Concentration-response curves for ACh (10^{-10} - $3x10^{-4}$ M), in rings precontracted with FEN. Groups: CTL (n = 5), Dgal (n = 8), Dgal+C50 (n = 6); Dgal+C100 (n= 7). C) Concentration-response curves for SPN (10^{-12} - $3x10^{-6}$ M) in rings precontracted with Phe. Groups: CTL (n = 6), Dgal (n = 5), Dgal+C50 (n = 5); Dgal+C100 (n= 5)**.** *Results are expressed as mean ± s.e.m. Data were analyzed using one way ANOVA statistical test, followed by Bonferroni post-test. #P< 0.05 vs Dgal.*

Several theories have been created to explain the aging process and the factors involved. However, the common point in all theories is the role of reactive oxygen species and the oxidative stress phenomenon in promoting aging-associated diseases. Furthermore, several studies have shown that oxidative stress contributes to endothelial dysfunction in these diseases [26]. Therefore, oxidative stress levels were evaluated using the DHE probe in isolated mesenteric arteries of aged rats after 8 weeks of carvacrol treatment. The aged rats showed (Figure 2A and 2B) significant increase in fluorescence intensity by the DHE probe compared to the CTL group (CTL=100 ± 3.618%, n=5 and Dgal=167.7 ± 7.9%, n=5, respectively), suggesting an increase in the levels of reactive oxygen species (ROS) in the superior mesenteric artery of these animals. A similar result was found in the studies by Dehghani et al. (2018) [31], XU et al. (2019) [12], WU et al. (2017) [32], and Chang et al. (2017) [19], which used the D-galactose accelerated aging model, and it promoted an increase in fluorescence intensity by DHE by increasing ROS generation and oxidative stress in cardiovascular tissues of animals, in addition to decreasing the antioxidant capacity of the body. However, the levels of ROS in the carvacrol-treated groups were significantly attenuated in the Dgal+C50 (138.8 ± 4.5%, n=5) and Dgal+C100 (130.0 ± 5.5%, n=5) groups (Figure 2A and 2B). The results demonstrate that carvacrol treatment exerted a protective effect against oxidative stress at the mesenteric artery level.

A



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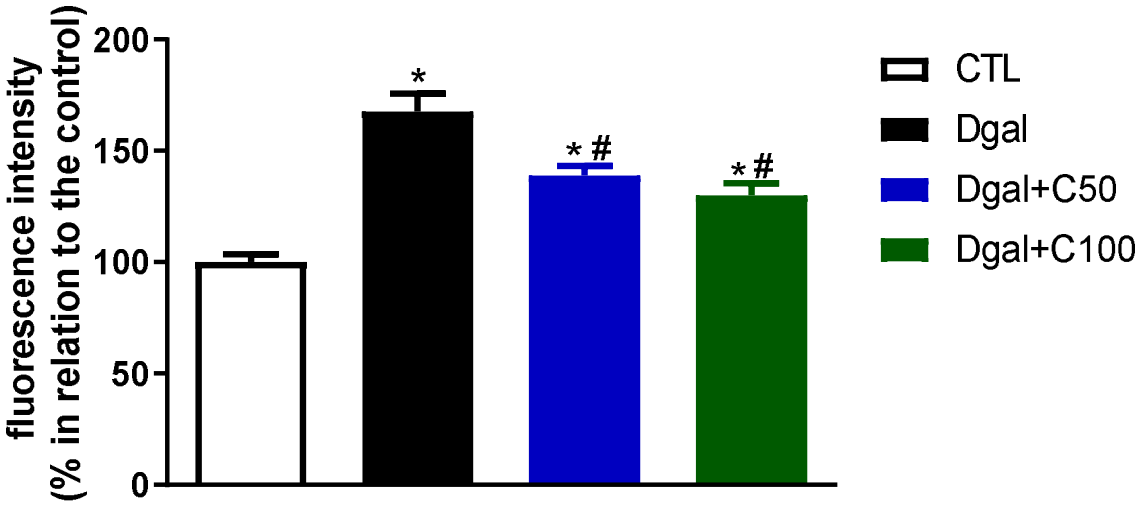

Figure 2 - Images (A) and statistical graph (B) of fluorescence intensity emitted by DHE probe in histological sections of aged rat mesenteric artery from different experimental groups treated with D-galactose and carvacrol for eight weeks (Objective 10x). Groups: CTL (control), Dgal (D-(+)-galactose 250 mg/kg), Dgal+C50 (D-(+)-galactose 250 mg/Kg + carvacrol 50 mg/Kg); Dgal+C100 (D-(+)-galactose + carvacrol 100 mg/Kg); 5n per group. *Data are expressed as mean values of percent fluorescence intensity relative to control ± p.e.m. Data were analyzed using one way ANOVA statistical test followed by Bonferroni post-test. *P<0.05 vs CTL, # P<0.05 vs Dgal.*

Evidence shows that oxidative stress may be an important factor in hypertension's pathophysiology. In addition, the disease becomes more prevalent with advancing age, with more than 60% of people over 60 presenting this pathology [33]. Thus, we evaluated the systolic blood pressure values in the four groups of animals studied.

Tail cuff is a common and non-invasive method to measure systolic blood pressure in conscious restrained rats. This method allows repeated SBP measurements to evidence the effect of a chronic drug administration with time and in comparison, with vehicle treated animals. The systolic blood pressure values in rats subjected to the D(+)Galactose-

induced aging model after carvacrol treatment for 8 weeks in the different groups were: CTL (135.9 ± 3.9 mmHg, n=6), Dgal (170.9 ± 2.0 mmHg, n=9), Dgal+C50 (137.9 ± 2.7 mmHg, n=5) and Dgal+C100 (124.6 ± 8.2 mmHg, n=5) (Figure 3).

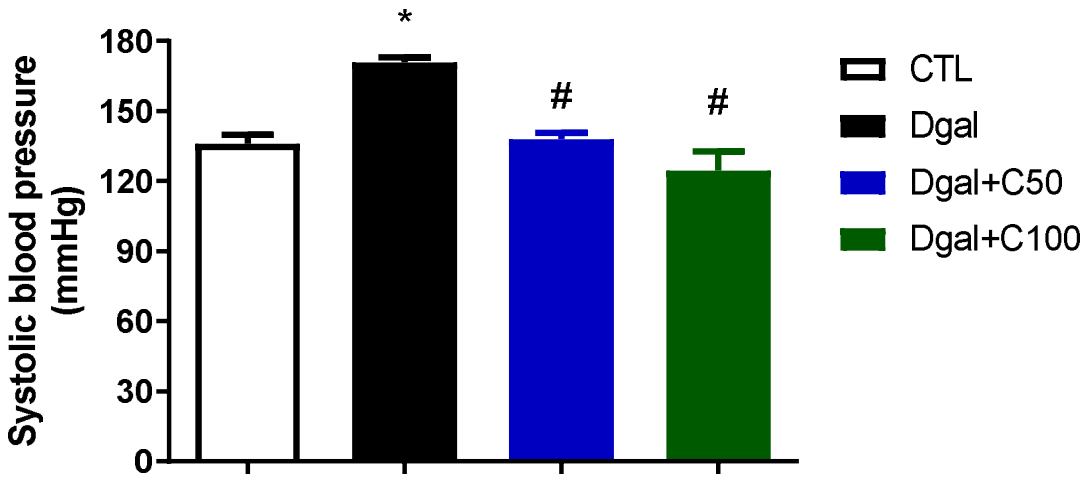

Figure 3 - Systolic blood pressure (SBP, in mmHg) measurement of the different experimental groups, during treatment for eight weeks. Groups: CTL (control, n = 6), Dgal (D-(+)-galactose 250 mg/kg, n = 9), Dgal+C50 (D-(+)-galactose 250 mg/Kg + carvacrol 50 mg/Kg, n = 5); Dgal+C100 (D-(+)-galactose + carvacrol 100 mg/Kg, n = 5). Results are expressed as mean ± p.e.m. Data were analyzed using one way ANOVA statistical test followed by Bonferroni post-test. *P<0.05 vs CTL; # P<0.05 vs Dgal.

Surprisingly, the animals in the Dgal group presented hypertension through the significant increase in SBP compared to the CTL group. These findings were similar to those of Dai et al. (2018) [28] and Dehghani et al. (2018) [31], who found that chronic exposure to accelerated aging induced by D-(+)-galactose resulted in increased blood pressure in rats via as-yet-unknown mechanisms, but with a massive presence of reactive oxygen species. Thus, due to its ability to mimic the senescent characteristics of natural aging, D-galactose-induced aging is potentially an ideal model for antiaging therapeutic intervention studies. Therefore, the decrease in SBP observed in this accelerated aging model in response to carvacrol treatment could be associated with its antioxidant properties. Interestingly, a recent study by Dias et al., (2022) also showed that thirty days of treatment with carvacrol induced an antihypertensive effect in SHR [34]. Additional studies are needed, however, to investigate additional hypotensive mechanisms in the Dgal model induced by chronic monoterpene treatment. One of the main limitations of the present study is that the Dgal-induced model poorly relates the actual physiological and biochemical changes. Furthermore, the present study did not measure inflammatory mediators nor the expression of aging-associated proteins, including p53-p21, PI3K/Akt, and AMPK/ULK1, which regulate cellular senescence.

4. CONCLUSION

Taken together, the results of the present study indicate that treatment with carvacrol significantly decreases the generation of ROS at the mesenteric artery level, in addition to the reduction in systolic blood pressure levels of aged rats by D-(+)-galactose administration. However, surprisingly, vascular reactivity was not altered after treatment with the monoterpene. Thus, future studies need to be conducted to investigate the mechanism of action by which carvacrol acts to promote such improvements in the cardiovascular system with an aging phenotype. Furthermore, this is a study that reveals the beneficial properties of carvacrol for the cardiovascular system of the elderly, opening an important window for the use of the monoterpene as an adjuvant in delaying cardiovascular aging since, currently, there are few therapeutic options useful for mitigating the effects caused by aging, especially on the cardiovascular system.

ETHICAL APPROVAL

All experimental protocols were performed according to the guidelines established by the National Council for the Control of Animal Experimentation - CONCEA submitted and previously approved by the Ethics Committee on Animal Use (Comissão de Ética no Uso de Animais - CEUA) of UFPB, n° 3183120919.

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COMPETING INTERESTS

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Sabine Helena Dantas, designed the study, conducted the research, analyzed the results, and drafted the manuscript. Arthur José Pontes de Almeida, Tays Amanda Felisberto Gonçalves, Mathania Silva de Almeida Rezende, conducted the research and analyzed the results. Antonia Leda Silva analyzed the results and helped to draft and review the manuscript. Islania G. A. Araújo and Robson C. Veras designed the study and helped to draft the manuscript. Isac A. de Medeiros took primary responsibility for the paper, conceived and coordinated the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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