ARTIGO ORIGINAL

Sperm parameters reflect the effects of topical use of Copaifera oil (*Copaifera* sp.) in Wistar rats

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ABSTRACT: Copaiba resin oil (CO) is used in the treatment of inflammatory skin injuries in humans. This study assessed the effects of topical use of CO ointment on sperm parameters. Sixty-three Wistar male rats were anesthetized and two wounds were inflicted on the back of each rat. The healing injuries were treated daily with petroleum jelly (control group), 0.01%, and 0.1% of CO ointment until the day they were euthanized: seven, fourteen, or twenty-one days later (D7, D14, or D21, respectively). Each group contained seven animals and the traits assessed in terms of sperm quality were sperm motility, integrity of plasma membrane, mitochondrial function, DNA integrity, and histopathological characteristics. Animals treated with CO at 0.01% and 0.1% for D7 had an increase in the number of cells with injured membranes (79.6% and 68.9%, respectively) and nonfunctional mitochondria (44.8% and 48.5%, respectively) when compared to the control group (43% of injured membrane and 19% of nonfunctional mitochondria). After D14 of treatment, we observed an increase ($P<0.05$) in the percentage of cells with damaged DNA and injured acrosome in the Copaiba-treated groups at 0.01% (27.8% and 43.8%, DNA and acrosome respectively) and 0.1% (23.7% and 41.4%, DNA and acrosome respectively), when compared to the control group (0% and 19.2%, DNA and acrosome respectively). We concluded that the topical use of CO decreased sperm quality in the period and at the concentrations studied.
Keywords: Sperm quality, Sperm motility, Mitochondria, DNA damage, Herbal medicine

RESUMO: Parâmetros espermáticos refletem os efeitos do uso tópico de óleo de copaíba (*Copaifera* sp.) em ratos Wistar. O óleo de copaíba (OC) é usado para o tratamento de lesões inflamatórias na pele em humanos. Este estudo avaliou os efeitos do uso tópico da pomada contendo OC sobre os parâmetros espermáticos em ratos Wistar. Sessenta e três ratos machos Wistar foram anestesiados e duas lesões foram feitas sobre o dorso de cada animal. As lesões em cicatrização foram tratadas diariamente com vaselina (grupo controle) e pomada de OC nas concentrações de 0,01% e 0,1% até o dia em que os animais foram eutanasiados: sete, quatorze ou vinte e um dias após o tratamento (D7, D14 ou D21, respectivamente). Cada grupo continha sete animais e foram analisadas as características sobre os parâmetros de qualidade espermática: motilidade espermática, integridade de membrana plasmática, funcionalidade de mitocôndria, integridade de DNA e histopatologia. Os animais tratados com OC a 0,01% e 0,1% por D7 tiveram um aumento no número de células com membranas lesadas (79,6% e 68,9%, respectivamente) e mitocôndrias não-funcionais (44,8% e 48,5%, respectivamente) quando comparados ao grupo controle (43% de membranas lesadas e 19% de mitocôndrias não-funcionais). Após o D14 de tratamento, foi observado um aumento ($P<0,05$) na porcentagem de células com DNA e acrossoma lesionados nos grupos tratados com copaíba a 0,01% (27,8% para o DNA e 43,8% para o acrossoma) e 0,1% (23,7% para o DNA e 41,4% para o acrossoma) quando comparados ao grupo controle (0% para o DNA e 19,2% para o acrossoma). Concluímos que o uso tópico de OC diminuiu a qualidade espermática no período e nas concentrações estudadas.
Palavras-chave: Dano ao DNA, medicamento fitoterápico, motilidade espermática, mitocôndria, qualidade espermática

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INTRODUCTION

Copaiba trees are native from Latin America and Western Africa. In Brazil, there are more than twenty species and the oil-resin is obtained by tapping the trunk of *Copaifera* L. trees. The oil-resin from *Copaifera* plants has biological properties, such as anti-inflammatory (Veiga Junior et al. 2007), antimicrobial (Tobouti et al. 2017), healing (Dias da Silva et al. 2013), analgesic (Carvalho et al. 2005), antibacterial (Pieri et al. 2012), antifungal (Deus et al. 2011; Svetlichny et al. 2015), antitumor (Lima et al. 2003).The use of copaifera oil-resin in herbal medicine is approved by Brazil's National Health Surveillance Agency (ANVISA), which recommends the external use of 10% Copaifera oil ointment in skin wounds three times a day on the affected area (Brasil 2011).

This oil is made up of different sesquiterpenes and diterpenes according to the *Copaifera* species. The main sesquiterpene found in *Copaifera* sp. is β-caryophyllene. This compound has spicy properties and it is commonly ingested with vegetables in an estimated daily intake of 10-200 mg (Gertsch et al. 2008). Moreover, it has antileishmanial (De Albuquerque et al. 2017), antimicrobial (Pieri et al. 2012), antioxidant, anti-inflammatory (Carvalho et al. 2005) and anticarcinogenic (Lima et al. 2003) properties.

The β-caryophyllene compound is supposed to be effective in the treatment of endometriosis (Abbas et al. 2013). However, this compound negatively affects sperm counts, motility and morphology, but it does not affect histological or ultrastructural features of testis and tail of epididymis of rats treated orally (Al-Alami et al. 2015).

As Santana et al. (2014) observed, in the Amazonian region 41% of the elderly interviewed used this oil as a healing agent. They also noted that respondents use the oil cautiously in low doses, reporting that the use in high doses can bring on health damages. Up until now, no study has reported the effect of topical use of copaiba oil-based products on sperm quality *in vivo*. Knowing that the recruitment of cells from stem cell lines occurs every 12.9 days (França et al. 1998; Du and Dianjun 2013), the aim of this study was to assess the effects of topical use of Copaifera oil-ointment on sperm parameters of rats after 7, 14, and 21 days of treatment.

MATERIALS AND METHODS

Collection and characterization of Copaifera oil

The copaifera resin oil (*Copaifera* sp.) was collected through puncture in the trunk of the tree *Copaifera* sp. (Herbarium – HFSL:6726). The collection was performed by Fundação Universidade Federal de Rondônia, in Rondônia State (RO-463, RO, 2.4 Km- NE-10.294235, -62.404860). After oil acquisition and botanical characterization, the chemical characterization was performed through gas chromatography (model GC/MS-QP 2010SE Shimadzu, Japan) using an auto-injector equipment (AOC-20i). The compound identification was determined by mass spectrometry using the library NIST 8 of GC/MS that stocked information of compounds previously identified and the quantity established by normalized area.

Dose setting up

Doses were defined from previous studies by our research group (unpublished data). Briefly, a cytotoxicity assay with Vero cells (African green monkey Kidney cells) was performed to determine which doses should be used in *in vivo* assay. Cells were cultured in minimal essential medium (MEM), containing 1% antibiotic solution and 10% fetal bovine serum (FBS) and kept in a humidified incubator at 37 ºC with 5% atmospheric CO_2. After confluent monolayer formation, aliquots were collected to perform the subculture at the bottom of a 96-well plate, in order to perform the cytotoxic effect test after 48 h through MTT (3-(4,5-dimethylthiazol-2-yl bromide)-2,5-diphenyltetrazolium). For cell treatment, the MEM was emulsified in dimetilsulfoxide solution (DMSO), thus allowing penetration of the copaiba oil resin into the cells at concentrations of 10%, 5%, 1%, 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001%. Then, plates were stored and kept in a humidified incubator at 37.0 ºC with 5.0% atmospheric CO_2. The assay was performed in triplicates using MEM as negative control (CC) and MEM emulsified in 1:200 DMSO as positive control (CDMSO). Cell viability was calculated using the formula: (mean absorbance of treated group/mean absorbance of control group) x 100 (Wang et al. 2011).

Animals

After the determination of doses in the *in vitro* assay, 63 Wistar male rats (90 days old) from the Central Vivarium of Federal University of Pelotas (UFPel) were used for *in vivo* assay. The rats were housed in pairs or threes in standard plastic cages with stainless-steel covers and wood shavings as bedding, and kept under controlled temperature (23 ± 2 ºC), relative humidity (maximum 70%), and a 12:12 h photoperiod with lights turned on at 07:00 a.m. A standard commercial diet for laboratory rats (Nuvilab CR-1® - Nuvital Curitiba-Brazil) and tap water were provided *ad libitum*. Rats were acclimatized for ten days before the experiment. The research protocol was approved by the Ethics and Animal Experimentation Committee of Federal University of Pelotas (CEEA-UFPEL), No. 9226.

Experimental design

Sixty-three male rats were divided into nine groups of seven animals each, according to the treatment received and the day of euthanasia. First, they were anesthetized with ketamine (100 mg/kg) and xylazin (10 mg/kg) for dorsum trichotomy and antisepsis, then two injuries were performed on the back of each rat using a punch number 8, according to Capella et al. (2016) (Figure 1). Rats were provided with a heated bed and received care until full recovery. The healing injuries were treated daily during three different periods:7 (D7), 14 (D14), and 21 (D21) days, with liquid Petroleum jelly (control group), 0.01% Copaifera oil (Herbário-HFSL:6726) ointment (0.01% Copaifera group), and 0.1% Copaifera oil ointment (0.1% Copaifera group). At the end of each experimental period, rats were euthanized with an overdose of ketamine and xylazin followed by cardiac ex sanguination. Afterwards, the testicles of each animal were collected and stored at 10% formalin solution. The epididymal tail was collected and stored in phosphate-buffered saline (PBS) preheated up to 37 °C. Sperm was collected through incisions with a hypodermic needle (40x12) in order to assess sperm parameters (Oyeyipo et al. 2018).

Sperm motility

To determine the sperm motility (0-100%), a droplet of the collected sperm was placed between a slide and cover slip and visualized under an optical microscope with a phase contrast and a heated plate at 37 °C (Olympus BX41-PH-III America Inc., São Paulo, Brazil) and 200X magnification in duplicates. Motility assessment ranged from zero to 100%.

Sperm concentration

To determine sperm concentration, 900 µl of formol saline was added to a 100 µl of sperm aliquot. The suspension was mixed thoroughly and placed into a Neubauer counting chamber. The total sperm count was determined by counting cells inside five 1 mm^2 squares and multiplying by $5x10^4$, in order to express the number of spermatozoa per ml.

Microscopical analyses of cell structure

Samples were incubated for 5 minutes in the dark, and 200 cells were observed by epifluorescence microscopy (Olympus BX 51, América Inc., São Paulo, SP). Analyses that were performed are as described according to Table 1.

Histological analysis

Testes samples were stored in 10% formalin and sent to the Laboratory of Histology of the Department of Animal Pathology, UFPel. Tissue fragments were processed, embedded in paraffin, subsequently cut into five-micron-thick sections, and stained with hematoxylin and eosin. Tissues were assessed for the presence/absence of degenerative, inflammatory, and proliferative wounds.

Statistical Analysis

Data are presented as the mean ± standard error (SE). Data normality and homogeneity of variances were assessed using the Shapiro-Wilk test. Mean values of dependent variables with a normal distribution were subjected to analysis of variance

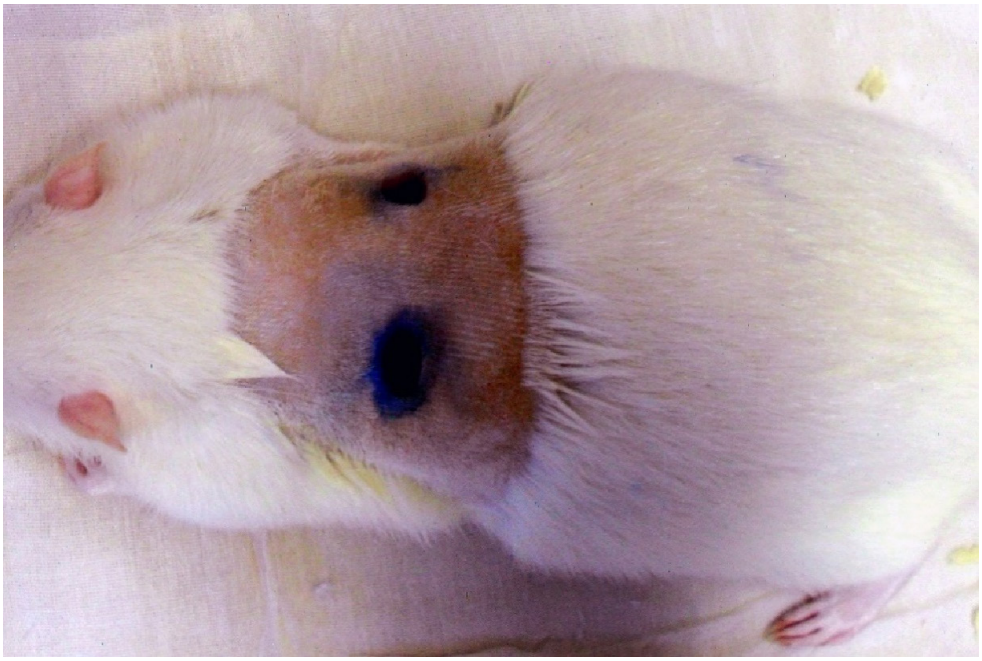

Figure 1. Rat with two punch wounds (blue staining) of 8mm length on the back (dorsal view).

Table 1. Assessments in sperm cell structures (integrity of plasma membrane, mitochondrial function, acrosome integrity, and DNA integrity) using fluorescent probes, observed by epifluorescence microscopy and its observation parameters (emission, excitation, and magnification).

Assessment	Fluorescent probe	Classification	Fluorescence emission	Emission	Excitation	Magnification	Reference
Integrity of Plasma membrane	Carboxyfluorescein diacetate (C4916 – 25 mg, Sigma Chemical Company, St. Louis, MO, USA)	Intact	Green fluorescence	516 – 617 nm	450 – 490 nm	400X	Harrison and Vickers (1990)
		Injured	Red fluorescence				
Mitochondrial function	Rhodamine 123 (R8004-5mg, Sigma Chemical Company, St. Louis, MO, USA),	Functional	Mid-piece intense green fluorescence	516 – 617 nm	450 – 490 nm	400X	Evenson et al. (1982)
		Non-functional	Mid-piece with few or without green fluorescence				
Acrosomal integrity	Conjugate of Arachis hypogaea lecithin-FITC	Intact	Acrosome with green fluorescence and normal form	520 nm	450 – 490 nm	1000X	Kawamoto et al. (1999)
		Injured	Acrosome without green fluorescence or abnormal form				
DNA integrity	Acridine orange (Molecular Probes Inc., Eugene, OR, USA)	Normal	Green fluorescence	520 nm	450 – 490 nm	1000X	Evenson et al. (1982)
		Injured	Red or yellow fluorescence				

(ANOVA) followed by Tukey's test. All analyses were performed using Statistic 9.0 ®software.

RESULTS AND DISCUSSION

Routinely, products of topical application are used indiscriminately, with the belief that they are not capable of carrying systemic consequences to the body. However, the absorption of a drug depends mainly on the vehicle used for emulsion. Petroleum jelly, an oily solution, which is indicated for preparations with terpenes, such as copaiba oil, showed absorption in all layers of the whole skin in just one hour (Cal 2006). The topical use of copaifera oil ointment on open wounds, even at a lower dose than that recommended by ANVISA, was toxic to sperm cells of rats under the studied conditions. The literature points out the cytotoxic potential of copaifera oil against microorganisms such as *Leishmania amazonensis* (Soares et al. 2013; De Albuquerque et al. 2017), *Streptococcus* spp. (Diefenbach et al. 2018), *Staphylococcus* spp. (Dias et al. 2015), *Pseudomonas aeruginosa* and *Escherichia* spp. (Mendonça and Onofre 2009). In addition, the resin oil also exhibits larvicidal (Silva et al. 2007) and acaricidal (Fernandes et al. 2016) activities. Some of its compounds are said to be responsible for this capacity, such as sesquiterpenes caryophyllene (Diefenbach et al. 2018) and humulene (Govindarajan and Benelli 2016).

In the chromatographic assessment the following compounds were identified: caryophyllene (78.6%), humulene (11%), bergamoptene (2.9%), copaene (2%), muurolene 4%), elemene (1.2%), cadinene (1%) and 1H-cycloprop [e] azulene (0.2%). The cytotoxicity assay with MTT *Copaifera* sp. demonstrated that the doses presented viability equal to or less than 0.01%. In the present study,

we chose to use the first inoculum dose (0.01%) and the first cytotoxic group (0.1%).

Although histological differences were not observed in the studied groups, the topical use of copaifera oil in both concentrations showed toxic effects to rat spermatozoa at the three observation times. After D7 of treatment with *Copaifera* oil ointment in the groups at 0.01% and 0.1% concentrations, the motility of the sperm cells were 38.0% and 41.6%, respectively, exhibiting a significant decrease in the percentage of mobile cells when compared to the control group (67.1%) ($p<0.05$). However, after D14 and D21 of treatment, the motility of sperm cells of copaifera group 0.01% were 42.5% and 42.0%, respectively, and values were equal to those of the control group (56.6% and 54.6%, respectively) ($p<0.05$). Meanwhile, the motility of sperm cells of copaifera group at 0.1% (23.3% and 17.5%, respectively) continued to decrease ($p<0.05$). The values of sperm motility are shown in Figure 2.

In the sperm concentration analysis, we observed that after D7 of treatment, the control group ($157.4x10^4$ sptz/ml) had a significant increase in cell concentration when compared to the copaifera -treated groups: 0.01% group with $65x10^4$ sptz/ml; and 0.1% group with $81.5x10^4$ sptz/ml ($p<0.05$). This trend was inverted after D14 of treatment, when the copaifera groups (0.01%, $126.7x10^4$ sptz/ml; and 0.1%, $100.8x10^4$ sptz/ml) presented a significant increase when compared to the control group ($9.33x10^4$ sptz/ml; $p<0.05$). After D21 of treatment, 0.1% copaifera group exhibited a higher sperm concentration ($167.9x10^4$ sptz/ml) when compared to control ($74.8x10^4$ sptz/ml) and 0.01% copaifera ($69.9x10^4$ sptz/ml) groups ($p<0.05$) (Figure 3).

Data published by Al-Alami et al. (2015) showed that caryophyllene, the main compound found in the resin oil used in this study (78.6%), adversely affects sperm count, motility and morphology, without affecting the testicular structure and the tail of the epididymis. In the present study, it was also possible to observe a negative effect on motility, especially in the treatment with higher concentration of resin oil, and sperm concentration after D14 of treatment. Caryophyllene has agonistic activity on cannabinoid receptors type 2 (CB2R), present in T cells of the immune system and testicular germ cells and, when activated, they are involved in the process of spermatogenesis. CB2R is highly found in cells at differentiation stages, which are poorly found in spermatids and undetectable in spermatozoa, and have pro-differentiation properties (Grimaldi et al. 2009; Al-Alami et al. 2015).

Regarding the impact of the CO in cell membranes, the copaifera group at 0.01% and 0.1% after D7 of treatment had a significantly increase in the number of cells with injured membranes (43.0%, 79.6%, and 68.9%, respectively) when compared to the control group. Moreover, with respect to the injured membrane, no statistical significant difference was observed between the studied groups after D14 of treatment ($P>0.05$). When animals were treated to D21, there was a significant decrease in the percentage of injured membrane cells of 0.1% copaifera group (48.1%) when compared to the control (70.6%) and 0.01% copaifera groups (68.8%; $p<0.05$) (Figure 4).

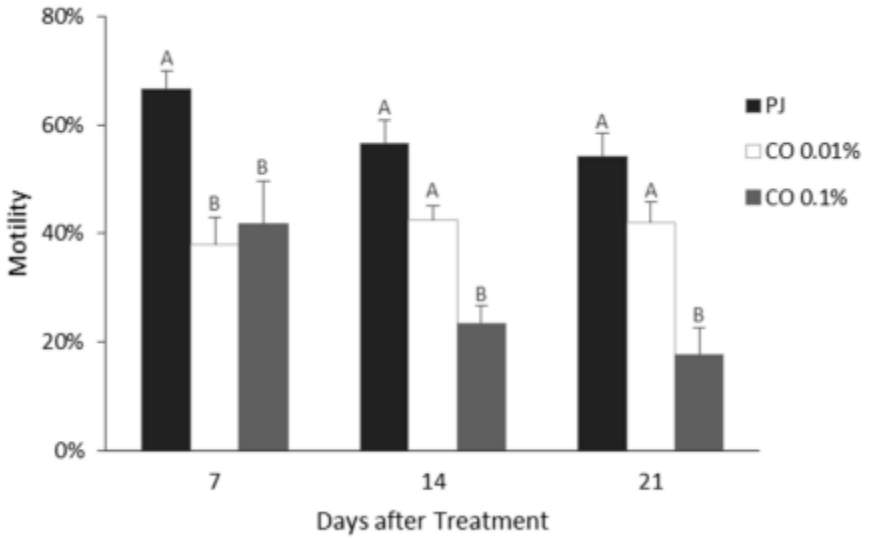

Figure 2. Effect on the motility of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

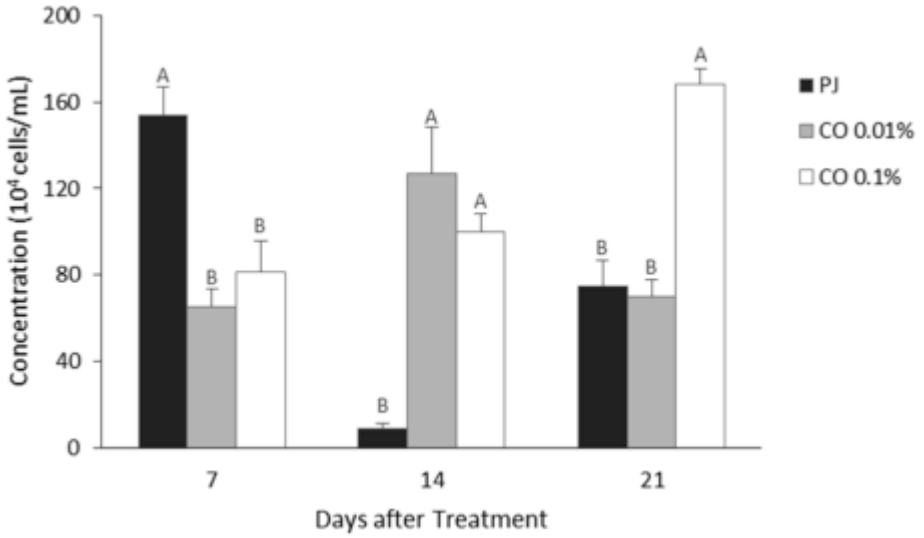

Figure 3. Effect on the sperm concentration of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; copaifera 0.01%: D7 n=5, D14 n=4, D21 n=11; copaifera 0.1%: D7 n=6, D14 n=6, D21 n=12; p<0.01. Different letters represent significative diferences between treatments in the observed dates (p<0.05).

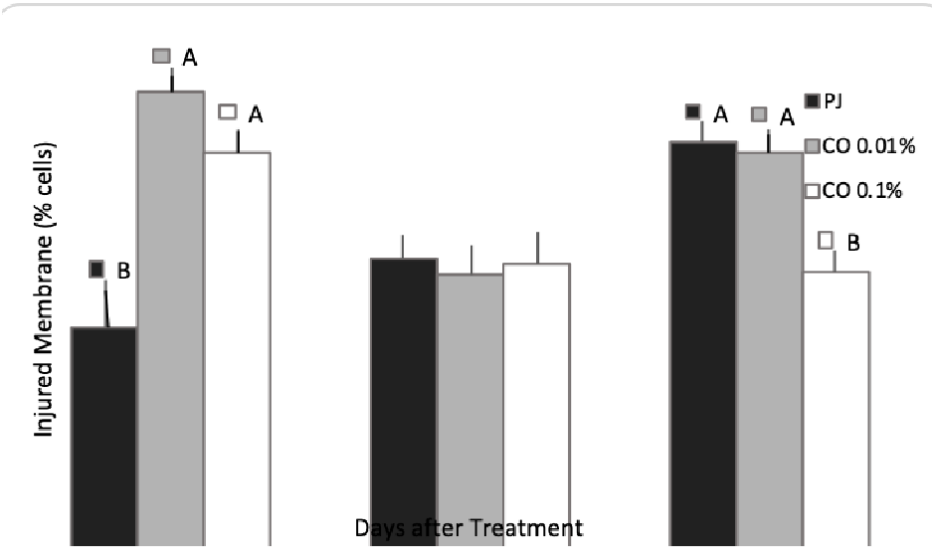

Figure 4. Effect on injured membrane of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; copaifera 0.01%: D7 n=5, D14 n=4, D21 n=11; copaifera 0.1%: D7 n=6, D14 n=6, D21 n=12; p<0.01. Different letters represent significative diferences between treatments in the observed dates (p<0.05).

With respect to mitochondrial function, the copaifera oil component β–caryophyllene caused an alteration in the mitochondrial potential of the protozoan *Trypanosoma cruzi*, decreasing the functionality of this organelle (Izumi et al. 2012; De Albuquerque et al. 2017). In our study, the control group exhibited a decreased percentage of cells with nonfunctional mitochondria (19.0%) when compared to 0.01% copaifera group (44.8%; p<0.05) after D7 of treatment (p<0.05). Moreover, the control group presented 25.2% of cells with nonfunctional mitochondria and the 0.1% copaifera group presented 40.2% after D14 of treatment. At D21 after treatment, the 0.1% copaifera group presented a significant increase in the percentage (59.9%) of cells with nonfunctional mitochondria when compared to the other groups (p<0.05) (Figure 5). These results corroborate the previous statement of possible mitochondrial alteration. Castro-e-Silva et al. (2004) demonstrated that copaifera resin oil

could uncouple oxidative phosphorylation in the mitochondrial respiratory chain and increase the rate of basal cellular respiration. However, the compound responsible for this action has not been established yet. These results agree with sperm motility findings, since the energy required for cell movement is stored in the mitochondria, which synthesizes ATP through the electron transport chain. We observed that the alterations in mitochondrial function are reflected in the motility changes.

Studies have shown that along with mitochondrial function and sperm motility, acrosome integrity is essential for fertilization (Kasai et al. 2002; Urióstegui-Acosta et al. 2014). The copaifera group at 0.01% exhibited a higher percentage of cells with injured acrosomes. After D7 of treatment, we observed a significant increase in the percentage of cells with injured acrosomes in the copaifera 0.01% and 0.1% groups (43.8% and 41.4%, respectively) when compared to the control group (19.2%) ($p<0.05$). After D21 of treatment, both groups treated with copaifera (0.01% and 0.1%) had a significantly increased percentage of cells with injured acrosome, 59.2% and 49.6% respectively ($p<0.05$) (Figure 6).

In the DNA integrity assessment, we found no statistical significant difference between the studied groups after D7 of treatment ($p>0.05$) (Figure 2A). After D14 of treatment, we observed a significant increase in the percentage of cells with injured DNA in the copaifera groups at 0.01% and at 0.1% (27.75% and 23.67%, respectively), when compared to the control group (0%; $p<0.05$), although there was no significant statistical difference. After D21 of treatment, the copaifera group at 0.1% presented a significant increase in the percentage of cells with injuries in the DNA (20.0%; $p<0.05$) (Figure 7). Dahham et al. (2015) demonstrated *in vitro* that colorectal cancer cells exposed to β-caryophyllene had their nuclear morphology altered using the DNA fragmentation analysis. At 10 µM concentration, this compound caused significant nuclei condensation after 6 h of treatment, indicating apoptosis. This compound activated caspase-3 in tumor cell lines. Induction of caspase-3 activity in turn leads to chromatin condensation, degradation and dissolution (Amiel et al. 2012).

The damage in the sperm cell DNA along with mitochondria and acrosome changes can lead to decreased sperm motility, reducing the capacity of semen fertilization. The recruitment of committed cells from stem-cell line occurs every 12.9 days (França et al. 1998; Du and Dianjun 2013), and our results demonstrated that the topical use of copaifera oil can cause damages to sperm cells. These changes stimulated the production of sperm cells after both D14 and D21 after exposure to copaifera resin oil. These findings can be the responses of the organism against the toxic effects such as compensation.

This study showed that the use of copaifera

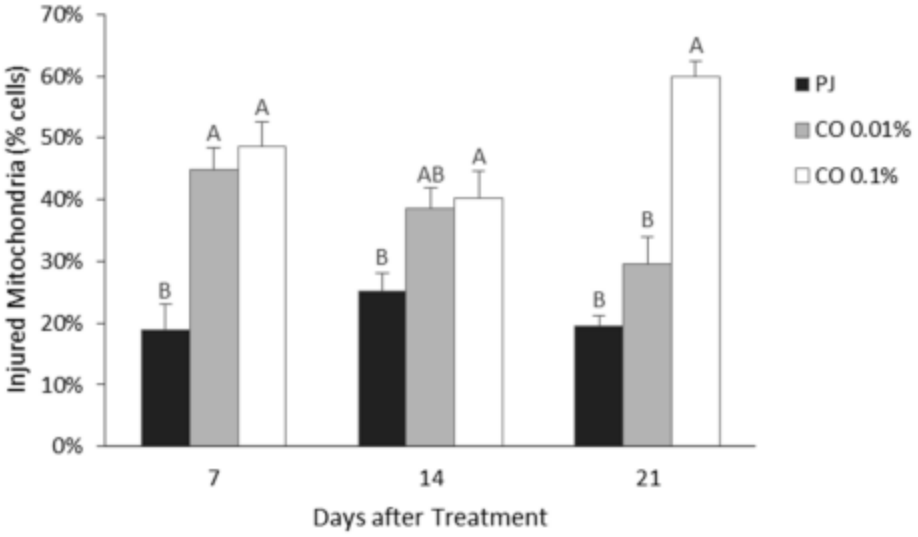

Figure 5. Effect on mitochondria of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; copaifera 0.01%: D7 n=5, D14 n=4, D21 n=11; copaifera 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

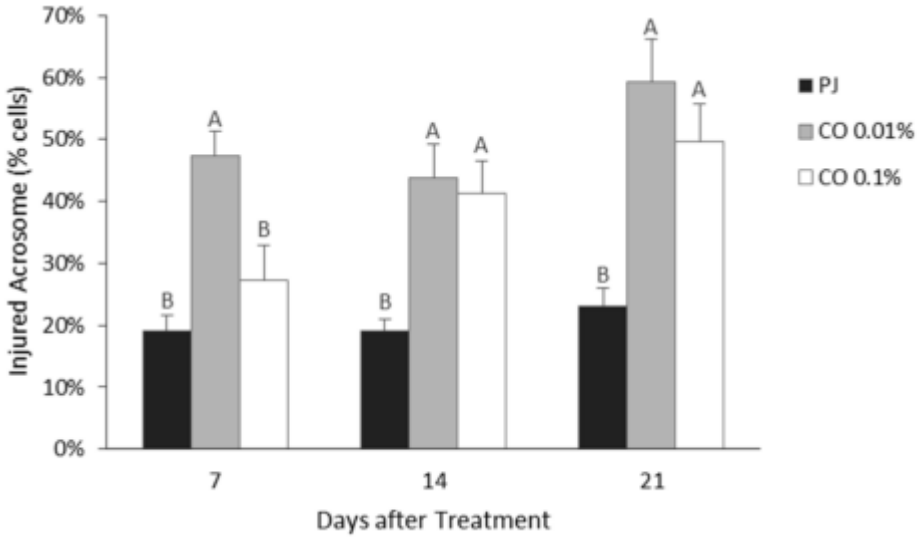

Figure 6. Effect on acrosome of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; copaifera 0.01%: D7 n=5, D14 n=4, D21 n=11; copaifera 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

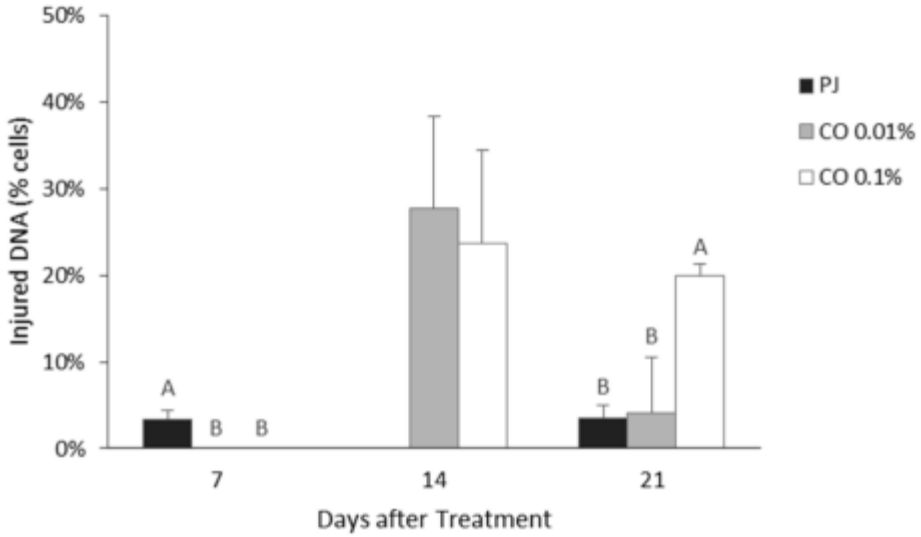

Figure 7. Effect on DNA of the different concentrations of copaifera oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; copaifera 0.01%: D7 n=5, D14 n=4, D21 n=11; copaifera 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

ointment at 0.01% and 0.1% concentrations may lead to poor sperm quality both in the acrosome and mitochondria, and at the DNA level. Thus, it is necessary to evaluate a possible grace period for the use of this formulation, in order to obtain an herbal product with efficacy and safety.

CONCLUSION

The topical use of copaifera oil decreased sperm quality at the studied concentrations. Our results showed that exposure to Copaifera oil at 0.01% and 0.1% for up to D14 caused alterations in differentiated sperm cells. Moreover, after D21, we noted that the use of 0.1% copaifera oil ointment caused changes in structures formed during initial spermatogenesis, such as DNA and mitochondria.

CONFLICTS OF INTEREST

The authors have declared that there are no conflicts of interest.

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REFERENCES

Abbas MA, Taha MO, Zihlif MA, Disi AM (2013) Beta-Caryophyllene causes regression of endometrial implants in a rat model of endometriosis without affecting fertility. Eur J Pharmacol 702:12-19. 10.1016/j.ejphar.2013.01.011

Al-Alami ZM, Shraideh ZA, Taha MO (2015) β-Caryophyllene as putative male contraceptive: enhances spermatogenesis but not spermiogenesis in albino rats. Med Chem Res 24 (11):3876-3884. 10.1007/s00044-015-1428-3

Amiel E, Ofir R, Dudai N, Soloway E, Rabinsky T, Rachmilevitch S (2012) β-Caryophyllene, a compound isolated from the biblical balm of gilead (*Commiphora gileadensis*), is a selective apoptosis inducer for tumor cell lines. J Evid Based Complement Altern Med 2012:872394. 10.1155/2012/872394.

Brasil. Formulário de Fitoterápicos da Farmacopeia Brasileira. ANVISA: ANVISA**:** 126 p. 2011. https://www.gov.br/anvisa/pt-br/assuntos/farmacopeia/formulario-fitoterapico.

Cal K (2006) How does the type of vehicle influence the in vitro skin absorption and elimination kinetics of terpenes? Arch Dermatol Res 297(7):311-315.

Capella S, Tillmann M, Félix A, Fontoura E, Fernandes C, Freitag R, Santos M, Félix S, Nobre M (2016) Therapeutic potential of *Bixa orellana* L. in skin wounds: a study in the rat model of open wound healing. Arq Bras Med Vet Zootec 68(1):104-112.

Carvalho JC, Cascon V, Possebon LS, Morimoto MS, Cardoso LG, Kaplan MA, Gilbert B (2005) Topical antiinflammatory and analgesic activities of *Copaifera duckei* Dwyer. Phytother Res 19(11):946-50.

Castro-e-Silva O, Zucoloto S, Ramalho FS, Ramalho LN, Reis JM; Bastos ÁA, Brito MV (2004) Antiproliferative activity of *Copaifera duckei* oleoresin on liver regeneration in rats. Phytother Res 18(1):92-94.

Dahham SS, Tabana YM, Iqbal MA, Ahamed MB, Ezzat MO, Majid AS, Majid AM (2015) The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. Molecules 20(7):11808-11829.

De Albuquerque KC, Da Veiga AD, Silva JV, Brigido HP, Ferreira EP, Costa EV, Marinho AM, Percario S, Dolabela MF (2017) Brazilian amazon traditional medicine and the treatment of difficult to heal *Leishmaniasis* wounds with *Copaifera*. Evid Based Complement Alternat Med 2017:8350320.

Deus R, Alves C, Arruda M (2011) Avaliação do efeito antifúngico do óleo resina e do óleo essencial de copaíba (*Copaifera multijuga* Hayne). Rev Bras Plantas Med 13(1):1-7.

Dias-da-silva MA, Pereira AC, Marin MC, Salgado MA (2013) The influence of topic and systemic administration of copaiba oil on the alveolar wound healing after tooth extraction in rats. J Clin Exp Dent 5(4):169-73

Dias FGG, Casemiro LA, Martins CHG, Dias LGGG, Pereira LDF, Nishimura LT, Souza FF, Honsho CS (2015) Endodontics pastes formulated with copaiba oil: action on oral microbiota and dentin bridge formation in dogs. Cien Rural 45:1073-1078.

Diefenbach AL, Muniz FWMG, Oballe HJR, Rösing CK (2018) Antimicrobial activity of copaiba oil (*Copaifera* ssp.) on oral pathogens: Systematic review. Phytother Res 32(4):586-596.

Du J, Dianjun G (2013) Cell cycle specificity of spermatogenic cell apoptosis in rats with experimental varicocele. Clin Lab 59(7-8):851-859.

Evenson D, Darzynkiewicz Z, Melamed M (1982) Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Citochem 30(3):279-280.

Fernandes COM, Félix SR, Nobre MO (2016) Toxicidade dos fitoterápicos de interesse do SUS: uma revisão. Semin Ciênc Biol Saúde 37(1):91-104.

França LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59(6):1371-1377.

Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann KH, Karsak M, Zimmer A (2008) Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci USA 105(26):9099-104.

Govindarajan M, Benelli G (2016) α-Humulene and β-elemene from *Syzygium zeylanicum* (Myrtaceae) essential oil: highly effective and eco-friendly larvicides against *Anopheles subpictus*, *Aedes albopictus*, and *Culex tritaeniorhynchus* (Diptera: Culicidae). Parasitol Res 115(7):2771-2778.

Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, Geremia R, Petrocellis L, Di Marzo V (2009) The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. Proc Natl Acad Sci USA 106(27):11131-11136.

Harrison R, Vickers SE (1990). Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J Reprod Fert 88(1):343-352.

Izumi E, Ueda-Nakamura TN, Veiga Junior VF, Pinto AC, Nakamura CV (2012) Terpenes from *Copaifera* demonstrated in vitro antiparasitic and synergic activity. J Med Chem 55(7):2994-3001.

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K (2002) Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian J Androl 4(2):97-104.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn

K, Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol 19(9):6318-6322.

Lima SR, Junior VF, Christo HB, Pinto AC, Fernandes PD (2003). In vivo and in vitro studies on the anticancer activity of *Copaifera multijuga* hayne and its fractions. Phytother Res 17(9):1048-53.

Mendonça DE, Onofre SB (2009) Antimicrobial activity of the oil-resin produced by copaiba *Copaifera multijuga* Hayne (Leguminosae). Rev Bras Farmacogn 19(2B):577-581.

Oyeyipo IP, Skosana BT, Everson FP, Strijdom H, Du Plessis SS (2018) Highly active antiretroviral therapy alters sperm parameters and testicular antioxidant status in diet-induced obese rats. Toxicol Res 34(1):41.

Pieri FA, Mussi MC, Fiorini JE, Moreira MA, Schneedorf JM (2012) Bacteriostatic effect of copaiba oil (*Copaifera officinalis*) against *Streptococcus mutans*. Braz Dent J 23(1):36-8.

Santana SR, Bianchini-Pontuschka R, Hurtado FB, Oliveira CA, Melo LPR, Santos GJ (2014) Uso medicinal do óleo de copaíba (*Copaifera* sp.) por pessoas da melhor idade no município de Presidente Médici, Rondônia, Brasil. Acta Agronómica 63(4):361-366.

Silva HHG, Geris R, Rodrigues Filho E, Rocha C, Silva IG (2007) Larvicidal activity of oil-resin fractions from the Brazilian medicinal plant *Copaifera reticulata* Ducke (Leguminosae-Caesalpinoideae) against *Aedes aegypti* (Diptera, Culicidae). Rev Soc Bras Med Trop 40:264-267.

Soares DC, Portella NA, Ramos MFS, Siani AC, Saraiva EM (2013) Trans-β-caryophyllene: An effective antileishmanial compound found in commercial copaiba oil (*Copaifera* spp.). Evid-Based Complement Altern Med 2013:761323.

Svetlichny G, Kulkamp-guerreiro IC, Cunha SL, Silva FE, Bueno K, Pohlmann AR, Fuentefria AM, Guterres SS (2015). Solid lipid nanoparticles containing copaiba oil and allantoin: development and role of nanoencapsulation on the antifungal activity. Pharmazie 70(3):155-64.

Tobouti PL, Martins TCA, Pereira TJ, Mussi MCM (2017) Antimicrobial activity of copaiba oil: A review and a call for further research. Biomed Pharmacother 94:93-99.

Urióstegui-Acosta M, Hernández-Ochoa I, Sánchez-Gutiérrez M, Piña-Guzmán B, Rafael-Vázquez L, Solís-Heredia M, Martínez-Aguilar G, Quintanilla-Vega B (2014) Methamidophos alters sperm function and DNA at different stages of spermatogenesis in mice. Toxicol Appl Pharmacol 279(3):391-400.

Veiga Junior VF, Rosas EC, Carvalho MV, Henriques MGMO, Pinto AC (2007) Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne - A comparative study. J Ethnopharmacol 112(2):248-254.

Wang JP, Ruan JI, Cai YI, Luo Q, Xu HX, Wu YX (2011) In vitro and in vivo evaluation of the wound healing properties of *Siegesbeckia pubescens*. J Ethnopharmacol 134(3):1033-1038.