Reproductive Toxicity Assessment of *Origanum vulgare* Essential Oil on Male Wistar Rats*

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**ABSTRACT**

**Background:** The oregano essential oil (*Origanum vulgare* L.) is rich in phenolic compounds with therapeutic actions such as antimicrobial, antifungal and antioxidant. Based on the therapeutic potential and clinical use of oregano essential oil, *in vivo* toxicology studies of oregano essential oil are scarce and the current researches focus on the genotoxicity of several species of oregano; however, toxicological and complementary studies are needed to ensure safety of formulations containing this oil. Through values of Minimum Inhibitory Concentration (MIC) of oregano essential oil against *Candida* species, and increased in an exponential scale, the initial dose was obtained. This study aims to evaluate male fertility through the reproductive aspects of rats chronically treated with oregano essential oil.

**Materials, Methods & Results:** The rats were divided into five groups with 10 males and 30 females each; three groups were treated with oregano essential oil at a concentration of 3% Vol/Vol (GO3%), of 9% Vol/Vol (GO9%) and of 27% Vol/Vol (GO27%). The negative control group received the vehicle, 0.001% Vol/Vol Tween 80 (GC-) and the positive control group was treated with thymol and terpinen-4-ol, at the same concentration found in the oregano essential oil, detected by gas chromatography (3% + 3% Vol/Vol) (GC+). Animals were allowed to adapt for at least ten days before the beginning of the experiment. They were maintained under controlled temperature (± 22ºC), 50% ± 5 relative humidity and lighting conditions (12 L, 12 D photoperiod) and had free access to food and water. All animals were treated daily by gavages. Male rats were treated for 91 days (70 days before mating and 21 during mating) and females were treated before mating (14 days) and during mating (21 days), pregnancy (21 days), and lactation periods (21 days). The reproductive variables were evaluated: the total number of sperm cells, daily sperm production, sperm morphology, serum testosterone dosage, organ histopathology and animal growth were evaluated. The results showed that the essential oil of oregano interfered decreasing the number of sperm cells, the total number of sperm stored in the epididymis tail and serum testosterone levels (*P* < 0.05), in the sperm morphology in the treated groups, dose dependent manner, also in the positive control (*P* < 0.001).

**Discussion:** The *Origanum vulgare* L. essential oil, as well as other essential oils, has potential to be used in the development of new drugs as recent researches claim. However, the essential oil acted directly on sexual organs, reducing their weight and causing tissue injury of the testes. At the highest dose, these changes may be associated with metabolic disorders, as testosterone levels are also decreased. The direct action of oregano essential oil at the highest dose (27% Vol/Vol) on the testicle is also responsible for the reduction in sperm concentration and production of abnormal gametes. It may also play an adverse role on the Leydig cell, affecting testosterone production. These outcomes corroborate the results of other essential oils. The oregano essential oil, only at the highest tested dose and its positive control (3% / 3% Vol/Vol), compared to a negative control, interfered in sperm and hormonal parameters evaluated, causing infertility in male Wistar rats under the conditions tested.

**Keywords:** essential oil, fertility, *Origanum vulgare*, rats, reproductive toxicity.
INTRODUCTION

Oregano (*Origanum vulgare* L.) is an important plant which is rich in phenolic compounds with therapeutic actions. While oregano is a native plant from Mediterranean area, it grows successfully in worldwide throughout the year. Belonging to terpenes series; the oregano essential oil has predominance of monoterpenes and sesquiterpenes with multiple biological activities such as insecticides, expectorant, antimicrobial, antifungal, anti-inflammatory and antioxidant [3,6,24].

Previous studies have shown that oregano has many uses. It can be used as food preservative of animal origin, controller of microorganisms and endoparasites and as a growth promoter in farm animals [1,3,12,13,20].

In *vivo* toxicology studies of oregano essential oil are scarce and the current researches focus on the genotoxicity of some oregano species. Only the genotoxic and antigenotoxic effects of oregano essential oil (*Origanum onites* L.) and carvacrol, through the Ames *Salmonella* / Microsomal tests were found [17].

In view of the potential use of oregano essential oil, toxicological studies are needed and complementary to ensure safety of formulations containing this oil; the present study was therefore designed to evaluate the effects on the male fertility, through the reproductive aspects of rats chronically treated with the oregano essential oil.

MATERIALS AND METHODS

*Plant material, extraction and chromatographic analysis*

The dried *Origanum vulgare* L. leaves were purchased from a commercial distributor originally from Peru. The essential oil of oregano leaves was extracted by the method known as steam distillation using equipment adapted for extraction without volatile solvents. The chemical analysis of the essential oil was carried out using gas chromatography-mass spectrometry (GC - MS) system with a split-splitless injector (QP 5050A). The following chromatographic conditions were employed: 60°C - 240° to 3°C / min at 10°C / min to 280°C, Td = 180°C, Dye = 240°C, split = 1: 10 [22].

*Animals*

Adult male (n = 50, 120 days old) and female (n = 150, 90 days old) Wistar rats were supplied by the Center of Reproduction and Experimentation of Laboratory Animal (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil), were housed in polypropylene cages (40 x 33 x 16.5 cm); maintained under controlled temperature (± 22°C), 50% ± 5 relative humidity and lighting conditions (12 L, 12 D photoperiod). Feed and tap water were provided *ad libitum*. Males and females were acclimated to the laboratory for 10 days prior to the start the experiment. All rats were treated daily by oral gavage, male rats were treated by 70 days before and 21 days during the mating period, and females were treated by at least 56 days before the mating period, 14 days during the mating period, 21 days of gestation and 21 days of lactation. Housing, handling, treatment and euthanasia of animals were carried out in accordance with norms of the Brazilian College of Animal Experimentation and the study was approved by the Committee for Animal Research of University.

**Experimental Design**

The animals were divided in five groups composed of 10 males and 30 females each. Initially was used a therapeutic dose of oregano essential oil from the values defined on Minimum Inhibitory Concentration (MIC) obtained in *in vitro* tests against *Candida* isolates. The treated groups received the concentrations of 3% (GO3%), 9% (GO9%) and 27% vol / vol (GO27%) of oregano essential oil, a combination of the major phenolic compounds thymol and terpinen-4-ol at the concentration found in the oregano essential oil (3% of each compound; positive control group; CG+) and an emulsion containing distilled water and 0.001% Tween 80 (negative control group, CG-), which was used as vehicle in the other groups.

**Fertility evaluation**

For mating male rats were housed individually in a cage were three nulliparous females were placed for 2 h (6:00 am to 8:00 am) after vaginal smears were collected and examined for the presence of sperm, if positive, the following first 24 h was designated as day 0 of gestation. The mating procedure was repeated from Monday to Friday for 3 weeks. After the end of mating and pregnancy period, reproductive index were calculated to measure the fertility of treated males:

- Mating index: number of sperm positive females / number of mated females X 100;
Pregnancy index: number of pregnant females / number of sperm positive females X 100;

Birth index: total number of pup alive / total number of pup born (live and / or dead) X 100.

Male examination procedure
At the following day of the end of the treatment, all male rats were euthanized by decapitation after anesthesia using tiletamine / zolazepam 50% (50 mg / kg) intraperitoneally. The organs were removed: testes, epididymides, seminal vesicles and prostate, kidneys, liver, spleen and heart of each animal were inspected macroscopically, weighed and fixed (with the exception of testes and epididymides) in 10% neutral buffered formalin for routine processing and light-microscopic evaluation of sections stained with hematoxylin-eosin. One testicle per group was fixed in Bouin’s solution, for histological examination.

After decapitation, blood was collected from the ruptured cervical vessels in a non-heparinized tube to determine serum testosterone levels. The serum was obtained after centrifugation and was frozen at -20°C until hormonal determination. Serum testosterone levels were determined by chemiluminescence using testosterone kit Immulite 1000® equipment.

Spermatid and sperm numbers
Each testis and epididymis tail were crushed and homogenized according to the according to the protocol described by Hollenbach et al. [15]. The final volume obtained by counting the total number of sperm (epididymis tail) and the number of spermatides per animal (testis) was performed using a Neubauer chamber under an optical microscope with 40 X magnification [8].

Sperm morphology assessment
To evaluate the percentage of morphologically abnormal sperm (defects in head, or tail piece) the ducts deferens were rinsed with 1mL 0.9% NaCl and a sperm suspension was subsequently obtained. An aliquot of sperm suspension was stained with 2% eosin to assess the percentage of morphologically abnormal sperm. Two hundred sperm/animal were analyzed microscopically (X 400 total magnification). The head alterations categories were outstanding, malformation and missing, the tail alterations categories were outstanding, broken and intense folding.

Statistical analysis
Values are expressed as mean ± standard error of the mean (SEM). Quantitative variables were analyzed by analysis of variance (ANOVA) followed by the Tukey test, whereas qualitative variables were analyzed using the chi-square test. The significance level was 99% (P < 0.001) for the chi-square test and 95% (P < 0.05) for the ANOVA.

RESULTS

Chromatographic analysis
The chromatographic peaks shown in Figure 1 represents the oregano essential oil major compounds in the analyzed sample using chromatographic standards, cis-sabinene hydrate (27.46%), thymol (17.97%), terpinen-4-ol (10.55%). The terpinen-4-ol and thymol is represented by the peaks 15 and 22.

Figure 1. Total ion chromatogram (GC / MS) of the essential oil of oregano sample, prepared in a solution of 1000 mg / L¹ in hexane. Major peaks identified as: 11 (cis-sabinene hydrate), 15 (terpin-4-ol) and 22 (thymol).
Body weight gain

The oregano essential oil dose orally given during 91 days, prior and during the mating period (at 3%, 9% and 27% Vol/Vol) did not induce death or systemic toxicity. Animals treated with different concentrations of oregano essential oil showed no reduction in body weight gain. In the Table 1 are presented the final body weight and organ relative weights of rats.

Serum testosterone levels

The serum testosterone levels shown in Figure 2 decreased significantly in GO9% and GO27% groups in comparison to the negative control group.

Reproductive index

As Table 2 shows pups number, body weight and the birth index did not differ between groups, the highest doses, GO9% and GO27% showed statistically significant difference in mating index, but only GO27% presents statistical difference in pregnancy index.

Sperm parameters

The number of sperm cells in epididymis tail in male rats treated with emulsion containing oregano essential oil at different concentrations resulted in statistically significant differences at the higher dose (27% Vol/Vol) and the positive control (GC+) [Table 3]. Spermatid number and daily sperm productions also resulted in statistically significant differences at the median and the higher dose (9% and 27% Vol/Vol) and the positive control (GC+).

Histology

Light microscopic evaluation showed morphological alterations in testicular tissue of male rats treated with oregano essential oil at 27% (GO27%). Degenerative alterations in testes included areas of seminiferous tubules with cellular rarefaction, vacuolization of cells of the base and absence of sperm within the tubules.

![Figure 2. Serum testosterone levels of rats treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative control (GC-) and positive control (GC+) groups. *Indicate significant (P < 0.05) differences compared to the negative control group. Data are expressed as mean ± SEM.](image)

Table 1. Final body weight and organ relative weights of male rats treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative control (GC-) and positive control (GC+) groups.

<table>
<thead>
<tr>
<th>Organ</th>
<th>GC- (n = 10)</th>
<th>GC+ (n = 10)</th>
<th>GO3% (n = 10)</th>
<th>GO9% (n = 10)</th>
<th>GO27% (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final B. W. (g)</td>
<td>432.1 ± 13.8</td>
<td>417.9 ± 10.3</td>
<td>431.4 ± 15.9</td>
<td>435.3 ± 16.4</td>
<td>408.7 ± 18.9</td>
</tr>
<tr>
<td>Heart</td>
<td>0.134 ± 0.01</td>
<td>0.262 ± 0.01</td>
<td>0.194 ± 0.01</td>
<td>0.193 ± 0.02</td>
<td>0.213 ± 0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.086 ± 0.02</td>
<td>0.167 ± 0.01</td>
<td>0.113 ± 0.01</td>
<td>0.133 ± 0.01</td>
<td>0.145 ± 0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>1.743 ± 0.19</td>
<td>3.138 ± 0.09</td>
<td>1.898 ± 0.03</td>
<td>2.432 ± 0.17</td>
<td>2.762 ± 0.29</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.221 ± 0.01</td>
<td>0.422 ± 0.12</td>
<td>0.291 ± 0.01</td>
<td>0.327 ± 0.03</td>
<td>0.366 ± 0.02</td>
</tr>
<tr>
<td>Kidney right</td>
<td>0.156 ± 0.01</td>
<td>0.293 ± 0.01</td>
<td>0.190 ± 0.01</td>
<td>0.243 ± 0.02</td>
<td>0.229 ± 0.02</td>
</tr>
<tr>
<td>Kidney left</td>
<td>0.157 ± 0.01</td>
<td>0.284 ± 0.01</td>
<td>0.182 ± 0.01</td>
<td>0.235 ± 0.02</td>
<td>0.234 ± 0.02</td>
</tr>
<tr>
<td>Testicle right</td>
<td>0.208 ± 0.01</td>
<td>0.354 ± 0.01</td>
<td>0.354 ± 0.01</td>
<td>0.292 ± 0.02</td>
<td>0.193 ± 0.03</td>
</tr>
<tr>
<td>Testicle left</td>
<td>0.215 ± 0.01</td>
<td>0.353 ± 0.01*</td>
<td>0.264 ± 0.01</td>
<td>0.293 ± 0.02</td>
<td>0.196 ± 0.01*</td>
</tr>
<tr>
<td>Epididymis right</td>
<td>0.076 ± 0.01</td>
<td>0.133 ± 0.01*</td>
<td>0.092 ± 0.01</td>
<td>0.114 ± 0.01</td>
<td>0.101 ± 0.02</td>
</tr>
<tr>
<td>Epididymis left</td>
<td>0.070 ± 0.01</td>
<td>0.138 ± 0.01*</td>
<td>0.297 ± 0.01</td>
<td>0.118 ± 0.01</td>
<td>0.108 ± 0.02</td>
</tr>
<tr>
<td>Ventral prostate</td>
<td>0.064 ± 0.01</td>
<td>0.105 ± 0.01</td>
<td>0.066 ± 0.01</td>
<td>0.102 ± 0.01</td>
<td>0.104 ± 0.02</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>0.093 ± 0.01</td>
<td>0.150 ± 0.01*</td>
<td>0.115 ± 0.01</td>
<td>0.115 ± 0.02</td>
<td>0.129 ± 0.02</td>
</tr>
</tbody>
</table>

*Indicate significant (P < 0.05) differences from negative control group. Data are expressed in mean ± SEM.
Table 2. Reproductive indexes of female’s rats treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative control (GC-) and positive control (GC+) groups.

<table>
<thead>
<tr>
<th></th>
<th>GC-</th>
<th>GC+</th>
<th>GO3%</th>
<th>GO9%</th>
<th>GO27%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating index (%)</td>
<td>76.7</td>
<td>73.3</td>
<td>56.7</td>
<td>40*</td>
<td>40*</td>
</tr>
<tr>
<td>Pregnancy index (%)</td>
<td>91.3</td>
<td>95.5</td>
<td>100</td>
<td>83.3</td>
<td>8.3*</td>
</tr>
<tr>
<td>Birth index (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pups per litter</td>
<td>9.1 ± 0.5</td>
<td>8.9 ± 0.7</td>
<td>10.4 ±0.5</td>
<td>8.8 ± 0.6</td>
<td>3* a</td>
</tr>
<tr>
<td>Pups body weight (g)</td>
<td>6.3 ± 0.1</td>
<td>6.2 ± 0.2</td>
<td>6.1 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>6.2 a</td>
</tr>
</tbody>
</table>

*Indicate significant (P < 0.001) differences from negative control group. aSymbol indicate values obtained from single offspring born in that group. Data are percentages and means/group ± SEM.

Table 3. Sperm parameters of rats treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative control (GC-) and positive control (GC+) groups.

<table>
<thead>
<tr>
<th></th>
<th>GC-</th>
<th>GC+</th>
<th>GO3%</th>
<th>GO9%</th>
<th>GO27%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatid number (x 10⁶ / testis)</td>
<td>280.5 ± 8.5</td>
<td>160 ± 9.3*</td>
<td>273.4 ± 19.4</td>
<td>193.5 ±2.9*</td>
<td>94.9 ± 11.1*</td>
</tr>
<tr>
<td>Daily sperm production (x 10⁶ / testis / day)</td>
<td>46 ± 1.4</td>
<td>25.9 ± 1.6*</td>
<td>55.6 ± 3.3</td>
<td>31.6 ± 0.7*</td>
<td>15.5 ± 2.6*</td>
</tr>
<tr>
<td>Number total of spermatozoa (x 10⁶/epididymis)</td>
<td>1478.5 ± 52.1</td>
<td>699.6 ± 63.3*</td>
<td>1597 ± 58</td>
<td>1347.9 ± 56.4</td>
<td>396 ± 80.4*</td>
</tr>
<tr>
<td>Sperm with morphological Alterations (Caput / Cauda) (%)</td>
<td>4.2 ± 0.3</td>
<td>14.6 ± 0.7*</td>
<td>3.4 ± 0.3</td>
<td>8.9 ± 0.6 **</td>
<td>17.9 ± 0.8**</td>
</tr>
</tbody>
</table>

*Indicate significant (*P < 0.05; **P < 0.001) differences from negative control group. Data are means/group ± SEM.

**DISCUSSION**

As part of reproductive toxicology evaluation of essential oil, the present manuscript aimed to discover the influence of oregano essential oil (Origanum vulgare) administration on sperm parameters and fertility of male rats.

The weight monitoring during treatment period provides an indication of animal’s health status, which can provide important information regarding toxicity. Weight analysis revealed no difference in weight gain between the three groups treated with essential oil and control groups, this result supports the hypothesis of no systemic toxicity in treated animals during the treatment period.

The results of the current study demonstrated that the essential oil acted directly on sexual organs, reducing their weight and causing tissue injury of the testes. At the highest dose, these changes may be associated with metabolic disorders, as testosterone levels are also decreased. The direct action of oregano essential oil at the highest dose (27% V / V) on the testicle is also responsible for the reduction in sperm concentration and production of abnormal gametes. It may also play an adverse role on the Leydig cell, affecting testosterone production as suggested by Chen et al. [5]. Changes in Leydig cells functions, in the blood-testicular barrier, in the testicular vascular system as well as local immune system reactions may be considered as indirect effects resulting in a reduced fertility [21].

The fertility of males treated with oregano essential oil decreased in a dose dependent way. In the intermediate dose (9%) only the mating index was affected, but at the highest dose (27%), had low mating rates, the males were able to mate but failed to fertilize female whereas that only one pregnancy occurred. This is explained by the low daily sperm production, number of sperm stored and the high abnormal sperm rate. If the number of normal sperm per ejaculation is very low, fertilization is unlikely and an infertile condition exists. Chenoweth [7] asserted that sperm structure could play a substantial role in both fertilization and pregnancy outcome.
This result corroborates to the found by Sharma & Jacob [25], which evaluated the contraceptive efficacy of the Mentha arvensis leaves, also from Lamiaceae family. They found that the daily oral administration of the extract aqueous solution (10 mg/day/mice) during 20, 40 and 60 days caused fertility inhibition but in these animals, the normal sexual behavior is maintained. As the treatment duration increase occurred a corresponding decrease in the weight mean of testes and accessory reproduction organs, decreased sperm concentration, motility and viability in the caudal epididymis.

The short-term administration of rosemary (Rosmarinus officinalis L. - Lamiaceae) aqueous solution on reproductive system, vital organs and sperm production of mature male Wistar rats was assessed, the results showed that the acute administration of the R. officinalis extract, at a dose of 291.2 and 582.4 mg / kg body weight, for five days, the animals treated with the higher dose showed significant weight increase of the seminal vesicle but no significant alteration of the other variables [23]. Illayperuma [16] tested the Citral, another monoterpene, on male rats’ reproductive system, concluded that Citral in intraperitoneal injection for 14 consecutive days, in a dose of 300 mg/kg, leads to weight reduction of testes, epididymides, seminal vesicles, prostate and decreased plasma testosterone levels.

The positive control group, treated with a combination of the major phenolic compounds thymol and terpinen-4-ol at the concentration found in the oregano essential oil had similar results comparing to the groups treated with the essential oil as regards of: sperm counts, morphological changes in the sperm and the serum testosterone concentration (the serum testosterone without statistical significance but decreased). This confirms that some compounds such as thymol, when used alone may cause toxicity [2,4]. However, literature review demonstrate that the terpinen-4-ol has toxic effect when is given alone, just in high concentrations [11]. Still, as in this essay was administered a relatively low dose, positive control group rats have not had their fertility affected. With these results, the female reproductive index was not affected.

In the positive control group was seen a weight increase in seminal vesicle, epididymides and testes. A weight increase of reproductive organs could suggest a disturbance of the reproductive endocrine functions, as these organs are controlled by hormones [10]. The pituitary gland is responsible for secreting follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on the testicle. Any damage to this gland could interfere with the male reproductive system, affecting the production of sex steroid hormones and male gametes [19].

The Origanum vulgare L. essential oil as well as other essential oils has potential to be used in development of new drugs as claim recent research [14,25].

CONCLUSION

This study confirms that under the conditions tested the oil only is harmful for the fertility of rats when used in very high doses, thus, the safety of the oil-containing preparations are in moderate doses.

REFERENCES


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