RENNER PHILIPE RODRIGUES CARVALHO

EFFECTS OF EUGENOL ON DIGESTIVE GLANDS, KIDNEYS, AND MALE REPRODUCTIVE ORGANS: A BIOCHEMICAL, OXIDATIVE, AND MORPHOLOGICAL STUDY

Thesis submitted to the Cellular and Structural Biology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Mariana Machado Neves

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ABSTRACT

CARVALHO, Renner Philipe Rodrigues, D.Sc., Universidade Federal de Viçosa, May, 2023. **Effects of eugenol on digestive glands, kidneys, and male reproductive organs: a biochemical, oxidative, and morphological study.** Adviser: Mariana Machado Neves.

Eugenol is a phenolic compound found in clove oil and is extensively used in traditional medicine. Although there is extensive research on the toxicity of natural products, studies related to the toxic effects of eugenol are still relatively scarce. Thus, we aimed to evaluate the effects of eugenol on biochemical, oxidative, and morphological parameters in healthy Wistar rats' digestive glands, kidneys, and male reproductive organs. Forty adult rats were divided into four groups (n=10/group). Control rats received 2% Tween-20 (eugenol vehicle), whereas the other animals received 10, 20, and 40 mg Kg^{-1} eugenol through gavage daily for 60 days. The liver, pancreas, submandibular and sublingual glands, kidneys, testes, epididymides, and sperm were analyzed under microscopic, biochemical, and functional approaches. Our results showed that eugenol treatment, regardless of dose, did not alter body and organ weights. However, eugenol at 20 and 40 mg Kg^{-1} altered serum levels of albumin, urea, creatinine, uric acid, testosterone, and alkaline phosphatase and aspartate transaminase activities. Lipase activity and sodium, potassium, and chloride serum levels were affected only in rats treated with 40 mg Kg^{-1} eugenol. In the liver, 20 and 40 mg Kg^{-1} eugenol caused structural and functional damage, reducing Na^+/K^+ ATPase activity, increasing glycogen content, and oxidative and nitrosative metabolites. In the pancreas, submandibular and sublingual glands, 40 mg Kg^{-1} eugenol altered most of the biochemical and oxidative parameters, whereas only submandibular glands presented histological changes. In the kidney, 40 mg Kg^{-1}, eugenol reduced Na^+/K^+ ATPase activity and apical bush-border of renal tubules and modulated oxidative parameters. Still, at 10 mg Kg^{-1}, eugenol decreased the total antioxidant capacity and increased the volumetric proportion of blood vessels and nitric oxide content in the kidneys. All doses of eugenol negatively impacted epididymal sperm parameters and modified the oxidative pattern in male organs with no influence on their histology. In summary, eugenol treatment, particularly at higher doses, can modulate biochemical and oxidative parameters leading to structural and functional alterations to digestive glands, kidneys, and male reproductive organs. These findings highlight the importance of research focused on an accurate understanding of the molecular mechanisms involved in eugenol effects on these organs.

Keywords: Clove oil. *Syzygium aromaticum*. Toxicology. Antioxidant. Histology.

RESUMO

CARVALHO, Renner Philipe Rodrigues, D.Sc., Universidade Federal de Viçosa, maio de 2023. **Efeitos do eugenol nas glândulas digestivas, rins e órgãos reprodutivos masculinos: um estudo bioquímico, oxidativo e morfológico.** Orientadora: Mariana Machado Neves.

Eugenol é um composto fenólico encontrado no óleo de cravo amplamente utilizado na medicina tradicional. Embora existam extensas pesquisas sobre a toxicidade de produtos naturais, estudos relacionados aos efeitos tóxicos do eugenol ainda são muito limitados. Assim, objetivamos avaliar os efeitos do eugenol em parâmetros bioquímicos, oxidativos e morfológicos nas glândulas digestivas, rins e órgãos reprodutivos masculinos de ratos Wistar saudáveis. Quarenta ratos adultos foram divididos em quatro grupos (n=10/grupo). Ratos controles receberam Tween-20 a 2% (veículo eugenol), enquanto os demais receberam 10, 20 e 40 mg Kg^{-1} de eugenol por gavagem, diariamente, durante 60 dias. O fígado, pâncreas, glândulas submandibular e sublingual, rins, testículos, epidídimos e espermatozoides foram analisados sob abordagens microscópicas, bioquímicas e funcionais. Nossos resultados mostraram que o tratamento com eugenol, independentemente da dose, não alterou o peso corporal e dos órgãos. Entretanto, eugenol nas doses de 20 e 40 mg Kg^{-1} alterou as concentrações séricas de albumina, ureia, creatinina, ácido úrico, testosterona, fosfatase alcalina e aspartato transaminase. A atividade da lipase e as concentrações séricas de sódio, potássio e cloreto foram afetadas pelo eugenol em animais tratados com a maior concentração (40 mg Kg^{-1}). No fígado, 20 e 40 mg Kg^{-1} de eugenol causaram danos estruturais e funcionais, reduzindo a atividade da Na^+/K^+ ATPase, aumentando o conteúdo de glicogênio e metabólitos oxidativos. A dose de 40 mg Kg^{-1} de eugenol alterou a maioria dos parâmetros bioquímicos e oxidativos no pâncreas e nas glândulas submandibular e sublingual. Porém, apenas as glândulas submandibulares apresentaram alterações estruturais após a exposição. No rim, 40 mg Kg^{-1} de eugenol reduziu a atividade da Na^+/K^+ ATPase e a borda em escova dos túbulos e modulou parâmetros oxidativos. Ainda, na dose de 10 mg Kg^{-1}, o tratamento com eugenol diminuiu a capacidade antioxidante total e aumentou a proporção volumétrica dos vasos sanguíneos e o conteúdo de óxido nítrico. Todas as doses de eugenol impactaram negativamente os parâmetros espermáticos, modificando também parâmetros oxidativos nos órgãos masculinos sem influenciar sua arquitetura tecidual. Por fim, o tratamento com eugenol, principalmente em doses mais altas, pode modular parâmetros bioquímicos e oxidativos levando a alterações estruturais e funcionais nas glândulas digestivas, rins e órgãos reprodutivos masculinos. Esses

achados destacam a importância de pesquisas focadas em uma compreensão precisa dos mecanismos moleculares envolvidos nos efeitos do eugenol nestes órgãos.

Palavras-chave: Óleo de cravo. *Syzygium aromaticum*. Toxicologia. Antioxidante. Histologia.

SUMÁRIO

1. INTRODUCTION

Medicinal plants have been used for a long time as sources of traditional treatments, being considered the basis of modern medicine. Plant-derived compounds have been and still are an essential source of drugs (WHO, 2000; SALMERÓN-MANZANO et al., 2020). Among medicinal plants, the clove (*Syzygium aromaticum* (L.) (Myrtaceae Family) is a well-known plant and established herb in traditional medicine due to its extensive biological activities (CORTÉS-ROJAS et al., 2014; HARO-GONZÁLEZ et al., 2021). For instance, clove essential oil has been used as an antimicrobial, antiseptic, and antispasmodic in traditional medicine since ancient times. In addition, it is well-established as a flavoring agent for foods and a topical analgesic in dentistry (ZARI et al., 2021). This natural product has received considerable interest due to its wide application in the perfume, cosmetic, health, medicine, flavoring, and food industries (GÜLÇIN et al., 2012; MITTAL et al., 2014). Clove essential oil mainly comprises four compounds, eugenol, β-caryophyllene, α-humulene, and eugenyl acetate (HATAMI et al., 2010; YANG et al., 2014). Eugenol is the main compound responsible for the biological activities of this oil, representing more than 50% of the total extracted composition (PRAMOD et al., 2010).

Eugenol was first isolated in 1929 as a volatile compound from clove, and commercial production began in the United States of America in 1940 (TAMMANNAVAR et al., 2013; MARCHESE et al., 2017; ULANOWSKA et al., 2021). Similarly to *Syzygium aromaticum*, the oil of plants from Lamiaceae, Lauraceae, Myrtaceae, and Myristicaceae families are rich in eugenol. Several techniques have been developed to isolate eugenol in a laboratory, including conventional (hydrodistillation or steam distillation) and modern extraction (supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction) methods. Also, eugenol can be produced synthetically by the allylation of guaiacol with allylchloride (MORAES et al., 2020) and through biotechnological approaches, such as biotransformation using different microorganisms' participation (MISHRA et al., 2013; MOLINA et al., 2013).

Eugenol is named as 4-Allyl-2-Methoxyphenol according to IUPAC (International Union of Pure and Applied Chemistry) and has the chemical formula $C_{10}H_{12}O_2$. Some chemical properties include a molecular weight of 164.20 g mol^{-1}, a density of 1.0664 g L^{-1}, being a colorless or yellowish oil with a strong odor. Eugenol is classified as a phenylpropanoid, a weak acid slightly soluble in water, as well as in ethyl alcohol, ether, chloroform, and oil. WHO generally recognizes it as safe for consumption (BARCELOUX, 2008; KHALIL et al., 2017).

In the pharmaceutical field, eugenol has garnered attention due to its ability to act as a scavenger of free radicals, preventing the generation of reactive oxygen species and contributing to improving cellular antioxidant defenses (ITO et al., 2005; TALEUZZAMAN et al., 2021). Furthermore, studies have demonstrated the antimicrobial (DA SILVA et al., 2018), anti-inflammatory (BARBOZA et al., 2018), and antihyperglycemic (CARVALHO et al., 2021) activities of this compound. Thus, several studies have investigated the beneficial effects of eugenol in the treatment of nervous system diseases (IRIE et al., 2006), diabetes (CARVALHO et al., 2021), hypertension (MNAFGUI et al., 2013), inflammatory diseases (LOPES et al., 2018), and cancer (JAGANATHAN & SUPRIYANTO et al., 2012).

The various pharmacological properties exhibited by eugenol have led to investigations of its potential as a protective agent against injuries in multiple organs in preclinical studies. Eugenol showed hepatoprotective effects against ischemia/reperfusion injury at a dosage of 10 mg Kg^{-1} (MOTTELEB et al., 2014). In the pancreas, studies have also shown a single dose of 15 mg Kg^{-1}, and 100 or 200 mg Kg^{-1} for three days of eugenol exerted a protective effect against biliopancreatic ligation and L-arginine-induced pancreatitis, respectively (SOWJANYA et al., 2012; TSAROUCHA et al., 2021). In toxicity induced by gentamicin, treatment with 100 mg Kg^{-1} eugenol for 10 days restored normal renal functions and suppressed drug-induced oxidative stress and hypoxia in Wistar rats (SAID, 2011). Similarly, co-administration of 10 mg Kg^{-1} eugenol for 28 days also improved the toxic effects caused by metanil yellow on oxidative stress and renal function in male Wistar rats (SHARMA et al., 2019). In male reproduction, administration of 100 mg Kg^{-1} eugenol for five days exerted an anti-apoptotic and antioxidant effect against cisplatin-induced testicular damage (AKDEMIR et al., 2019) and protection against the harmful effects of the insecticide chlorpyrifos on the testis, increasing antioxidant capacity and improving sex hormones secretion, at a dosage of 250 mg Kg^{-1} (NIKBIN et al., 2020).

Despite the positive effects of eugenol, several studies have associated its consumption with some adverse effects, and there has been great concern about its toxicity in recent years (NEJAD et al., 2017). In vitro assays have shown that eugenol can cause hepatotoxicity due to the formation of methyl vinyl quinone during its metabolism (MIZUTANI et al., 1991; USTA et al., 2002). In male rats, eugenol treatment using 20 and 30 μg $100g^{-1}$ for 10 days caused an increase in alkaline phosphatase activity, transaminases, and lactate dehydrogenase, suggesting that eugenol has a toxic effect on the liver (SOUDRAN et al., 1994). In the oral cavity, adverse effects of eugenol have been previously reported, ranging from localized skin irritation to allergic contact dermatitis (JACOBSEN & HENSTEN-PETTERSEN, 1989; KANERVA et al.,

1998; SARRAMI et al., 2002). Different extracts of *Syzygium aromaticum* containing eugenol, when administered in doses above 20 mg Kg^{-1}, inhibited spermatogenesis with a consequent reduction in sperm production and epididymal secretory activity, affecting male fertility by decreasing litter size (SINGH & MISHRA, 2013; MISHRA & SINGH, 2016; CHOI et al., 2014). In humans, the toxicity of eugenol is even less studied. In only one case study of accidental ingestion by a two-year-old child, 5-10 ml of clove oil caused coma, convulsions, coagulopathy, and acute liver injury (HARTNOLL et al., 1993).

Interestingly, although there is extensive research on the toxicity of natural products, studies related to the toxic effects of eugenol are still relatively scarce (KAMATOU et al., 2012; NEJAD et al., 2017). However, balancing the therapeutic potential with the adverse effects of this compound could intensify its positive effects, favoring its subsequent clinical use. For this, it is necessary to investigate the safety of eugenol as well as the effects of its long-term exposure. Therefore, this thesis aimed to evaluate the effects of eugenol treatment using three different concentrations (10, 20, and 40 mg Kg^{-1}) for 60 days on morphological, biochemical, oxidative, and functional parameters of digestive glands (**Chapter 1:** *High doses of eugenol cause structural and functional damage to the rat liver*; **Chapter 2:** *Effect of eugenol on the pancreas, submandibular, and sublingual glands of Wistar rats: a biochemical, oxidative, and morphological study*), kidneys (**Chapter 3:** *Eugenol ingestion affects renal morphology and function in healthy Wistar rats*), and male reproductive organs (**Chapter 4:** *Eugenol reduces serum testosterone levels and sperm viability in adult Wistar rats*) from healthy Wistar rats.

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2. CHAPTER 1

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High doses of eugenol cause structural and functional damage to the rat liver

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High doses of eugenol cause structural and functional damage to the rat liver

Short title: High doses of eugenol lead to hepatic damage in Wistar rats

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Abstract

Eugenol is a phenolic compound found in clove extract and extensively used in traditional medicine. It is unclear whether its intake can cause positive or negative effects on liver morphology and physiology in healthy individuals. Thus, we aimed to evaluate liver parameters of rats treated with 10, 20, and 40 mg Kg^{-1} eugenol. After 60 days of treatment, liver samples were collected and analyzed by biometric, histological, biochemical, and oxidative analyses. Our results showed that 10, 20, and 40 mg Kg^{-1} eugenol did not alter body and liver weights, serum and hepatic ALT levels and catalase, glutathione-s-transferase, total, Ca^{2+}, and Mg^{2+} ATPases activities in treated animals. However, 20 and 40 mg Kg^{-1} eugenol reduced Na^{+}/K^{+} ATPase pump activity and blood glucose levels. They also increased hepatic glycogen content, superoxide dismutase activity, ferric reducing antioxidant power, and nitric oxide and malondialdehyde levels. Still, 20 and 40 mg Kg^{-1} eugenol caused structural and functional damage to the liver tissue of eugenol-treated rats. We concluded that 10 mg Kg^{-1} eugenol is a safe dose for consumption in long-term treatment for rats. Doses higher than 20 mg Kg^{-1} lead to hepatic damage that can impair vital processes of liver functionality.

Keywords: Clove, hepatocyte, histomorphometry, oxidative stress, phenolic compound, toxicology.

1. Introduction

Herbal drugs have been used for medicinal purposes for centuries, exhibiting an exponential increase in their consumption in the last decades [1,2]. The ingestion of plant extracts is considered safe by consumers since they believe that natural products cause no risk to human health [3,4]. However, these products may contain substances harming the functionality of body organs, relying upon the biochemical structure and concentrations [1]. The liver is one of the organs affected by exposure to plant extract and dietary supplements. The hepatotoxicity of these substances may be related to the formation of o-quinones, a reactive metabolite of several phenolic compounds produced after their oxidation or detoxifying processes. Once generated, these o-quinones can lead to detoxification, chemoprevention, and toxicity. For instance, o-quinones can deplete GSH content, causing oxidative stress through protein alkylation or oxidation [4,5,6,7,8].

Eugenol is a phenolic compound commonly found in clove extract and used in traditional medicine [9] against alcoholic liver disorders, liver cirrhosis, and drug-induced liver diseases [10,11,12]. It is rapidly absorbed and metabolized in the body after oral administration, exhibiting a half-life in the plasma of 14 h, with excretion occurring in the urine within 24 h [13,14,15]. Studies have demonstrated the potential of eugenol to exert antioxidant [16], antimicrobial [17], anti-inflammatory [18], and antidiabetic activities [19]. However, *in vitro* assays reported that eugenol caused hepatotoxicity due to the formation of methyl vinyl quinone during its metabolism [20,21,22,23]. Alteration in enzymatic parameters related to liver functions may also occur in eugenol-treated animals [24].

Despite its widespread use in traditional medicine [9,25,26], it is still unclear whether the eugenol treatment can cause positive and negative effects on liver morphology and physiology. In addition, no studies are reporting how this substance act on hepatocyte function in healthy individuals. In this framework, we aimed to evaluate the effects of purified eugenol in the liver of Wistar rats. To that end, rats ingested low (10 mg Kg^{-1}) and high (20 and 40 mg Kg^{-1}) concentrations of eugenol for 60 days [19]. We focused on morphological, functional, and oxidative parameters of the liver tissue.

2. Material and methods

2.1 Animals and ethics statement

Twenty male Wistar rats (70 days old; 230–250 g) were supplied by the Central Animal Facility of the Universidade Federal de Viçosa (UFV). They were housed individually in polypropylene cages under controlled photoperiod (12-12 h light/dark cycle) and temperature

(21 °C). The animals had free access to rat chow and drinking water. The study was approved by the Ethics Committee of Animal Use of UFV (protocol 61/2021) and was conducted in strict accordance with the ethical guidelines of Guide for the Care and Use of Laboratory Animals [27].

2.2 Experimental design

Animals were randomly divided into four experimental groups (n = 5 animals/group). The control group comprised rats receiving 2% Tween-20 added into distilled water (vehicle; 1 mL per gavage) daily for 60 d. Other groups, in turn, were composed of animals exposed to 10, 20, and 40 mg Kg^{-1} of purified eugenol (Sigma Aldrich Co., St. Louis, MO) diluted in 1 mL of the vehicle administered through gavage daily for 60 d. The concentrations were determined following our meta-analytical study published previously [19]. The monitoring of body weight and clinical signs of toxicity (e.g., diarrhea, vomiting, hair loss) occurred weekly and daily during the experiment.

2.3 Euthanasia, tissue collection, and biometric analysis

After 60 d of treatment, the rats were weighed and euthanized by deep anesthesia (ketamine 150 mg Kg^{-1} i.p. and xylazine 10 mg Kg^{-1} i.p) followed by cardiac puncture [28]. Blood was used to analyze glucose and serum enzyme levels using biochemical kits. The liver, in turn, was removed, weighed, and fragmented into four pieces quickly. While two fragments were frozen in liquid nitrogen and stored at −80 °C for liver enzyme and oxidative/nitrosative stress assays, the others were fixed and used for histological analysis. Liver somatic index (LSI) was calculated by normalizing the liver weight by the final body weight [29]. Water content was also measured using fresh hepatic tissue. First, the liver was dried at 60 °C for 96 h to obtain the dry liver weight. The water content (%) resulted from the difference between wet and dry liver weight [30].

2.4 Functional markers of hepatic damage

Blood samples (n = 5/ group) were centrifuged at 2,000 x*g* for 15 min. Moreover, frozen fragments of liver tissue (100 mg; n = 5/ group) were homogenized in 1 mL of phosphate buffer saline (PBS; pH 7.4, 0.2 M) and centrifuged at 2,000 x*g* for 10 min at 4 °C) [31]. Then, serum samples and the supernatant of frozen liver tissues were used to assess the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and

albumin using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) following the manufacturer's instructions.

2.5 Oxidative/nitrosative stress markers in the liver

For this analysis, 100 mg of liver tissue (n = 5/ group) were homogenized in PBS and centrifuged at 3,500 x*g* for 10 min at 4 ºC. The supernatant was used to quantify the activity of superoxide dismutase [32], catalase [33], glutathione-S-transferase [34], and ferric reducing/antioxidant power (FRAP) assay [35]. Total protein concentration was measured following the Lowry method [36], whereas the occurrence of lipid peroxidation was determined by measuring malondialdehyde levels [37]. Nitric oxide levels were determined by detecting nitrite/nitrate levels in the liver following the Griess methodology [38].

2.6 Histological analysis of hepatic tissue

Liver fragments (n = 5/group) were immersed in 10% formalin solution for 24 h. Fragments were dehydrated in crescent series of ethanol (70, 80, 90, and 100%) and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Sections at the thickness of 3 μm were obtained in semi-series, using one in every 20 sections, and stained with hematoxylin and eosin (HE) for histopathological and stereological analyses. Other sections were stained with periodic acid Schiff (PAS) method for determining tissular glycogen presence, modified toluidine blue staining (TB) for detecting mast cells, and Sirus red for identifying collagen fibers [39]. Slides were mounted with Entellan (Merck, Germany) [40,28]. Histological fields, and the acquisition of histological images, were analyzed using a photomicroscope (Olympus BX53, Tokyo, Japan).

The two-stage stereological analysis of liver components' volume was performed in 20 histological images (200 x magnification), using a test system of 266 points in standard test areas (2.38×10^6 μm^2). Coincident points were recorded in the hepatic parenchyma (sinusoidal capillaries and hepatocytes) and stroma (biliary ducts, venous and arterial vessels, and periadventitial connective tissue) from HE-stained sections [41,42]. The results obtained for the parenchyma volume were used as reference values to perform the second step of this analysis. The volume of each parenchyma component was obtained by counting 266 intersection points in standard test areas (6.05×10^5 μm^2) per animal projected onto 20 images of the parenchyma (400 x magnification). Matching points were counted on the hepatocyte nuclei and cytoplasm, sinusoidal capillaries, and Kupffer cells. Glycogen-containing cytoplasmic inclusions, observed in PAS-stained sections, were quantified by counting 266 intersection points projected onto 20

histological images (200 x magnification) per animal [43,28]. Moreover, collagen fibers stained with Sirius red staining, associated with hepatic fibrosis, were quantified using a grid of 266 intersection points projected onto 20 histological images (200 x magnification) per animal. Finally, 20 histological images (200 x magnification) per animal were used to count matching points on inflammatory infiltrate, sinusoidal dilatation, congestion, and hydropic degeneration observed in HE-stained sections (200 x magnification) per animal [28]. All stereological analyses were performed using ImageJ software (National Institutes of Health), and the volume of each component was calculated using the following formula: $V = PP / PT \times Vo$, where PP represents the number of points over the interest structure, PT is the total test points in the histological area, and Vo is the organ volume [28,43,44,45].

The quantification of mast cells in the liver was performed using ten histological fields stained with toluidine blue. Their number was determined in each histological field with a total area (AT) of 1.96 mm^2 and determined following the formula $QA = \Sigma$ mast cells/AT [43,46].

2.7 Activities of total, Ca^{2+}, Na^{+}/K^{+}, and Mg^{2+} ATPases

Liver tissue (100 mg) was homogenized in Tris-HCl buffer (0.1 M, pH 7.4) and centrifuged at 1,500 x*g* for 10 min at 5 ◦C. The supernatant was used for the determination of the total [47], Ca^{2+} [48], Na^{+}/K^{+} [49], and Mg^{2+} [50] ATPase activities. The ATP solution (0.01 M) was used as a substrate to generate free phosphate by the activity of ATPases. The reaction was arrested by adding 500 μL of a cold solution of 10% TCA. The tubes were centrifuged at 1,500 xg for 10 min. Finally, the supernatant was used to measure the phosphorous content using a biochemical kit (Bioclin Laboratories, Belo Horizonte, MG, Brazil) as described by the manufacturer's instructions. The pellet, in turn, was used to quantify the level of total proteins by the Bradford method [51]. The ATPase activity was expressed as micrograms of phosphorous liberated per minute per milligram of protein.

2.8 Statistical analysis

Results had their normality evaluated by the Shapiro-Wilk test. Later on, they were analyzed by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. Differences were considered significant when $P < 0.05$. The statistics and graphics were performed using the GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as means ± standard deviation (mean ± SD).

3. Results

3.1 Biometry and biochemical analysis

Rats treated with eugenol showed no alteration in their clinical signs and body and liver weights, regardless of the concentration tested ($p > 0.05$; Table 1). Blood glucose levels were lower in animals treated with 20 and 40 mg Kg^{-1} eugenol than in control rats ($p < 0.05$; Table 1). Also, rats receiving 20 and 40 mg Kg^{-1} eugenol showed a higher percentage of water content in their liver than the control animals ($p < 0.05$; Table 1). Further, serum and hepatic ALT levels did not change after eugenol treatment ($p > 0.05$; Fig. 1). On the other hand, animals treated with 20 and 40 mg Kg^{-1} eugenol presented lower serum ALP levels and higher serum AST levels than control animals ($p < 0.05$; Fig. 1). Within the liver, eugenol-treated rats with 20 and 40 mg Kg^{-1} showed high levels of ALP and low levels of AST compared to their controls ($p < 0.05$; Fig. 1). Ultimately, albumin levels were lower in the serum and liver of rats treated with 20 and 40 mg Kg^{-1} eugenol than in control animals ($p < 0.05$; Fig. 1).

Table 1. Body and liver weights, blood glucose levels, and hepatic water content in Wistar rats treated with eugenol for 60 days.

		Eugenol		
Parameters	Control	10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Body weight	383.8 ± 32.9	386.2 ± 29.1	399.4 ± 11.9	386.0 ± 22.7
Liver weight (g)	11.42 ± 0.82	10.70 ± 0.93	11.40 ± 0.93	10.64 ± 1.13
Liver somatic index (%)	2.98 ± 0.26	2.78 ± 0.27	2.85 ± 0.19	2.75 ± 0.23
Glucose (mg dl^{-1})	168.8 ± 37.8	118.0 ± 11.5	109.0 ± 5.1*	100.8 ± 32.1*
Water content (%)	30.6 ± 1.83	31.98 ± 1.73	35.10 ± 1.92*	37.77 ± 1.61*

Mean ± SD. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. *Significant differences ($p < 0.05$) between control and treated groups by Tukey's test ($n = 5$ rats/group).

3.2 Hepatic oxidative/nitrosative stress markers

The activity of superoxide dismutase was higher in rats receiving 40 mg Kg^{-1} eugenol, whereas FRAP increased in animals treated with 20 and 40 mg Kg^{-1} eugenol ($p < 0.05$; Fig. 2). In contrast, eugenol did not alter the activity of catalase and glutathione S-transferase, regardless of its concentration ($p > 0.05$; Fig. 2). Additionally, the liver of animals treated with 20 and 40mg Kg^{-1} eugenol exhibited higher malondialdehyde and nitric oxide levels than the liver of control rats ($p < 0.05$; Fig. 2).

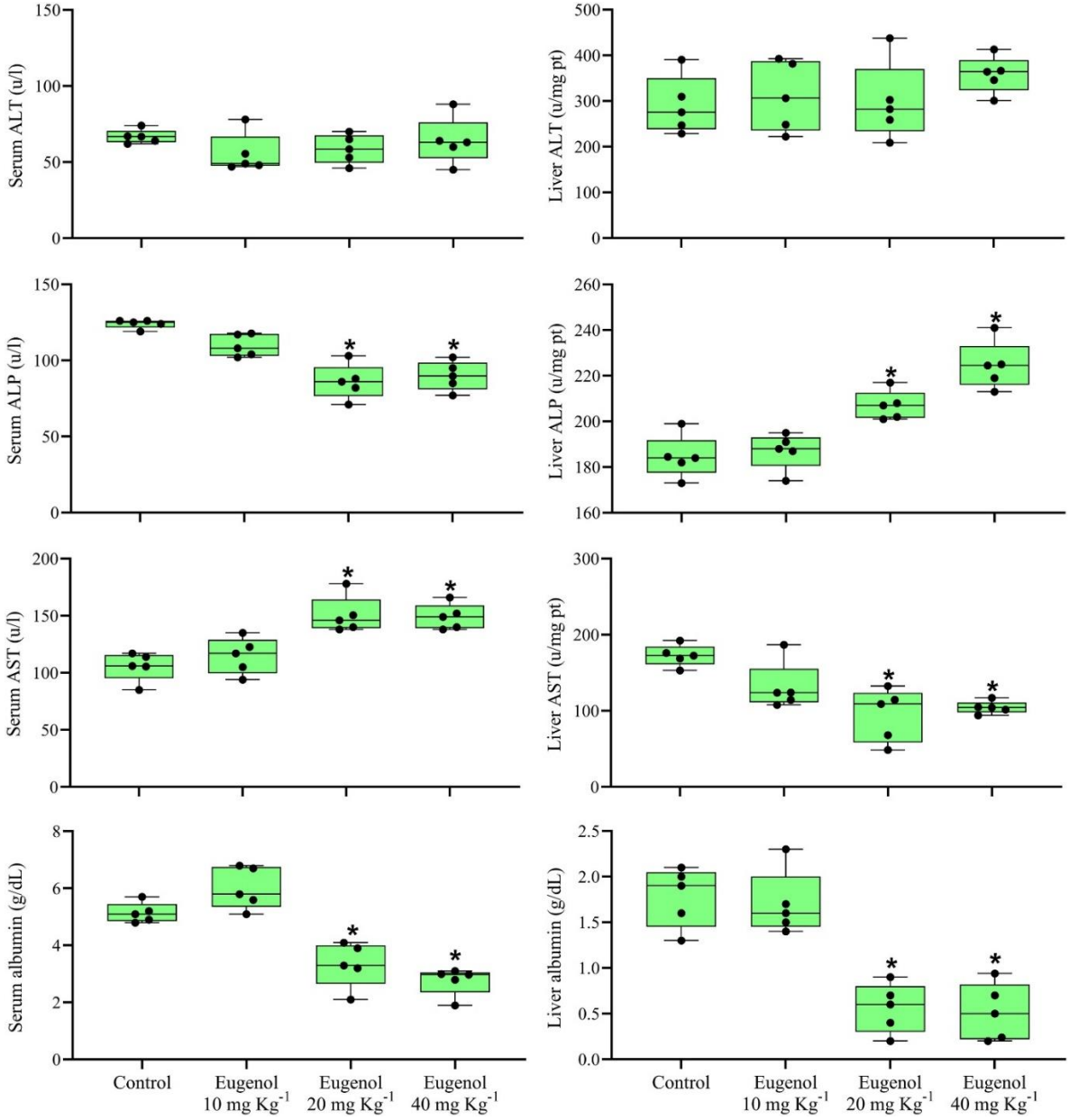

Fig. 1. Serum and hepatic levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and albumin from Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. The box represents the interquartile interval with the median indicated (horizontal line), whiskers represent the minimum and maximum data, and dots represent each data point. *Significant difference ($p < 0.05$) between control and treated groups by Tukey's test. (n = 5 rats/group).

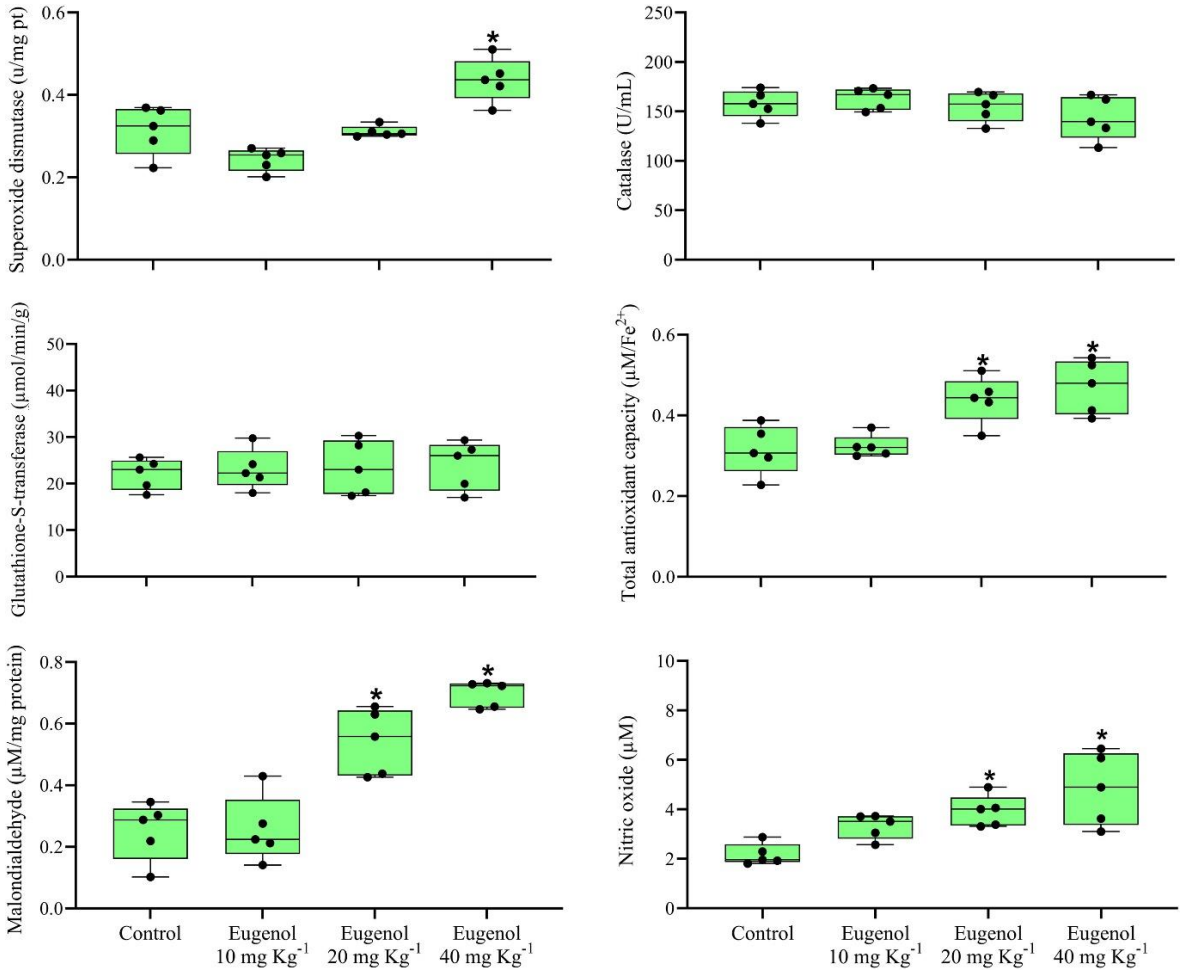

Fig. 2. Antioxidant enzymes activity and oxidative/nitrosative stress markers in the liver from Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. The box represents the interquartile interval with the median indicated (horizontal line), whiskers represent the minimum and maximum data, and dots represent each data point. *Significant difference ($p < 0.05$) between control and treated groups by Tukey's test. (n = 5 rats/group).

3.3 Liver histology

The liver of control rats and animals receiving 10 mg Kg^{-1} eugenol showed a normal histological appearance, with lobules composed of hepatocytes arranged into cords and surrounded by sinusoidal capillaries toward central veins (Fig. 3). In contrast, the liver of rats treated with 20 and 40 mg Kg^{-1} eugenol presented histological changes, including a periportal mixed inflammatory infiltrate, congestion, sinusoidal dilatation, and hydropic degeneration (Fig. 3). The volume of these four histological alterations was higher in animals receiving 20 and 40 mg Kg^{-1} eugenol than in their controls ($p < 0.05$; Fig. 3). Moreover, the liver of rats receiving the higher doses of eugenol (20 and 40 mg Kg^{-1}) presented a higher volume of stroma,

mainly venous and arterial vessels, and sinusoidal capillaries when compared to the liver of control animals ($p < 0.05$; Table 2). Animals treated with 40 mg Kg^{-1} eugenol presented a reduced volume of cytoplasmic hepatocytes ($p < 0.05$; Table 2), whereas the volume of hepatocyte nucleus and macrophages remained unchanged between experimental groups ($p > 0.05$; Table 2).

Table 2. Volume of liver components from Wistar rats treated with eugenol for 60 days.

Parameters	Control	Eugenol		
		10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Stroma	300.4 ± 19.08	287.8 ± 18.40	342.9 ± 11.43*	365.9 ± 16.16*
Biliary ducts (mm^3)	44.26 ± 7.86	43.90 ± 6.30	49.32 ± 4.54	50.96 ± 7.72
Venous and arterial vessels (mm^3)	202.5 ± 8.05	191.9 ± 13.44	230.6 ± 12.83*	256.5 ± 13.48*
Connective tissue (mm^3)	53.58 ± 4.64	52.05 ± 7.52	62.95 ± 4.75	58.52 ± 4.42
Parenchyma	7542.0 ± 171.5	7443.9 ± 173.9	7725.0 ± 261.4	7722.8 ± 218.2
Sinusoidal capillaries (mm^3)	1633.1 ± 218.5	1836.7 ± 118.3	2095.3 ± 196.1*	2282.6 ± 284.1*
Cytoplasm hepatocyte (mm^3)	5184.9 ± 325.9	4854.7 ± 186.4	4855.2 ± 151.4	4650.2 ± 299.5*
Nuclei hepatocyte (mm^3)	694.2 ± 36.68	681.0 ± 29.34	708.5 ± 25.18	719.7 ± 24.03
Macrophages (mm^3)	75.83 ± 8.23	70.98 ± 8.99	66.57 ± 7.48	70.17 ± 11.48

Mean ± SD. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. *Significant differences ($p < 0.05$) between control and treated groups by Tukey's test (n = 5 rats/group).

Moreover, there was an increase in the volume of glycogen-containing cytoplasmic inclusions and collagen deposition in the liver of rats treated with 20 and 40 mg Kg^{-1} eugenol compared to their controls ($p < 0.05$; Fig. 4). The number per area of mast cells only increased in the liver of rats treated with 40 mg Kg^{-1} eugenol ($p < 0.05$; Fig. 4).

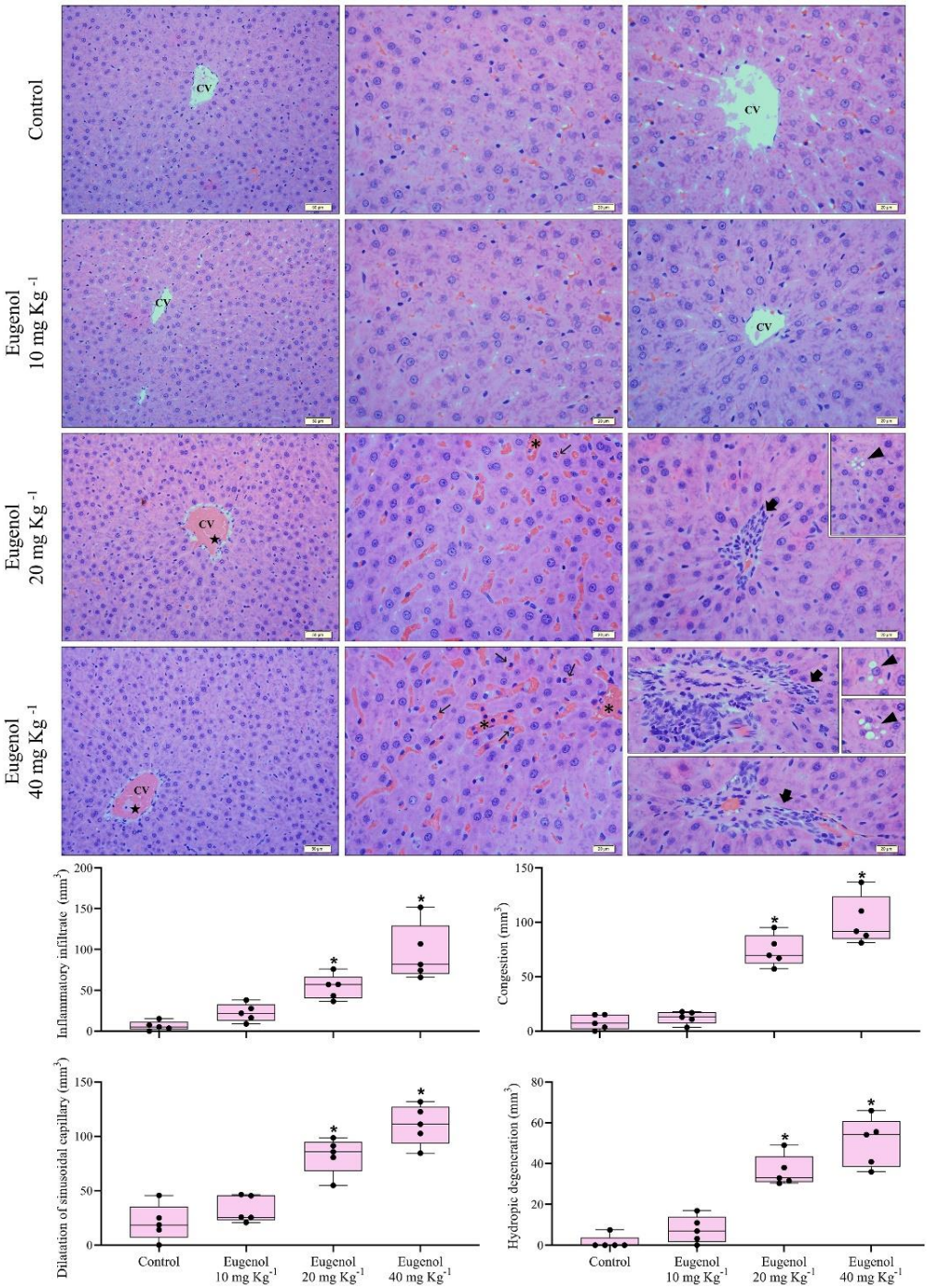

Fig. 3. Photomicrographs of liver pathologies in Wistar rats treated with eugenol, showing congestion (star), sinusoidal dilatation (asterisk), leukocyte cells in sinusoidal capillary (thin arrows), periportal mixed inflammatory infiltrate (thick arrows), and hydropic degeneration (triangle). CV: central vein. HE staining. The box plot show the results of stereological analysis. The box represents the interquartile interval with the median indicated (horizontal line), whiskers represent the minimum and maximum data, and dots represent each data point. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. *Significant differences ($p < 0.05$) between control and treated groups by Tukey's test (n = 5 rats/group).

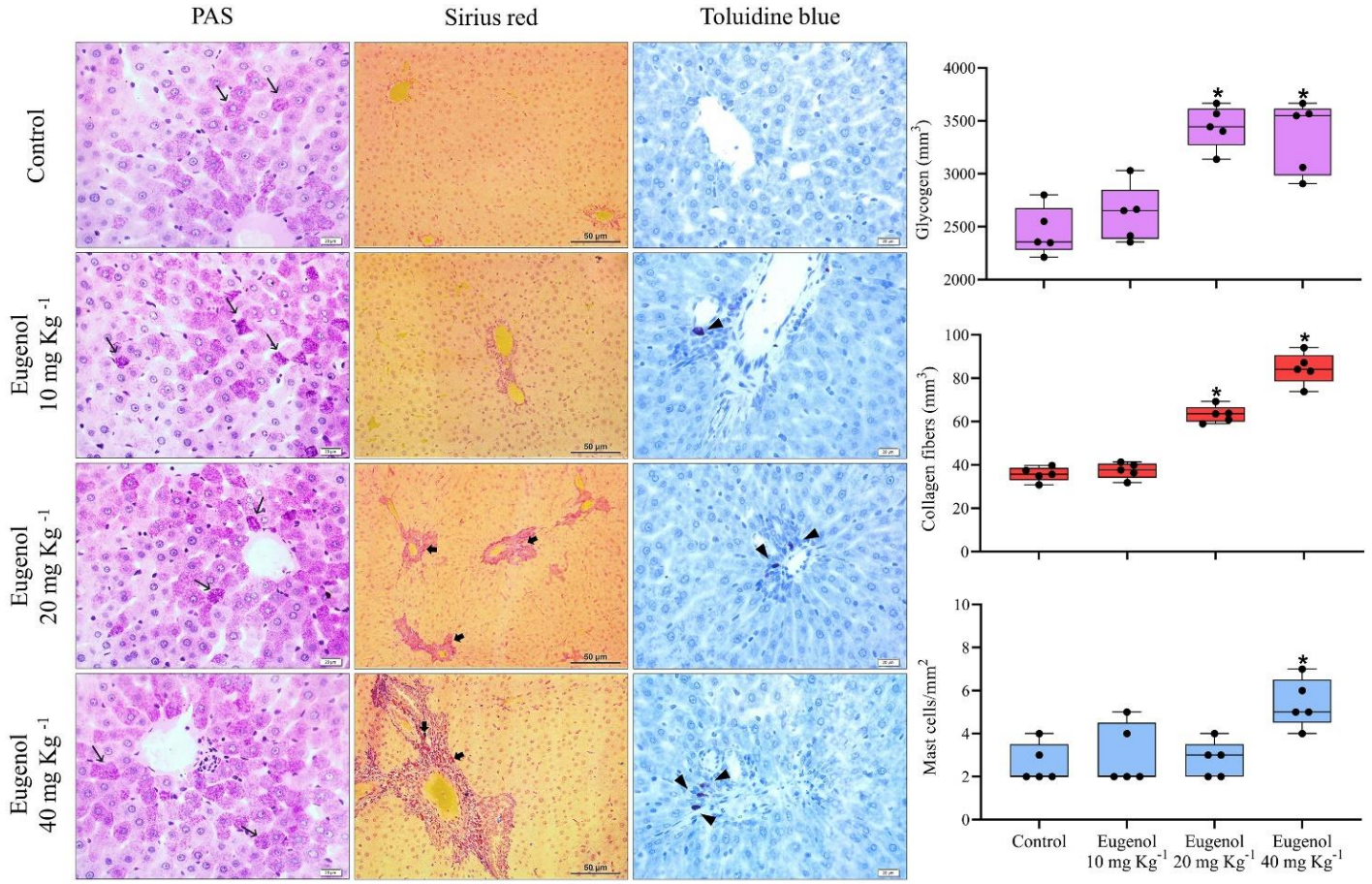

Fig. 4. Histological and stereological analyses of the liver from Wistar rats treated with three concentrations of eugenol, showing the presence of glycogen cytoplasmic inclusions (thin arrows; PAS staining), mast cells (triangle; toluidine blue staining), collagen fibers (thick arrows; Sirius red staining). The graphics show the results of stereological analysis. The box represents the interquartile interval with the median indicated (horizontal line), whiskers represent the minimum and maximum data, and dots represent each data point. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. *Significant differences ($p < 0.05$) between control and treated groups by Tukey's test (n = 5 rats/group).

3.4 Activity of total, Ca^{2+}, Mg^{2+}, and Na^{+}/K^{+} ATPases

Rats receiving 20 and 40 mg Kg^{-1} eugenol showed a reduced activity of Na^{+}/K^{+} ATPase pump in the liver tissue ($p < 0.05$; Table 3), with no alteration in the total, Ca^{2+}, and Mg^{2+} ATPases activity ($p > 0.05$; Table 3).

Table 3. Activity of total, Ca^{2+}, Na^{+}/K^{+} and Mg^{2+} ATPases in the liver of Wistar rats treated with eugenol for 60 days.

		Eugenol		
Parameters (Pi/min/mg protein)	Control	10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Total ATPase activity	0.39 ± 0.07	0.36 ± 0.08	0.38 ± 0.13	0.41 ± 0.08
Ca^{2+} ATPase activity	0.07 ± 0.03	0.07 ± 0.02	0.07 ± 0.03	0.08 ± 0.03
Na^{+}/K^{+} ATPase activity	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.01*	0.03 ± 0.01*
Mg^{2+} ATPase activity	0.30 ± 0.04	0.25 ± 0.04	0.25 ± 0.07	0.30 ± 0.03

Mean ± SD. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. *Significant differences ($p < 0.05$) between control and treated groups by Tukey's test. ($n = 5$ rats/group).

4. Discussion

Our results provided the first evidence of the impact of daily ingestion of eugenol in the liver of healthy rats after 60 days of treatment. High doses of eugenol (20 and 40 mg Kg^{-1}) caused morphological and functional disruption in this organ. However, animals receiving the lowest concentration (10 mg Kg^{-1}) did not present hepatic damage. Previous studies indicated that eugenol acted as a protective agent in animals with pre-existent diseases in a dose-dependent manner. For instance, 10 mg Kg^{-1} eugenol mitigated hepatic damages caused by ischemia injury, whereas 100 mg Kg^{-1} of this phenolic compound did not protect the rat liver and caused tissue alterations [52]. Harb et al. [53] reported that eugenol administration for four weeks reduced LDL cholesterol and hepatic steatosis in hypercholesterolemic rats. These authors concluded that 10 mg Kg^{-1} was more effective than 100 mg Kg^{-1}, possibly due to hepatotoxicity associated with the higher dose [53]. Rats submitted to gastric ulcer induction using acetic acid and ethanol presented an improvement in the mucosa histology and antioxidant enzyme activity after treatment with 1, 5, and 10 mg Kg^{-1} eugenol, but not 100 mg Kg^{-1}, which aggravated gastric lesions [54,55].

We observed that 20 and 40 mg Kg^{-1} eugenol reduced blood glucose levels. The elevation of liver glycogen content may have influenced this result. Indeed, treatment with 40 mg Kg^{-1} eugenol for 15 weeks [56] and 80 mg Kg^{-1} for 30 days [57] reduced blood glycemic levels of diabetic rodents. In animals with this metabolic disease, eugenol prevented glucose intolerance by stimulating insulin secretion and hexokinase activity in the liver, which may have improved glucose consumption for energy production and hepatic glycogen formation [19,58,59]. Otherwise, the intracellular accumulation of glycogen in the liver of healthy animals may be a sign of hepatotoxicity and disturbance in the carbohydrate metabolism [60]. Interestingly, serum glucose and liver glycogen content did not alter in animals treated with 10 mg Kg^{-1} eugenol, as reported by Srinivasan et al. [59] and Prasad et al. [61]. Thus, we may suggest that high concentrations of eugenol can impair glucose homeostasis in healthy individuals.

Our results revealed that 20 and 40 mg Kg^{-1} eugenol increased FRAP, malondialdehyde, and nitric oxide levels. Additionally, the superoxide dismutase activity increased in animals treated with 40 mg Kg^{-1}. These findings indicate that both high doses of eugenol acted as prooxidants and generated oxidative and nitrosative stress. Superoxide dismutase is a well-known metalloproteinase involved in the dismutation of superoxide anions (O_2^-) to O_2 and hydrogen peroxide (H_2O_2), representing the first line of enzymatic defense against ROS [62]. FRAP, in turn, indicates the total amount of non-enzymatic antioxidants [63]. Despite the increase in antioxidant parameters, they did not prevent the formation of byproducts from lipid peroxidation (malondialdehyde) [64] and nitrosative damage (nitric oxide) [65]. High concentrations of eugenol are associated with oxidative stress generation [52,66] because this phenolic compound undergoes direct two-electron oxidations to an electrophilic quinone methide responsible for oxidative damage to cells [8]. Otherwise, eugenol is also a potent antioxidant substance, mainly at low doses [52,55,66]. Its molecular structure presents an abundance of electrons that favors this antioxidant activity by directly donating electrons to reactive oxygen species or reacting to free radicals via hydrogen atom transfer [16].

The oxidative stress generated by high doses may be involved in the Na^+/K^+ ATPase inhibition in the liver of eugenol-treated animals. The determination of ATPases is relevant by indicating potential changes in membranes under pathological conditions [67]. The activity of Na^+/K^+ ATPase is highly susceptible to free radicals and membrane lipid peroxidation [68,69]. Besides, any decrease in Na^+/K^+ ATPase activity will lead to obstacles in intracellular energy production and ion transport, with consequent disturbance in cell function and increment in cell damage [70]. Previous studies showed that Na^+/K^+ ATPase in rats is more sensitive than Mg^{2+}

ATPase and Ca^{2+}ATPase [71,72], which may explain the maintenance of Mg^{2+} ATPase and Ca^{2+}ATPase activities observed in this study.

Both occurrences of oxidative stress and dysregulation of Na^{+}/K^{+} ATPase may cause structural and functional abnormalities in the liver [73,74,75]. The low volume of cytoplasm hepatocytes accompanied a significant elevation of AST in the serum of rats treated with 20 and 40 mg Kg^{-1} eugenol. These results indicate possible damage to hepatocytes' membranes. Meanwhile, ALT levels did not alter in serum and liver tissue. Serum ALT and AST are markers of hepatocellular injury, but AST levels are mainly higher during hepatic injuries [76,77]. The enzyme ALP, in turn, is another suitable marker of liver disorders [78]. Its levels were high in the tissue, as reported in hepatic diseases [78,79,80], and lower in the serum of rats treated with 20 and 40 mg Kg^{-1}. Unlike in humans, serum ALP isozymes in rats are mainly of the bone type [81]. Therefore, the decrease in serum ALP activity might be associated with the eugenol effect in osteoblasts [82]. Decreased hepatic and serum albumin levels also corroborate the functional tissue damage caused by treatment with 20 and 40 mg Kg^{-1} eugenol. One of the most important causes of reduced albumin levels is the reduced protein production by the injured liver [83]. We also observed an occurrence of hydropic degeneration in this study, which may indicate an impairment in the cell membrane transport system that culminated in excess water within the cell [84]. This pathology may explain the increase in water content in the liver tissue of animals treated with high doses of eugenol. Collectively, these findings indicated that 20 and 40 mg Kg^{-1} eugenol exerted adverse effects on hepatocytes by altering biochemical and histological parameters.

The increase in the volume of sinusoidal capillaries observed in rats treated with 20 and 40 mg Kg^{-1} might be related to sinusoidal dilatation. This finding is relevant because the sinusoids play a pivotal role in the blood flow supply to hepatocytes, mainly during hepatic regeneration [28]. In addition, the increased blood vessel volume observed in these animals may be a consequence of the sinusoidal congestion reported here. These vascular disorders can be responsible for cellular extravasation, characterized by inflammatory infiltrate associated with vascular congestion, sinusoidal dilatation, and the high number of mast cells [85,28]. The high levels of nitric oxide detected in the liver of rats treated with 20 and 40 mg Kg^{-1} eugenol may be involved in the etiology of vascular disorders. Nitric oxide plays an important role in modulating hepatic circulation and mediating inflammation under pathological conditions [86]. There is evidence that inflammation and oxidative stress are related to liver fibrosis that, in the end, may lead to organ failure [87,88,89,90]. Taking these findings together, we may suggest that the tissue damage caused by eugenol was related to oxidative stress.

Despite the damages caused by high doses of eugenol, this compound was safe and did not elicit hepatic changes after treatment with 10 mg Kg^{-1} daily for 60 days. Notwithstanding, eugenol ingestion did not positively affect liver biochemistry, morphology, and functionality. The intake of eugenol for aphrodisiac purposes [91], for example, may not lead to liver dysfunctions. Overall, eugenol is not a reactive compound *per se*, being metabolized within the hepatocytes. Distinct byproducts are produced after animal exposure to low and high concentrations of this compound. Sutton et al. [92] reported that high concentrations of this compound yield glucuronides in rats, whereas sulfates are metabolites formed in animals that ingested low concentrations of eugenol. Moreover, in *in vitro* cultured rat liver cells, the toxic effects caused by exposure to high concentrations of eugenol were attributed to the metabolite quinone methide [21]. Thus, we may suggest that low concentrations of eugenol did not generate toxic metabolites and not alter liver parameters in healthy individuals.

5. Conclusion

Our results showed that eugenol affected hepatic parameters in a dose-dependent manner. High doses (20 and 40 mg Kg^{-1}) of this phenolic compound reduced blood glucose levels and Na^{+}/K^{+} ATPase pump activity. They also increased hepatic glycogen content, FRAP, nitric oxide, and malondialdehyde. Consequently, liver tissue presented structural and functional damage with potential implications for hepatic dysfunction. Eugenol treatment using 10 mg Kg^{-1} caused no deleterious effects on liver histology and functions. We may conclude that 10 mg Kg^{-1} is a safe dose for use in future studies focused on the therapeutic effects of eugenol. Additional studies should investigate the impact of eugenol on molecular pathways and gene expression involved in liver function.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding this article.

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3. CHAPTER 2

Effect of eugenol on the pancreas, submandibular, and sublingual glands of Wistar rats: a biochemical, oxidative, and morphological study

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Effect of eugenol on the pancreas, submandibular, and sublingual glands of Wistar rats: a biochemical, oxidative, and morphological study

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Highlights

- Eugenol at 40 mg kg^{-1} decreased lipase and increase pancreatic amylase activity.
- The submandibular gland was more sensitive to the effects caused by eugenol.
- In the sublingual gland, eugenol changed only NO levels and Mg^{2+} ATPase activity.
- Eugenol modulates the oxidative profile and ATPases activities in the pancreas.

Abstract

Objective: We evaluated the effects of eugenol on histological, enzymatic, and oxidative parameters in the pancreas, submandibular, and sublingual glands of healthy Wistar rats.

Design: Twenty-four adult rats were assigned into four groups (n = 6/group). Control rats received 2% Tween-20 (eugenol vehicle), whereas the other animals received 10, 20, and 40 mg kg^{-1} eugenol through gavage daily for 60 d. Salivary and pancreatic glands were weighed and preserved fixed for microscopic analysis and frozen for *in vitro* assays.

Results: Eugenol did not alter glands' weight and serum amylase activity regardless of the concentration. The highest dose of eugenol caused an increase in pancreatic amylase activity and a reduction of lipase activity from serum and pancreas. Eugenol at 40 mg kg^{-1} diminished the activity of SOD and FRAP in the submandibular gland and CAT and FRAP in the sublingual gland. However, it did not exert any effect on GST regardless of the gland. Additionally, 40 mg kg^{-1} eugenol increased MDA levels in pancreatic and submandibular glands and NO levels in the sublingual. The concentrations of eugenol induced distinct responses in the glands regarding the activity of Na^{+}/K^{+}, Mg^{2+}, and total ATPase activity. They also affected histomorphometrical and histochemistrical parameters in the submandibular gland only.

Conclusions: Results indicated that 40 mg kg^{-1} eugenol altered most of the biochemical and oxidative parameters of digestive glands. Only submandibular glands presented histological changes after eugenol exposure suggesting potential implications for its function. The sublingual gland was the less responsive gland to eugenol action.

Keywords: salivary glands; histomorphometry; toxicology; clove.

1. Introduction

Eugenol is a light-yellow oil mainly found in essential oils and extracts from *Syzygium aromaticum*, previously known as *Eugenia caryophyllata* (Bendre & Rajput, 2016; Laghari & Khan, 2022). This phenolic compound was first isolated in 1929, with commercial production beginning in the United States eleven years later (Kamatou et al., 2012). Eugenol has a clove odor and pungent taste interesting for food purposes, besides multiple pharmacological properties (Silva et al., 2018) including antioxidant (Nagababu et al., 2010), neuroprotective (Kabuto & Yamanushi, 2011), anti-inflammatory (Lopes et al., 2018), anticancer (Jaganathan & Supriyanto, 2012), and antidiabetic activities (Carvalho et al., 2021).

The antidiabetic potential of eugenol is related to its ability to inhibit the activity of digestive amylases involved in energy metabolism (Singh et al., 2016). Amylases are responsible for the hydrolysis of starch, initiating the digestion of carbohydrates in the oral cavity and, subsequently, in the intestine. In humans, the main sources of amylase are salivary glands and the exocrine portion of the pancreas (Bonnefond et al., 2017). Lipase is another important digestive enzyme involved in the hydrolysis of triglycerides into glycerol and free fatty acids (de-Madaria et al., 2021). Despite being mainly secreted by the pancreas, small rates of lipolysis in the oral cavity may occur due to the presence of enzymes of microbial or endogenous origin, produced in the oral cavity by the major or minor salivary glands or even due to refluxed gastric contents (Besnard et al., 2016; Lai et al., 2019). Biochemical assays have shown that eugenol inhibits the activity of amylases and lipases *in vitro* (Adefegha & Oboh, 2012; Jelenković et al., 2014; Oboh et al., 2015; Tahir et al., 2016). In rats, other studies demonstrate a protective effect of eugenol against biliopancreatic ligation and L-arginine-induced pancreatitis (Sowjanya et al., 2012; Tsaroucha et al., 2021). Both studies attributed this therapeutic effect to free radical scavenging promoted by eugenol.

Nevertheless, the role of eugenol in the pancreas and salivary glands of healthy animals has not been elucidated yet. Humans use eugenol in perfumes, mouthwashes, dental analgesics, and dental materials, such as impression materials, filling materials, dental cement, endodontic sealers, periodontal dressing materials, and dry socket dressings (Sarrami et al., 2002; Amiri et al., 2008; Aburel et al., 2021). Adverse effects of eugenol in the oral cavity have been previously reported, varying from localized skin irritation to allergic contact dermatitis (Jacobesen & Henste-Pettersen, 1989; Kanerva et al., 1998; Sarrami et al., 2002). Even though, little is known about the effects of eugenol exposure on the metabolism of salivary glands responsible for physiological balance in the oral environment. Once gland tissues are susceptible to exogenous agents, salivary glands can be negatively impaired through their toxic potential, long exposure

duration, direct contact with the mucous membrane, and their absorption in the circulating blood, resulting in volumetric, morphological, and even functional impairment of the organ (Aragão et al., 2017; Carvalho et al., 2022a).

In this scenario, it is relevant to better understand the effects of eugenol on the pancreas, submandibular, and sublingual glands, verifying whether this phenolic compound exerts a potential deleterious or therapeutic role in their functions and morphology. Therefore, this study aimed to evaluate the effects of eugenol (10, 20, and 40 mg kg^{-1}) on histological, biochemical, and oxidative parameters of the pancreas, submandibular, and sublingual glands of healthy Wistar rats.

2. Material and methods

2.1 Animals and ethics statement

This study is part of a detailed and comprehensive work concerning the effects of eugenol on healthy adult rats (Carvalho et al., 2022a; Carvalho et al., 2022b). Twenty-four male Wistar rats (70 days old; 230-250 g) were provided by the Central Animal Facility of the Universidade Federal de Viçosa (UFV) and housed individually in polypropylene cages. The animals were maintained under controlled photoperiod (12-12h light/dark cycle) and temperature (21°C) receiving rat chow and drinking water *ad libitum*. This study was conducted strictly following the ethical guidelines of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) and approved by the Ethics Committee of Animal Use of UFV (protocol 61/2021).

2.2 Experimental design

The animals were randomly divided into four experimental groups (n=6/group). Rats from the control group ingested 2% Tween-20 (vehicle) diluted in 1 mL distilled water by gavage daily for 60 d. The other animals were exposed to 10, 20, and 40 mg kg^{-1} eugenol (Sigma Aldrich Co., St. Louis, MO) diluted in 1 mL of the vehicle by gavage daily for 60 d. The treatment period lasted 60 days. The concentrations and the period of treatment were based on previous studies (Carvalho et al., 2021).

2.3 Euthanasia, tissue collection, and biometry

On day 61 of the experiment, animals were weighed and euthanized by deep anesthesia with ketamine hydrochloride (150 mg kg^{-1} i.p.) and xylazine (10 mg kg^{-1} i.p.), followed by cardiac puncture. Blood samples were collected to obtain the serum for biochemical analysis.

The pancreas, submandibular and sublingual glands were removed and weighed (absolute and relative weight). The relative weight of each organ was calculated by normalizing the organ weight by the final body weight. Fragments from each gland were immersed in 10 % formalin solution for histological analyses, whereas other fragments were frozen in liquid nitrogen and stored at -80 °C for assessing biochemical and oxidative stress assays.

2.4 Activity of serum and pancreatic amylase and lipase

Blood samples (n = 6/group) were centrifuged at 2000 × g for 15 min to obtain the serum. In addition, fragments of frozen pancreatic tissue (n = 6/group) were homogenized in phosphate buffer (PBS; pH 7.4, 0.2 M) and centrifuged at 2000 × g for 10 min at 4 °C to recover the supernatant. Both serum and supernatant samples were used to evaluate the activity of amylase and lipase enzymes. Amylase activity was determined by the colorimetric method described by Caraway et al. (1959), using a substrate of starch and iodine/potassium iodide as a color reagent. Spectrophotometry measured absorbances at 660 nm, and amylase activity was expressed as residual starch (U/mg of protein). *In turn, l*ipase activity assay was conducted using the 2,3-dimercapto-1-propanol tributyrate (DMPTB)-dithionitrobenzoic (DTNB) method with minor modifications (Furukawa et al., 1982). Briefly, samples were mixed with DTNB and phenylmethylsulfonyl fluoride (PMSF) and incubated at 37 °C for 2 min. A DMPTB solution was added and incubated at 37 °C for 30 min. The reaction was stopped by adding acetone. Absorbances were measured by spectrophotometry at 410 nm, and lipase activity was expressed as U/mg of protein.

2.5 Adenosine triphosphatase (ATPases) activity in gland tissues

Frozen fragments of the pancreas, submandibular, and sublingual glands (50 mg; n = 6/group) were homogenized in 500 µL of Tris-HCl buffer (100 mM, pH 7.4) and centrifuged at 10,000 ×g for 10 min at 4 ◦C. Aliquots of tissue homogenates were transferred to a centrifuge tube to remove the endogenous phosphate present in samples. For this, a saturated solution of ammonium sulfate was added to a final concentration of 3.2 M for protein precipitation. After resting on ice for 20 min, samples were centrifuged at 10000 ×g at 4°C for 10 min. The supernatant was discarded, and the pellet was resuspended back to its original volume in Tris-HCl buffer (100 mM, pH 7.4) and used to determine the activity of membrane-bound ATPase activities. For each total (Evans, 1969), Ca^{2+} (Hjertén & Pan, 1983), Na^{+}/K^{+} (Bonting et al., 1962), and Mg^{2+} (Ohnishi et al., 1982) ATPase, an incubation medium was prepared in Tris-HCl buffer. ATP solution was used as a substrate to generate free phosphate by the ATPases.

For each reaction, 50 μL of the sample was added and incubated at 37 °C for 30 min. The reaction was arrested by adding 500 μL to a cold solution of 10% TCA. The tubes were centrifuged at 1500 ×g for 10 min. The phosphorous content in the supernatant was determined by a reaction using ammonium molybdate and ascorbic acid as a reductor. The intensity of color developed is proportional to the concentration of phosphorus in the sample. Absorbances were measured by spectrophotometry at 650 nm, and ATPase activity was expressed as micrograms of phosphorous liberated per hour per microgram of protein. The pellet, in turn, was used to quantify the level of total proteins by the Bradford method (Bradford, 1976).

2.6 Antioxidant enzymes activity

Fragments (50 mg) of the pancreas, submandibular, and sublingual glands (n = 6/group) were dissected and homogenized in PBS (pH 7.4). The homogenate was centrifuged at 3500 ×g for 10 min at 4 °C. The supernatant was used to analyze the activity of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and ferric-reducing antioxidant power (FRAP). SOD activity was assessed by the pyrogallol autoxidation method based on the ability of this enzyme to catalyze the dismutation of superoxide (O_2^-) into O_2 and hydrogen peroxide (H_2O_2) (Marklund & Marklund, 1974). Absorbances were measured by spectrophotometry at 320 nm. CAT activity was measured as described by Hadwan and Abed (2016) with modifications. The supernatant was incubated with the substrate (50 mM PBS, pH 7.4, with 20 mM H_2O_2). Subsequently, ammonium molybdate was added to stop the reaction, and the absorbance was measured at 374 nm. The values were calculated from a standard curve using a known concentration of H_2O_2. The results of both assays were expressed as U/mL. GST activity, in turn, was measured as described by Habig et al. (1974). The method monitors the formation of the conjugate of CDNB (1-chloro-2,4-dinitrobenzene) with reduced glutathione (GSH). The increase in absorbance was monitored at 340 nm, data were expressed as μM/min. *Finally, a*ntioxidant power was performed using the FRAP method as described by Benzie & Strain (1996). The FRAP assay consisted of a colorimetric measurement of the reduction of ferric-tripyridyltriazine complex (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by sample antioxidants. Absorbances were measured by spectrophotometry at 570 nm and results were expressed as μM/Fe^{2+}.

2.7 Oxidative and nitrosative metabolites

Malondialdehyde (MDA), an important marker for monitoring lipid peroxidation, and nitric oxide (NO) production, involved in nitrosative stress, was measured in the same

homogenate by biochemical assays. MDA was quantified using the thiobarbituric acid reactive substance (TBARS) solution added into aliquots of supernatant and in a water bath, cooled, and centrifuged (Buege & Aust, 1978). The supernatant was used for estimation at 535 nm in a spectrophotometer, and MDA levels were expressed in μM/mg protein. In addition, NO production was quantified indirectly through the nitrite/nitrate (NO_2/NO_3) content by the standard Griess reaction (Tsikas, 2007). The homogenate supernatant was incubated with an equal volume of Griess reagent at room temperature. The absorbance was measured at 570 nm, and the nitrite concentration was calculated regarding the standard curve using a known concentration of sodium nitrite ($NaNO_2$). Levels of NO are expressed in μM.

2.8 Histological processing

Fragments of the pancreas, sublingual, and submandibular glands fixed in 10 % formalin solution for 24 h underwent dehydration processing with ethanol (70, 80, 90, and 100 %) for embedding in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Histological slides containing sections with 3 μm thickness were stained with toluidine blue, mounted with Entellan (Merck, Germany), and analyzed under light microscopy. Additionally, histological sections of submandibular and sublingual glands were stained with periodic acid-Schiff (PAS), which labels neutral mucins (magenta color), and alcian blue (AB) at pH 2.5 that stain acidic mucins (blue color). Tissue regions labeled with the mixture of these two stains (PAS+AB) evidence the presence of both acidic and neutral mucins (dark purple color) (Cerri & Sasso-Cerri, 2003). The acquisition of histological images was made in a photomicroscope (Olympus BX53, Tokyo, Japan).

2.9 Morphometric analyses

The morphometries were performed on 20 histological images (200x magnification) in the ImageJ software (National Institutes of Health) using the "free hands" tool to delimit the areas occupied by parenchymal structures (ducts and acini). The stromal area was considered the total image area minus the parenchyma area. The total area of the image was quantified at 100%. After averaging the images, the resulting values were submitted to a rule of three to calculate the percentage of each structure analyzed (Santos et al., 2022). In sections of the submandibular and sublingual glands stained with PAS + AB, mucin detection was determined on 20 histological images (200x magnification) per animal, using the ImageJ color threshold plug-in (1.43h, Wayne Rasband WS, US National Institute of Health, NIH, Bethesda, MD, http://rsb.info.nih.gov/ij/). Values were normalized by dividing the total number of positive

pixel counts for acidic (AB^+), neutral (PAS^+), or mixtures of mucins (PAS^+/ AB^+) by the total pixel count for the sampled area. These values were expressed as percentages (Rose et al., 2011).

2.10 Statistical analysis

The normality of the results was evaluated by the Shapiro-Wilk test. Data with normal distribution were analyzed by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. Differences were considered significant when $p < 0.05$. The GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) was used for the statistics and graphics creation. Results were expressed as mean ± standard deviation (mean ± SD).

3. Results

3.1 Biometry and biochemical analysis

Rats treated with the three concentrations of eugenol did not show differences in the absolute or relative weights of the pancreas, submandibular, and sublingual glands when compared to control animals ($p > 0.05$; Table 1). Moreover, 10 and 20 mg kg^{-1} eugenol did not affect the activity of any of the serum and pancreatic enzymes analyzed here ($p > 0.05$; Table 1). Conversely, 40 mg kg^{-1} eugenol increased the activity of pancreatic amylase in treated animals, as well as decreased the activity of their serum and pancreas lipases, when compared to control rats ($p < 0.05$; Table 1). Serum amylase activity was not altered with the administration of 10, 20, and 40 mg kg^{-1} eugenol ($p > 0.05$; Table 1).

Table 1. Biometric and biochemical parameters of Wistar rats treated orally with three concentrations of eugenol for 60 d.

Parameters	Control	Eugenol		
		10 mg kg^{-1}	20 mg kg^{-1}	40 mg kg^{-1}
Pancreas				
Weight (g)	0.93 ± 0.20	0.93 ± 0.16	0.95 ± 0.18	0.95 ± 0.18
Relative weight (%)	3.78 ± 0.70	3.73 ± 0.77	3.77 ± 0.84	3.67 ± 0.81
Submandibular gland				
Weight (g)	0.37 ± 0.02	0.38 ± 0.02	0.39 ± 0.04	0.37 ± 0.03
Relative weight (%)	1.51 ± 0.19	1.53 ± 0.16	1.55 ± 0.19	1.43 ± 0.14
Sublingual gland				
Weight (g)	0.13 ± 0.03	0.13 ± 0.03	0.15 ± 0.03	0.14 ± 0.03
Relative weight (%)	0.55 ± 0.17	0.56 ± 0.17	0.60 ± 0.12	0.53 ± 0.11
Serum amylase (U/l)	511.0 ± 18.6	515.3 ± 9.6	528.6 ± 15.7	536.3 ± 12.7
Pancreatic amylase (U/mg protein)	19.5 ± 3.6	21.5 ± 3.7	19.7 ± 4.4	26.3 ± 1.0*
Serum lipase (U/l)	14.1 ± 4.6	10.4 ± 3.7	8.99 ± 2.6	7.49 ± 1.6*
Pancreatic lipase (U/mg protein)	1.02 ± 0.13	0.94 ± 0.19	0.94 ± 0.14	0.74 ± 0.16*

Mean ± SD. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. *Significant differences ($p < 0.05$) between control and eugenol groups by one-way ANOVA and Tukey's test (n = 6 rats/group).

3.2 Activity of total, Ca^{2+}, Mg^{2+}, and Na^{+}/K^{+} ATPases

The activity of the pancreatic total and Mg^{2+}ATPases increased in rats treated with 20 and 40 mg kg^{-1} eugenol than in the pancreas of control animals ($p < 0.05$; Table 2). No changes in Ca^{2+} and Na^{+}/K^{+} ATPase activities were observed in the pancreas of any eugenol-treated rats ($p > 0.05$; Table 2). The submandibular gland, in turn, presented an elevated activity of total, Na^{+}/K^{+}, and Mg^{2+} ATPases in rats receiving 10 mg kg^{-1} eugenol than in control rats ($p < 0.05$; Table 2). The activity of submandibular total ATPase reduced after the administration of 40 mg kg^{-1} eugenol ($p < 0.05$; Table 2). Only Ca^{2+} ATPase did not exhibit alteration in its activity after eugenol treatment ($p > 0.05$; Table 2). Similarly, the consumption of eugenol did not affect the activity of the sublingual enzymes total, $Ca^{2+}$and Na^{+}/K^{+} ATPases ($p > 0.05$; Table 2). The intake of 40 mg kg^{-1} eugenol reduced the activity of Mg^{2+} ATPase in the sublingual gland ($p < 0.05$; Table 2).

Table 2. Activity of total, Ca^{2+}, Na^{+}/K^{+}, and Mg^{2+} ATPases in the pancreas, submandibular, and sublingual glands of Wistar rats treated orally with three concentrations of eugenol for 60 d.

Enzyme activity (Pi/min/mg protein)	Control	Eugenol		
		10 mg kg^{-1}	20 mg kg^{-1}	40 mg kg^{-1}
Pancreas				
Total ATPase	0.080 ± 0.007	0.077 ± 0.006	0.095 ± 0.004*	0.095 ± 0.004*
Ca^{2+} ATPase	0.024 ± 0.001	0.018 ± 0.004	0.024 ± 0.002	0.022 ± 0.005
Na^{+}/K^{+} ATPase	0.021 ± 0.005	0.017 ± 0.003	0.023 ± 0.004	0.023 ± 0.003
Mg^{2+} ATPase	0.024 ± 0.002	0.024 ± 0.002	0.032 ± 0.006*	0.032 ± 0.005*
Submandibular gland				
Total ATPase	0.063 ± 0.004	0.076 ± 0.003*	0.065 ± 0.008	0.045 ± 0.003*
Ca^{2+} ATPase	0.023 ± 0.007	0.026 ± 0.003	0.024 ± 0.006	0.021 ± 0.004
Na^{+}/K^{+} ATPase	0.015 ± 0.001	0.022 ± 0.001*	0.014 ± 0.003	0.013 ± 0.001
Mg^{2+} ATPase	0.018 ± 0.005	0.028 ± 0.007*	0.020 ± 0.002	0.016 ± 0.002
Sublingual gland				
Total ATPase	0.075 ± 0.013	0.071 ± 0.013	0.069 ± 0.011	0.073 ± 0.005
Ca^{2+} ATPase	0.023 ± 0.006	0.024 ± 0.004	0.023 ± 0.005	0.021 ± 0.007
Na^{+}/K^{+} ATPase	0.018 ± 0.003	0.019 ± 0.004	0.021 ± 0.003	0.018 ± 0.002
Mg^{2+} ATPase	0.021 ± 0.003	0.020 ± 0.004	0.020 ± 0.003	0.015 ± 0.003*

Mean ± SD. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. *Significant differences ($p < 0.05$) between control and eugenol groups by one-way ANOVA and Tukey's test (n = 6 rats/group).

3.3 Oxidative and nitrosative stress markers and antioxidant enzymes

In the pancreas, the activity of SOD was lower, and CAT was higher, in rats receiving 10 mg kg^{-1} eugenol than their controls ($p < 0.05$; Fig. 1). Eugenol treatment did not alter GST activity and FRAP regardless of the concentration administered ($p > 0.05$; Fig. 1). On the other hand, animals treated with 40 mg kg^{-1} eugenol presented high MDA and low NO levels in contrast to the values observed in the pancreas of control rats ($p < 0.05$; Fig. 2). Submandibular glands exhibited lower SOD activity in rats receiving 20 and 40 mg kg^{-1} eugenol than in control animals ($p < 0.05$; Fig. 1). FRAP was lower in rats treated with 40 mg kg^{-1} eugenol ($p < 0.05$; Fig. 1). Moreover, MDA levels increased only in the submandibular gland of rats treated with 40 mg kg^{-1} eugenol ($p < 0.05$; Fig. 2). In this organ, eugenol treatment did not alter CAT and GST activities and NO levels, regardless of concentration ($p > 0.05$; Fig. 1 and 2). Finally, rats treated with 40 mg kg^{-1} eugenol showed higher SOD activity and NO levels in the sublingual gland than in control animals ($p > 0.05$; Fig. 1 and 2). In addition, CAT activity and FRAP decreased after the ingestion of 40 mg kg^{-1} eugenol ($p < 0.05$; Fig. 1). GST activity and MDA levels were not altered in animals treated with the three concentrations of eugenol ($p > 0.05$; Fig. 1 and 2).

3.4 Histology and histomorphometry

Histological sections of the pancreas, submandibular, and sublingual glands from control and eugenol-treated rats exhibit regular tissue architecture. The pancreatic parenchyma showed acini composed of densely packed serous (tubuloacinar) glands and closely spaced pancreatic islets surrounded by stroma with connective tissue (Fig. 3). In the histology of the submandibular gland showed tubuloacinar structures, with its secretory portion comprised of ducts and mucous and serous cells (Fig. 4). Tubuloacinar structures formed by predominantly mucous cells and ductal systems were also observed in the sublingual gland (Fig. 5). Septa of connective tissue penetrated the organ supporting blood vessels, lymphatic vessels, and nerves.

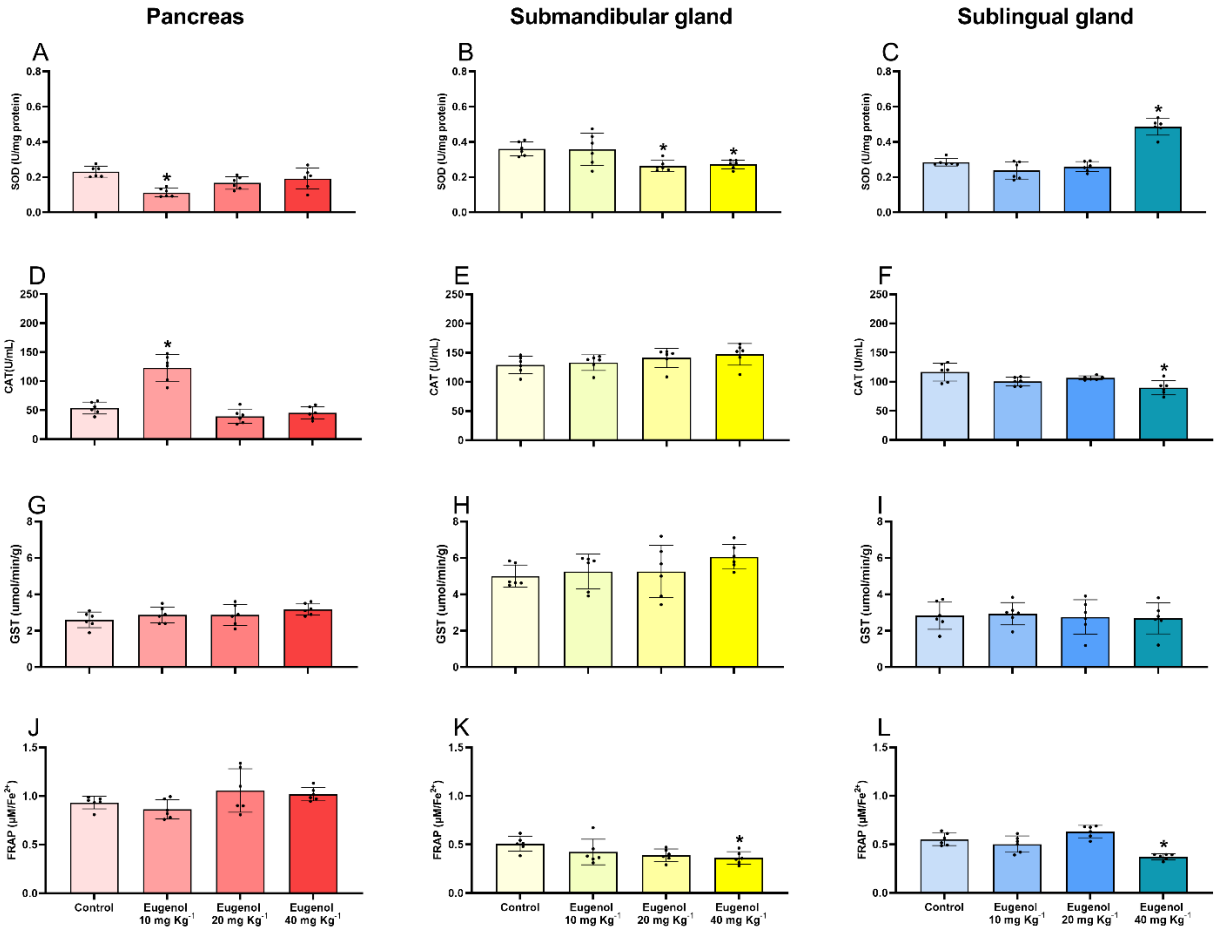

Fig 1. Antioxidant enzymes activity in pancreas, submandibular, and sublingual glands from Wistar rats treated with three concentrations of eugenol for 60 d. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. (A-C) SOD: superoxide dismutase; (D-F) CAT: catalase; (G-I) GST: glutathione S-transferase; (J-L) FRAP: ferric reducing antioxidant power. *Significant difference ($p < 0.05$) between control and eugenol groups by one-way ANOVA and Tukey's test. (n = 6 rats/group).

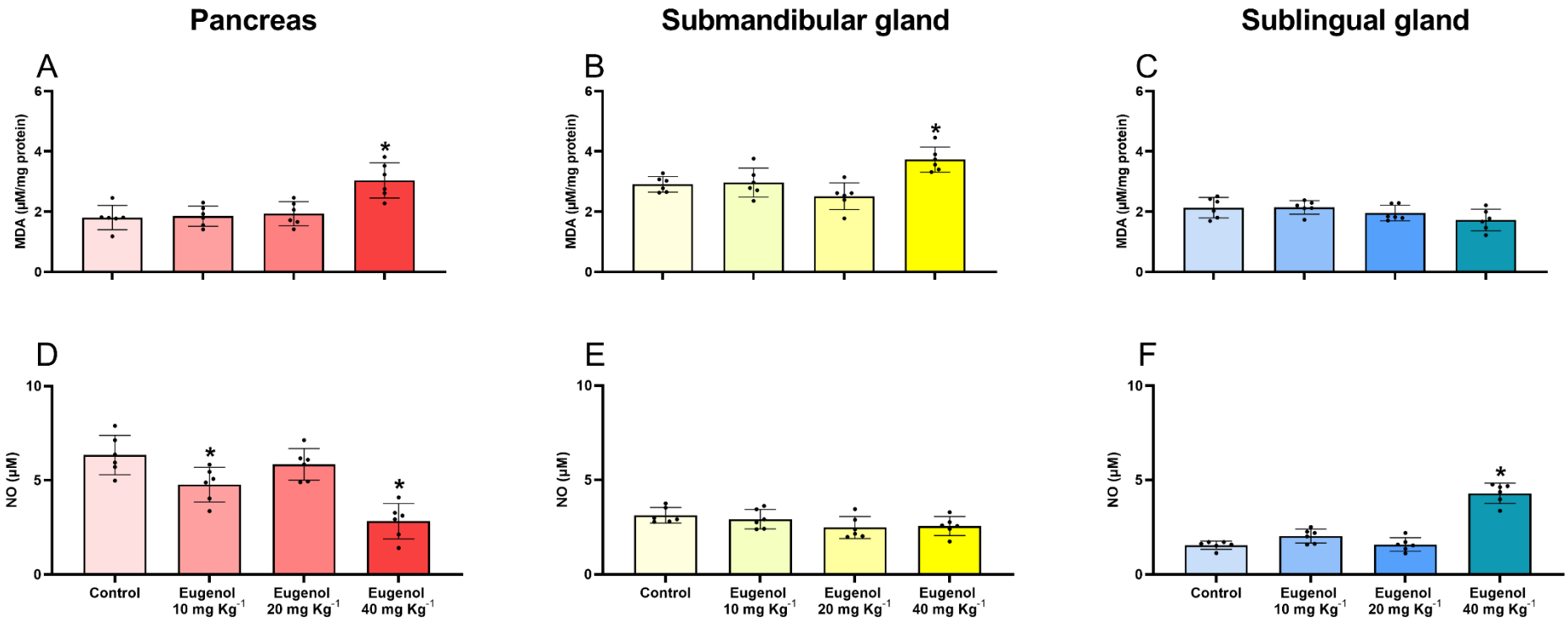

Fig 2. Oxidative/nitrosative stress markers in pancreas, submandibular, and sublingual glands from Wistar rats treated with three concentrations of eugenol for 60 d. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. (A-C) MDA: malondialdehyde; (D-F) NO: nitric oxide. *Significant difference ($p < 0.05$) between control and eugenol groups by one-way ANOVA and Tukey's test. (n = 6 rats/group).

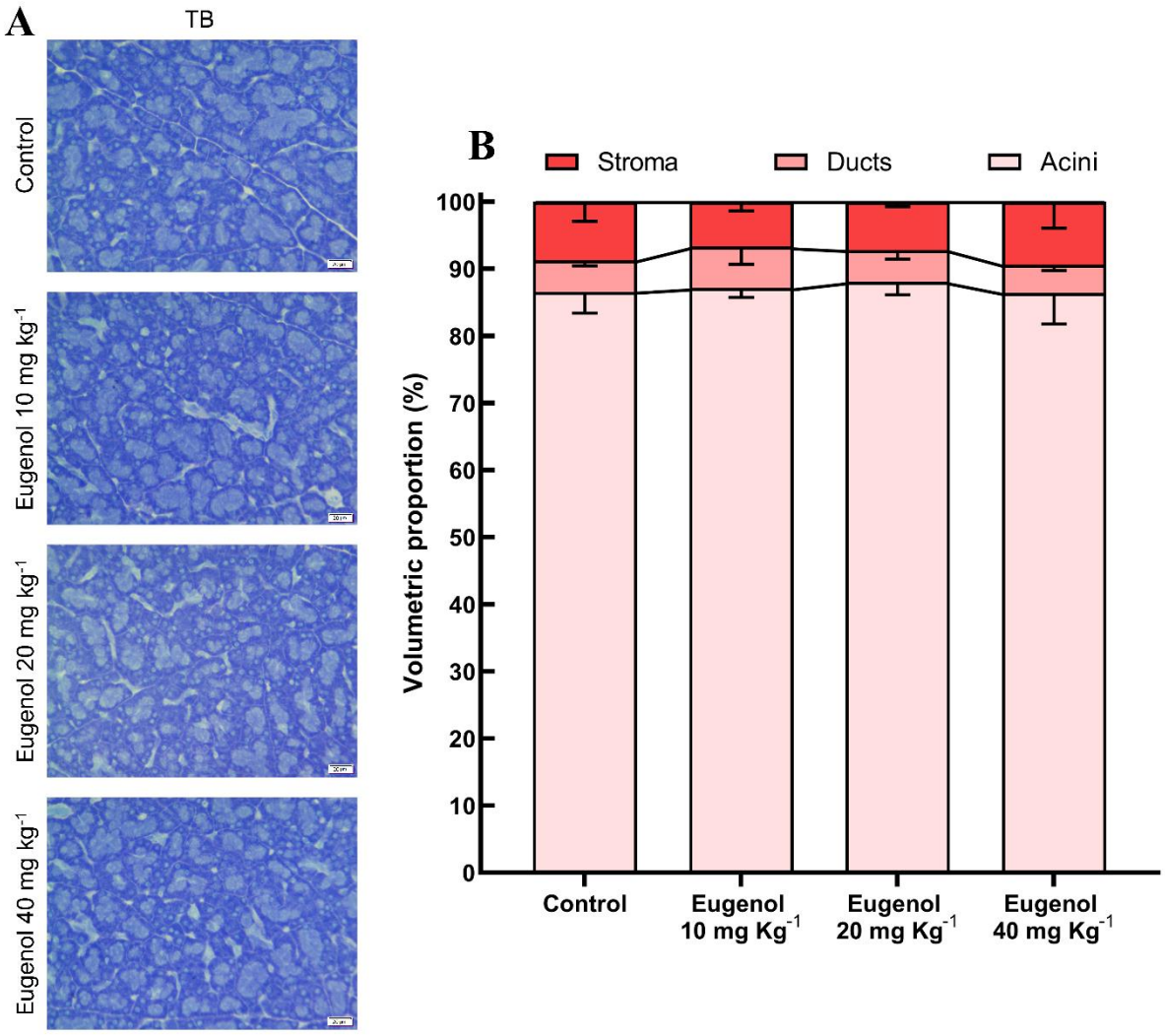

Fig 3. Histological sections (A) and volumetric proportion of pancreatic components, acini, ducts, and stroma (B), from Wistar rats treated with three concentrations of eugenol for 60 d. TB: Toluidine blue staining. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. $p > 0.05$. (n = 6 rats/group).

The proportion of pancreatic and sublingual gland components, including acini, ducts, and stroma, was similar between the control and eugenol groups ($p > 0.05$; Fig. 3 and 5). On the other hand, the submandibular gland from rats treated with 10 and 40 mg kg^{-1} eugenol showed a decrease in the percentage of acini accompanied by an increase in the percentage of stroma and ducts ($p < 0.05$; Fig. 4). Animals treated with 20 mg kg^{-1} eugenol presented only a reduction in the proportion of acini in relation to the acini percentual found in control rats ($p < 0.05$; Fig. 4).

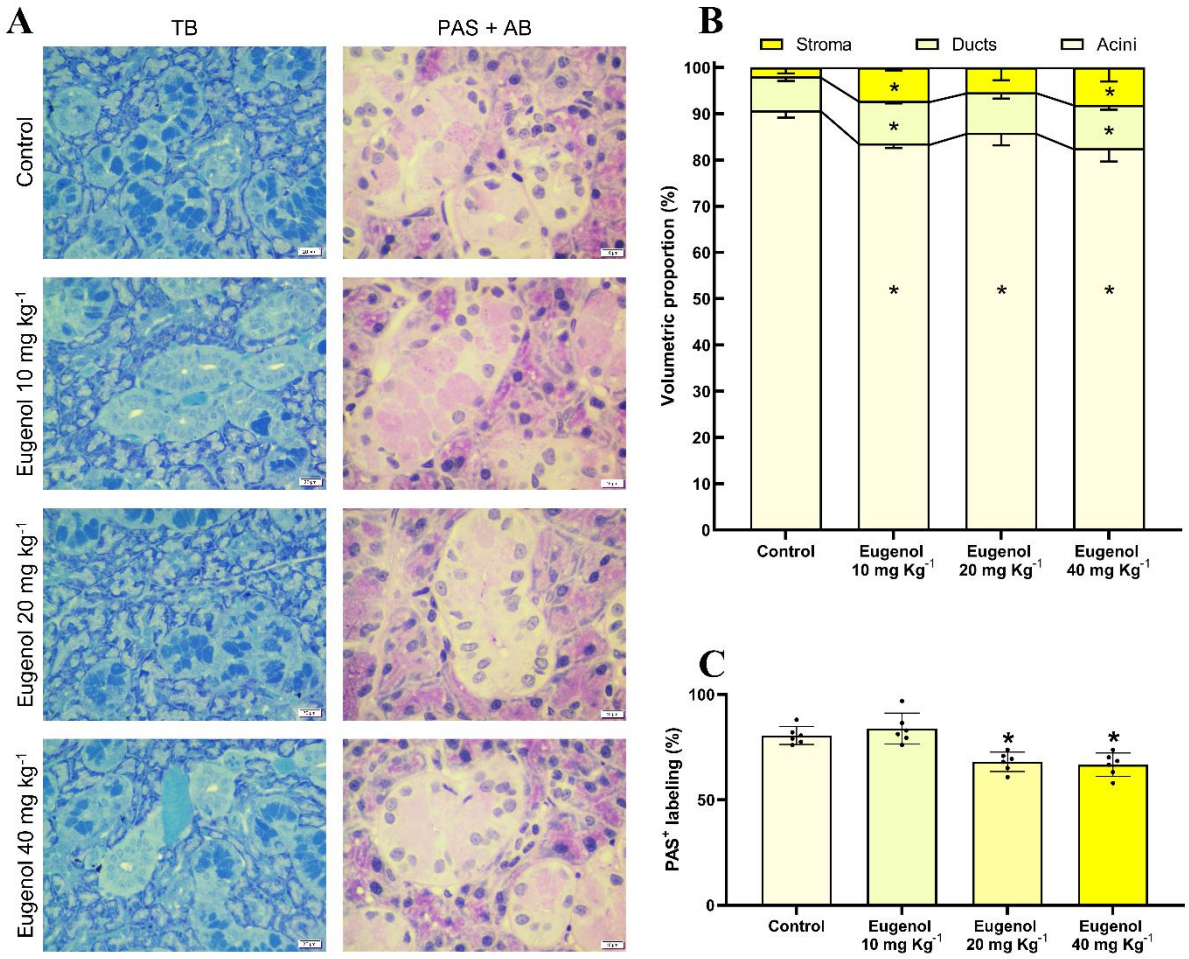

Fig 4. Histological sections (A) and volumetric proportion of acini, ducts, and stroma (B) from submandibular glands of Wistar rats treated with three concentrations of eugenol for 60 d. (C) Bar graphic showing the percentage of areas positively labeled for periodic acid-Schiff (PAS). TB: Toluidine blue; AB: Alcian blue (AB). Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. *Significant difference ($p < 0.05$) between control and eugenol groups by one-way ANOVA and Tukey's test. (n = 6 rats/group).

Histological sections of the submandibular and sublingual glands were stained with PAS and AB for identification of mucin content. This staining revealed the presence of neutral mucins (PAS^+) and the absence of acid mucins in the submandibular gland (AB^-; Fig. 4). The percentage of tissue components PAS^+-labeled was lower in glands from animals treated with 20 and 40 mg kg^{-1} eugenol than in glands from their controls ($p < 0.05$; Fig. 4). In the sublingual gland, in turn, it was possible to observe the presence of neutral mucins (PAS^+), acidic mucins (AB^+), as well as regions containing a mixture of acidic and neutral mucins (PAS^+/AB^+). The

ingestion of 10, 20, and 40 mg kg^{-1} eugenol did not change the percentage of areas labeled with PAS and AB in the sublingual glands from control and treated animals ($p > 0.05$; Fig. 5).

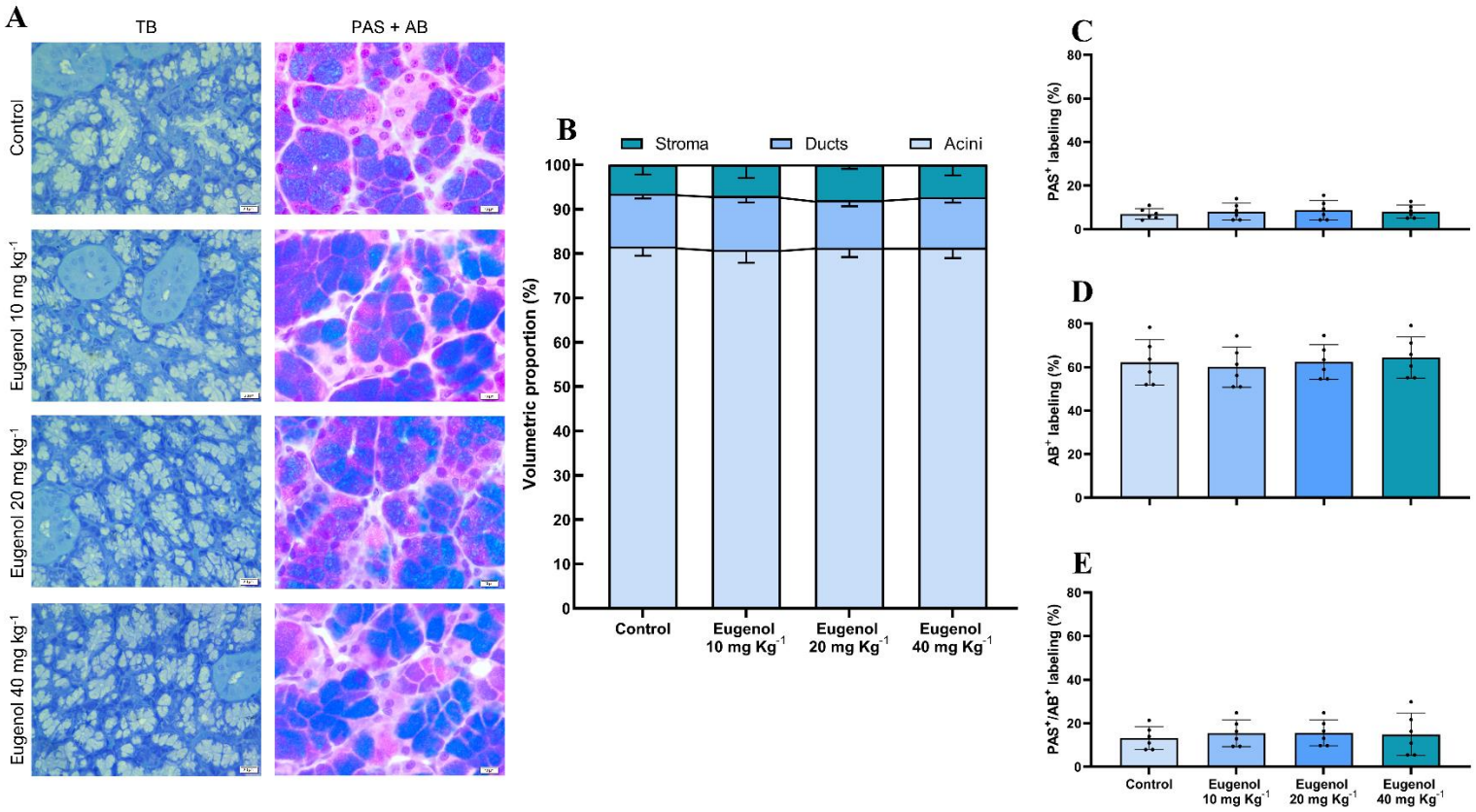

Fig 5. Histological sections (A) and volumetric proportion of acini, ducts, and stroma (B) from sublingual glands of Wistar rats treated with three concentrations of eugenol for 60 d. TB: Toluidine blue stain; PAS + AB: periodic acid-Schiff (PAS) + alcian blue (AB) staining. Bar graphics show the percentage of areas PAS^+ (C), AB^+ (D), and PAS^+/AB^+ staining (E). Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. $p > 0.05$. (n = 6 rats/group).

4. Discussion

Our findings provide pioneer information concerning the responsiveness of digestive glands to a subchronic exposure to 10, 20, and 40 mg kg^{-1} of eugenol. Herein, the highest concentration (40 mg kg^{-1}) of this compound caused most of the changes observed in the pancreas, submandibular, and sublingual glands. The other concentrations (10 and 20 mg kg^{-1}) affected oxidative and enzymatic parameters, which might indicate a potential modulation of eugenol on their functions. Moreover, this phenolic compound did not exert any effect on glands' weight but altered some biochemical and oxidative parameters in a gland- and dose-dependent manner. Notably, microscopic alterations were observed only in the submandibular tissue. This gland seems to be the most sensitive to eugenol, whereas the sublingual gland was the least responsive to its stimulus.

A decrease in serum and pancreatic lipase activity was observed only in animals treated with 40 mg kg^{-1} eugenol. Lipase is a digestive enzyme responsible for breaking dietary fat into smaller molecules that, in turn, are absorbed by the intestine (Ros, 2000). Besides the pancreas, the mouth and stomach also contribute to the production of lipase to facilitate fat digestion. However, lipase serum levels mainly reflect the pancreatic production of this enzyme (Tietz & Shuey, 1993). The inhibition of pancreatic lipase has been a relevant target to obtain controlled lipid absorption for hypotriglyceridemic and hypocholesterolemic therapies (Sheng et al., 2006). Mnafgui et al. (2013) reported that eugenol inhibited pancreatic lipase activity *in vitro* and reduced serum lipase activity in diabetic rats. These effects resulted in a notable decrease in serum levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol, with an increase in high-density lipoprotein-cholesterol (Mnafgui et al., 2013). These effects may justify the use of eugenol as a lipid-lowering agent, reducing fat and cholesterol absorption by inhibiting pancreatic lipase activity.

Although eugenol did not affect the activity of serum amylase, the concentration of 40 mg kg^{-1} increased the pancreatic amylase activity. Interestingly, an inhibitory effect of eugenol on these carbohydrate-metabolizing enzymes, such as amylases and glycosidases, was previously reported in diabetic rodents (Carvalho et al., 2021). For instance, Mnafgui et al. (2013) reported that eugenol treatment using 80 mg kg^{-1} for 30 days reduced amylase activity in serum, pancreas, and intestine in alloxan-induced diabetic Wistar rats. Under diabetic conditions, the inhibitory mechanism of eugenol occurs due to the ability of hydroxyl groups from eugenol molecules to interact with active sites of enzymes and delay carbohydrate absorption (Carvalho et al., 2021). To date, no studies evaluated the effects of eugenol on pancreatic amylase activity in healthy animals. Thus, we might speculate that, under

physiological conditions, eugenol may modulate amylase patterns differently from the observed in animals exhibiting metabolic disorders. The mechanism, however, needs to be further investigated.

Furthermore, the concentration of 40 mg kg^{-1} elicited the main alterations in the activity of antioxidant enzymes in the submandibular and sublingual glands. Indeed, high concentrations of eugenol are associated with oxidative stress generation (Fujisawa, 2002; Motteleb et al., 2014; Carvalho et al., 2022a). Overall, SOD, CAT, and FRAP showed distinct activity patterns in the submandibular and sublingual glands, resulting in high levels of MDA and NO in their respective tissues. SOD is a metalloproteinase involved in the dismutation of superoxide anions (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), representing the first line of enzymatic defense against reactive oxygen species (ROS; Inal et al., 2001). The second line of protection is composed of CAT and GST enzymes, which are responsible for converting H_2O_2 into water. Regardless of the concentration administered, eugenol did not influence GST activity from the pancreas, submandibular, and sublingual glands. Thus, low SOD, CAT, and FRAP activity promotes ROS and H_2O_2 overload with their consequent attack on lipids, proteins, and nucleic acids (Bandyopadhyay et al., 1999). This fact may explain the high level of MDA in the submandibular tissue, which is a byproduct of lipid peroxidation (Kheradmand et al., 2009), and NO_2/NO_3 levels in the sublingual tissue indicating a high production of NO, a by-product of nitrosative stress (Ridnour et al., 2004). Hence, our findings revealed the potential of eugenol at the concentration of 40 mg kg^{-1} to elicit oxidative and nitrosative stress in the submandibular and sublingual glands, respectively.

Once created an oxidative environment within the cell, disturbances in ATPase activity may occur in the gland. Rats treated with 40 mg kg^{-1} of eugenol presented low total ATPase activity in the submandibular gland and low Mg^{2+} ATPase activity in the sublingual gland. Still, in the submandibular gland, 10 mg kg^{-1} of eugenol increased the activity of total, Na^+/K^+, and Mg^{2+} ATPases. The determination of ATPase activity can show potential changes in cell membranes (Kempaiah & Srinivasan, 2006). Accordingly, changes in their activity can cause obstacles to intracellular energy production and ion transport, leading to cell dysfunction and tissue damage (Sone & Horsier, 1992). Salivary secretion depends on the coordinated activity of several membrane transport proteins that use the ion gradient generated by ATPases located in the basolateral membranes of epithelial cells, including salivary acinar cells and ducts (Roussa, 2011). For instance, eugenol at 40 mg kg^{-1}, as well as 10 and 20 mg kg^{-1}, diminished the intensity of PAS labeling and altered the volumetry of the submandibular gland's components by reducing the functional portion of the gland and increasing its structural

components (stroma and duct). Eugenol consumption may develop atrophy of this gland by affecting the acini area responsible for producing mucous and serous secretion (Pedersen et al., 2018). In the end, its reduction could result in salivary and digestive changes. Moreover, the weak intensity of PAS labeling observed in the submandibular gland of rats treated with 20 and 40 mg kg^{-1} eugenol can be attributed to the reduction in the concentration of neutral mucins in acinar cells (Hassabou & Elseweidy, 2021), corroborating the hypothesis that treatment with eugenol may impair the synthesis and secretion activities of this gland.

Otherwise, the sublingual gland showed changes in oxidative and biochemical parameters in 40 mg kg^{-1} eugenol-treated animals, with no impairment to its histology and histochemistry. Onopiuk et al. (2021) reported resistance of the sublingual gland against oxidative stress induced by cadmium when treated with black chokeberry extract. The distinct susceptibility of submandibular and sublingual glands observed here has already been justified by aspects of their morphology and physiology in studies evaluating salivary glands from animals exposed to other compounds, such as ethanol and methylmercury (Fagundes et al., 2016; Lima et al., 2018). Sublingual glands secrete a mixed fluid that is mostly mucus, which accounts for only 5% of total salivary production (Fouani et al., 2021). Submandibular glands, in turn, contribute approximately two-thirds of salivary production (Bachmeier et al., 2019). Such particularities can guarantee different biochemical, regulatory, and secretory mechanisms that result in different responses to harmful exposures (Lopes et al., 2020).

Concerning the pancreas, eugenol-treated rats presented distinct alterations in their oxidative, biochemical, and histological parameters depending on the concentration administered. Changes in the levels of oxidative metabolites (low NO and high MDA production) occurred after treatment using 40 mg kg^{-1}. In contrast, 10 mg kg^{-1} of eugenol reduced SOD and increased CAT activity, besides altering the percentual between functional (acini) and structural (duct and stroma) components of the pancreatic tissue. Variation in antioxidant enzyme activity reflects the concentration of free radicals and H_2O_2 available in the tissue, as well as the efforts of antioxidant enzymes to neutralize them. In rats ingesting the highest dose, the antioxidant defense system got to neutralize nitrosative stress rather than oxidative stress. Peroxidation of membrane phospholipids can be accompanied by changes in the structural and functional characteristics of membranes, affecting the function of ATPase activities (Rauchová et al., 1995), and causing tissue damage (Altavilla et al., 2003). Herein, rats treated with 40 mg kg^{-1} of eugenol, as well as 20 mg kg^{-1}, presented an increased activity of ATPases (total and Mg^{2+}). This finding may be a response to a membrane dysfunction to maintain membrane potential and provide the driving force for pancreatic secretion (Wang et

al., 2015). It is worth mentioning that the high activity of ATPases observed in rats receiving 20 mg kg^{-1} occurred without any sign of oxidative damage. We might speculate that eugenol is exerting another impact on the membranes that is stimulating the activity of these enzymes. However, based on the knowledge currently available on this topic, it is difficult to hypothesize potential signaling vias in which eugenol could affect pancreatic cell membranes.

Despite its widespread use in traditional medicine (Silva et al., 2018) and in dentistry (Aburel et al., 2021), our findings highlight possible detrimental effects of eugenol on the functionality and biochemistry of the pancreas, sublingual, and submandibular glands. Some limitations of our study need to be considered. The main limitation refers to the eugenol effect on these glands based on an experiment carried out in male Wistar rats. Considering the presence of sexual dimorphism in salivary glands regarding histomorphometric parameters, besides probable differences in their functionality (Lima et al., 2004), it seems necessary to investigate in a deeper study whether these effects are also present in females. Another limitation relates to the scarcity of studies performing functional, oxidative, and biochemical analyses in salivary gland tissues, which made it impossible to obtain data directly related to their activity. Bearing in mind the paucity of information regarding eugenol and its effect on the pancreas and salivary glands, the exact molecular mechanisms by which eugenol may affect these tissues must be investigated.

5. Conclusion

Our findings revealed that eugenol ingestion did not affect the biometry of the pancreas, submandibular and sublingual glands. However, ingestion of 40 mg kg^{-1} eugenol decreased serum and pancreatic lipase activity and increased pancreatic amylase activity. Depending on the organ, treatment with 10, 20, and 40 mg kg^{-1} eugenol induced changes in the activity of antioxidant enzymes, metabolites of oxidative stress, and the activity of ATPases. Despite this, only the submandibular gland showed morphological and histochemical changes by treatment, suggesting potential implications for its function.

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CRediT authorship contribution statement

Renner Philipe Rodrigues Carvalho: Conceptualization, Formal analysis, Investigation, Writing - original draft. Isadora Ribeiro de Carvalho: Conceptualization, Formal analysis, Investigation, Writing - original draft. Rosiany Vieira da Costa: Investigation, Formal analysis. Luiz Otávio Guimarães Ervilha: Investigation, Formal analysis. Mariana Machado-Neves: Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4. CHAPTER 3

Eugenol ingestion affects renal morphology and function in healthy Wistar rats

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Eugenol ingestion affects renal morphology and function in healthy Wistar rats

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Abstract

Eugenol is a natural phenolic compound with multiple pharmacological properties. Several studies have used eugenol as a renoprotective agent against nephrotoxic drugs. However, it is unclear whether its intake can cause positive or negative effects on kidney morphology and physiology in healthy individuals. Thus, we aimed to evaluate the kidney parameters of rats treated with 10, 20, and 40 mg Kg^{-1} eugenol. After 60 days of treatment, kidney samples were collected and analyzed under histological, biochemical, and oxidative approaches. Our results showed that 20 and 40 mg Kg^{-1} of this phenolic compound reduced serum concentrations of urea, creatinine, and uric acid. In addition, the treatment using 40 mg Kg^{-1} eugenol increased serum sodium, potassium, and chloride electrolytes, reduced kidney Na^{+}/K^{+} ATPase activity and thinned apical brush border in proximal tubules. Still, this concentration increased glutathione-S-transferase activity and decreased catalase activity and the concentration of renal oxidative metabolites. Eugenol at 20 mg Kg^{-1}, in contrast, caused no deleterious effects on renal histology and functions. The low dose (10 mg Kg^{-1}) of eugenol decreased the total antioxidant capacity and increased the volumetric proportion of renal blood vessels and nitric oxide content. We concluded that eugenol causes distinct effects on kidney functions and morphology in a dose-dependent manner.

Keywords: clove oil; *Syzygium aromaticum;* toxicology; kidney.

1. Introduction

The use of plants with medicinal properties for treating human diseases has been applied since ancient times (Jamshidi-Kia et al., 2018). These natural products have long been considered safe and effective without severe toxicity and side effects. Currently, their exponential long-term use has raised more attention to adverse reactions and toxic events (Rao et al., 2022). A broader view of the available literature regarding plant toxicity in traditional medicine reveals that most of these plants contain toxic substances (Bussmann et al., 2011; Mounanga et al., 2015). Furthermore, it is known that consuming natural products without assessing their efficacy and safety can lead to unexpected toxic reactions with negative consequences to the functionality of body organs, such as the liver, heart, and nervous system (Shaw, 2010; Carvalho et al., 2022a). Specifically, the potential for kidney damage caused by natural products has also become a genuine concern (Nauffal and Gabardi, 2016; Rao et al., 2022).

Syzygium aromaticum, commonly known as clove, is globally recognized for its medicinal and culinary qualities (Browmik et al., 2012; Kumar et al., 2021; Vicidomini et al., 2021). Despite the advantages of consuming clove extracts and essential oils, there is also a risk of toxicity (Nirmala et al., 2022; Özbek and Ergönül, 2022). For instance, the administration of *Syzygium aromaticum* extracts at concentrations greater than 1,000 mg Kg^{-1} for two weeks caused deleterious effects on the kidney and liver of healthy Wistar rats (Goyal et al., 2023). Moreover, a case report of accidental ingestion of 5-10 mL of clove oil by a two-year-old child caused coma, convulsions, coagulopathy, and acute liver injury (Hartnoll et al., 1993). Even so, due to a lack of information on the possible toxic effects of cloves, they are still used indiscriminately in traditional medicine (Özbek and Ergönül, 2022; Goyal et al., 2023).

The primary bioactive compound with pharmacological properties found in clove was eugenol (4-all-methoxyphenyl; $C_{10}H_{12}O_2$) (Rajput et al., 2017; Silva et al., 2018). Preclinical studies reported the effectiveness of eugenol against negative health conditions, including blood glucose and cholesterol disturbances (Harb et al., 2019; Carvalho et al., 2021), microbial infections (Khalil et al., 2017), hypertension (Mnafgui et al., 2013), inflammations (Ahmad et al., 2019), as well as reproductive (Helmy et al., 2022) and nervous system disorders (Kabuto and Yamanushi, 2011). Importantly, the eugenol use as a renoprotective agent has been widely reported. Treatment with 100 mg Kg^{-1} of eugenol for 10 days restored normal renal functions in Wistar rats, as well as suppressed hypoxia and oxidative stress, induced by the nephrotoxic antibiotic gentamicin (Said, 2011). Barhoma (2018) described the positive effects of eugenol

on urea and creatinine levels, renal antioxidants, and TNF-α content in rats intoxicated with potassium dichromate. Conversely, the administration of 10 mg Kg^{-1} of eugenol for 28 days mitigated the deleterious effects of metanil yellow on oxidative parameters and renal function in male Wistar rats (Sharma et al., 2019).

Even with all those beneficial effects, there is lack information about the safety of eugenol intake on renal parameters in healthy animals. The kidney is the route of excretion of most natural compounds. The high blood flow rate and large endothelial surface area of the kidneys ensure the delivery of large amounts of these chemicals to the parenchyma (Jha, 2010). In addition, the renal impairment associated with using traditional medicines can take several forms, including acute kidney injury, defects in tubular function, dyselectrolytemia, systemic hypertension, chronic kidney disease, renal papillary disease, and urolithiasis (Jha and Chugh, 2003; Bagnis et al., 2004; Gabardi et al., 2007). In this framework, we aimed to evaluate the effects of eugenol on the kidney of healthy rats. To that end, rats ingested 10, 20, and 40 mg Kg^{-1} eugenol for 60 days. We focused on the kidney's morphological, functional, and oxidative parameters.

2. Material and methods

2.1. Animals and ethics statement

This study is part of a comprehensive work concerning the effects of eugenol treatment in rats (Carvalho et al., 2022a; Carvalho et al., 2022b; Carvalho et al., 2023[not published]). Twenty-four male Wistar rats (70 days old; 230–250 g) were supplied by the Central Animal Facility of the Universidade Federal de Viçosa (UFV). They were housed individually in polypropylene cages under controlled photoperiod (12–12 h light/dark cycle) and temperature (21 °C). The animals had free access to rat chow and drinking water. The study was approved by the Ethics Committee of Animal Use of UFV (protocol 61/2021) and was conducted in strict accordance with the ethical guidelines of Guide for the Care and Use of Laboratory Animals (National Research Council, 2010).

2.2. Experimental design

Animals were randomly divided into four experimental groups (n = 6/group). The control group comprised rats receiving 2% Tween-20 added into distilled water (vehicle; 1 mL per gavage) daily for 60 days. Other groups, in turn, were composed of animals exposed to 10, 20, and 40 mg Kg^{-1} of purified eugenol (Sigma Aldrich Co., St. Louis, MO) diluted in 1 mL of the vehicle administered through gavage daily for 60 days. The monitoring of body weight and

clinical signs of toxicity (e.g., diarrhea, vomiting, hair loss) occurred weekly and daily during the experiment.

2.3. Euthanasia and tissue collection

After 60 days of treatment, the rats were weighed and euthanized by deep anesthesia (ketamine 150 mg Kg^{-1} i.p. and xylazine 10 mg Kg^{-1} i.p.) followed by cardiac puncture. The blood was collected by cardiac puncture, and the kidneys were removed. One was frozen in liquid nitrogen and stored at − 80 °C for enzyme and oxidative/nitrosative stress assays. The other kidney, in turn, was fixed and used for histological analysis.

2.4 Biochemical analysis

2.4.1 Markers of renal function and serum electrolytes

Blood samples (n = 6/group) collected by cardiac puncture were centrifuged at 2000×g for 15 min. The serum was used for quantification of markers of renal function. The determination of serum urea, creatinine, and uric acid, using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) in accordance with the manufacturer's instructions. The serum electrolytes sodium, potassium, chloride, and bicarbonate levels were determined using colorimetric methods using biochemical kits (Bioclin Laboratories, Belo Horizonte, MG, Brazil) in accordance with the manufacturer's instructions.

2.4.2 Antioxidant enzyme activity and oxidative stress markers in the kidney

The activity of antioxidant enzymes was evaluated in the supernatant of 100 mg of the frozen kidney (n = 6/group) homogenized in ice-cold phosphate buffer saline (PBS) and centrifuged at 3500×g at 4 °C for 10 min. The activity of superoxide dismutase (Madesh and Balasubramanian, 1998), catalase (Aebi, 1984), glutathione-S-transferase (Habig et al., 1974), and ferric reducing/antioxidant power assay (Benzie and Strain et al., 1996) were accessed. Protein oxidation was analyzed by the quantification of protein carbonyls in kidney tissue pellets using the 2.4-dinitrophenylhydrazine (DNPH) method (Levine et al., 1990). The occurrence of lipid peroxidation was determined by measuring malondialdehyde levels (Buege and Aust, 1978) whereas nitric oxide levels were determined by detecting nitrite/nitrate levels in the liver following the Griess methodology (Tsikas, 2007). Total protein concentration was measured following the Lowry method (Lowry et al., 1951).

2.4.3 ATPases activity in the kidney

Briefly, 50 mg of frozen kidney fragments (n = 6/group) were homogenized in 500 µl of Tris-HCl buffer (100mM, pH 7.4) and centrifuged at 10000 ×g for 10 min at 4 ◦C. Aliquots of tissue homogenate were transferred to a centrifuge tube to remove the endogenous phosphate present in the samples. A saturated solution of ammonium sulfate was added to a final concentration of 3.2 M. After resting on ice for 20 minutes, the samples were centrifuged at 10000 ×g at 4°C for 10 min. The supernatant was discarded, and the pellet was resuspended back to its original volume in Tris-HCl buffer and used to determine the activity of membrane-bound adenosine triphosphatase (ATPases) activities. For each Total (Evans, 1969), Ca^{2+} (Hjertén and Pan, 1983), Na^{+}/K^{+} (Bonting et al., 1962) and Mg^{2+} (Ohnishi et al., 1982) ATPase, an incubation medium was prepared in Tris-HCl buffer. ATP solution was used as a substrate to generate free phosphate by the ATPases. For each reaction, 50µl of the sample was added and incubated at 37 °C for 30 min. The reaction was arrested by adding 500µl to a cold solution of 10% TCA. The tubes were centrifuged at 1500 g for 10 min. The phosphorous content in the supernatant was determined by a colorimetric test using a biochemical kit (Bioclin Laboratories, Belo Horizonte, MG, Brazil). The pellet, in turn, was used to quantify the level of total proteins by the Bradford method (Bradford, 1976). The ATPase activity was expressed as micrograms of phosphorous liberated per hour per micrograms of protein.

2.5 Histological analysis of kidney

Kidney fragments (n = 6/group) were immersed in 10 % formalin solution for 24 h. Fragments were dehydrated in crescent series of ethanol (70, 80, 90, and 100 %) and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Sections at the thickness of 3 µm were obtained in semi-series, using one in every 20 sections, and stained with hematoxylin/eosin (HE) and Periodic Acid Schiff (PAS).

From each kidney, 25 histological fields, obtained from different HE-stained sections of the renal cortex, were photographed with a photomicroscope (Olympus BX53, Tokyo, Japan) for examination (200× magnification) using a test system of 266 points in standard test areas (2.38 × 106 µm2) (Carvalho et al., 2022). Coincident points were recorded in the glomeruli, blood vessels, tubules, and interstitium (Molitoris and Sutton, 2004; Alghamdi et al., 2020). The volume density [Vv] occupied by these components was calculated using the following formula: Vv = PP / PT, where PP is the number of points over the interest structure and PT represents the total test points (Mandarim-de-Lacerda et al., 2003). In PAS-stained sections,

histochemical examination was performed under light microscopy and documented using a photomicroscope (Olympus BX53, Tokyo, Japan).

2.6 Statistical analysis

Results had their normality evaluated by the Shapiro-Wilk test. Later on, they were analyzed by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test. Differences were considered significant when $P < 0.05$. The statistics and graphics were performed using the GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as means ± standard deviation (mean ± SD).

3. Results

3.1 Biochemical analysis

Rats treated with 20 and 40 mg Kg^{-1} of eugenol presented lower serum urea, creatinine, and uric acid levels than control animals ($p < 0.05$; Fig. 1). Also, rats receiving 40 mg Kg^{-1} of eugenol showed a higher serum content of sodium, potassium, and chloride than the control animals ($p < 0.05$; Fig. 2). In contrast, eugenol did not alter the serum bicarbonate, regardless of the concentration ($p > 0.05$; Fig. 2).

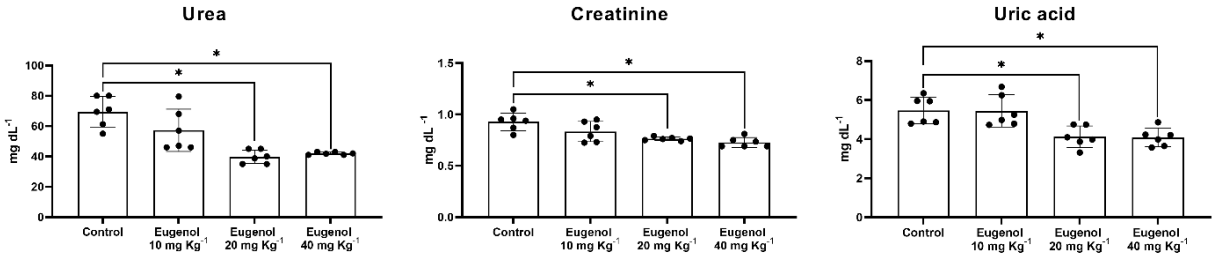

Fig. 1. Serum urea, creatinine, and uric acid from healthy Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Values are expressed as mean ± SD and dots represent each data point. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group).

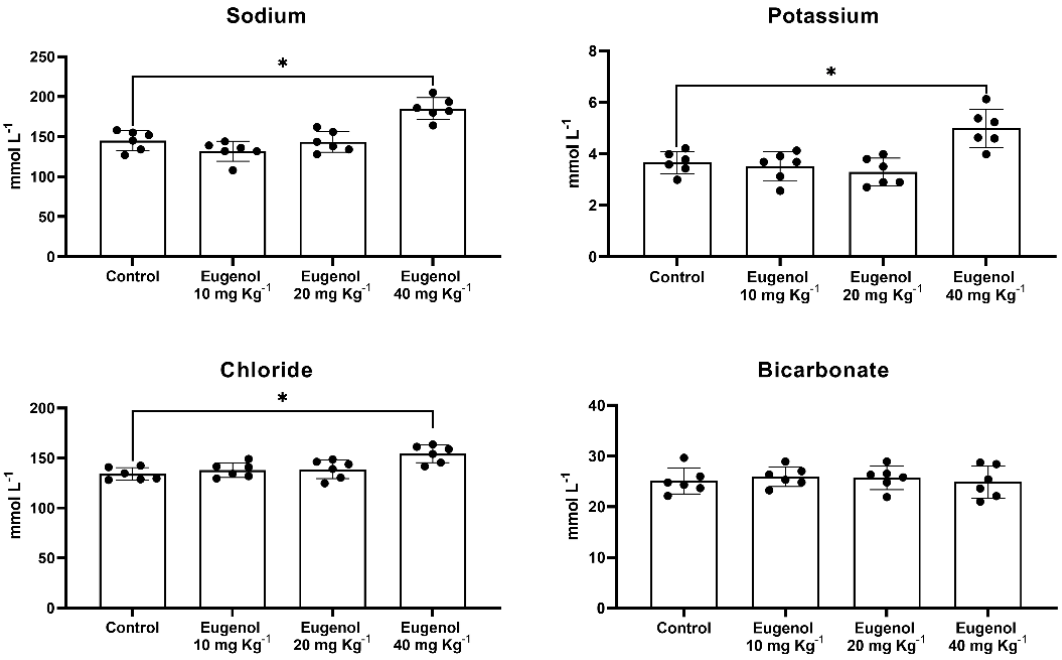

Fig. 2. Serum electrolytes sodium, potassium, chloride, and bicarbonate levels from healthy Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Values are expressed as mean ± SD and dots represent each data point. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group).

3.2. Kidney oxidative/nitrosative stress markers and antioxidant enzymes

The activity of catalase was higher in rats receiving 40 mg kg^{-1} of eugenol, whereas total antioxidant capacity decreased in animals treated with 10 mg Kg^{-1} ($p < 0.05$; Fig. 3). In contrast, the three concentrations of eugenol did not alter the activity of superoxide dismutase ($p > 0.05$; Fig. 3). The kidney of animals receiving 20 mg Kg^{-1} of eugenol did not show any alteration in the activity of antioxidant enzymes and total antioxidant capacity ($p < 0.05$; Fig. 3).

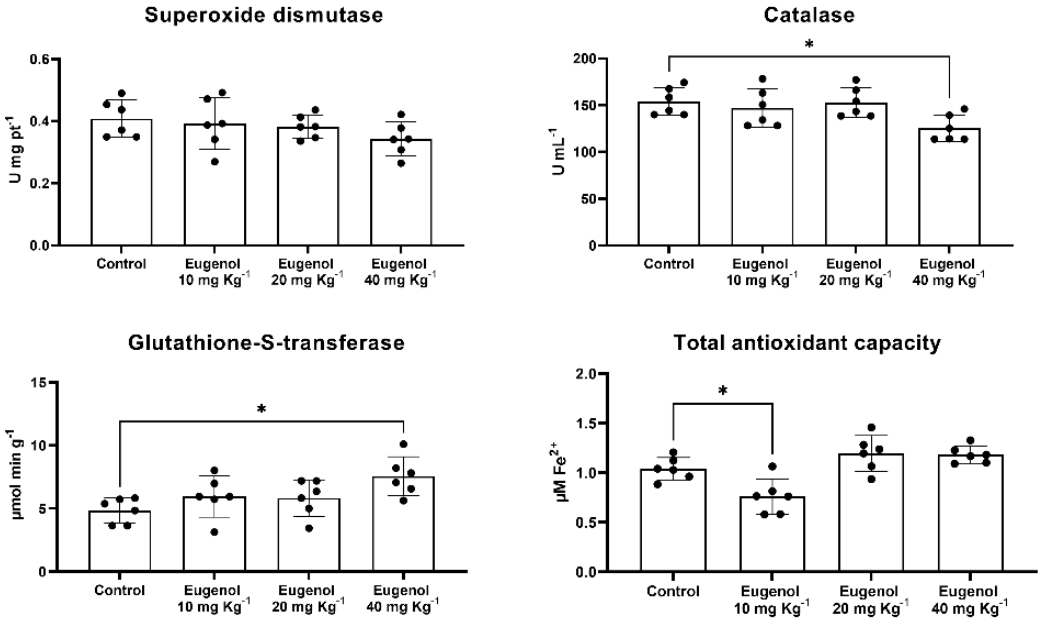

Fig. 3. Antioxidant enzyme activity and total antioxidant capacity in the kidneys of healthy Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Values are expressed as mean ± SD and dots represent each data point. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group).

The renal malondialdehyde content only decreased in rats treated with 40 mg Kg^{-1} of eugenol ($p < 0.05$; Fig. 4). The protein carbonyl levels, in turn, were lower in rats receiving 20 and 40 mg Kg^{-1} of eugenol than their controls ($p < 0.05$; Fig. 4). Ultimately, nitric oxide content was higher in the kidneys of rats treated with 10 mg Kg^{-1} of eugenol than in control animals ($p < 0.05$; Fig. 4).

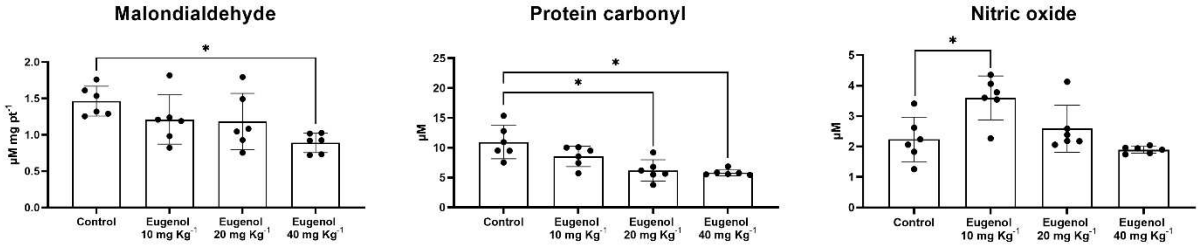

Fig. 4. Malondialdehyde, protein carbonyl and nitric oxide levels in the kidneys from Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Values are expressed as mean ± SD and dots represent each data point. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group).

3.3. Activity of total, Ca^{2+}, Mg^{2+}, and Na^{+}/K^{+} ATPases in the kidneys

Rats receiving 20 and 40 mg Kg^{-1} eugenol showed a reduced activity of Na^{+}/K^{+} ATPase pump in the kidney tissue ($p < 0.05$; Fig. 5), with no alteration in the total, Ca^{2+}, and Mg^{2+} ATPases activity ($p > 0.05$; Fig. 5).

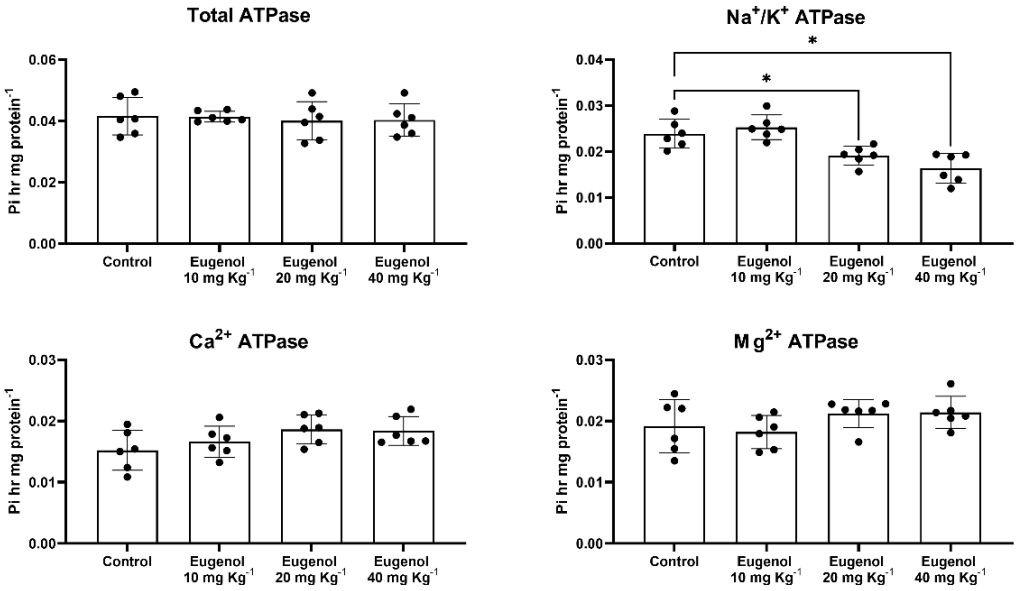

Fig. 5. Activity of total, Ca^{2+}, Na^{+}/K^{+} and Mg^{2+} ATPases in the kidneys from Wistar rats treated with three concentrations of eugenol for 60 days. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Values are expressed as mean ± SD and dots represent each data point. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group).

3.4. Kidney histology

HE-stained histological sections of the renal cortex from control and eugenol-treated rats show neither any apparent pathology nor morphology alteration in renal tubules, glomerulus, blood vessels, or interstitium (Fig. 6). In contrast, the kidneys of rats receiving the lower dose of eugenol (10 mg Kg^{-1}) presented a higher volumetric proportion of blood vessels and a reduced volumetric proportion of glomeruli than control rats ($p < 0.05$; Fig. 6). The volumetric proportion of renal tubules and interstitium remained unchanged between experimental groups ($p > 0.05$; Fig. 6).

Regarding PAS-stained histological sections, the renal cortex sections revealed a PAS-positive reaction along the prominent apical brush borders of the renal proximal tubular cells, as well as at their basement membrane (Fig. 7). Only in the kidneys of rats treated with 40 mg Kg^{-1} eugenol, PAS-stained sections revealed the renal proximal tubular cells with an apparent weak reaction and thinning at their brush border (Fig. 7).

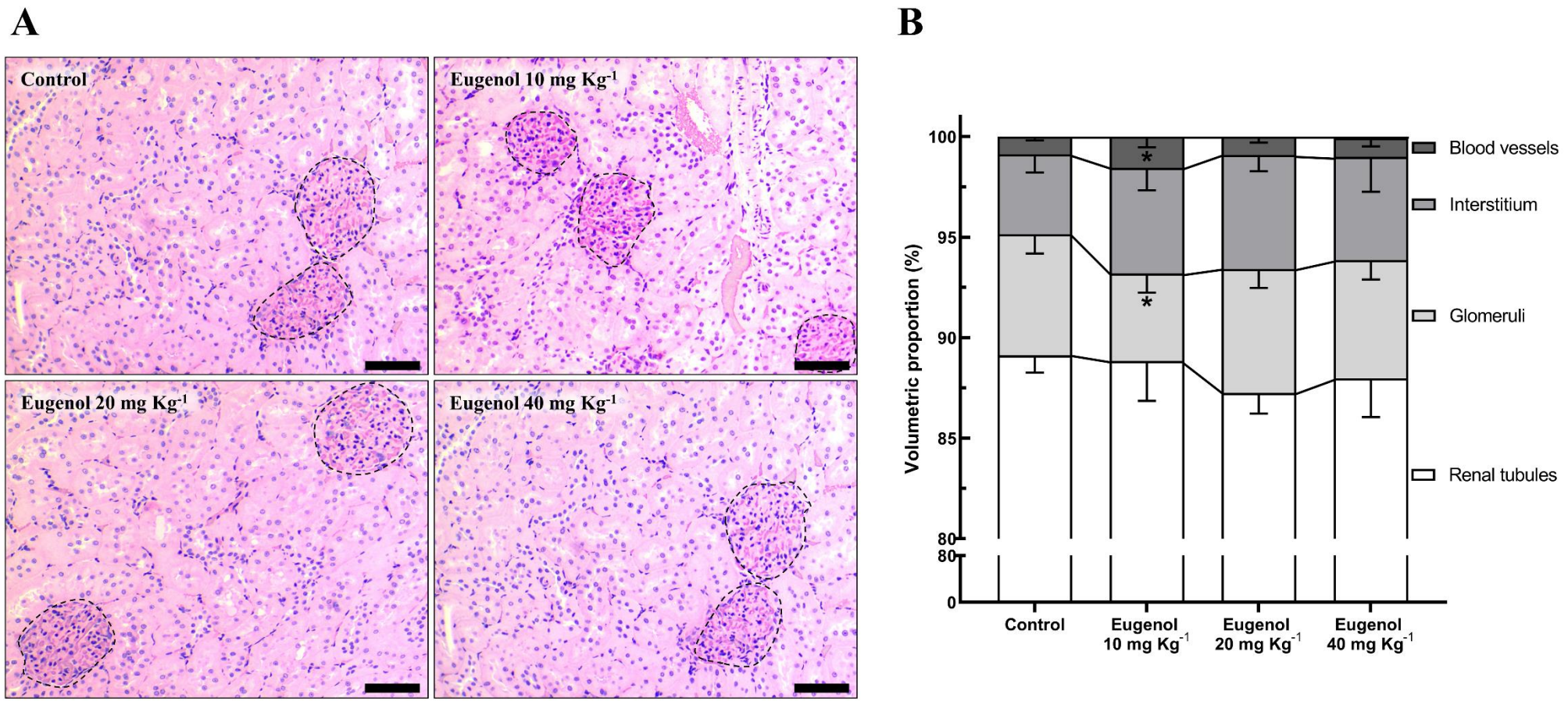

Fig. 6. Histological sections (A) and volumetric proportion of kidney components, renal tubules, glomeruli, interstitium, and blood vessels (B), of Wistar rats treated with three concentrations of eugenol for 60 days. The glomeruli are delimited by the dotted line. Control group: 2 % Tween-20; Eugenol groups: eugenol diluted in 2 % Tween-20. Values are expressed as mean ± SD. *Significant differences ($p < 0.05$) between control and treated groups by ANOVA and Tukey's test (n = 6 rats/group). Hematoxylin-eosin staining. Scale bar: 20 µm.

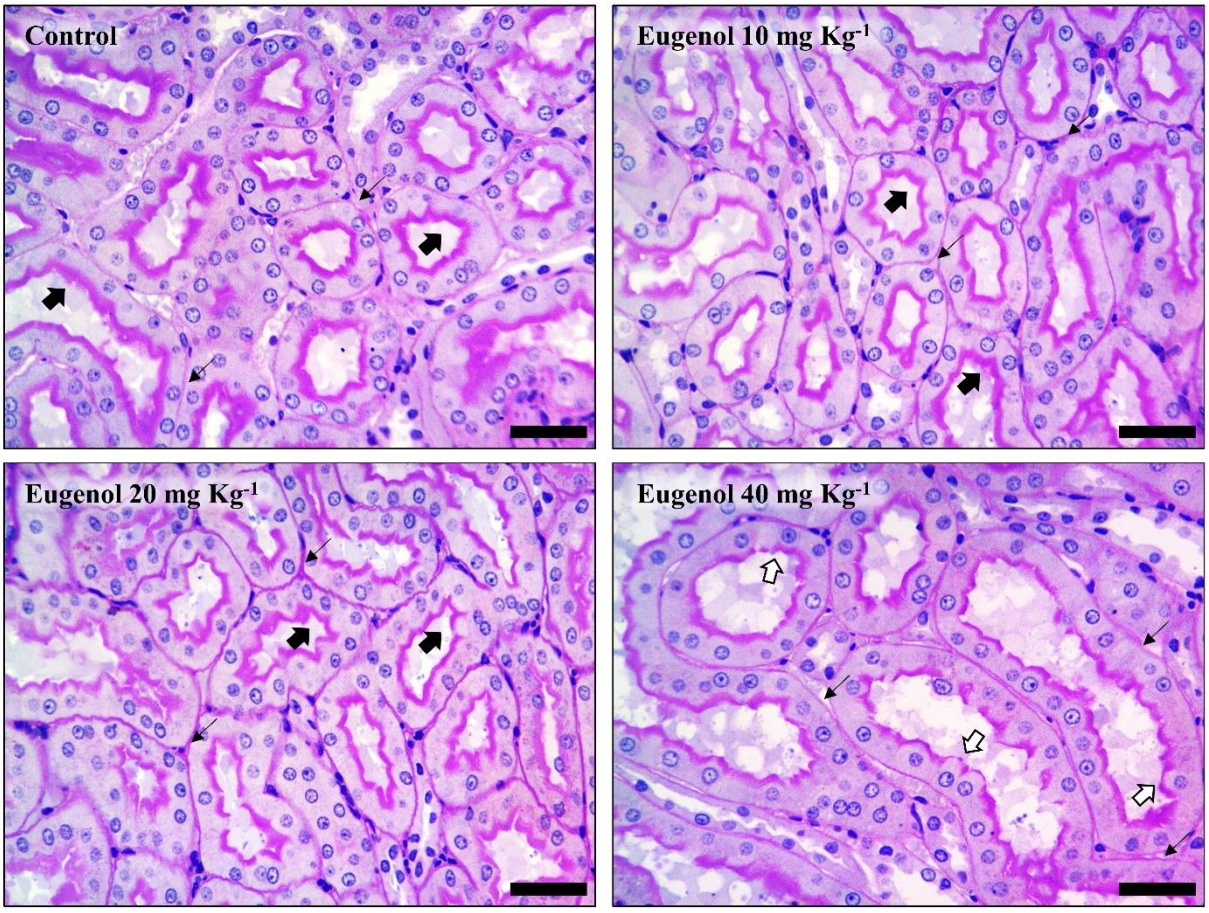

Fig. 7. Histological sections of the proximal tubules in renal cortex of Wistar rats treated with three concentrations of eugenol for 60 days. Thick and thin arrows show glycogen deposits in the brush border, basement membrane, and renal tubules, respectively. Thick white arrows indicate thinning of the apical brush border. Control group: 2% Tween-20; Eugenol groups: eugenol diluted in 2% Tween-20. Periodic Acid Schiff staining. Scale bar: 10 μm.

4. Discussion

The results provide pioneer information regarding the effect of eugenol on the kidney's biochemical, oxidative, and morphological parameters in healthy Wistar rats. This information is valuable for the understanding of the safety, toxicity, and potential therapeutic applications of eugenol. The highest dose (40 mg Kg^{-1}) of eugenol probably impaired fluid transport in the the kidneys, causing consequent loss of serum electrolyte homeostasis. Even so, there was a modulation of antioxidant enzyme activity as well, preventing oxidative stress in the kidney tissue. Conversely, treatment with the lowest dose of eugenol (10 mg Kg^{-1}) may have induced a vasodilator response in the kidneys with no alteration in the physiological levels of renal function markers and electrolyte homeostasis.

Our results showed a reduction in the serum levels of urea, creatinine, and uric acid after 60 days of treatment using 20 and 40 mg Kg^{-1} of eugenol. The concentration of these substances in the blood reflects the balance between their production and elimination through the urine. Although they are considered good markers of renal function (Gowda et al., 2010), a reduction in their serum levels is less common and clinically less significant than an increase in these markers (Lum and Leal-Khouri, 1989). This reduction can be caused by their low production, high urinary excretion, or both (Levey et al., 1988; Musso et al., 2012; Higgins, 2016). In the liver, hepatic enzymes convert ammonia to urea. Meanwhile, creatine is synthesized and stored in skeletal muscle before being eliminated as creatinine (Salazar, 2014). Additionally, serum uric acid is a product of purine metabolism, originating from hypoxanthine after double enzymatic catalysis by hepatic xanthine oxidase (Lombardi et al., 2016). Given the liver's role in the synthesis of these markers of renal function, we hypothesize that the decrease in urea, creatinine, and uric acid is a secondary effect of liver damage caused by eugenol treatment at doses of 20 and 40 mg Kg^{-1}, as we recently reported (Carvalho et al., 2022a).

Herein, our results evidenced neither histological damage or alteration in the volumetry of kidney components in eugenol-treated rats using the doses of 20 and 40 mg Kg^{-1}. The only alteration found in the kidney of animals from the 40 mg Kg^{-1} group was a reduction in the glycogen labeling in the apical brush border of the proximal tubules. This finding may have influenced the absorption surface and led to plasma electrolyte imbalances, which were reflected in the altered levels of serum sodium, potassium, and chloride detected here. Indeed, the proximal renal tubule is responsible for water, electrolyte, and nutrient reabsorption. The apical brush border dramatically increases its surface area and contains multiple glycoproteins contributing to its functions and cell responses, depending on the physiological state (Walmsley et al., 2010). These dynamic functions are crucial in maintaining renal electrolyte transport homeostasis and are frequently impaired in kidney disturbances (Dhondup and Qian, 2017).

The inhibition of Na^+/K^+ ATPase activity observed in animals treated with 40 mg Kg^{-1} of eugenol was another critical factor involved in the electrolyte disturbance observed in this study. Previous reports have described the potential of eugenol to inhibit intestinal and renal Na^+/K^+ ATPase (Kreydiyyeh et al., 2000), as well as in the liver (Carvalho et al., 2022a). Renal transport of electrolytes can also be disturbed when the tubular flow is altered (Verschuren et al., 2020). Various transport proteins mediate these processes, such as Na^+/K^+ ATPase, one of the essential basolateral transport proteins in the proximal tubules (Jaitovich and Bertorello, 2006) and crucial for maintaining the resting potential across tubular cell membranes (Silva and Soares-da-Silva, 2009). Besides the electrolyte imbalance, the inhibition of Na^+/K^+ ATPase

pump can impair the production of intracellular energy, resulting in a consequent disturbance in cell function, generation of oxidative stress, and cell damage (Mishra et al., 1989; Sone and Horsier, 1992; Fuller, 2003; Carvalho et al., 2022a).

In this light, the decrease in catalase and increase in glutathione-S-transferase activity observed in the kidney suggested that the highest dose of eugenol elicited an increased production of reactive oxygen species. The elevation in glutathione-S-transferase activity probably acted as a compensatory mechanism for suppressing catalase activity. The decrease in catalase activity can be worrying because catalase converts H_2O_2 into water and molecular oxygen, decreasing the concentration of H_2O_2 and preventing damage to cellular components such as lipids, proteins, and nucleic acids (Keher, 2000; Bagnyukova et al., 2005). As an adaptive response to reduced catalase activity, both glutathione-S-transferase and glutathione peroxidase may act to detoxify xenobiotics and aldehyde products of lipid peroxidation caused by increased levels of reactive species (Storey, 2004). At the same time, the decrease in malondialdehyde and carbonyl protein content and the absence of histopathological findings suggest that the tissue's antioxidant defense successfully neutralized the oxygen reactive species.

Animals treated with the lowest dose of the compound (10 mg Kg^{-1}) showed an increase in the proportion of blood vessels and an increase in nitric oxide levels, suggesting a vasodilator response in the kidneys. Nitric oxide is a crucial regulator of vascular tone, which can increase blood vessel dilation leading to increased blood flow to the kidney (Giles et al., 2012; Ahmad et al., 2018; Krishnan et al., 2018; Carlstrom, 2021). This change can have positive effects, as greater renal blood flow improves the glomerular filtration rate (Konda et al., 2016; Tholén et al., 2021). Another finding found in animals that received the lowest dose of eugenol was the decrease in total antioxidant capacity, measured by the ferric reducing/antioxidant power assay. Although this result suggests a reduction in non-enzymatic antioxidant capacity in the tissue (Benzie and Devaki, 2017), no effects were observed on the activity of antioxidant enzymes, demonstrating that at a dose of 10 mg Kg^{-1}, eugenol differentially modulates enzymatic and non-enzymatic antioxidant defense. Indeed, studies show that there may be differences in the modulation of enzymatic and non-enzymatic antioxidant defense depending on experimental conditions (Dadkhah et al., 2006; Nonato et al., 2016). Furthermore, unaltered levels of malondialdehyde and protein carbonyl and the absence of tissue and biochemical changes suggest that tissue antioxidant defense effectively protects against oxidative damage.

Interestingly, the only alterations observed in kidneys of animals treated with the intermediate dose of eugenol (20 mg Kg^{-1}) were the inhibition of the Na^+/K^+ ATPase pump and

the reduction in the carbonyl protein content. Although Na^+/K^+ ATPase inhibition may represent the direct effects of eugenol on this enzyme, other effects were not observed, suggesting tissue adaptation. Considering the absence of alterations in the other parameters evaluated in these animals, one possibility is that the treatment of eugenol generated trifurcated responses depending on the dosage used. For example, it is possible that 10 mg Kg^{-1} of eugenol primarily activates specific pathways in blood vessels that result in vasodilation in the kidneys. In comparison, the higher dose effectively activates other pathways in the renal tubules that lead to inhibition of Na^+/K^+ ATPases with thinning apical brush border and cause electrolytes imbalance. In this context, we can hypothesize that the intermediate dose was insufficient to activate either of the two mechanisms. Previous studies have indicated that eugenol can act differentially, according to the administered dose. The oral treatment with eugenol at 1 mg Kg^{-1} reduced acetic acid-induced ulcers in rats, favoring antioxidant imbalance and mucus production. However, eugenol at 100 mg Kg^{-1} worsens the ulceration process (Longo et al., 2021). In another study, the treatment with eugenol yielded bifurcated responses depending on the dose employed. While eugenol at 10 mg Kg^{-1} appeared to mitigate effects from subsequent liver ischemia/reperfusion, 100 mg Kg^{-1} of eugenol treatment led to an amplification of liver injury in the rats (Motteleb et al., 2014). Given the scarce information about eugenol and its effect on renal parameters, future research should seek a deeper understanding of the exact molecular mechanisms involved in these responses.

5. Conclusion

Our results showed that eugenol affected renal parameters in a dose-dependent manner. High doses (20 and 40 mg Kg^{-1}) of this phenolic compound reduced serum concentrations of urea, creatinine, and uric acid, probably as a secondary response to liver damage. In addition, the highest dose (40 mg Kg^{-1}) increased serum sodium, potassium, and chloride electrolytes, reduced Na^+/K^+ ATPase activity, thinned apical brush border in proximal tubules, increased glutathione-S-transferase activity, and decreased catalase activity and the concentration of renal oxidative metabolites in the kidney. Differently, rats ingesting 10 mg Kg^{-1} of eugenol showed a decrease in the total antioxidant capacity and an increase in the volumetric proportion of blood vessels and nitric oxide levels in the kidneys.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5. CHAPTER 4

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Eugenol reduces serum testosterone levels and sperm viability in adult Wistar rats

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Eugenol reduces serum testosterone levels and sperm viability in adult Wistar rats

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Highlights

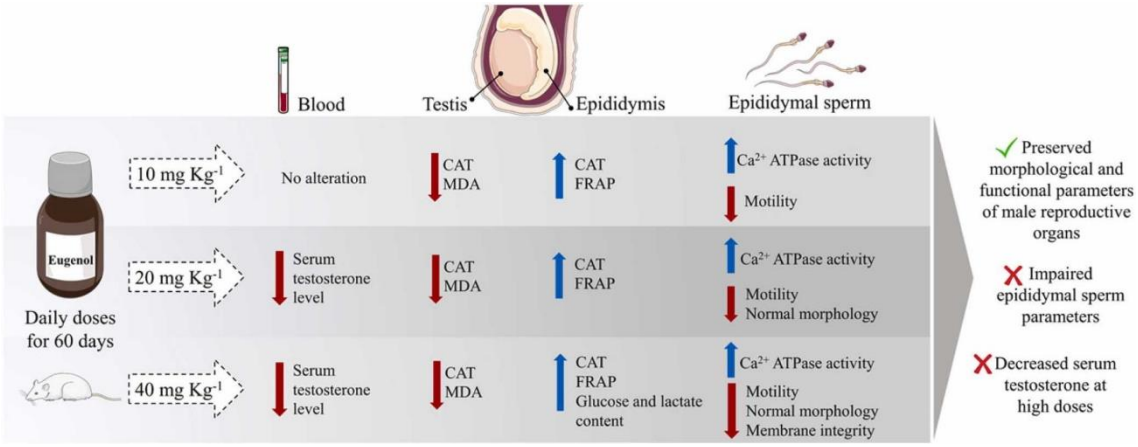
- Eugenol treatment did not affect the integrity and functionality of testis tissue
- Serum testosterone levels reduced in animals treated with 20 and 40 mg Kg^{-1} eugenol
- Eugenol intake did not alter the epididymis histology and sperm transit time
- Eugenol impaired sperm viability in a dose-dependent trend

Abstract

Eugenol is the main constituent of clove extract. It is a remarkably versatile molecule incorporated as a functional ingredient in several food products and widely applied in the pharmaceutical industry. Men consume natural products enriched with eugenol for treating sexual disorders and using as aphrodisiacs. Nevertheless, there is no information about the impact of eugenol intake on the reproductive parameters of healthy males. Therefore, we provided 10, 20, and 40 mg Kg^{-1} pure eugenol to adult Wistar rats for 60 days. Testis, epididymis, and spermatozoa were analyzed under microscopic, biochemical, and functional approaches. This phenolic compound did not alter testicular and epididymal biometry and microscopy. However, 20 and 40 mg Kg^{-1} eugenol reduced serum testosterone levels. The highest dose altered lactate and glucose concentrations in the epididymis. All the eugenol concentrations diminished CAT activity and MDA levels in the testis and increased FRAP and CAT activity in the epididymis. Epididymal sperm from rats receiving 10, 20, and 40 mg Kg^{-1} eugenol presented high Ca^{2+} ATPase activity and low motility. In conclusion, eugenol at low and high doses negatively impacted the competence of epididymal sperm and modified oxidative parameters in male organs, with no influence on their microscopy.

Keywords: Antioxidant, aphrodisiac, clove, phenolic compound, spermatozoa, *Syzygium aromaticum*

Graphical abstract



1. Introduction

Eugenol has been attracted the attention of researchers due to its functional attributes. This phenolic compound is recognized as the main component of clove (*Syzygium aromaticum*), being found in other herbal plants, such as cinnamon, tulsi, pepper, ginger, and oregano [1,2,3]. Eugenol has been commonly used as a culinary spice worldwide, and a spicy flavoring agent in whisky, ice cream, baked goods, and candies [4,5,6]. Its wide range of applications includes its addition in perfumes, mouthwashes, and dental analgesics [7]. For instance, eugenol, either isolated from natural sources or synthesized in laboratories, have been applied in the food industry because of its antimicrobial properties, preservative ability, and possible use in the development of packaging materials [8,9,10,11,12].

Moreover, eugenol has relevant health-promoting functions that raise the interests of the pharmaceutic industry [13,14,15]. It may act as a free radical scavenger by preventing reactive oxygen species (ROS) generation and increasing the cellular antioxidant defenses [16,17]. Hence, several studies have investigated the positive effect of eugenol in the treatment of nervous system diseases [18], hypercholesterolemia [19], diabetes [20], hypertension [21], inflammatory diseases [22], and cancer [23]. Interestingly, few studies reported the effects of pure eugenol in male reproductive parameters. The recent literature showed that eugenol improved the hormone production and antioxidant defense in rat testis after exposure to the chemotherapeutic agent cisplatin [24] and the insecticide chlorpyrifos [25]. However, the benefits of eugenol treatment were not seen in mouse sperm damaged by exposure to the nano-pollutant C60 fullerene [26].

To the best of our knowledge, there are no studies evaluating the effect of pure eugenol on the male reproduction of healthy individuals. Men may ingest natural products enriched with eugenol, widely used in traditional medicine, to treat sexual disorders or act as aphrodisiacs [27,28,29,30]. Clove extract is an example of eugenol source in its free and acetate form, but also contains other substances, such as caryophyllene, sesquiterpene ester, phenylpropanoid, and β caryophyllene [27,31,32]. Studies have documented a dose-dependent effect of clove extracts on male fertility. In mice, clove extracts at concentrations up to 15 mg Kg^{-1} stimulated the production of testosterone through activation of steroidogenic enzymes 3-β and 17-β hydroxysteroid dehydrogenase in the testis [33,34], the secretion of sialic acid by epididymis and seminal vesicle [34], and improved the motility and morphology of sperm harvested from the cauda epididymis [34,35]. Conversely, concentrations higher than 20 mg Kg^{-1} inhibited spermatogenesis with a consequent reduction in sperm production, reduced the secretory activity of epididymis, and affected male fertility by diminishing the litter size [32,34,35]. It is

tempting to attribute these effects to eugenol. However, distinct therapeutical activities between extracts and isolated compounds may occur due to the synergistic, additive, or unknown interactions with their compounds [14,30,36].

Considering the importance of eugenol to medical and pharmaceutical purposes, as well as the paucity of information regarding its role on male reproductive functions, we aimed at analyzing the impact of this phenolic compound on male reproductive organs and gametes of healthy adult rats. To this end, we investigated the influence of low (10 mg Kg^{-1}) and high doses (20 and 40 mg Kg^{-1}) of eugenol in morphological, biochemical, and functional parameters of testis, epididymis, and spermatozoa. Also, we assessed oxidative stress markers in these organs, including antioxidant enzymes activity and oxidative metabolites production. Our findings may contribute to understanding the impact of long-term exposure to eugenol on reproductive changes that may affect male fertility.

2. Material and methods

2.1. Animals and ethics statement

This study is part of a comprehensive work concerning the effects of eugenol on the morphology and function of kidney and digestory glands [37]. Forty male adult Wistar rats (70 days old; 200 ± 250 g) were supplied by the Central Animal Facility of the Center of the Biological and Health Sciences of the Universidade Federal de Viçosa (UFV). They were maintained individually in polypropylene cages under controlled photoperiod (12 h light/dark cycle) and temperature (21 °C) with free access to rat chow and water *ad libitum*. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals [38]. All experimental procedures were reviewed and approved by the Ethics Committee for the Use of Experimental Animals of UFV (CEUA; protocol 61/2021).

2.2. Experimental design

After one week of acclimatization, the animals were randomly divided into four groups (n = 10/group). The control rats received 1 mL of vehicle (2% Tween 20 diluted in distilled water), and the treated rats received 10, 20, and 40 mg Kg^{-1} of eugenol (Sigma Aldrich Co., St. Louis, MO) diluted in 1 mL of vehicle. Each solution was administered by gavage every day for 60 days. The eugenol concentrations were based on studies systematically reviewed by our research group [20]. The treatment period was determined using the duration of one cycle of the seminiferous epithelium in rats [39]. Body weight and clinical changes (e.g., diarrhea, vomiting, hair loss) were evaluated in rats from all groups during the experiment.

2.3.Euthanasia, tissue collection, and biometric analysis

After 60 d of treatment, the rats were weighed and euthanized by deep anesthesia (ketamine 150 mg Kg^{-1} i.p. and xylazine 10 mg Kg^{-1} i.p.) followed by cardiac puncture [40]. Blood was obtained for serum testosterone dosage. Testis and epididymis were removed, dissected, and the weights (absolute and relative weight to the body) recorded. The left testis and epididymis were used for histology, whereas the right ones were processed for sperm evaluation and biochemical analyses.

2.4. Determination of serum testosterone levels

Blood samples (n = 5/group) were centrifuged at 2,000 × g for 15 min. The obtained serum was used for quantifying serum testosterone levels by chemiluminescence methodology at the Laboratório Diagnósticos do Brasil (https://www.diagnosticosdobrasil.com.br/). The results were expressed in ng dL^{-1}.

2.5. Quantification of glucose and lactate levels

Fragments (100 mg) of testis and epididymis (n = 5/ organ per group) stored at -80 ºC were homogenized in 1 mL of PBS (pH 7.4; 0.2 M) and centrifuged at 2,000 × g for 10 min at 4 °C. The supernatant was used for determining glucose and lactate levels. All tests were performed using kits from Bioclin® (Belo Horizonte, MG, Brazil) following the manufacturer's recommendations. The results were expressed as mg g^{-1} tissue.

2.6. Assessment of oxidative stress markers

The analysis of oxidative stress markers was performed using 100 mg of testis parenchyma and epididymis (n = 5/ organ per group). The tissues were homogenized in PBS and centrifuged at 3,500 g for 10 min at 4 ºC. The supernatant was then used to measure the activity of superoxide dismutase (SOD) [41], catalase (CAT) [42], glutathione-S-transferase (GST) [43], and total antioxidant capacity by iron reduction method (FRAP) [44]. Total protein concentration was measured following the Lowry method [45], using bovine serum albumin as the standard curve. The nitric oxide (NO) levels were indirectly determined through the detection of nitrite/nitrate levels in the tissues according to the Griess methodology [46]. The status of lipid peroxidation was determined by analyzing the tissue levels of malondialdehyde (MDA) [47].

2.7. Histological processing and histomorphometrical analysis

Left testes and cauda epididymides (n = 5/group) were immersed in 10% formalin solution for 24 h. After the fixation period, the fragments were dehydrated in crescent series of ethanol (70, 80, 90, and 100%) and embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica Microsystems, Nussloch, Germany). Semi-serial sections of 3 μm thickness were obtained using a rotating microtome (RM 2255, Leica, Nussloch, Germany), stained with toluidine blue/sodium borate (1%), and mounted with Entellan (Merck, Germany). Qualitative analyses were performed using a light microscope at 10, 20, and 40 x magnification. Tissue sections were analyzed by the organization and integrity of tubular and intertubular compartments in the testis [48] and the tissue architecture from the cauda epididymis [49].

For morphometric analyses, ten digital images of the testis and cauda epididymis were obtained using a photomicroscope (Olympus BX53, Tokyo, Japan) and analyzed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD). Seminiferous tubules were used for obtaining the tubular diameter, luminal diameter, and epithelium height in 30 tubular cross-sections per animal, as described by Souza et al. [50]. Moreover, 50 nuclei of Leydig cells were chosen randomly in either circular or elliptical form, and their volume was measured using image analysis software Leica Q-win (version 3) with the aid of the software ImageJ 1.48. The nuclear volume of Leydig cells was determined by the mathematical formula: [Diameter^3 × π × 1/6] [51]. The epididymal duct, in turn, presented the ductal diameter, luminal diameter, and epithelium height by measuring 30 cross-sections per animal [52].

2.8. Analysis of daily sperm production (DSP), sperm number, and transit time in the epididymis

Homogenization-resistant testicular spermatids (HRTS) at stage 19 of spermiogenesis and sperm in the caput/corpus epididymis and cauda epididymis were counted as described by Robb et al. [53] with adjustments [54]. Firstly, the testis (n = 5/group) was decapsulated, weighed, and homogenized in 5 mL 0.9% NaCl containing 0.05% Triton X-100. After a 10-fold dilution, the sample was transferred to Neubauer chambers to count mature spermatids (four fields per animal). DSP was assessed by dividing the number of HRTS per testis by 6.1 (the number of days those spermatids are present in the seminiferous tubules). Likewise, portions of caput/corpus and cauda epididymis (n=5/group) were cut into small fragments and homogenized in 0.9% NaCl containing 0.05% Triton X-100 (1:20), and sperm were counted as described for testis. The transit time of sperm through the epididymis was determined by dividing the number of sperm in each epididymal portion by the DSP.

2.9. Analysis of sperm parameters

Freshly dissected portions of the cauda epididymis (n = 5/group) were cut into small pieces and placed in a Petri dish containing 1 mL Biggers-Whitten-Whittingham (BWW) medium [55]. Fragments were maintained immersed in this medium for 5 min at 37 °C to enable the release of spermatozoa. Aliquots of this fluid were used for sperm cells analysis. Thus, sperm motility was assessed using 10 µL of cauda epididymal fluid placed between the slide and coverslip, previously heated to 37 °C. Two hundred sperm cells were evaluated under a phase-contrast microscope (L-1000B, Bioval, São Paulo, Brazil) at 400× magnification and classified as motile or immotile cells [56]. Moreover, sperm morphology was evaluated using 50 µL of epididymal fluid fixed in 100 µL 4% buffered formaldehyde. The samples were smeared onto glass slides and analyzed under a phase-contrast microscope (Bioval L-1000B) at 1000× magnification, and 200 gametes were analyzed. Morphological abnormalities were classified as defects in the head, midpiece, and tail [57]. In addition, 10 µL of epididymal fluid were incubated in a solution of 4% buffered formaldehyde plus buffer citrate, carboxyfluorescein diacetate (CFDA), and propidium iodide (PI) for 8 min at 37 °C [58]. Two hundred cells were counted by epifluorescence microscope at 400 x magnification and filter of 480 at 610 nm. They were classified into two categories: cells with intact plasma and acrosomal membranes ($CFDA^{+}/PI^{-}$), and cells with damaged plasma and acrosomal membranes ($CFDA^{-}/PI^{+}$). Results were expressed as percentage. Finally, Ca^{2+}-ATPase activity was determined in sperm samples by measuring the inorganic phosphate (Pi) released following the method described by Hjertén and Pan [59]. The incubation mixture contained 50 µL of $CaCl_2$ 0.1M, ATP 0.01M, and water. After equilibrating the tubes at 37 °C for 10 min, the reaction was initiated by the addition of 50 µL of sample. The contents were incubated at 37 °C for 30 min. The reaction was arrested by addition 500 µL of a cold solution of 10% TCA. The tubes were centrifuged at 1,000 g for 10 min, and the supernatant containing the phosphorous content was analyzed using a commercial kit (Bioclin Laboratories, Belo Horizonte, MG, Brazil) under the manufacturer's instructions.

2.11. *In silico* network analysis

Chemical-protein interaction networks with the eugenol structure for *Homo sapiens* and *Rattus norvegicus* were elaborated to elucidate potential mechanisms of action of this compound, using the STITCH software (available for free at http://stitch.embl) [60].

2.12. Statistical analysis

All results had their normality evaluated by the Shapiro-Wilk test. Data were submitted to One-way ANOVA followed by the *post hoc* Tukey test. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using GraphPad Prism (version 6.0, Graph Pad Software Inc., San Diego, CA, USA). Results were expressed as means ± standard deviation (SD).

3. Results

3.1. Biometric parameters did not change after eugenol treatment

The treatment with eugenol did not cause any alteration in clinical signs of animals during the experiment. In addition, animals receiving 10, 20, and 40 mg Kg^{-1} eugenol did not show changes in their body and male organs' weight ($p > 0.05$; Table 1).

3.2.Eugenol altered hormonal and biochemical parameters in treated rats

The rats treated with 40 mg Kg^{-1} eugenol presented lower serum testosterone levels, followed by animals receiving 20 mg Kg^{-1}, than control rats and animals treated with 10 mg Kg^{-1} eugenol ($p < 0.05$; Table 1). Testicular glucose and lactate levels did not alter after eugenol intake ($p > 0.05$; Table 1). In the epididymis, in turn, the concentration of lactate was higher in rats receiving 40 mg Kg^{-1} eugenol compared to animals from the other groups. Also, glucose levels were higher in the epididymis of rats treated with 40 mg Kg^{-1} eugenol than in controls rats and animals treated with 10 mg Kg^{-1} of this compound ($p < 0.05$; Table 1).

Table 1. Biometric, hormonal, and biochemical parameters of Wistar rats treated with eugenol for 60 days.

Parameters	Control	Eugenol		
		10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Body weight (g)	407.7 ± 40.50[a]	398.1 ± 32.82[a]	398.6 ± 38.85[a]	384.5 ± 25.72[a]
Testis weight (g)	1.72 ± 0.09[a]	1.75 ± 0.11[a]	1.69 ± 0.14[a]	1.68 ± 0.05[a]
Relative weight of testis (g/ 100 g)	0.42 ± 0.02[a]	0.45 ± 0.01[a]	0.42 ± 0.04[a]	0.42 ± 0.01[a]
Epididymis weight (g)	0.71 ± 0.06[a]	0.72 ± 0.05[a]	0.69 ± 0.04[a]	0.70 ± 0.06[a]
Relative weight of epididymis (g/ 100 g)	0.17 ± 0.01[a]	0.18 ± 0.02[a]	0.17 ± 0.01[a]	0.18 ± 0.01[a]
Serum testosterone (ng dL^{-1})	688.7 ± 154.7[a]	619.0 ± 118.2[a]	329.3 ± 57.38[b]	291.6 ± 82.61[c]
Testicular lactate (mg g^{-1} tissue)	28.56 ± 1.85[a]	31.19 ± 4.70[a]	34.23 ± 6.00[a]	32.02 ± 6.05[a]
Testicular glucose (mg g^{-1} tissue)	3.18 ± 0.36[a]	3.05 ± 0.77[a]	3.47 ± 0.83[a]	3.71 ± 0.91[a]
Epididymal lactate (mg g^{-1} tissue)	34.34 ± 3.56[a]	32.36 ± 6.20[a]	33.14 ± 1.29[a]	43.87 ± 4.61[b]
Epididymal glucose (mg g^{-1} tissue)	5.23 ± 1.16[a]	5.37 ± 1.15[a]	5.51 ± 0.95[ab]	7.98 ± 1.42[b]

Mean ± SD. Control group: Rats receiving vehicle (2% Tween 20) by gavage; Eugenol groups: Rats treated with eugenol (diluted in 2% Tween 20) by gavage. Different superscript letters (a,b,c) in the same row indicate differences among the groups ($p < 0.05$) by *post hoc* Tukey's test (n = 5 rats/group).

3.3. Eugenol altered oxidative stress markers in the testis and epididymis tissue

Table 2. Antioxidant enzymes and oxidative metabolites in testis and epididymis of Wistar rats treated with eugenol for 60 days.

Parameters	Control	Eugenol		
		10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Testis				
Superoxide dismutase (U/mg protein)	0.34 ± 0.08^{a}	0.45 ± 0.12^{a}	0.43 ± 0.04^{a}	0.38 ± 0.08^{a}
Catalase (U/mL)	127.5 ± 7.90^{a}	105.5 ± 14.32^{b}	81.31 ± 6.43^{c}	86.85 ± 6.31^{c}
Glutathione-S-transferase (µmol/min/g)	19.50 ± 3.64^{a}	16.44 ± 1.70^{a}	17.23 ± 3.73^{a}	19.92 ± 2.90^{a}
Total antioxidant capacity (µM/Fe^{2+})	0.59 ± 0.08^{a}	0.55 ± 0.07^{a}	0.61 ± 0.13^{a}	0.60 ± 0.06^{a}
Nitric oxide (µM)	1.41 ± 0.23^{a}	1.18 ± 0.23^{a}	1.21 ± 0.07^{a}	1.58 ± 0.25^{a}
Malondialdehyde (µM/mg protein)	4.51 ± 0.85^{a}	2.00 ± 0.51^{b}	1.77 ± 0.25^{b}	1.18 ± 0.15^{b}
Epididymis				
Superoxide dismutase (U/mg protein)	0.49 ± 0.25^{a}	0.42 ± 0.04^{a}	0.45 ± 0.17^{a}	0.44 ± 0.06^{a}
Catalase (U/mL)	41.37 ± 2.62^{a}	59.08 ± 9.10^{b}	59.20 ± 6.26^{b}	119.7 ± 3.93^{c}
Glutathione-S-transferase (µmol/min/g)	6.82 ± 2.59^{a}	7.81 ± 0.66^{a}	6.97 ± 1.37^{a}	7.26 ± 1.66^{a}
Total antioxidant capacity (µM/Fe^{2+})	0.46 ± 0.08^{a}	0.76 ± 0.04^{b}	0.73 ± 0.08^{b}	0.66 ± 0.03^{b}
Nitric oxide (µM)	1.61 ± 0.84^{a}	1.98 ± 0.57^{a}	2.49 ± 0.83^{a}	1.41 ± 0.46^{a}
Malondialdehyde (µM/mg protein)	3.57 ± 1.65^{a}	2.82 ± 0.44^{a}	3.48 ± 1.26^{a}	2.64 ± 0.42^{a}

Mean ± SD. Control group: Rats receiving vehicle (2% Tween 20) by gavage; Eugenol groups: Rats treated with eugenol (diluted in 2% Tween 20) by gavage. Different superscript letters (a,b,c) in the same row indicate differences among the groups ($p < 0.05$) by *post hoc* Tukey's test (n = 5 rats/group).

In the testis, the lowest activity of CAT was observed in eugenol-treated rats from 20 and 40 mg Kg^{-1} groups ($p < 0.05$; Table 2), followed by the 10 mg Kg^{-1} group ($p < 0.05$; Table 2). The other enzymes did not present changes in their activity after eugenol treatment ($p > 0.05$; Table 2). Moreover, the levels of MDA were lower in the testis of rats from all eugenol groups than in animals from the control group ($p < 0.05$; Table 2). NO and FRAP levels did not differ among treatments ($p > 0.05$; Table 2).

The highest activity of CAT was observed in the epididymis of animals receiving 40 mg Kg^{-1}. The activity of this antioxidant enzyme was also higher in rats treated with 10 and 20 mg Kg^{-1} eugenol than in the control animals ($p < 0.05$; Table 2). Epididymis from rats receiving 10, 20, and 40 mg Kg^{-1} eugenol showed increased FRAP compared to their controls ($p < 0.05$; Table 2). No differences among groups were observed regarding SOD, GST, MDA, and NO parameters ($p > 0.05$; Table 2).

3.4. Eugenol caused no histological alteration in the testis and epididymis tissue

Histological sections of the testis from control and eugenol-treated rats showed intact tissue architecture. The tubular compartment exhibited concentric seminiferous tubules containing regular germ cell layers in their seminiferous epithelium and sperm in the lumen (Fig. 1). The intertubular compartment, in turn, was composed of Leydig cells, lymphatic and blood vessels, and connective tissue (Fig. 1). The morphometry of tubular diameter, luminal diameter, and epithelium height, as well as the nuclear volume of Leydig cells, did not differ among groups ($p > 0.05$; Table 3). Likewise, the tissue architecture of the cauda epididymis did not alter from control and eugenol-treated rats. Overall, cauda epididymis displayed characteristic tubular arrangement, with a pseudostratified epithelium composed of the principal, clear, and basal cells and a lumen filled with spermatozoa ($p > 0.05$; Fig. 1). Also, the morphometry of ductal and luminal diameters and epithelium height in cauda epididymis was similar among groups ($p > 0.05$; Table 3).

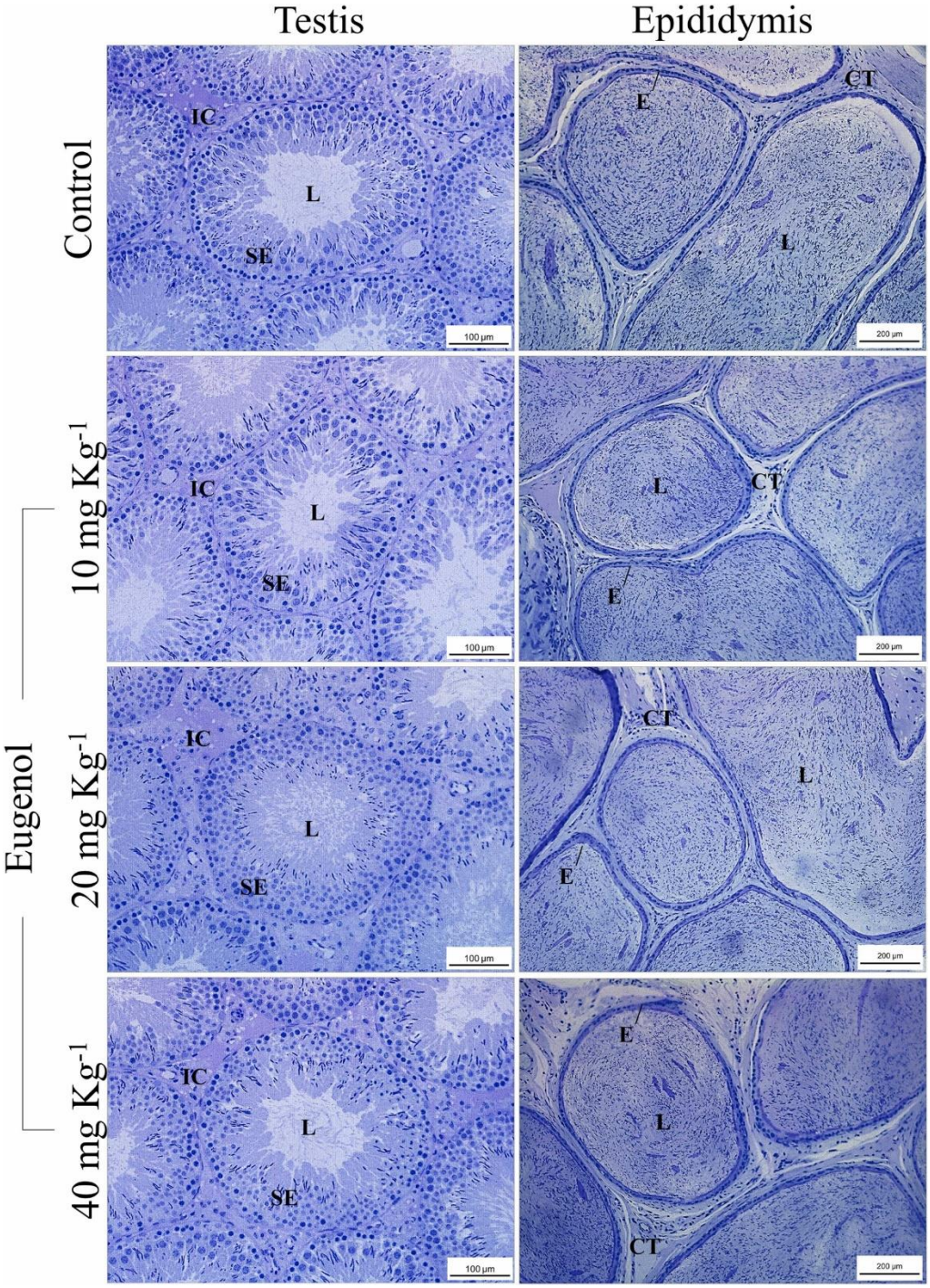


Figure 1. Histological sections of testis and cauda epididymis of Wistar rats treated with eugenol at three concentrations. Sections stained with toluidine blue and analyzed under light microscopy. IC: intertubular compartment; L: lumen; SE: seminiferous epithelium; LC: Leydig cell; SP: sperm; BV: blood vessels; E: epithelium; CT: connective tissue; L: lumen; SM: smooth muscle cells; BV: blood vessels. (n = 5 animals/group).

Table 3. Testicular and epididymal morphometry of Wistar rats treated with eugenol for 60 days.

Parameters	Control	Eugenol		
		10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Testis				
Tubular diameter (μm)	314.3 ± 6.28[a]	313.9 ± 5.46[a]	309.2 ± 1.69[a]	311.1 ± 1.84[a]
Lumen diameter (μm)	117.0 ± 5.02[a]	122.0 ± 2.77[a]	117.6 ± 4.41[a]	116.3 ± 7.09[a]
Epithelium height (μm)	100.2 ± 4.68[a]	96.37 ± 2.33[a]	97.76 ± 3.04[a]	100.2 ± 0.92[a]
Leydig cell nuclear volume (μm^3)	110.0 ± 3.52[a]	115.3 ± 5.62[a]	109.0 ± 3.31[a]	114.9 ± 4.57[a]
Cauda epididymis				
Ductal diameter (μm)	448.4 ± 44.2[a]	465.4 ± 33.8[a]	458.1 ± 14.9[a]	440.2 ± 20.6[a]
Lumen diameter (μm)	421.7 ± 42.6[a]	430.5 ± 33.2[a]	428.5 ± 17.8[a]	412.3 ± 20.1[a]
Epithelium height (μm)	14.88 ± 2.0[a]	16.75 ± 2.3[a]	14.78 ± 2.2[a]	13.98 ± 1.2[a]

Mean ± SD. Control group: Rats receiving vehicle (2% Tween 20) by gavage; Eugenol groups: Rats treated with eugenol (diluted in 2% Tween 20) by gavage. [a]Superscript letters in the same row indicate non-significant difference among groups ($p > 0.05$) by one-way ANOVA. (n = 5 rats/group).

3.5.Eugenol exerted negative impact in sperm parameters of treated rats

Our results showed that eugenol did not alter the number of mature spermatids per testis and gram of testis and the DSP ($p > 0.05$; Table 4). Similarly, the number of sperm count and the sperm transit time in caput/corpus and cauda epididymis did not differ among groups ($p > 0.05$; Table 4). The percentage of sperm motility was lower in rats receiving 10, 20, and 40 mg Kg^{-1} eugenol than in their controls. The lowest motility percentual was observed in epididymal sperm from animals treated with 40 mg Kg^{-1} eugenol ($p < 0.05$; Table 4). Further, rats treated with 20 and 40 mg Kg^{-1} eugenol showed a lower percentage of sperm with normal morphology and a higher percentage of sperm with midpiece defects than gametes from control animals ($p < 0.05$; Table 4). No differences were observed among groups concerning the percentage of sperm cells with head and tail defects ($p > 0.05$; Table 4). Moreover, only sperm from rats treated with 40 mg Kg^{-1} eugenol showed low integrity of acrosome and sperm membranes ($p < 0.05$; Table 4). Finally, the activity of Ca^{2+}-ATPase pump was higher in epididymal sperm from rats receiving 10, 20, and 40 mg Kg^{-1} eugenol than in control animals ($p < 0.05$; Table 4).

Table 4. Sperm count parameters in testis and epididymis of Wistar rats treated with eugenol at three concentrations for 60 days.

Parameters	Control	10 mg Kg^{-1}	20 mg Kg^{-1}	40 mg Kg^{-1}
Spermatid number ($\times 10^6$/testis)	113.6 ± 30.31^a	99.22 ± 12.66^a	93.06 ± 14.68^a	91.83 ± 6.92^a
Daily sperm production ($\times 10^6$/testis/day)	20.9 ± 6.1^a	19.1 ± 5.9^a	17.5 ± 4.2^a	16.3 ± 3.1^a
Caput/corpus epididymis sperm number ($\times 10^6$/organ)	59.3 ± 9.8^a	53.4 ± 13.9^a	51.8 ± 6.6^a	51.6 ± 8.5^a
Sperm transit in caput/corpus epididymis (days)	3.26 ± 0.67^a	3.13 ± 0.46^a	3.45 ± 0.62^a	3.81 ± 0.86^a
Cauda epididymis sperm number ($\times 10^6$/organ)	103.6 ± 37.06^a	113.2 ± 21.50^a	92.96 ± 10.99^a	99.30 ± 15.23^a
Sperm transit time in the cauda epididymis (days)	6.46 ± 1.40^a	6.72 ± 0.74^a	5.93 ± 0.43^a	6.49 ± 1.42^a
Sperm motility (%)	81.8 ± 5.8^a	68.6 ± 5.3^b	60.8 ± 2.9^{bc}	52.8 ± 7.3^c
Sperm with normal morphology (%)	89.5 ± 2.17^a	85.2 ± 1.2^{ab}	81.40 ± 4.98^b	79.40 ± 5.17^b
Sperm with head defects (%)	4.20 ± 1.30^a	5.40 ± 2.79^a	6.80 ± 1.48^a	6.80 ± 2.16^a
Sperm with midpiece defects (%)	1.10 ± 0.22^a	2.56 ± 0.93^{ab}	3.20 ± 1.64^b	4.00 ± 0.70^b
Sperm with tail defects (%)	5.20 ± 0.83^a	6.80 ± 2.16^a	8.60 ± 3.13^a	9.80 ± 3.89^a
Sperm with intact membranes (%)	74.20 ± 6.53^a	66.80 ± 3.89^a	66.20 ± 6.14^a	53.20 ± 3.63^b
Ca^{2+} ATPase activity (Pi/min/mg protein)	0.05 ± 0.01^a	0.18 ± 0.05^b	0.16 ± 0.02^b	0.17 ± 0.03^b

Mean ± SD. Control group: Rats receiving vehicle (2% Tween 20) by gavage; Eugenol groups: Rats treated with eugenol (diluted in 2% Tween 20) by gavage. Different superscript letters (a,b,c) in the same row indicate differences among the groups ($p < 0.05$) by *post hoc* Tukey's test (n = 5 rats/group).

3.6. Eugenol interacts with proteins from UGT family, caspase 9, NAD(P)H dehydrogenase, and cation channel receptor

The *in silico* network performed using STITCH software for *Homo sapiens* (Fig. 2a) showed interactions between eugenol and enzymes from the UGT family. Eugenol also interacts with caspase 9 and NAD(P)H dehydrogenase, quinone 1. When investigating the network built for *Rattus norvegicus* organism (Fig. 2b), besides the interaction with enzymes from the UGT family, the eugenol also interacts with the transient receptor potential cation channel subfamily A member 1 (Trpa1).

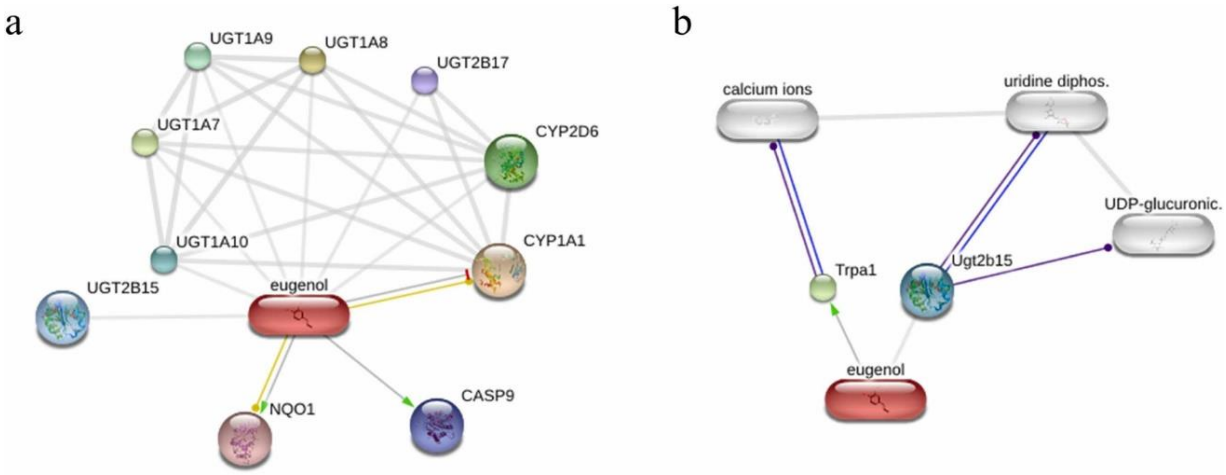


Figure 2. Bioinformatics predictions of proteins interacting with eugenol (using STITCH, http://stitch.embl.de/). (a) *Homo sapiens*. NQO1: NAD(P)H dehydrogenase, quinone 1; CASP9: caspase 9; CYP1A1: cytochrome P450, family 1, subfamily A, polypeptide 1; CYP2D6: cytochrome P450 family 2, subfamily D, polypeptide 6; UGT2B17: UDP glucuronosyltransferase 2 family, polypeptide B17; UGT1A8: UDP glucuronosyltransferase 1 family, polypeptide A8; UGT1A9: UDP glucuronosyltransferase 1 family, polypeptide A9; UGT1A7: UDP glucuronosyltransferase 1 family, polypeptide A7; UGT1A10: UDP glucuronosyltransferase 1 family, polypeptide A10; UGT2B15: UDP glucuronosyltransferase 2 family, polypeptide B15. (b) *Rattus norvegicus*. Ugt2b15: UDP-glucuronosyltransferase 2B15 precursor; UDP-glucuronic.: Uridine diphosphate glucuronic; Uridine diphos.: Uridine diphosphate; Trpa1: transient receptor potential cation channel subfamily A member 1.

4. Discussion

The current study provides pioneer information concerning the effects of eugenol on male reproductive functions. This phenylpropanoid compound did not change the testis and epididymis biometry, histology, and histomorphometry. Likewise, eugenol did not alter sperm production and number in healthy rats. However, 10, 20, and 40 mg Kg^{-1} eugenol were harmful

to sperm parameters, whereas the higher doses (20 and 40 mg Kg^{-1}) affected serum testosterone levels. Eugenol modulated some oxidative and biochemical parameters relying upon the organ and the concentration.

Eugenol was not toxic to the animals once they did not exhibit alterations in their clinical sign and body weight. Analysis of body weight supplies relevant information about the toxic potential of compounds and the possible implications for body homeostasis [61]. The similarity of final body weight between animals treated with eugenol and their controls indicates no apparent systemic consequences to animal health, which corroborates the results reported by other studies using continuous doses of 10, 20, and 100 mg Kg^{-1} eugenol [62,63]. Also, the absolute and relative weight of testis and epididymis did not alter in eugenol-treated rats. These parameters indicate drug side effects in male reproductive organs [50, 64].

Healthy rats treated with 10, 20, and 40 mg Kg^{-1} eugenol presented preserved testicular histology and histomorphometry. The maintenance of testis histoarchitecture is directly related to intratesticular testosterone levels. Testosterone production is influenced by the pituitary gland through the secretion of luteinizing gonadotropin hormone, which activates steroidogenic enzymes in Leydig cells [65,66]. Unfortunately, there are no studies evaluating the effect of purified eugenol on the hypothalamic-pituitary-testicular axis. Only one study described the positive effects of eugenol on the central nervous system by protecting neuronal cells against excitotoxicity and acting as an anti-stressor agent modulating the hypothalamic-pituitary-adrenal axis [67]. Regarding Leydig cells, their nuclear volume did not change in eugenol-treated rats, which is a sensitive indicator of cellular secretory activity [68]. Once secreted, testosterone crosses the wall of seminiferous tubules and binds to androgen receptors in Sertoli cells. Hence, these cells can support germ line cells during spermatogenesis and secrete the luminal fluid. Also, Sertoli cells produce androgen binding proteins (ABP) through follicle-stimulating hormone stimulation [69,70,71,72]. ABP binds to testosterone that, in turn, becomes less lipophilic and highly concentrated within the luminal fluid of the seminiferous tubules, enabling the spermatogenic process [73]. It is worth mentioning that the intratesticular concentration of testosterone is 10-fold higher than its serum levels [74]. Thus, the maintenance of tubular morphometry, integrity of seminiferous epithelium, and sperm production observed in eugenol-treated animals reflect an adequate testosterone concentration within the seminiferous tubules.

Notwithstanding, our results showed that serum testosterone levels decreased dramatically in animals treated with 40 mg Kg^{-1} eugenol, as well as 20 mg Kg^{-1}. The detrimental effect of eugenol on serum testosterone levels was observed in female Wistar rats treated with

0.4 ml of this compound through intramuscular injections for 15 days [75]. *In silico* analysis showed that eugenol interacts with NAD(P)H dehydrogenase and enzymes from the UDP-glycosyltransferases (UGT) family. For instance, enzymes from this family are highly expressed in the liver and intestine of eugenol-treated rats [76]. UGTs catalyze the covalent addition of sugars to a wide range of lipophilic molecules, eliminating exogenous chemicals and byproducts of endogenous metabolism. Also, UGTs are involved in the main androgen-metabolizing pathway. The glucuronidation of androgens is an irreversible event. It can act locally in male reproductive organs modulating androgen levels and shutting down androgen/receptor signaling. Additionally, UGTs act in the liver to control serum androgen levels [77,78]. Iwano et al. [76] observed a positive effect of eugenol on the mRNA expression of the UGT gene family in the liver of treated rats. Therefore, we may suggest that eugenol induces testosterone glucuronidation in the liver and accelerates its excretion culminating in low serum levels. Future studies may investigate the effects of eugenol treatment on testosterone biosynthesis and metabolism.

Eugenol treatment did not affect the epididymis histology, epididymal sperm number, and sperm transit time. Adequate testicular functions involving testosterone, sperm, and luminal fluid production are essential for epididymis integrity and functionality [79,80]. The epididymis is well-known for its dependence on androgen stimulus and its role in sperm maturation, which is crucial for gamete fecundity [81]. Once male gametes are immature after testis releasing, they acquire motility and fertility ability during their passage throughout the epididymis [82,83]. The transit within the epididymal duct lasts between 7 and 10 days in rats, and any alteration in this period may affect sperm fertility [84].

Nevertheless, sperm cells harvested from cauda epididymis were affected by 10, 20, and 40 mg Kg^{-1} eugenol after 60 days of treatment, exhibiting reduced motility mainly at 40 mg Kg^{-1}. Similarly, other studies have reported low motility of sperm from mice and catfish exposed to eugenol-rich extracts and pure eugenol, respectively [35,85]. Al-Alami et al. [86] also reported an impairment in sperm motility in animals treated with β-caryophyllene, a substance present in the clove extract, despite the maintenance of structural and tissular integrity in testis and epididymis. The increased activity of Ca^{2+} pumps in spermatozoa of rats from all eugenol groups and the interaction between eugenol and Trpa1, shown by *in silico* assay, support the hypothesis that the decrease in sperm motility occurred by a disturbance in the Ca^{2+} homeostasis. *Previous studies have reported the importance of this event as an early marker of fertility disorders (Schuh et al., 2004; Kumosani et al., 2008; Lestari et al., 2017*). In fact, eugenol induces cytosolic Ca^{2+} elevations resulting from the Ca^{2+}influx across the plasma

membrane in nerve cells [87], immune-responsive cells [88], and yeast models [89]. The activation of TRP channels may be responsible for the cytosolic Ca^{2+} increase caused by eugenol [90, 91]. A large set of TRP channels regulate the influx of Ca^{2+} and the release of Ca^{2+} from storage sites in the sperm [92]. Although Ca^{2+} is necessary for sperm motility, it must be at adequate levels to allow progressive motility [93]. On the other hand, Ca^{2+}-ATPase may act as a fine-tuner of Ca^{2+} efflux and maintain Ca^{2+} homeostasis in other mammalian cells [94]. The ablation of this enzyme reduces sperm motility in mice, probably resulting from Ca^{2+} overload and mitochondrial damage [95]. Even though an abnormally high concentration of intracellular Ca^{2+} can alter the function of Ca^{2+}-ATPase [96], it seems that the sperm plasma membrane from cauda epididymis is not fully saturated with Ca^{2+}-ATPase before ejaculation [97]. This fact may explain the high sperm Ca^{2+}-ATPase activity observed in eugenol-treated animals. We assume that Ca^{2+}-ATPase exerted a cellular defense function in the sperm of the eugenol-treated groups, working up to its threshold to release Ca^{2+} extracellularly to maintain Ca^{2+} homeostasis.

Moreover, we observed a decrease in membrane and acrosome integrity of sperm from animals treated with 40 mg Kg^{-1} eugenol and a high percentage of sperm exhibiting abnormal morphology in animals treated with 20 and 40 mg Kg^{-1}. Oehninger et al. [98] reported abnormal levels of Ca^{2+} in infertile patients with a high incidence of cells with abnormal morphology. Indeed, the sperm plasma membrane contributes to cellular homeostasis, whereas the acrosome is critical for mammalian fertilization. Sperm must undergo acrosomal exocytosis to penetrate the zona pellucida. During these processes, notable changes in ionic homeostasis occur, such as a transient increase in Ca^{2+} [99]. Therefore, we may associate the effect of eugenol with the loss of sperm membrane integrity due to a dysregulation of Ca^{2+}.

In this study, we observed increased glucose and lactate levels in the epididymis of healthy rats treated with 40 mg Kg^{-1} eugenol, differently in the testis. Glucose uptake elevation may be an adaptive response for maintaining high glycolysis and lactate production [100]. Accordingly, epithelial cells lining the duct may improve the secretion of protons and lactate into the epididymal fluid to maintain sperm cells in their quiescent state [101]. Another hypothesis that is worth to be mentioned is the direct effect of eugenol on glucose transporters. Several transporters were identified in the epididymis of rodents, such as GLUT 1, 3, 4, and 8 [102,103]. Eugenol may play a role in the stimulus of glucose uptake by translocating GLUT 4 [104].

All eugenol-treated groups showed low CAT activity and MDA levels in the testis. CAT is one of the enzymes responsible for converting H_2O_2 into water, whereas MDA is a byproduct

of lipid peroxidation produced by excessive generation of H_2O_2 in the tissue [105]. The inhibition of lipid peroxidation provided by eugenol and the simultaneous decrease in some antioxidant enzyme activity [20,106] may indicate the antioxidant activity of this compound. Likewise, our findings showed that eugenol treatment did not cause oxidative damage in the epididymis. The high activity of CAT and FRAP observed in rats treated with 10, 20, and 40 mg Kg^{-1} eugenol may have neutralized reactive oxygen/nitrogen species and promoted an optimal cellular redox state, which favors the activity of antioxidant enzymes [107,108].

Interestingly, pure eugenol at low (10 mg Kg^{-1}) and high doses (20 and 40 mg Kg^{-1}) did not cause injuries to male reproductive organs, unlike the studies evaluating male exposure to clove extracts [32,34,35]. Our results evidence the effects of isolated eugenol rather than a complex mixture of substances often found in plant extracts. Their distinct effect may be related to the synergistic or additive effects of the multiple compounds present in extracts [30]. The negative impact of pure eugenol observed in body organs, such as the liver and stomach [37,109,110,111], is mainly related to metabolites (e.g., quinone methide) produced during its metabolism in animals exposed to high doses [112]. On the other hand, low eugenol doses are usually considered safe and effective for their therapeutic activities [3,19,37,113]. However, the intake of 10 mg Kg^{-1} showed an initial deleterious effect of eugenol on sperm parameters that may intensify in long-term treatment. *Many studies have reported the potential importance of this event as an early marker of fertility disorders (Schuh et al., 2004; Kumosani et al., 2008; Lestari et al., 2017).* Sperm damage has been documented by authors testing low doses of natural substances, such as acaciaside [114,115] and saponins [116,117]. These substances are primarily involved in the pharmacological benefits of the herbs *Acacia auriculiformis, Calendula officinalis, and Cestrum parqui*. These compounds, for example, showed spermicide activity in humans and animals at low concentrations [118,119]. Therefore, our findings highlight the potential detrimental effect of low doses of eugenol on sperm viability, which may trigger alterations in their physiology and affect male fertility.

In conclusion, our findings revealed that eugenol intake did not affect testis and epididymis biometry, microscopy, as well as daily sperm production and number. However, the ingestion of 20 and 40 mg Kg^{-1} eugenol diminished serum testosterone levels. Moreover, 10, 20, and 40 mg Kg^{-1} eugenol were harmful to sperm motility and Ca^{2+} ATPase, reducing the percentage of gametes with normal morphology and intact membranes at higher doses. Collectively, these alterations compromise cell viability and may affect sperm fertilizing ability. Bearing in mind the scant information regarding eugenol and its effect on male reproductive

parameters, the exact molecular mechanisms by which eugenol may affect spermatozoa must be investigated.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding this article.

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6. CONCLUSION

Based on the findings presented in this thesis, it can be concluded that eugenol in different doses has significant and distinct effects on biochemical, oxidative, and morphological parameters in multiple organs of healthy Wistar rats, including the liver, pancreas, submandibular, and sublingual glands, kidneys, and male reproductive organs. Treatment with eugenol, especially at higher doses, can cause structural and functional damage to these organs. On the other hand, the lowest evaluated dose can be considered in future studies aiming to explore the therapeutic potential of eugenol. Furthermore, our findings highlight the importance of an in-depth assessment of eugenol toxicity and potential risks and possible mechanisms that modulate organ-specific responses to treatment with this compound.