SUBACUTE TREATMENT OF COPAIBA AND WHITE ROSIN ESSENTIAL OILS USING INTEGRATED BIOMARKER RESPONSES ON THE REPRODUCTIVE SYSTEM OF WISTAR RATS EXPOSED TO VALPROIC ACID

TRATAMENTO SUBAGUDO DOS ÓLEOS ESSENCIAIS DE COPAÍBA E BREU BRANCO USANDO RESPOSTAS DE BIOMARCADORES INTEGRADOS NO SISTEMA REPRODUTIVO DE RATOS WISTAR EXPOSTOS AO ÁCIDO VALPRÓICO

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ABSTRACT

Valproic acid (VPA) is a short-chain fatty acid used as a treatment for epilepsy. Treatment with valproic acid presents several side effects, such as male subfertility, causing reversible changes in sperm motility, morphology, sperm count, and testicular cytoarchitecture. The objective was to understand how the use of valproic acid during pregnancy can affect the fertility of male offspring, and whether the use of copaiba, white rosin essential oils, or their combination can reverse the damage caused. Female rats in the negative control group received water, while those in the experimental groups received valproic acid (600 mg/kg) intraperitoneally, in a single dose. The male offspring were divided into six groups: negative and positive controls, vehicle, treated with copaiba oil, white rosin oil, and a combination of copaiba and white breurosin oils. The TERRA® oils were administered via gavage for 30 consecutive days. Results showed oxidative and nitrosative stress in the testis and epididymis, along with alterations in antioxidant enzyme activity. Mild testicular changes and cribiform areas in the epididymis were observed. It was concluded that exposure to valproic acid during the intrauterine period causes oxidative damage and structural alterations in both the testis and epididymis. However, the use of essential oils at this dose and duration was not sufficient to reverse these damages, even though it altered the activity of antioxidant enzymes.

KEYWORD: toxicology; testis; epididymis; valproic acid.

RESUMO

O ácido valpróico é um ácido graxo de cadeia curta, utilizado como tratamento para epilepsia. O tratamento com ácido valpróico apresenta vários efeitos colaterais como subfertilidade masculina, causando alterações reversíveis na motilidade do espermatozoide, morfologia, contagem de espermatozoides e citoarquitetura dos testículos. Objetivou-se entender como o uso do ácido valpróico durante a gestação pode afetar a fertilidade da prole masculina, e se o uso de óleos essenciais de copaíba, breu branco ou a associação deles pode reverter os danos causados. as ratas do grupo controle negativo receberam água e as dos grupos experimentais receberam ácido valpróico (600 mg/kg), por via intraperitoneal, uma única vez. Os filhotes machos foram divididos em seis grupos: controles negativo e positivo; veículo; tratado com óleo de copaíba; com óleo breu branco e com óleo de copaíba associado ao breu branco. Os óleos do TERRA® foram administrados via gavagem, por 30 dias consecutivos. Como resultados observamos estresse oxidativo e nitrosativo testicular e epididimário, e uma alteração da atividade das enzimas antioxidantes. Alterações testiculares leves e áreas cribiforme epididimária. Conclui-se que a exposição ao ácido valpróico durante o período intrauterino casa danos oxidativos e alterações estruturais tanto testiculares como epididimário, porém o uso dos óleos essenciais nesta dose e tempo não foram suficientes para reverter esses danos mesmo alterando a atividade das enzimas antioxidantes.
PALAVRAS-CHAVE: toxicologia; testículo; epidídimo**;** ácido valpróico.

INTRODUCTION

Valproic acid (VPA) is a short-chain fatty acid used as a treatment for epilepsy in both adults and children[1]. It is also employed in the treatment of other conditions such as bipolar disorders, migraine prophylaxis, schizophrenia, and severe depression[2]. When patients are pregnant or planning to become pregnant, antiepileptic drugs cannot be discontinued due to the risk of seizures during pregnancy. VPA treatment presents various side effects, mainly affecting the gastrointestinal, neurological, and hematological systems[3].

Although the most critical period is between three and eight weeks of gestation, several organs continue to develop beyond this period and may still be affected by teratogens. Examples include the heart[4] and the reproductive system[5]. It is known that VPA use can cause male subfertility, leading to reversible alterations in sperm motility, morphology, sperm count, and testicular histoarchitecture[6]. The pathogenesis of these VPA-associated toxicities is not well

understood. However, recent studies have highlighted its role in inducing oxidative stress, a critical factor in cellular damage[7].

Essential oils (EOs) are secondary metabolites of plants, colorless liquids that are primarily aromatic and naturally occurring volatile organic compounds found in all parts of the plant[8]. They are commonly included as medical products, perfumes, cosmetics, and dietary supplements in various countries. EOs exhibit specific medicinal benefits, such as antibacterial and antifungal activities[9]. Part of their biological effects is attributed to their antioxidant properties[10]. Among these essential oils, copaiba oil and white rosin oil are widely used in Brazil for their antioxidant properties and potential beneficial effects on diseases associated with oxidative stress[11].

Studies with EOs have shown positive results regarding some changes. Using the EO origanum marjoram suggests that it can control male infertility induced by pesticides[12]. Another study confirms that using the EO Lavandula dentata L. has different activities to improve the progression of histopathological changes. However, if used in combination with antiepileptic drugs, it can slow the progression of epileptogenesis[13]. Finally, a recent study showed that using EOs of copaiba and white rosin oil led to a picture of oxidative and nitrosative stress, and the combination of the two oils has promising results in reversing changes in cardiac microstructure caused by VPA[4].

Considering that prolonged use of VPA is widely associated with the induction of oxidative stress in several organs and tissues, which can compromise essential physiological functions — including fertility —it becomes relevant to understand the implications of this exposure during critical periods of development, such as gestation. Given that, in many clinical cases, interruption of VPA treatment during pregnancy is not feasible due to its efficacy in controlling seizures, this study aimed to investigate the effects of gestational exposure to VPA on the fertility of male offspring, evaluating the therapeutic potential of copaiba and white rosin essential oils, alone or in combination, in attenuating the induced reproductive damage, with a focus on their antioxidant properties.

MATERIALS AND METHODS

Thirty reproductive-age Wistar rats (120 days old) were used, consisting of 20 females and 10 males, with an average weight of 236.55 g. The animals were sourced from the pharmacy vivarium of UFPE (Federal University of Pernambuco) and housed in the anatomy department's vivarium during the entire experimental phase. The rats were kept in polypropylene cages, with two animals per cage, in an environment with an average temperature of 22°C (± 2°C), humidity between 60-70%, and a 12:12 h inverted light-dark cycle. All animals had access to commercial feed and water *ad libitum.*

The experimental procedures adhered to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee on Animal Use of the Federal University of Pernambuco (CEUA/UFPE - protocol number 126/2022).

After an adaptation period, the animals were mated in a 2:1 ratio of females to males. Vaginal smears were performed to confirm mating, with the presence of spermatozoa in the smear used as the criterion for determining the first day of gestation[14]. From the 13th day of gestation, the pregnant rats were randomly assigned to experimental groups, categorized as induced or non-induced. The induced females received a single intraperitoneal injection of valproic acid (VPA) at a dose of 600 mg/kg, diluted in water, with a total volume of 0.5 mL[4]. The control group females experienced only the stress of restraint and injection but received only water in the same volume as the other groups.

Fetal sexing was conducted at birth, and the litters were adjusted to ensure better nutritional control of the offspring, aiming for a consistent standard among the pups. The animals were weaned at 21 days postnatal and randomly assigned to the experimental groups, each containing 10 males: Negative Control Group (NCG): Animals not exposed to VPA, receiving 0.5 mL of water; Positive Control Group (PCG): Animals exposed to VPA, receiving 0.5 mL of water; Vehicle Group (VG): Animals exposed to VPA, receiving 0.5 mL of fractionated coconut oil;

Copaiba Essential Oil Group (CEOG): Animals exposed to VPA, receiving 0.5 mL of fractionated coconut oil + 50 mg/kg of copaiba essential oil; white rosin Essential Oil Group (BBEOG): Animals exposed to VPA, receiving 0.5 mL of fractionated coconut oil + 100 mg/kg of white rosin essential oil; copaiba and white rosin combined group (C+BBG): Animals exposed to VPA, receiving 0.5 mL of fractionated coconut oil + 50 mg/kg of copaiba essential oil and 100 mg/kg of white rosin essential oil.

The oils administered were commercial products from TERRA® and were delivered via gavage[15] for 30 consecutive days starting at 30 days of age. The duration and doses of treatment were based on guidelines and protocols from previous studies using copaiba and white rosin essential oils.

After 24 hours from the last treatment, the animals were weighed, sedated, and anesthetized using xylazine hydrochloride (10 mg/kg, intraperitoneal) and ketamine hydrochloride (25 mg/kg, intraperitoneal), respectively. During the anesthesia phase, blood was collected via cardiac puncture and centrifuged at 4,119 g for 20 minutes to obtain serum. The serum samples were properly labeled and stored in microtubes at -80°C. Subsequently, a longitudinal incision was made in the animal's abdominal cavity along the linea alba to facilitate dissection and collection of the liver, kidneys, and spleen. These organs were sectioned, with portions fixed in a formalin solution for histological processing and light microscopy. Other portions were frozen at -80°C for oxidative stress analysis**.**

The organs were dehydrated using a graded ethanol series and subsequently embedded in paraffin (Paraffin, Leica Microsystems, Nussloch, Germany). Tissue sections of 3 μm thickness were obtained using a rotary microtome (RM 2255, Leica Biosystems, Nussloch, Germany), with approximately 39 μm intervals between sections. For sample analysis, micrographs were captured using a bright-field microscope (Axio Imager.M2m/Zeiss) equipped with a camera (AXIO Cam HRc/Zeiss) and connected to the ZEN 2 PRO (Blue edition) imaging software. All images were analyzed using the ImageJ® software (National Institutes of Health, USA).

After dissection, each animal was individually weighed, and its respective organs—liver, kidney, and spleen—were also weighed on a precision scale (0.01g, AS500, Marte) immediately after the euthanasia process. Throughout the experiment, the animals were weighed once a week at fixed intervals starting from the beginning of the treatment (30 days). After dissection, each animal was individually weighed, and its respective organs—liver, kidney, and spleen—were also weighed.

Testis and epididymis (it was divided into proximal and distal parts) homogenates were prepared in phosphate buffer + EDTA. The analysis of oxidative stress markers and antioxidant activity was performed[14]. Catalase (CAT) activity was measured by the hydrogen peroxide (H_2O_2) decomposition rate. Superoxide dismutase (SOD) activity was evaluated using the xanthine oxidase method, based on H_2O_2 production and reduction of nitroblue tetrazolium. Total protein content was measured using the Bradford method. The oxidative status of the cells was assessed by the amount of malondialdehyde (MDA) and glutathione (GSH) available in the homogenized hearts. Total antioxidant capacity (FRAP) was measured by the reducing capacity of iron. Nitric oxide (NO) levels in the supernatant were measured indirectly by quantifying nitrite/nitrate levels in the hearts, according to the Griess method.

To integrate the results from different biomarkers and understand the overall responses, the Integrated Biomarker Response (IBR). The overall mean (m) and standard deviation (s) of all data for a given biomarker were calculated, followed by standardization for each condition to obtain Y, where $Y = (X - m)/s$, and X is the mean value for the biomarker at a given concentration. Instead of transforming each biphasic biomarker into two variables with positive and negative values, we used the squared form of the control score, thus $Z = (YControl - Y)^2$. Star plots were then used to display the score results (S) and to calculate the Integrated Biomarker Response (IBR).

To estimate the proportions of normal and pathological seminiferous tubules, 200 tubules were counted in random fields in the histological preparations

of the testes from each animal. The alterations in the seminiferous tubules were classified based on the score modified by Dias [14,16].

Qualitative analyses were performed under a light microscope with 100x, 200x, and 400x magnification. Tissue sections were analyzed for the organization and integrity of the epididymal compartments. The lumen, epithelium, and interstitial tissue were examined according to morphology and the occurrence of alterations, such as germ cells in the lumen, changes in the epithelium, morphology of clear cells, and inflammatory infiltrates in the interstitium[17].

The results were evaluated for normality using the Shapiro-Wilk test, followed by analysis of variance (ANOVA) and the Student Newman-Keuls test. The STATISTICA software for Windows 3.11 was used, and a significance level of $p \leq 0.05$ was considered. All results were expressed as mean ± standard deviation.

Principal Component Analysis (PCA) was performed to verify possible groupings, eliminate redundancies, and define the most important variables during the separation of experimental groups. For this, the data were transformed (varied) for standardization due to differences in scale magnitudes. The importance level of each variable was determined by the eigenvector values, with substantial correlation values determined for each attribute in relation to principal components (PC) 1 and 2. The importance level of each PC was determined using the Broken-stick method, where eigenvalues exceeding expected values were retained for interpretation. The analyses were performed using the Fitopac 2.1.2.85 software.

RESULTS

The initial weight of the animals showed no differences between the groups, while the final weight was lower in all experimental groups; therefore, the weight gain did not differ among them. Regarding the weights of the tests, epididymis, and seminal vesicles, no alterations were found, nor were there changes in the relative weight of these organs (Table 1).

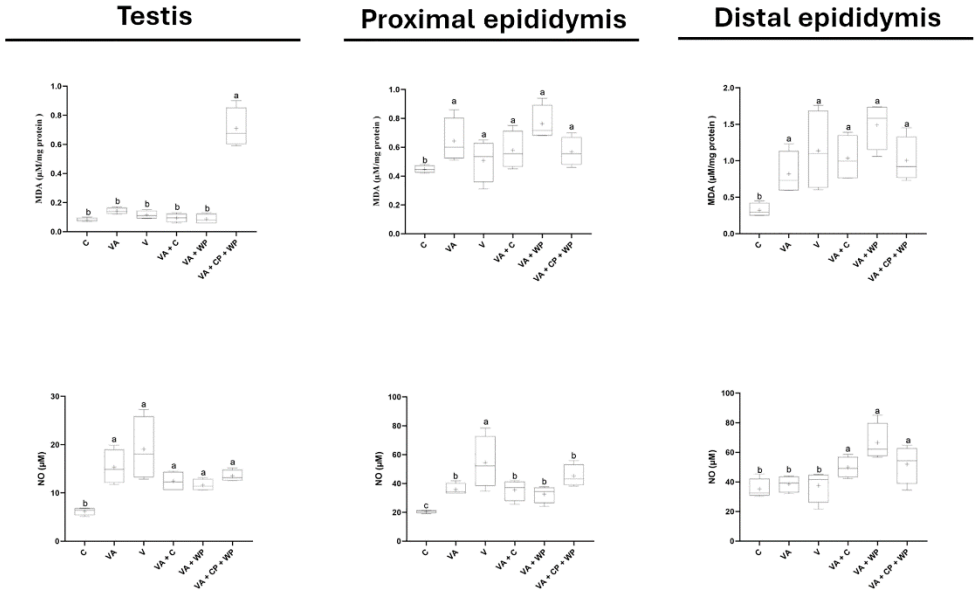
Table 1. Somatic and reproductive organ biometrics of male wistar rats prenatally exposed to valproic acid and postnatally treated with copaiba oil and white rosin oil.

	C	VA	V	AV + CP	AV + WP	VA + CP +WP
BW i	107±11 [a]	103±7 [a]	97±29 [a]	92±22 [a]	92±23 [a]	94±5 [a]
BW f	301,8±14,1[a]	276,7±10,7[b]	252,7±76,7 [b]	253,8±56,4 [b]	256,2±64,0 [b]	248,6±60,7 [c]
WG	195±13 [a]	174±14 [b]	157±47 [b]	161±38 [b]	165±42 [b]	167±21 [b]
TW	1,52±0,13 [a]	1,43±0,09 [a]	1,36±0,41 [a]	1,34±0,31 [a]	1,37±0,34 [a]	1,34±0,32 [a]
TRW	0,51±0,05 [a]	0,52±0,03 [a]	0,50±0,15 [a]	0,49±0,12 [a]	0,50±0,12 [a]	0,50±0,13 [a]
EW	0,45±0,04 [a]	0,42±0,06 [a]	0,40±0,12 [a]	0,38±0,09 [a]	0,40±0,11 [a]	0,39±0,10 [a]
ERW	0,15±0,02 [a]	0,15±0,02 [a]	0,15±0,04 [a]	0,14±0,03 [a]	0,15±0,04 [a]	0,15±0,04 [a]
SVW	0,65±0,27 [a]	0,70±0,15 [a]	0,58±0,18 [a]	0,57±0,22 [a]	0,67±0,24 [a]	0,62±0,23 [a]
SVRW	0,21±0,09[a]	0,25±0,05 [a]	0,21±0,07 [a]	0,21±0,08 [a]	0,24±0,09 [a]	0,23±0,10 [a]

BW i - Initial body weight; BW f - Final body weight. GW- Weight gain.TRW - Relative testicular weight; ERW- Epididymal relative weight; SVRW - Seminal vesicle relative weight; SVW- Seminal vesicle weight; EW - Epididymis weight; TW- Testicular weight; GCN – Negative control group; GAV – Group treated with valproic acid; GV – Vehicle group; GC – Group with valproic acid + copaiba oil; GBB – Group with valproic acid + white rosin oil; GC + BB – Group with valproic acid + copaiba oil + white rosin oil.

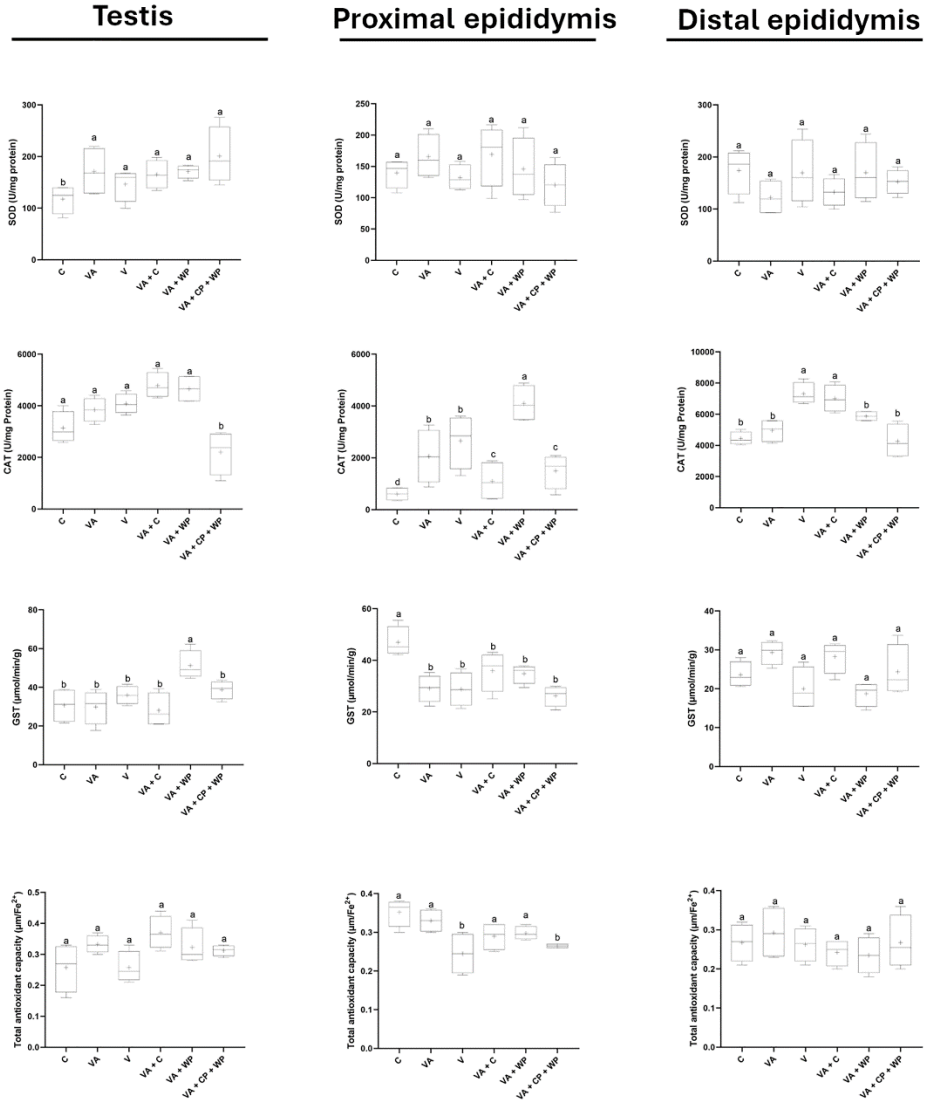
Testicular malondialdehyde levels increased only in the group treated with copaiba and white rosin. In both the proximal and distal epididymis, malondialdehyde levels were elevated in all groups compared to the control. Testicular nitric oxide levels were higher in all groups compared to the control. In the proximal epididymis, nitric oxide levels were also higher in all groups compared to the control, with the coconut oil-treated group showing the highest levels. In the distal epididymis, the levels were elevated only in the groups treated with copaiba oil, white rosin, and the combination of copaiba and white rosin (Figure 1).

Figure 1. Nitric oxide (NO) and malondialdehyde (MDA) levels in the testis and epididymis of male Wistar rats prenatally exposed to valproic acid and postnatally treated with copaiba oil and/or white rosin oil. GCN - negative control group; GAV - valproic acid group; GV - vehicle group; GC: valproic acid + copaiba oil group; GBB - valproic acid + white pitch oil group; GC + BB - valproic acid + copaiba oil + white pitch oil group. Data are presented as mean ± standard deviation. $p < 0.05$ compared to GCN.



Superoxide Dismutase (SOD) activity was increased in all groups, but only in the testes, showing no changes in the proximal or distal epididymis. Testicular catalase activity decreased only in the group treated with copaiba oil and white rosin. In the proximal epididymis, catalase activity increased in the VPA, coconut oil, and white rosin groups, with the white rosin group showing even higher activity than the other two. In the distal epididymis, catalase activity increased only in the coconut oil and copaiba oil groups. Testicular glutathione levels increased only in the white rosin group. In the distal epididymis, glutathione levels decreased in all groups compared to the control, while no changes were observed in the proximal epididymis. Total antioxidant activity was unchanged in both the testes and distal epididymis. However, in the proximal epididymis, glutathione activity was reduced in the coconut oil and copaiba oil + white rosin-treated groups (Figure 2).

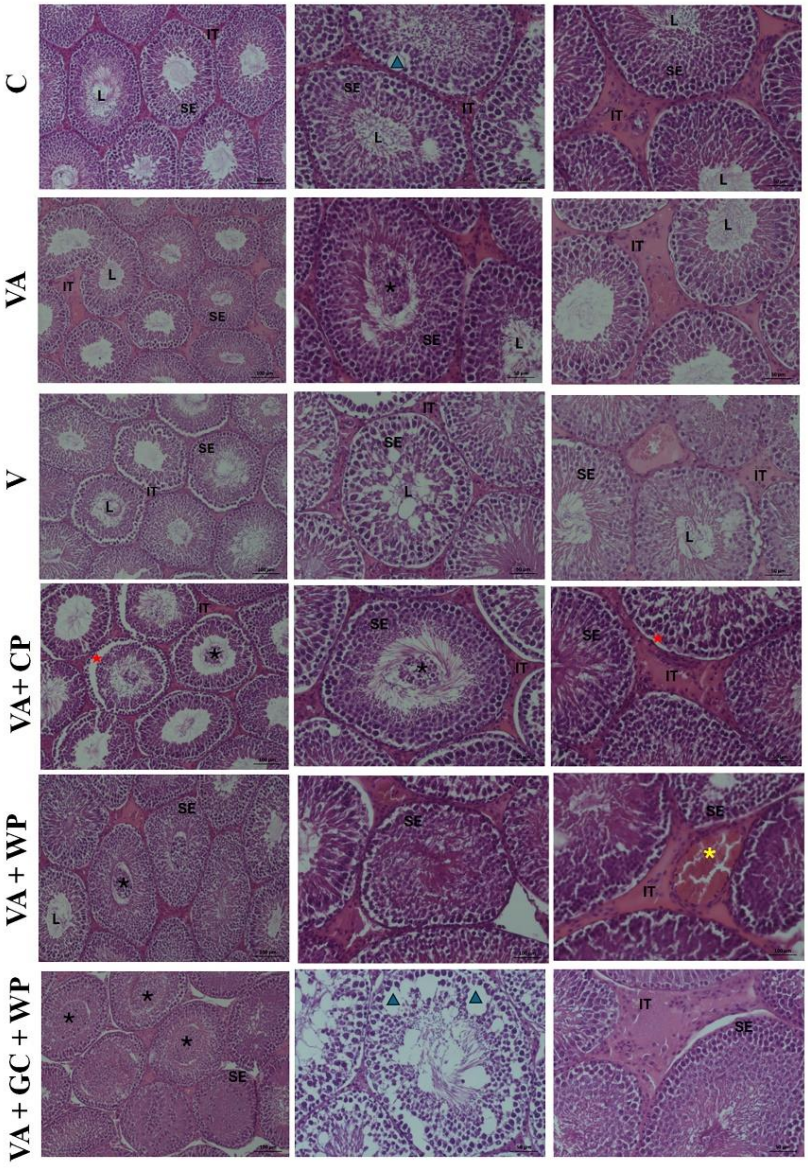
Figure 2. Antioxidant enzyme activity in the testis and epididymis of male Wistar rats prenatally exposed to valproic acid and postnatally treated with copaiba oil and/or white rosin oil. The activities of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and total antioxidant capacity (TAC) were evaluated. GCN - negative control group; GAV - valproic acid group; GV - vehicle group; GC: valproic acid + copaiba oil group; GBB - valproic acid + white pitch oil group; GC + BB - valproic acid + copaiba oil + white pitch oil group. Data are presented as mean ± standard deviation. $p < 0.05$ compared to GCN.



Overall, vacuoles were observed at both the base and apex of the tubules in all experimental groups. However, only in the group treated with copaiba oil and white rosin was there an increase in the number of tubules exhibiting vacuoles in

both regions. The group treated with copaiba oil and white rosin also showed an increased number of tubules with detachment of cells (Figure 3).

Figure 3. Photomicrographs of testicular tissue from male Wistar rats prenatally exposed to valproic acid and postnatally treated with copaiba oil and/or white pitch oil. SE: seminiferous epithelium; IT - intertubular region; L - lumen; cells in the lumen; red star: epithelial detachment; yellow asterisk- vascular congestion; blue triangle - cytoplasmic vacuolization. GCN - negative control group; GAV - valproic acid group; GV - vehicle group; GC: valproic acid + copaiba oil group; GBB - valproic acid + white pitch oil group; GC + BB - valproic acid + copaiba oil + white pitch oil group. Bar = 100 µm.



The epididymal duct in rats from all experimental groups showed regular tissue organization, with a single epithelial layer composed of typical cells (principal cells, basal cells, and clear cells) and a lumen filled with spermatozoa in all four epididymal regions. No changes were observed in the luminal diameter or tissue structure of the blood vessels in the rat epididymis. The presence of inflammatory infiltrates in the interductal compartment of the epididymis was noted in the groups treated with valproic acid and those receiving copaiba oil and white rosin, but in a focal manner (Figure 4). All experimental groups exhibited a cribriform epithelium, which was most evident in the group treated with only copaiba oil (Figure 4). Another alteration observed was the presence of cells in the epididymal lumen in the groups treated with valproic acid, copaiba oil, and copaiba oil + white rosin (Figure 4).

The star plots for the IBR of the testes, proximal epididymis, and distal epididymis are shown (Figure 5). The testes exposed to copaiba oil and white rosin showed increased overall damage, while the group treated only with white rosin showed reduced overall damage. In the proximal epididymis, exposure to valproic acid and copaiba oil + white rosin increased global damage, while in the distal epididymis, all groups showed increased overall damage except for the group treated with coconut oil (Figure 6).

The total variation in the data was 56.13%, with the most important attributes for distinguishing the groups having correlation values greater than 0.6 (Figure 7). For PC1 (horizontal axis), the most relevant attributes and their respective correlation values were: initial weight (-0.1952); body weight (-0.2708); weight gain (-0.2395); testis weight (-0.2466); IGS (0.2371); epididymal weight (-0.1963); SOD-T (0.1989); NO-T (0.2012); MDA-T (0.1872); NO-EP (0.2257); FRAP-EP (-0.2144); MDA-EP (0.2028); GST-EP (-0.2270); NO-ED (0.1695); MDA-ED (0.2140); normal (-0.1848); congestion (-0.2464).

Figure 4. Photomicrographs of epididymal tissue from male Wistar rats prenatally exposed to valproic acid and postnatally treated with copaiba oil and/or white pitch oil. E - epithelium; L - lumen; Ct - connective tissue; blue arrowhead - cribriform area. GCN - negative control group; GAV - valproic acid group; GV - vehicle group; GC - valproic acid + copaiba oil group; GBB - valproic acid + white pitch oil group; GC + BB - valproic acid + copaiba oil + white pitch oil group.

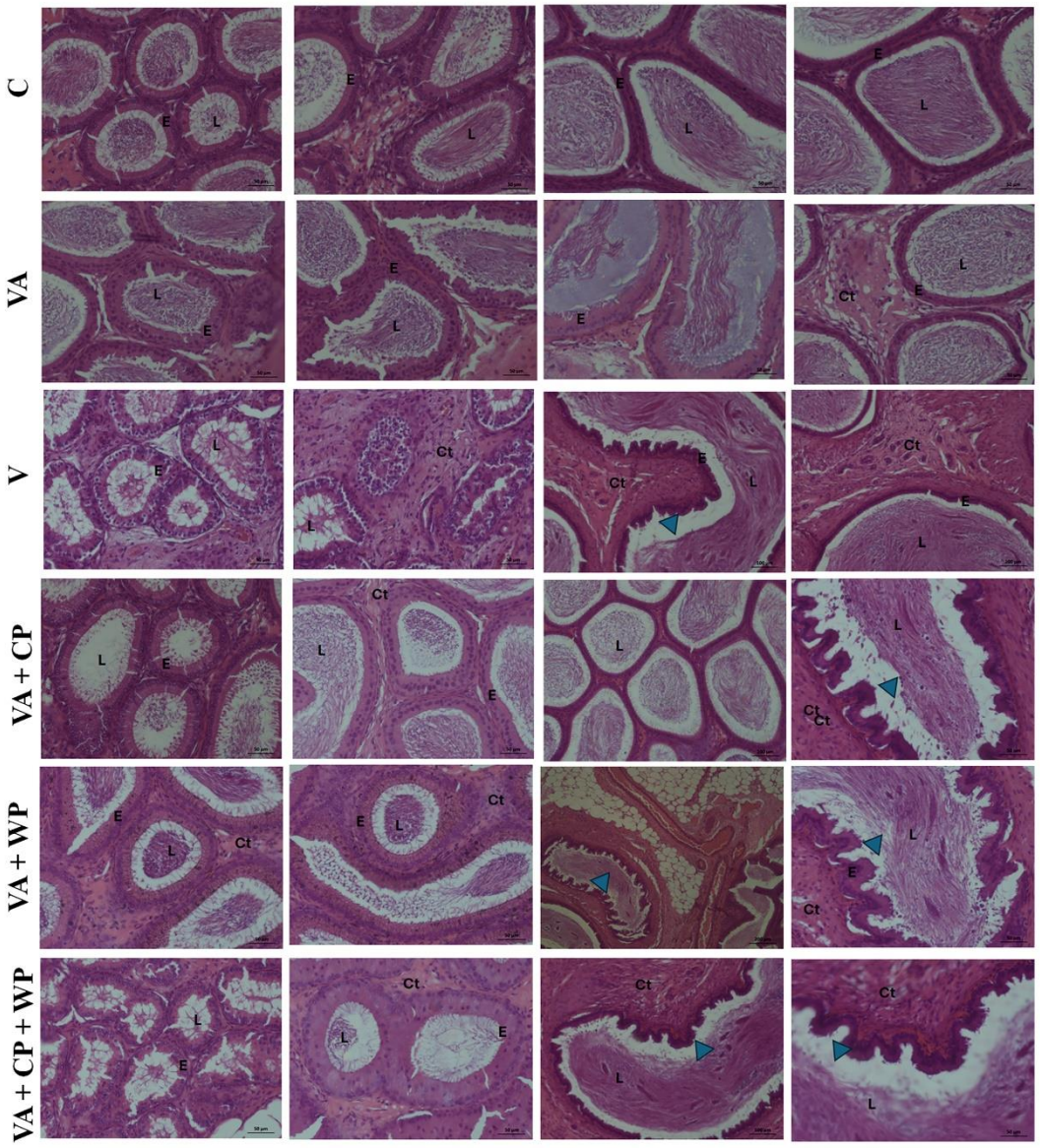


Figure 5. Radar plot representing the exposed tissues' response to essential oils. The total area of the radar plot corresponds to the IBR (Index of Biological Response) value.

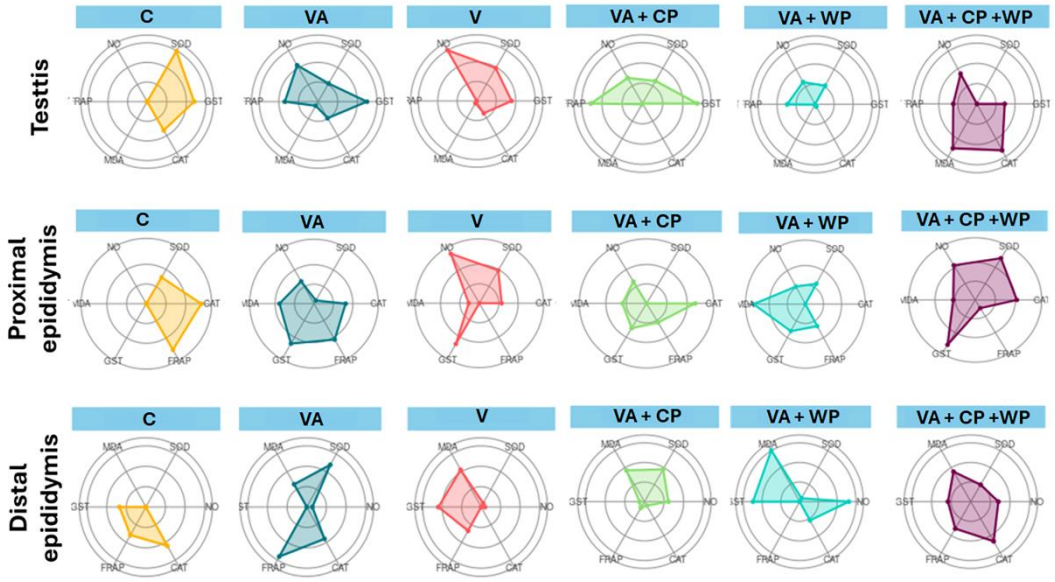
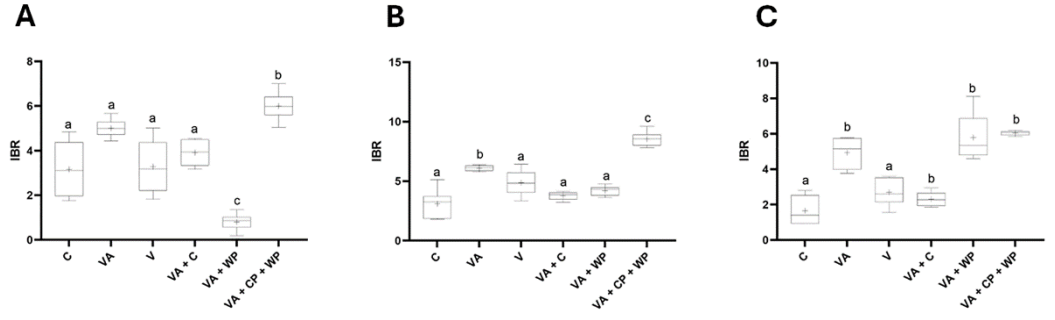


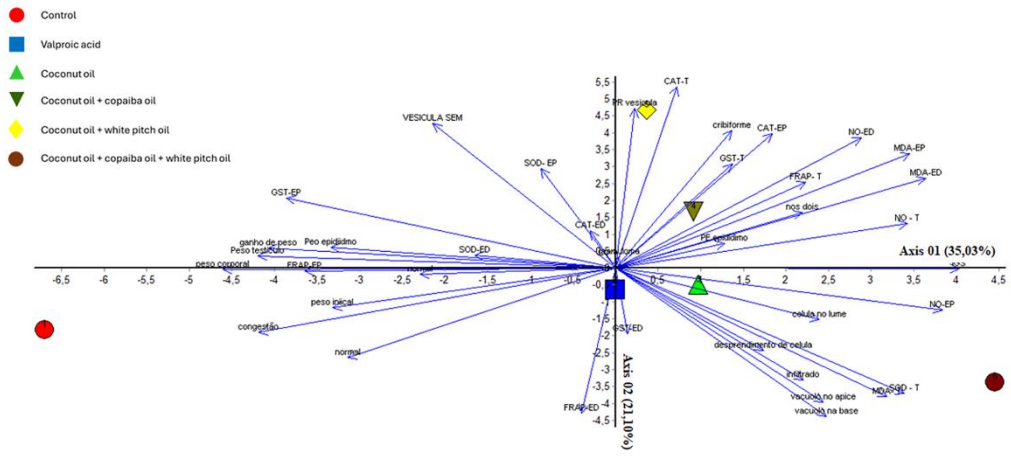
Figure 6. Integrated Biomarker Response (IBR) index. Global analysis of detoxification and damage biomarkers in male Wistar rats exposed to treatments. Data are presented as mean ± standard error of the mean (SEM). $p \leq 0.05$ compared to the control group.



For PC2 (vertical axis), the treatments were primarily separated by seminal vesicle (0.2516); PR vesicle (0.2778); SOD-T (-0.2174); MDA-T (-0.2241); CAT-T (0.3150); MDA-EP (0.1993); CAT-EP (0.2342); NO-ED (0.2270); FRAP-ED (-0.2521); vacuoles at the base (-0.2579); vacuoles at the apex (-0.2334); cribriform (0.2389); infiltrate (-0.1939). It is evident that all groups differ from each other, but the groups treated with valproic acid, coconut oil, and copaiba oil are closer to each

other than the others, with the group treated with copaiba and white rosin being the farthest from the negative control group.

Figure 7. Main component analysis, highlighting the separation between the experimental groups



DISCUSSION

The loss of body weight leads to the mobilization of compounds into the bloodstream, which are then exposed to various organs, including those of the reproductive system[18], potentially resulting in functional damage. Although no biometric changes were observed in the organs, the passage of compounds into the bloodstream leads to alterations in the individual, with one of the first being a disruption in the homeostasis between the production of reactive species and antioxidant activity[16]. The production of reactive oxygen species (ROS) is essential in many healthy processes in the body[19], and there is a balance between their production and degradation. This degradation is carried out through the body's antioxidant defense, which is a combination of enzymatic and non-enzymatic processes.

Oxidative stress is characterized by an imbalance between these oxidizing and antioxidizing agents[20], leading to an increase in ROS levels. Reactive oxygen species are highly reactive molecules that are naturally generated in small amounts

through metabolism and can damage cellular molecules, playing an important role in the etiology and progression of various diseases [19]. The increase in nitric oxide (NO) is one of the key indicators of this oxidative stress and/or cell death, as NO is a functional mediator or promoter of redox imbalance that leads to cell death[21]. The observed increase in nitric oxide levels in both the testes and in the proximal and distal epididymis points to a condition of nitrosative stress. The increase in malondialdehyde (MDA) levels observed in both the testes and epididymis indicates a condition of oxidative stress. MDA is a byproduct of lipid peroxidation and is a toxic compound that reacts with proteins and phospholipids[22]. The increase in MDA can signal damage to the cell membrane, affecting cellular integrity and functionality, which can impact sperm maturation[17]. Increased levels of NO and MDA were also observed in the cardiac tissue of animals that received copaiba essential oil, white pitch or a combination of both, suggesting that the administration of these oils causes a systemic effect in these animals[4].

In the context of oxidative and nitrosative stress induced by the administration of oils, modulation of antioxidant activities both in the testes and the epididymis occurred. Antioxidant activity is divided into two phases, superoxide dismutase (SOD) and catalase (CAT). These phases represent the first line of antioxidant defense, and their balance plays a critical role in the elimination of free radicals[23]. Regarding this first phase of antioxidant defense, distinct outcomes were observed between the testes and epididymis. In the tests, no changes in SOD activity were noted, but a reduction in CAT activity was observed. On the other hand, the epididymis showed no alteration in SOD activity, but there was an increase in CAT activity in both the proximal and distal parts. In a study observing the effect of essential oils of white pitch, copaiba or their association in cardiac tissue, a reduction in the activity of SOD and CAT was also observed and the authors associated this reduction with the doses used in the study being lower than in other studies with the same essential oils[4]. As observed in studies that evaluated the effect of *Lavandula dentata's* and *Origanum majorana* Oil at doses of 300mg/kg and

200mg/kg, an increase in the activity of antioxidant enzymes and a consequent reduction in MDA and NO levels were observed[12,13].

The second phase of defense refers to the activity of glutathione, which is considered an important indicator in cellular detoxification since it catalyzes the dismutation of H_2O_2 into H_2O and O_2[24]. Its induction is a response to oxidative stress. This detoxification occurs through the conjugation of glutathione with xenobiotics and aldehydic products produced during lipid peroxidation[25]. In this study, the testicular glutathione activity was increased only in the white pitch oil group, reduced in the distal epididymis, and showed no change in the proximal epididymis. In cardiac tissue only white pitch caused an increase in GST activity[4]. In this way, it is understood that each tissue responds differently to the effects of the oil, being more or less susceptible to its effects.

The treatment did not cause changes in non-enzymatic antioxidant activities in either the testes or the epididymis. This non-enzymatic activity was assessed through the ferric reducing antioxidant power (FRAP) assay. FRAP measures the antioxidant capacity of all antioxidants in a biological sample, rather than the capacity of a single compound[26]. Despite the increase in antioxidant parameters, they did not prevent the formation of lipid peroxidation by products (malondialdehyde) and nitrosative damage (nitric oxide).

The oxidative and nitrosative stress induced by the administration of the oils, affecting both testicular and epididymal tissues, may lead to alterations in the microstructure of these organs. It was observed that, in general, the treatment with essential oils resulted in increased epithelial vacuolization. However, the group treated with both oils combined showed the highest vacuolization index, culminating in the detachment of epithelial cells. This increase in microstructural alterations is possibly linked to changes in Sertoli cells. Vacuolization is an early sign of damage to Sertoli cells[27]. Sertoli cells are resistant to cell death and respond to these exogenous agents with biochemical alterations rather than cell death[12]. The microvacuolization in the basal region of Sertoli cells causes displacement and disorganization of germ cells. This cellular detachment occurs due to the loss of

cellular adhesion, followed by transport to the rete testis and epididymis[28]. These pathologies reduce Sertoli cells' ability to maintain the arrangement of germ cells, leading to disorganization in the testicular microstructure. Most studies with essential oils show that they mitigate the damage caused by some frog or treatment, as is the case with *Origanum majorana* essential oil[12] ou *Satureja edmondi* Briq[29]. The difference in the effect of essential oils may be justified by the dose administered or the vehicle used. In this study, the oils were diluted in fractionated coconut oil as indicated by the oil suppliers, and the studies cited used PBS or Tween 80%.

Most toxicities induced by drugs or xenobiotics tend to occur in the testicles. Understanding how these compounds affect the epididymis is crucial, as it is in the epididymis that post-testicular differentiation and sperm maturation occur. It is also where sperm is stored until ejaculation, protected from immune reactions by the blood-epididymal barrier[30]. The most noticeable epididymal alteration observed in this study was the presence of cribriform epithelium, with the group treated with the combination of copaiba oil and white rosin showing the highest occurrence of this alteration. Cribriform alteration is a form of epithelial hyperplasia, where the epithelium extends to fold over itself, forming pseudoglandular structures. This change has been associated with testicular toxicity, often reflecting a lack of sperm or testicular fluid, leading to disruption of the epididymal microenvironment. It may be linked to androgen depletion, aging, testicular atrophy, cryptorchidism, and even testicular tumors, though its definitive cause remains undetermined[30]. Another alteration observed, albeit less frequently, was the presence of cells in the epididymal lumen in the groups treated with VPA, copaiba essential oil, and the combination of copaiba and white rosin essential oil. These cells serve as evidence of cellular detachment that occurred in the testicles.

The Integrated Biomarker Response (IBR) is indicative of the overall health status of an individual. In this study, a distinct profile was observed in the testes among the different groups. The group treated only with white rosin showed a reduction in the overall damage state, while the group treated with both copaiba and

white rosin oils exhibited increased damage. In both the proximal and distal epididymis, there was an increase in global damage. The IBR was used to provide visual and comprehensible representations of toxicity effects, highlighting the relativity of biomarkers in terms of sensitivity, magnitude, or severity of responses following exposure to the essential oils[31].

It was observed that the intrauterine administration of VPA and its association with copaiba oil, white rosin oil, or their combination led to oxidative stress in the epididymis. However, only the group receiving VPA and the combination of essential oils showed oxidative stress. On the other hand, nitrosative stress was evident in all experimental groups, both in the testes and in the proximal epididymis, with the groups receiving valproic acid and the oils showing nitrosative stress in the distal epididymis. The alteration in the activity of antioxidant enzymes likely helped contain the damage in some experimental groups. This control is evident in the absence of severe lesions in both the testicular and epididymal microstructures.

CONCLUSION

In conclusion, the administration of valproic acid during the intrauterine period induces testicular nitrosative stress and causes oxidative stress in the distal region of the epididymis, as well as nitrosative stress in both the distal and proximal regions of the epididymis. However, its association with essential oils, or the oils themselves, does not effectively reverse the damage caused by valproic acid and, in some cases, intensifies the damage, particularly in the group treated with both copaiba and white rosin oils. Furthermore, a higher number of microstructural alterations were observed in both the testicular and epididymal tissues in the group receiving the combination of oils, with greater epithelial vacuolization and cell detachment in the testes, as well as an increase in areas with cribriform epithelium in the epididymis, indicating a testicular toxicity profile. Finally, the principal component analysis and IBR further highlighted the separation between the

experimental groups, with the group treated with copaiba and white rosin oils, as well as the group treated only with white rosin, showing distinct differences. This was confirmed by the integrated biomarker response, which indicated a worsening of the overall health condition in the group treated with valproic acid alone, white rosin, and the combination of copaiba and white rosin oils.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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