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INTRA UTERINE GROWTH RETARDATION IN RATS TREATED WITH ESSENTIAL OIL OF *ROSMARINUS OFFICINALIS* LINN

Martha de Oliveira Guerra, Nathália Barbosa do Espírito-Santo Borges*, Pedro Martins Bellei*, Marco Aurélio Faria Elias*, Sâmia Martins da Costa Silveira*, Vera Maria Peters**

Abstract

The essential oil of *Rosmarinus officinalis* L. (EORO) is used in cosmetics, in the food industry and in medicines, presenting antimicrobial and antifungal activity. Aim of the study: to observe maternal toxicity and embryo development after treatment of dams with EORO. Inseminated rats were distributed in four groups (n =15): Control (C) (saline 0.5ml) and treated with 242 (T1), 484 (T2) and 968mg/Kg (T3) of EORO, from the fifth to the seventh dpc and sacrificed on the 15th dpc. Variables analyzed: maternal behavior, body weight gain, food consumption, kidneys, liver and ovaries weight, hemogram, number of corpora lutea, implants, live, dead, malformed fetuses and placenta and fetuses weight. Statistics: Dunnett and Chi-square tests (α=0.05). The EORO higher dose reduced ($p<0.05$) maternal number of erythrocytes; the haematocrit, hemoglobin concentration and the fetuses body weight. Conclusions: Highest dose of EORO reduce the fetuse´s body weight and induced anemia in the dams.

Keywords

Rosmarinus officinalis. Anemia. Blastocyst. Implantation. Rat.

1 INTRODUCTION

Rosmarinus officinalis L. is a bushy plant (its common name being rosemary), originally from the Mediterranean area of Europe (MARTINS et al., 1998) which carry out astringent, analgesic, antiseptic, antispasmodic, anti-inflammatory, antioxidant, aromatic, digestive, stimulant, tonic and vasodilator activities (FLAMINI et al., 2002). The essential oil of *R. officinalis* (EORO) is used in perfumes, cosmetics, in the food industry and in medicines, presenting antimicrobial and antifungal activity (SANTOYO et al., 2005).

Some of the chemical components of rosemary are: ascorbic acid, labiatic acid, rosmaniric acid, borneol, canphene, camphor, beta-carotene, cineol, elemol, eugenol, alpha-pinene, beta pinene, rosmadiol, rosmanol, rosmaricin, rosmarinol, sabinene, beta-sitosterol, tannin, alpha-terpinene, timol, alpha-tocoferol, carnosol, carnosic acid, epirosmanol and dimethyl-isorosmanol. The main components of the essential oil are alpha-pinene, borneol, (-) camphene, camphor, verbenone, bornyl-acetate, 1,8-cineole. 2-ethyl-4,5-dimethylphenol borneol (+) and alpha-terpineol (ANGIONI et al., 2004; ATTI-SANTOS et al., 2005).

Correspondence Author: Martha de Oliveira Guerra. Address: Rua São Mateus, 187/801, São Mateus, CEP: 30.025-001, Juiz de Fora, MG, Brazil. Tel.: 55-32-21023251; Fax: 55-32-21023255. E-mail: martha.guerra@ufjf.edu.br

* Centro de Biologia da Reprodução – Juiz de Fora Federal University, Caixa Postal 328, CEP 36001-970, Juiz de Fora, MG – Brazil.

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The pharmacological effects reported for *R. officinalis* were: antispasmodic (AL-SEREITI; ABU-AMER; SEN, 1999), hepatoprotective (SOTELO-FELIX et al., 2002), antineoplastic (HUANG et al., 2005), inhibition of *Trypanosoma cruzi* mobility in culture (ABE et al., 2002); antiulcerogenic activity (DIAS et al., 2000), anti-inflammatory and antinociceptive activity (GONZALEZ-TRUJANO et al., 2007) for treatment of diabetic complications (KIM, H. Y.; KIM, K., 2003); diuretic (HALOUI et al., 2000) and neuroprotective effects (KIM et al., 2006).

The essential oil of *R. officinalis* (EORO) presenting antimicrobial and antifungal activity (SANTOYO et al., 2005); inhibits tracheal smooth muscle contractions (AQEL, 1991); has hyperglycemic activity and inhibitory effects on the release of insulin (AL-HADER; HASAN; AQEL, 1994); antimutagenic and hepatoprotective activity (FAHIM et al., 1999).

Investigation of the reproductive toxicity of rosemary suggest the aqueous extract of rosemary inhibited blastocyst implantation (LEMONICA; DAMASCENO; DI-STASI, 1996) and the alcoholic extract of leaves reduced estrogenic hepatic metabolism and the uterotropic activity of the hormone in female mice (ZHU et al., 1998). It also reduces the production of prostaglandin E2 (AL-SEREITI; ABU-AMER; SEN, 1999), and hormone involved in the process of blastocyst implantation (PARIA; SONG; DEY, 2001). Domaracky et al. (2007) (DOMARACKY ET AL., 2007) shown that some essential oil reduced the number of cells and increased the incidence of cell death of mouse embryos.

The antimicrobial activity of the essential oils is produced in different ways, among them the sensibilization of the lipid cell

membrane and alterations in the activity of calcium channels, which increases the permeability of cells and releases intracellular constituents (PORTE; GODOY, 2001). These effects can interfere with the blastocyst implantation process, which depends on vascularization and on appropriate levels of estrogen, progesterone, prostaglandin and different proteins, like cadherin, a calcium-dependent protein related to cell adhesion, polarization and cell signaling [PARIA; SONG; DEY, 2001; ALIKANI, 2005]. Besides, to our concern, no matter about toxicity of essential oil of rosmarinus or upon embryo development was cited in the literature.

In this work we tried to assess maternal toxicity and embryo development in female rats treated in the implantation period with the essential oil of *R. officinalis* (EORO).

2 MATERIALS AND METHODS

The experimental approach followed the Organization for Economic Co-operation and Development (OECD) Guideline for the Testing of Chemicals 414 (OECD, 2001) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline S5 (ICH, 2005), with minor adaptations. The experimental protocol was approved by the Ethical Committee on Animal Experimentation of UFJF, MG, Brazil, which follows the guidelines of the International Council of Laboratory Animal Science (Protocol n. 53/2003 - CEEA).

2.1 EXPERIMENTAL DESIGN

The subjects used were three-month old female Wistar rats, nulliparous obtained from the vivarium of the Centro de Biologia da Reprodução of Juiz de Fora Federal University (Minas Gerais, Brazil), where they were raised and housed as described previously (PINTO et al., 2007).

2.2 MATING AND ORGANIZATION OF THE EXPERIMENTAL GROUPS

The animals were mated polygamously, with males of proven fertility, at the end of the afternoon (17:00h), in the proportion of three females to each male. The next morning, the inseminated animals were identified by the presence of spermatozoids in the vaginal smear; this day was designated as day one post-coitum (dpc-1).

2.3 EXPERIMENTAL PROCEDURE

The inseminated rats were randomly distributed in four groups of 15 animals each: Control (C) and Treated 1, 2 and 3 (T1, T2 and T3) which received, respectively: 242mg/Kg; 484mg/Kg and 968mg/Kg of essential oil of *R. officinalis* (Rosmarinho, OERO @, code STM0433, lot 0608433, Stratus, SP, Brazil). The treatment was applied from the fifth to the seventh day of pregnancy, which corresponds to the period of blastocyst implantation.

During the whole period of the experiment the body weight was measured every days, starting from the first day to the day of euthanasia; a daily estimate was made of the food consumption and an assessment of behavior indicating stress or maternal toxicity such as: piloerection, hypo- or hyper-motility inside the cage, stereotypy, diarrhea, vaginal bleeding, chromodacriorrhea and deaths (HOOD, 2006).

The euthanasia of animals were performed by exsanguinations by cardiac puncture under anesthesia (ketamine + xylazine) on the 15th dpc and the plasma concentration of aminotransferase (AST), alanin aminotransferase (ALT), urea and creatinine plasmatic concentration were measured.

The exsanguinated animals were laparohysterectomized, and the internal organs were checked to identify lesions. Kidneys, liver and ovaries were removed and weighed. In the ovaries the corpora lutea were counted and in the uterine cornua, the implants, the resorptions and the live, dead and malformed fetuses. The corrected body weight was obtained by subtracting the weight of the uterus and its content from the weight of whole rats.

The 15-day fetuses were fixed for 30min in Bouin solution and later examined under a stereomicroscope in order to observe the face, members and the closure of the neural tube. Fetuses and placentas were weighed in groups of litter, later obtaining the mean weight of the litter.

2.4 STATISTICAL ANALYSIS

The collected data was submitted to one-way variance analysis and Dunnett test for continuous variables and homocedastic samples. Discontinuous data was analyzed by Chi-square or Kruskal-Wallis tests. Significance level of the tests: $\alpha=0.05$.

3 RESULTS

Female rats from the T2 group (484mg/kg EORO) and T3 (968mg/kg EORO) gained less body weight; the latter also had an increase in the absolute and relative weight of the liver compared to the control group. The weight of the ovaries was less ($p < 0.05$) in the T2 and T3 groups. The number of implants, corpora lutea, pre- and post-implantation losses did not differ among the groups (Table 1). The consumption of food was significantly reduced ($p < 0.05$) on day six (C: 16.4 ± 2.3 ; T3: 7.2 ± 3.1); day seven (C: 16.4 ± 2.3 ; T3: 4.8 ± 3.5) and day eight (C: 18.3 ± 1.6 ; T3: 6.4 ± 4.2) post-coitum. No other signs indicative of toxicity were observed.

Table 1: Gain in maternal body weight (g) and absolute and relative weight (g) of organs; number of implants and corpora lutea in control and EORO treated groups (T1 242mg/Kg; T2 484mg/Kg and T3 968mg/Kg).

Variables	**Control (n = 15)**	**T1 (n = 15)**	**T2 (n = 15)**	**T3 (n = 15)**
Body weight Day-1 p.c	191.45 ± 14.85	189.54 ± 7.58	186.3 ± 9.76	186.24 ± 7.90
Corrected final weight	211.41 ± 14.46	210.72 ± 10.71	199.64 ± 11.05	194.09 ± 14.43 [a]
Weight gaining	19.96 ± 6.37	21.18 ± 6.38	15.46 ± 7.84	13.39 ± 5.56 [a]
Absolute liver weight	9.23 ± 0.67	9.38 ± 0.63	9.09 ± 0.75	10.00 ± 1.11 [a]
Relative liver weight	4.37 ± 0.33	4.45 ± 0.23	4.55 ± 0.31	5.15 ± 0.31 [a]
Absolute kidney weight	1.47 ± 0.10	1.50 ± 0.12	1.42 ± 0.10	1.50 ± 0.13
Relative kidney weight	0.7 ± 0.04	0.71 ± 0.04	0.71 ± 0.04	0.78 ± 0.09
Ovaries weight	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01 [a]	0.06 ± 0.01 [a]
Total of corpora lutea	187	206	176	180
Mean corpora lutea / rats	12.47 ± 1.30	12.75 ± 0.93	11.73 ± 1.10	12.00 ± 1.60
Total of implants	171	171	158	159
Mean implants / rats	11.4 ± 2.56	10.69 ± 1.78	10.53 ± 1.99	10.6 ± 1.50
Pre-implantation loss (%)	9.10 (58 – 7)	16.00 (47– 8)	8.70 (23 – 8)	9.10 (43 – 7)
Post-implantation loss (%)	8.00 (27 – 7)	9.10 (62 – 8)	10.00 (23 – 8)	17.00 (40 – 9)

Results expressed as mean ± standard deviation, except pre and post-implantation losses expressed as median and range. [a] $p < 0.05$.

An increase was observed in the absolute and relative weight of the liver among the animals treated with a higher dose of EORO and a reduction in the weight of the ovaries in groups T1 and T2.

Table 2 shows the results obtained from the hematological and biochemical analyses of the control rats and those treated with EORO.

Table 2: Data of the blood count and biochemical analyses in control and EORO treated rats (T1 242mg/Kg; T2 484mg/Kg and T3 968mg/Kg).

	Control	**Treated 1**	**Treated 2**	**Treated 3**
Erithrocyte count	6.6 ± 0.7 *x* 10^6/ µ l	6.6 ± 0.7 x 10^6/ µ l	6.0 ± 0.5 x10^6/ µ l [b]	5.6 ± 0.6 x 10^6/ µ l [b]
Haematocrit	41.80 ± 2.83	40.93 ± 1.33	42.53 ± 3.74	38.19 ± 4.04 [b]
Leukocyt	6310.00 ± 2104.09	5784.61 ± 1203.19	6306.67 ± 1534.58	5825.00 ± 1621.32
Lymphocyte	59.07 ± 8.75	57.00 ± 6.07	54.07 ± 6.77	58.69 ± 12.56
Segmented Neutrophil	34.55 ± 8.29	37.00 ± 5.93	41.33 ± 6.83	36.19 ± 10.46
Hemoglobin	12.75 ± 0.76	12.67 ± 0.68	13.16 ± 0.48	11.86 ± 0.89 [b]
MCV	63.13 ± 5.97	62.13 ± 6.01	70.27 ± 5.43 [b]	67.69 ± 6.93
M Hb C	19.40 ± 1.64	19.33 ± 1.99	22.00 ± 2.17 [b]	21.00 ± 2.00
MCHbC	30.80 ± 1.26	31.07 ± 1.67	31.27 ± 2.94	31.25 ± 1.91
Cholesterol	65.19 ± 8.96	64.95 ± 9.81	74.55 ± 15.20	66.38 ± 10.38
Triglicerides	100.41 ± 30.80	89.39 ± 18.23	79.32 ± 29.02	105.21 ± 56.98
AST	62.53 ± 35.55	58.81 ± 20.15	54.80 ± 12.50	53.50 ± 19.45
ALT	33.67 ± 4.89	38.37 ± 5.89	40.33 ± 15.72	31.00 ± 4.77
Urea	53.53 ± 8.50	43.00 ± 6.78 [b]	52.00 ± 15.11	64.75 ± 10.21 [b]
Creatinine	0.57 ± 0.06	0.57 ± 0.12	0.60 ± 0.10	0.63 ± 0.15

Results expressed as mean ± standard deviation [b] $p < 0.05$. MCV = Mean Corpuscular Volume; MCHb = Mean corpuscular hemoglobin; MCHbC = Mean Corpuscular Hemoglobin Concentration. AST = aminotransferase. ALT = alanin aminotransferase.

The animals treated with 484mg/Kg and 968mg/Kg of EORO presented a reduction in the number of erithrocytes; those with the highest dose were also observed to have reductions in haematocrit and hemoglobin levels. The urea plasmatic concentration was reduced in the treated group with the smallest dose (T1) and it increased with the largest dose (T3).

Table 3 shows the data relating to the development of the pregnancy and the placenta weight.

Table 3: Number of live and malformed fetuses, weights of litter and placentas in control rats and those treated with EORO (T1 242mg/Kg; T2 484mg/Kg and T3 968mg/Kg).

Variables	**Control**	**T1**	**T2**	**T3**
Total of live fetuses	159	164	142	115
Live fetuses / mothers[1]	10.60 ± 2.75	10.25 ± 2.49	9.47 ± 2.67	8.85 ± 1.77
Total of resorptions	10	4	8	11
Body weight (g)[1]	1.79 ± 0.49	1.71 ± 0.47	1.55 ± 0.50	1.36 ± 0.35[2]
Placental weight (g)[1]	1.47 ± 0.35	1.44 ± 0.33	1.30 ± 0.40	1.15 ± 0.33
Total of malformations	0	1	0	4

[1] Results are expressed as mean ± standard deviation
[2] $p < 0.05$.

Live fetuses, resorptions, malformations and placenta weight did not present any significant difference among the groups, but the body weight of animals in group T3 was less ($p < 0.05$) when compared to the control group.

4 DISCUSSION

The maternal toxicant effects are normally detected through clinical observations, such as reductions of more than 10% of the animal's initial body weight (HOOD, 2006). The groups treated with 484 (T2) and 968mg (T3) of EORO/Kg gained less body weight, but none of the animals lost weight during the pregnancy. The reduction in food consumption, particularly in the group that received the highest dose may explain the smaller increase in body weight.

Hepatomegaly was observed among the animals that received the highest dose of EORO and the same group presented anemia, characterized by the reduction in the number of erithrocytes, and the haematocrit value and hemoglobin concentration. Considering that the descriptions in the literature refer to the hepatoprotective effect of rosemary, hepatomegaly might not indicate toxicity (AL-SEREITI.; ABU-AMER; SEN, 1999; FAHIM et al., 1999), a fact that seems to be corroborated, because there are no alterations related to aminotransferase (AST/TGO) and alanin aminotransferase (ALT/TGP). The liver material is being processed for histopathologic analyses to explain the hepatomegaly.

The data observed in mothers suggest that the highest dose of EORO induced anemia. It is known that the antimicrobial activity of essential oils is produced, among other mechanisms, through the sensibilization of the lipid layers of the cellular membrane and alterations in the activity of calcium channels, which increases the permeability of cells and releases intracellular constituents [PORTE; GODOY, 2001]. It could be suggested, speculatively, that EORO produced this effect on the erythrocytes, which would explain the anemia with a reduction in the hemoglobin concentration observed in the group treated with large doses.

The smallest dose of EORO produced low urea concentrations, being followed, later, with the highest dose, by an increase in its plasmatic concentration. This data may be compatible with nefrotoxicity, which needs to be verified through histopathologic analyses.

It is known that some plants extracts can interfere with the preimplantation development of embryos, for instance the aqueous extract of *Ruta graveolens* induced abnormal embryos in mice (DE FREITAS; AUGUSTO; MONTANARI, 2005); *Maytenus ilicifolia* caused embryonic loss before the implantation period (MONTANARI; BEVILACQUA, 2002) and higher dose of hydroalcoholic extract of *Coleus barbatus* is embryotoxic to rats (ALMEIDA; LEMONICA, 2000).

When the embryo development is evaluated, it is verified that the maternal consumption of EORO during the phase of blastocyst implantation does not prevent the implantation and the subsequent development of the embryo from taking place, since the index of post-implantation losses and the average of implants, resorptions and live fetuses per mother were similar among the groups.

Domaracky et al., 2007 (DOMARACKY, 2007) observed reduced growth of mice embryo when the dams were treated with essential oil of *Salvia officinalis* that contains cineole and borneole, chemicals that also are present in EORO. It is not known if these chemical components can inhibit the embryo growth. Another possible cause of embryo growth reduction is the fetal hypoxia caused by maternal anemia.

In conclusion, the highest doses EORO induced anemia in pregnant rats and a reduction in fetuses' body weight, but had no effect on the implantation or in the normal morphogenesis of the embryo.

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