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## Pre- and postnatal evaluation of offspring rats exposed to *Origanum vulgare* essential oil during mating, gestation and lactation

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**ABSTRACT:** Despite the increasing use of oregano (*Origanum vulgare* L.) essential oil for therapeutic purposes, pre- and postnatal development of animals offspring exposed to this oil has not yet been evaluated. In line with previous concerns of genotoxicity, in this study adult rats were exposed to different doses of oregano essential oil (3, 9 and 27% vol/vol) during pre-mating, mating, gestation and lactation. Prenatal screening included fetal development and uterine inspection, where the reproductive rate of females such as breeding, pregnancy, delivery, viability and post-implantation loss rate were measured. Postnatal evaluation of rat offspring included motor development, neuroendocrine and behavioral assessment. Body weight of rat dams and signs of dystocia were evaluated daily. Development of physic characteristics and reflex tests of puppies were also assessed. Additionally, these rats, when adults, were submitted to sexual and open field behavioral tests. The main differences among the groups were observed in the indices of mating, pregnancy and post-implantation loss ( $P < 0.01$ ). Results demonstrated that the treatment of parental generation with oregano essential oil has the potential to affect the developing fetuses at the highest dose used, but without causing maternal toxicity and changes in general behavior and development of the progeny.

**Key words:** oregano, reproductive toxicity, progeny, reproduction, rats.

## Avaliação pré e pós-natal da prole de ratos expostos ao óleo essencial de *Origanum vulgare* durante acasalamento, gestação e lactação

**RESUMO:** Apesar do aumento do uso do óleo essencial de orégano (*Origanum vulgare* L.) para fins terapêuticos, o desenvolvimento pré e pós-natal da progênie de animais expostos a este óleo ainda não foi avaliado. Partindo das suspeitas prévias de genotoxicidade desse óleo, neste estudo, ratos Wistar adultos foram expostos a diferentes doses do óleo essencial de orégano (3, 9 e 27% vol/vol) durante o acasalamento, a gestação e a lactação. Para a avaliação pré-natal, o desenvolvimento gestacional foi observado e os úteros inspecionados, assim como os índices reprodutivos das fêmeas, como a taxa de acasalamento, de gestação, parto e perdas pós-implantação. Na avaliação pós-natal, observou-se o desenvolvimento motor, neuroendócrino e comportamental da prole. Observou-se, diariamente, o peso das ratas e sinais de distocia. Após o parto, as características de desenvolvimento e testes de reflexos dos filhotes foram avaliadas, enquanto que, na puberdade, foram realizadas análises histopatológicas e dosagem hormonal. Adicionalmente, na idade adulta, esses ratos foram submetidos ao teste de comportamento em campo aberto e ao comportamento sexual. As principais diferenças entre os grupos foram nos índices de acasalamento, de gestação e de perdas pós-implantação ( $P < 0,01$ ). Os resultados demonstram que o tratamento da geração parental com óleo essencial de orégano tem potencial para afetar os índices reprodutivos das ratas e o desenvolvimento dos fetos na maior dose utilizada, mas sem causar toxicidade materna e alterações no desenvolvimento geral e comportamental da sua progênie.

**Palavras-chave:** orégano, toxicidade reprodutiva, progênie, reprodução, ratos.

## INTRODUCTION

Oregano (*Origanum vulgare* L.) essential oil contains terpene compounds such as thymol, carvacrol, monoterpene alcohols,  $\alpha$ -terpineol and  $\gamma$ -terpinene which are responsible for the known antimicrobial, antifungal and antioxidant properties of this oil (BUSATTA et al., 2007, CLEFF et al., 2010). Moreover, several studies have mentioned the use of oregano essential oil as a preservative in foods of animal origin, in the control of

endoparasites and as a growth promoter in farm animals (ALLAN & BILK, 2003; GIANNENAS et al., 2003; FUKAYAMA et al., 2005;).

Studies on the toxicity of oregano essential oil are scarce. KARPOUHTSIS et al. (1998) evaluated the genotoxic potential of oregano essential oil and its main constituents, carvacrol and thymol, using the somatic mutation and recombination test in *Drosophila*. Authors reported that only thymol showed genotoxic activity. Genotoxic effects of

thymol were also investigated in human peripheral lymphocytes. A decrease in the mitotic index was observed at the highest concentration used, showing a significant clastogenic effect (BUYUKLEYLA & RENCUZOGULLARI, 2009).

In view of its potential genotoxicity, the use of *O. vulgare* essential oil depends on a toxicological evaluation, as recommended by the Food and Drug Administration (FDA, 2007). The aim of this study was to evaluate the pre- and postnatal development, concerning gestational, motor, neuroendocrine and behavioral development, of the offspring of rat dams exposed to different concentrations of oregano essential oil throughout mating, pregnancy and lactation.

## MATERIALS AND METHODS

### *Extraction and chromatographic analysis of the essential oil*

Essential oil was extracted from *O. vulgare* L. leaves (Oseca Laura Elisabeth Company, Tacna, Peru) by the method known as steam distillation using equipment adapted for extraction without volatile solvents (SCHULTZ et al., 1977). The essential oil was chemically characterized using a gas chromatography-mass spectrometry (GC-MS) system with a split-splitless injector (Shimadzu QP-5050A) as follows: 60°C to 240°C at 3°C min<sup>-1</sup>, to 280°C at 10°C min<sup>-1</sup>; Td=180°C; Dye=240°C; split=1:10 (RODRIGUES et al. 2004).

### *Animals and experimental design*

Groups of 10 males and 30 females were formed: GO3%, GO9% and GO27%, which were treated with oregano essential oil (vol/vol) at the concentrations of 3%, 9% and 27%, respectively; GC+ (positive control), treated with a combination of the major compounds present in oregano essential oil (3% thymol and 3% terpinen-4-ol); GC- (negative control), which received distilled water and 0.001% Tween 80 emulsion (the same vehicle for the other groups).

Adult male (n=50, 120 days old) and female (n=150, 90 days old) Wistar rats were treated daily by oral gavage. Male rats were treated for 70 days before and 21 days during the mating period, and females were treated for 14 days before the mating period, 21 days during the mating period, 21 days of gestation and 21 days of lactation. Housing, handling, treatment and euthanasia of the animals were performed in accordance with norms published by the Brazilian College of Animal Experimentation.

For mating, three naive females were placed into one male's cage for 2h every morning. At the end of the period, the presence of spermatozoa (sptz) in vaginal washings was evaluated. The mating procedure was conducted for a period of 21 days, corresponding to three cycles of five consecutive days and intervals of two days between each cycle.

Pregnant females were weighed daily and observed for signs of resorption, dystocia and prolonged duration of pregnancy. On day 21 of pregnancy, half of the females of each group were assigned to give birth to their offspring and the other half were assigned to teratogenicity studies. After the end of the lactation period, remaining progenitors were euthanized and each uterus was inspected for uterine implantation sites and sent to histopathological evaluation. The following reproductive rates were calculated (x100): mating (females with sptz in vaginal smear / mated); pregnancy (pregnant females / females with sptz in vaginal smear); delivery (parous females / pregnant); birth (live pups born/ pups born); viability (live pups on day fourth of lactation / live pups born); post-implantation loss (implantation sites - fetuses born / implantation sites).

### *Postnatal development assessment*

From Day 21 of pregnancy onwards, the cages were inspected for births and the Day 1 of birth was designed as Day 0 after birth. Number of rat pups born alive or dead was recorded and pups were sexed and weighed. All litters with more than eight puppies were standardized at eight pups, four males and four females. Each pup was individually weighed at Days 0, 7, 14, 21 and litters were daily weighed up to Day 36. Pup physical development was evaluated by the time of: a) pinna unfolding; b) development of primary coat of downy hair; c) hair growing; d) incisor eruption; e) eye opening; f) testes descent to scrotum; g) preputial separation; and h) vaginal opening. Individually daily tests in newborn rats were performed according to WHISHAW & KOLB (2005), and included: righting reflex, from the 2<sup>nd</sup> to the 5<sup>th</sup> postnatal day (PND); grip strength, from the 14<sup>th</sup> to the 17<sup>th</sup> PND; negative geotaxis, from the 7<sup>th</sup> to the 10<sup>th</sup> PND.

One male and one female from each litter were euthanized at puberty (Day 65) (males: GC-=6, GC+=9, GO3%=7, GO9%=5 and GO27%=2; females: GC-=6, GC+=9, GO3%=7, GO9%=5 and GO27%=1) for histopathological analysis and evaluation of hormonal and sperm parameters. Males were killed by decapitation after anesthesia and blood was collected for hormonal dosage. Females were

euthanized on the day of estrus, confirmed by vaginal cytology. For assessment of organs, kidney, liver and heart were collected, weighed and fixed in 10% formalin for histopathological analysis. The sexual organs (testes, epididymis, prostate, vas deferens and seminal vesicle) were collected and weighted, only the prostate and seminal vesicle were fixed in 10% formalin, and only one testicle per group randomly selected was fixed in Bouin solution, and submitted to histopathology.

The testes and epididymides that were not submitted to histopathology were crushed and homogenized individually and the total sperm count (epididymis tail) and the spermatid number per animal (testis) were evaluated from an aliquot of each organ using a Neubauer chamber. The number of spz and their daily production as well as the analysis of the percentage of morphological changes of head and tail of sperm collected from the vas deferens were determined according to HOLLENBACH et al. (2012). Testosterone levels were assessed by chemiluminescence assay using the Immulite 1000R automated equipment (Imunolab®, Porto Alegre, Brazil), using blood serum samples collected before euthanasia and centrifuged at 2400rpm for 20min.

The remaining animals were kept until adulthood for behavioral tests. In the 75<sup>th</sup> PND, one male for each litter was randomly assigned to the open field test. The GO27% group was not evaluated because all the animals were euthanized at puberty. A single rat was placed in the center of the arena and for three min, locomotion (the number of squares crossed with the four paws), frequency of rearing (posture sustained with hind-paws on the floor) and time (in seconds) spent on grooming were manually counted (AZEVEDO De et al., 2005).

Non-consanguineous couples were randomly formed within each experimental group for the sexual behavioral test, starting at day 100 after birth. Ten couples were formed in the groups GC-, GC+ and GO3%, and five couples were formed in the GO9% group. Acrylic boxes and a 15W red lamp for illumination during the dark period were used; the recording was started at the time of introduction of the female and lasted 20min. The mating procedure was repeated every day for three weeks. The behavior parameters observed were: female receptivity (measured by the ratio of lordosis to mount frequencies), male latency time to first approach, latency time to first intromission, incomplete mount number (without intromission) and number of intrusions (CHAHOUDE & FAQI, 1998).

### Statistical analysis

Quantitative symmetric variables such as body and organ weight, reflex tests, sperm counts, time to the onset of appearance of pups' physical characteristics and behavioral tests were submitted to analysis of variance (ANOVA) followed by the Tukey test. Qualitative variables such as morphological changes in the sperm and reproductive rates were analyzed using the Chi-square test. Values are expressed as mean  $\pm$  standard error of the mean (SEM). The significance level was set at  $P < 0.05$  for the Tukey test and  $P < 0.01$  for the Chi-square test.

## RESULTS AND DISCUSSION

As far as we know, no previous studies have evaluated the effects of administration of oregano essential oil on the development of pregnancy and on the development of rat offspring. In the present study, the administration of the three concentrations of oregano oil did not reveal signs of toxicity during pregnancy and lactation periods. However, a very low pregnancy rate was observed at the highest concentration of oregano essential oil (27%). No differences were detected regarding motor, neuroendocrine and behavioral development of rat offspring generated from parents exposed to different concentrations of oregano essential oil.

No signs of toxicity were apparent or deaths were induced in female rats treated orally during pre-mating, mating, pregnancy and lactation with the different concentrations of oregano essential oil (3%, 9% and 27%). There was no statistically significant difference between treated groups and the negative and positive control groups neither with respect to maternal weight and offspring nor about pregnancy weight gain observed at any dose level.

The litters per group and the number of pups per litter (within parentheses) were as follows: GC-, n=9 (58); GC+, n=9 (67); GO3%, n=7 (55); GO9%, n=5 (36); GO27%, n=1(3). GO27% had only one litter, with three pups born alive and eleven uterine implants. The other females in this group had spz in vaginal washings but did not have uterine implants. Table 1 shows outcomes of fertility tests and the reproductive indices of female rats with delivery at term. Significant differences were identified in the mating index in the GO9% and GO27% groups when compared to the negative control group; pregnancy and post-implantation loss indices were statistically different only in the GO27% in comparison to the others groups. The birth index of the CG+ group was statistically different from the other groups ( $P < 0.01$ ).



Table 1 - Outcomes of fertility tests and reproductive indexes of rat dams treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative (GC-) and positive (GC+) control groups.

Outcome	CG-	CG+	GO3%	GO9%	GO27%
Mated females (n)	30	30	30	30	30
Mated males (n)	10	10	10	10	10
Sperm-positive females (n)	23	22	17	12	12
Pregnant females (n)	21	21	14	10	1
Mating index (%)	76.6	73.3	56.7	40*	40*
Pregnancy index (%)	100	100	100	100	8.3*
Post-implantation loss index (%)	5.8	4.1	1.5	3.8	62.5*
-----Reproductive indexes-----					
Dams (pups) (n)	9 (58)	9 (67)	7 (55)	5 (36)	1 (3)
Offspring per litter <sup>a</sup> (mean)	6.4	7.4	7.8	7.2	3
Offspring body weight (g)	6.3 ± 0.1	6.2 ± 0.2	6.05 ± 0.1	6.4 ± 0.4	6.2 ± 0.1
Delivery index (%)	100	100	100	100	100
Viability index (%)	100	91.1	98.5	100	100
Birth index (%)	100	84.8	100	100	100

Data were analyzed by Chi-square test. <sup>a</sup>After standardization. \*Significantly different (P<0.01) from control group.

The extent to which maternal toxicity affects development has been widely discussed in the context of risk assessment. It has been widely debated whether embryo/fetal toxicity is secondary to maternal toxicity (CHAHOUDE et al., 1999). The treatment of GO3% and GO9% during pre-implantation, implantation and organogenesis did not affect uterine growth because the weight of the pups at birth showed no statistically significant difference. However, in the GO27% group only one out of 12 females that had spz in vaginal washings was pregnant, what could be called pre-implantation loss, since the females were diagnosed with pregnancy but did not carry the pregnancy to term and had no implantation sites in the uterus.

The pre-implantation period of pregnancy is considered to be “all or none”, the period during which maternal exposure to exogenous agents may cause embryo lethality (LEMONICA et al., 1996). According to STANLEY & BOWER (1996), at the undifferentiated stage of zygote proliferation and before implantation, exposure to a teratogen usually either kills the fertilized ovum, which results in a spontaneous resorption, or spares it completely.

The low mating index in GO9% and GO27% and the low pregnancy index in GO27% may also be related to changes reported in the sperm parameters of males treated with oregano essential oil, such as reduction in sperm concentration and production of abnormal spz, in line with the results reported by HOLLENBACH et al. (2015). Therefore,

it is possible to assign the apparent infertility of females to males. According to WORKING (1988), if the number of normal sperm per ejaculation is sufficiently low, fertilization is unlikely and an infertile condition exists. Moreover, sperm structure can play a substantial role in both fertilization and pregnancy outcome (CHENOWETH, 2005).

Results of our study suggested that the treatment with the highest concentration of oregano essential oil (GO27%) and with the main compounds of oregano (GC+) induced embryotoxicity; although, the high rate of post-implantation loss was calculated in the only one litter born. GC+ had a lower rate of live births, differing statistically from GC-, GO3% and GO9% groups. The evidence reported here is likely to have occurred due to toxic effects of the essential oil in the embryo (LEMONICA et al., 1996).

The weight of the progeny obtained from rats treated with the three concentrations of oregano essential oil did not show significant statistical differences in comparison to the non treated group during the 36 days of evaluation (P>0.05). There was no difference on the onset of appearance of physical characteristics in the offspring of the groups GO3%, GO9% and GC+ in comparison to offspring of the negative control (P>0.01). GO27% could not be statistically compared with the other groups because there was only one litter (Table 2).

The motor development of the offspring was assessed from the 2nd day of birth onwards. The righting reflex, negative geotaxis and grip strength tests showed

Table 2 - Mean time (days) for the appearance of general and sexual development characteristics of the progeny of rat dams treated with different concentrations of oregano essential oil (GO3% GO9% and GO27%) and of the negative (GC-) and positive (GC+) control groups.

Characteristics	GC- (n=58)	GC+ (n=67)	GO3% (n=55)	GO9% (n=36)	GO27% <sup>a</sup> (n=3)
Pinna unfolding	2.5 ± 0.5	3.0 ± 0.6	2.5 ± 0.5	1.5 ± 0.5	2 ± 0.02
Primary coat of downy hair	5.5 ± 0.5	5.5 ± 0.5	5.5 ± 0.5	5.5 ± 0.5	5 ± 0.04
Hair growing	8	7.5 ± 0.5	8	8	8
Incisor eruption	9 ± 0.5	10 ± 0.7	8.5 ± 0.5	9 ± 0.5	8 ± 0.03
Eye opening	13 ± 0.7	13 ± 0.7	13.5 ± 0.5	13.5 ± 0.6	11 ± 0.05
Testicular descent	16.5 ± 0.7	18.5 ± 0.6	16.5 ± 0.7	18.5 ± 0.5	18 ± 0.01
Preputial separation	36 ± 0.5	36.5 ± 0.7	36 ± 0.5	37 ± 0.6	36 ± 0.03
Opening of vaginal canal	37 ± 1	34.5 ± 1.1	35 ± 1	35 ± 0.9	32

<sup>a</sup>Values of the only one litter born in this group. Data were analyzed by the Tukey test (P>0.05).

no statistical differences when the treated groups were compared with the control group GC- (P>0.05).

The relative organ weight and the histopathological characteristics of male and female offspring euthanized at puberty were not altered by the treatment of the dams with oregano essential oil. The relationship between organ weight and body weight showed no statistical difference in comparison to the GC- group (P>0.05), and the histopathological analysis did not show any lesion in the organs (data not shown).

The number of sperm cells, the daily sperm production, the total number of morphological changes in sperm and testosterone levels in the serum not showed statistical significance differences when treated groups were compared to the GC- control (P> 0.05) (data not shown). The GO27% group could not be compared to the other litters because the group was composed of only two animals from a single litter, assessed only by descriptive statistics, presenting normal values.

In line with the results reported in the open field test, the sexual behavior also did not show statistically significant differences (P>0.05) (data not shown). The results obtained in the open field test showed that the treatment of rat dams with oregano essential oil did not affect the emotional state of the offspring. Studies have shown that stressful stimuli during the period of sexual differentiation of the central nervous system, prenatal and soon after birth induce stable changes manifested by decreased sexual behavior in male and female rats (BENNETI et al., 2007).

No toxicity data of other essential oils in rat offspring were found in the literature. Moreover, the lack of weight difference and proper physical and motor development of the rat offspring in our study corroborates the results reported by MELLO et al.

(2008), who evaluated preclinical toxicity of several plant formulations where the treatment of dams before and during mating, pregnancy and lactation did not change the physical development of the progeny.

## CONCLUSION

Results demonstrated that treatment of the parental generation with oregano essential oil has the potential to affect the developing fetuses at the highest dose used (27%). Notwithstanding, the administration of oregano essential oil to adult rats during breeding at the concentrations of 3% and 9%, did not affect reproduction and offspring development. Moreover, these concentrations did not affect the physical, motor and neuroendocrine parameters of rat dams and of the offspring. Therefore, the data presented here are indicative of the safety of oregano essential oil when used at low concentrations.

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The project (N° 2008067) was approved by the Ethics Committee on the Use of Animals (CEUA/UFRGS).

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