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Chronic Administration of *Rosmarinus officinalis* Essential Oil Decreases Fertility in Female Rats

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ABSTRACT

Introduction: Introduction Most studies consider the useful aspects of herbal medicines, but the side effects that come with their long-term administration are not taken into account. *Rosemarinus officinalis*, an edible evergreen shrub from the Lamiaceae family, has long been used as a medicinal plant for its useful effects, most of which are proved scientifically. In the present study, we studied the effect that the long-term administration of *R. officinalis* essential oil has on ovaries.

Material and Methods: Female rats were divided into 4 groups, and for 21 days were injected intraperitoneally (i.p) with 0.2; 1; and 2% *R. officinalis* essential oil and solvent. Afterwards, the number of primordial, primary, secondary, graffian, and atretic follicles as well as the number of corpora lutea were assessed.

Results: Our results showed that the 2% concentration of *R. officinalis* essential oil decreased the number of graffian follicles and corpora lutea significantly when compared with the control group ($p < 0.05$). In addition, the number of atretic follicles showed significant increment.

Conclusion: This study suggested that the long-term administration of *R. officinalis* in food industries as a spice or preservative in animal and human nutrition should be taken with more caution.

Keywords: *Rosmarinus officinalis*; Ovary; Follicles; Toxicology.

Introduction

In the last years, the administration of herbal medicines has been growing fast. However, it has been shown that some of these plants may also have some adverse effects. For example, some medicinal plants such as *Curcurma longa* and *Salvia officinalis* have antifertility effects in both males and females, so they may have the potential to be used as contraceptives.[1,2]

Rosmarinus officinalis, an edible evergreen shrub from the *Lamiaceae* family, has long been used as a medicinal plant for its useful effects,[3] such as antimicrobial, antiinflammatory, and antioxidant action, which are proved scientifically.[4] It has also been used in the food industry as a spice and as a food preservative to prevent food poisoning.[1] Antiproliferative and anticancer activity of this plant is also reported.[3,5,6] There is a correlation between cell cycle and cancer because cell cycle signal pathways control cell proliferation, and cancer is a disease of inappropriate cell proliferation due to reduction in the sensitivity to signals that regulate cell adherence, differentiation, or die.[7] As in the gonads, the cell proliferation and the number of cell cycles are high, and *R. officinalis* exerts its antiproliferative and anticancer activities via interfering in cell cycle,[8,9] thus, we suspected that this plant might have some adverse effects in the gonads, including testis and/or ovaries. In this regard, there are reports that show this plant can decrease the number of primary and secondary spermatocysts and spermatids in addition to reduction of sperm motility and density in male rats.[10] In female rats, a high incidence of anomalous rat embryos, reduced number of uterine blastocycts,[11] embryo toxic effects in the preimplantation period and also no significant developmental alterations after the preimplantation period have been observed.[12] In addition, this plant can inhibit the uterotropic action of estradiol and estron.[13] Adverse effects of natural or synthetic chemicals on the ovaries can be defined by follicle quantitation and morphometric analysis.[14,15] As the cell cycle in the germinal cells of the ovaries is short, and the antiproliferative effect of this plant is reported, for the first time we evaluated the effects of *R. officinalis* essential oil on a number of different kinds of ovarian follicles. The essential oil used in the present study, according to its manufactured company, was composed of 48.70% 1,8-cineole.

Material and Methods

Animals

Adult female *Wistar* rats weighting between 180 to 220 g were used for this study. The animals were kept in the animal house of the Faculty of Veterinary Medicine at the Shahid Bahonar University of Kerman, Kerman, Iran. The animals were kept in plastic cages covered with wooden chips. They had free access to

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food and water under a standard light/dark cycle. The procedures of this experiment were based on the ethical principles of international committees that protect laboratory animals (Approval No. 20/07/95).

Experimental Design

The pure essential oil of the leaf of *R. officinalis* was purchased from Zardband Pharmaceuticals, Tehran, Iran. The concentrations of the essential oil used in this experiment, according to a previous work13 and a pilot study, were 0.2; 1; and 2%. One group was determined as control and received just the solvent solution. The number of rats in each group was 7. The solutions were prepared daily. All the injections were intraperitoneal and continued for 21 days, between 11:00 AM to 3:00 pm.

Histopatological Procedures

On day 21, the rats were sacrificed, the left ovaries were removed and fixed in 10% buffered formalin solution. Serial sections of 5 µm were prepared and stained with hematoxylin and eosin. In the sections, the ovaries were divided into 12 regions using Dino-Lite digital microscope, and the number of primordial, primary, secondary, antral, graffian, and atretic follicles as well as the number of corpora lutea were assessed.

Morphological Classification of Follicles

For better evaluation of the follicles, we used the Pedersen & Peters classification:[16] follicles were determined as primordial if they contained an oocyte surrounded by a partial or complete layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulosa cells. Follicles were determined secondary if they possessed more than one layer of granulosa cells with no visible antrum. Early antral follicles possessed generally only one or two small areas of follicular fluid (antrum), while graffian follicles possessed a single large antral space (Fig. 1). The earliest sign of atresia was the presence of 5% pyknotic granulosa cells in the largest cross section of the follicles.[17]

Statistical Analysis

The data was presented as mean ± standard error of the mean (SEM). They were analyzed with one-way analysis of variance (ANOVA) and Tukey posthoc test, using the SPSS software version 22 (IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

Results

Quantitative Evaluation of the Ovaries

The effect of *R. officinalis* essential oil on a number of different types of follicles and corpora lutea is summarized in Table 1. Our data showed that the 2% concentration of *R. officinalis* essential oil decreased the number of graffian follicles and corpora lutea significantly when compared with the control group (p < 0.05). In addition, the number of atretic follicles showed significant increment (p < 0.05).

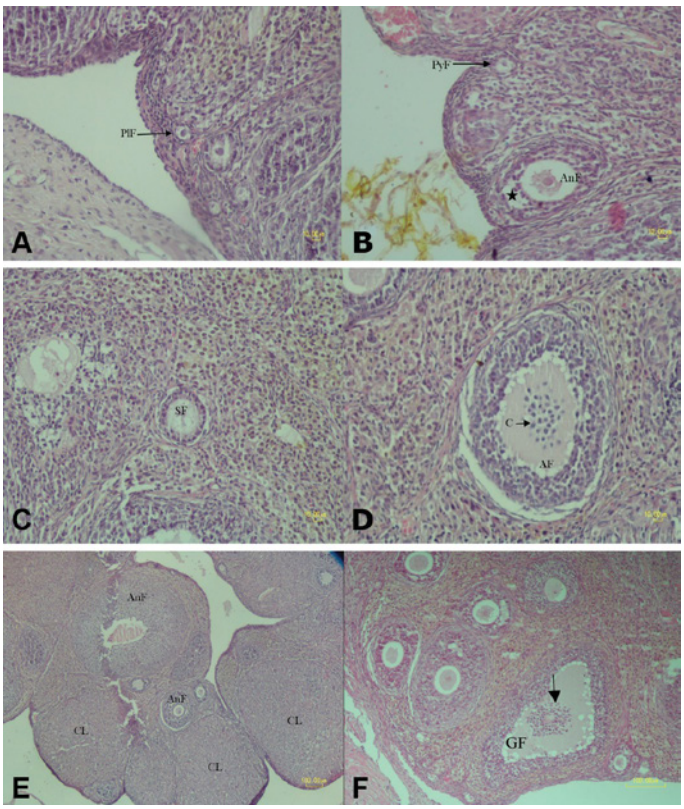

Figure 1. Different types of follicles used for analysis with hematoxylin and eosin (H&E) staining. The numbers show the objective lens magnification. (A) Primordial follicles (PIF): they contained an oocyte surrounded by a layer of squamous granulosa cells (10), (B)Primary follicles (PyF): an oocyte with a single layer of cuboidal granulosa cells, antral follicle (AnF) which possesses a single large antral space, the star shows the antrum (10) (C) Secondary follicle (SE) with a layer of granulosa cells with no visible antrum (20) (D)asampleofatretic follicle (AF), this figure shows the abnormal presence of cells (C) in the antrum (20) (E) corpus luteum (CL) and antral follicle (AnF) (4), (F) Graffian follicle (GF) have a single large antral space, the arrow shows a cumulusoophorus(10).

Table 1. The number of different types of follicles in control and treated groups

	Primordial follicles	Primary follicles	Secondary follicles	Antral follicles	Atresia follicles	Graffian follicles	Corpus luteum
Control	148.37 ± 11.35	107.27 ± 8.45	42.37 ± 2.25	32.21 ± 2.84	11.14 ± 1.25	7.13 ± 1.41	14.8 ± 0.75
0.2%	152.07 ± 12.13	104.32 ± 9.12	45.52 ± 2.84	30.75 ± 2.53	12.7 ± 1.28	6.82 ± 1.12	15.37 ± 0.14
1%	145.84 ± 9.85	112.57 ± 9.47	39.74 ± 3.01	31.08 ± 2.75	11.35 ± 0.97	6.92 ± 1.25	13.4 ± 0.9
2%	140.72 ± 11.22	113.48 ± 8.52	40.85 ± 2.97	21.08 ± 2.31*	14.7 ± 0.68*	2.7 ± 0.42*	8.25 ± 0.13*

The number of different types of follicles in control and treated rats is presented as mean ± standard error of the mean (SEM).
*show a significant effect compared with the control group (p ::: 0.05).

Histopathological Findings

Histologic findings in the ovaries of the animals in the control group contained graffian follicles, developing follicles, newly ruptured follicles with blood clots, corpora lutea and corpora fibrosa. The oocytes were surrounded by epithelial cells. The ovaries contained many primordial follicles, which were mostly found around the edges of the cortex under the germinal layer. Zona pellucida and granulosa cells were formed clearly. Secondary and developing follicles were also all normal with respect to their components, such as antrum, cumulus oophorus, and internal and external theca (Fig. 1). Histopathologic findings in the ovaries of the animals in the treatment group (2%) revealed different degrees of pyknotic granulosa cells (Fig. 2D).

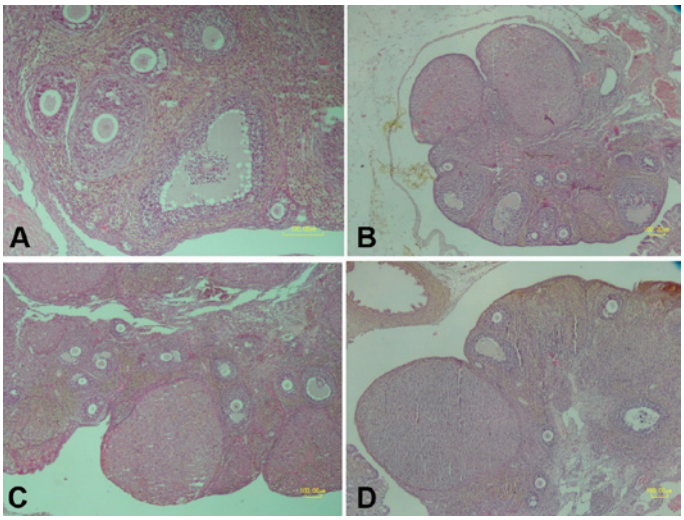

Figure 2. Histopathologic findings in control (A) and treatment groups (B): 0.2%, (C): 1% and (D):2%) with hematoxylin and eosin (H&E) staining and the objective lens magnification: 4). The ovaries in all groups are covered by germinal epithelium. Graffian follicles, devel-oping follicles, corpora lutea and corpora fibrosa, are present in all groups. The Zona pellucida and granulosa, secondary follicles with antrum, cumulus oophorus and internal and external theca are visible in all four groups.

Discussion

The study of follicular numbers can provide important information about the function of ovaries, in particular the relationship between folliculogenesis and the factors that regulate it.[15]

Ovarian toxicity can also be defined by follicle quantitation and morphometric analysis.[18] Our result showed that the long-term administration of *R. officinalis* essential oil increased the number of atretic follicles. Most of the ovarian follicles undergo atresia, a hormonally-controlled apoptotic process. Atresia can occur at any stages of follicle development.[19] There are many factors that prevent or help apoptosis. One of the most important factors are gonadotropins. In addition to triggering the ovulatory process, gonadotropins are required for the growth and development of ovarian follicles. Sufficient exposure of antral follicles to follicle-stimulating hormone (FSH) is the most critical stimulus for the follicles to escape atresia and reach the preovulatory (graffian) follicle stage.[20] Binding of gonadotropins to their membrane receptors in granulosa cells results in the activation of adenylate cyclase, accumulation of cAMP, and, subsequently, activation of the protein kinase, which is a signaling pathway.[21] The apoptosis-suppressing action of gonadotropins is augmented by some local factors, one of which is estrogen that, in turn, prevents apoptosis by activating the nuclear estrogen receptors.[19] Atretic follicles exhibit decreased estrogen production and a lower estrogen/androgen ratio in the follicular fluid, suggesting the importance of local estrogens for the maintenence of healthy follicles.[22] Zhu *et al*, in 1998, showed that the administration of 2% R. *officinalis* extract for 3 weeks, similar to our study, made the liver increase the microsomal metabolism of endogenous estrogens.[13] Our study also showed the reduction in the number of graffian follicles and corpora lutea. Estrogens are necessary for the growth and maturation of follicles. Treatment with estrogen increases the division of granulosa cells and increases ovarian weight. Therefore, the increased number of atretic follicles and decreased number of graffian follicles in this study may be related to the decreased amount of local endogenous estrogen because of its increased metabolism including, the oxidation or glucuronidation[13] by the liver. There are many reports that show that R. officinalis has antiproliferative effects.[8,23] R. *officinalis* interfered with different phases of the cell cycle. In a study by Tai *et al*, in 2012, down regulation in the expression of several antiapoptosis proteins, including Bcl-2, Bcl-x, clAP-1, HIF-α, and HO-1, was observed.[9]

The essential oil of *R. officinalis* is mainly composed of α-pinene, 1,8 cineole and camphor. Approximately 48%of the essential oil used in the present study is constituted of 1,8-cineole, according to its manufacturer's data sheet. Eucalyptol, which is another name for 1,8-cineole, is a monoterpene oxide found in the essential oils of many herbs. This compound also has some pharmacological effects, such as antimicrobial, antiinflammatory,[24] and antioxidant activity.[8] There are many studies that show the antiproliferative activity of this organic component too.[25,26] It is shown that 1,8-cineole inhibits cell proliferation in a dose-dependent manner,[25] and its mechanism of action is via induction of apoptosis.[27] Thus, the effect seen in the present study may be related to this component. In the 2016 study by Caldas *et al*, the chronic administration of 1,8-cineole in female rats caused reproductive toxicity, including reduction in the number of corpora lutea during the organogenesis.[28] This does not happen only in females; there are also some reports that show the toxicity of *R. officinalis* in male rats. In this regard, Nusier *et*

al, in 2007, reported that ingestion of this plant, at levels of 250 and 500 mg/kg body weight for 63 days, caused a significant decline in the number of primary and secondary spermatocytes and spermatids, which is attributed to a significant decrease in testosterone. Sperm motility and density were also significantly decreased in the cauda epididymis and in the testes of rosemary-treated male rats.[29]

Conclusion

In the end, our results show that the long-term administration of *R. officinalis* may have some adverse effects on the ovaries, especially the reduced number of graphing follicles, which may lead to decreased number of offsprings in the next meeting. As the leaves of this plant are commonly used as a spice for humans and as an evergreen plant that may be consumed by domestic animals, expenditures of it should be taken into account more cautiously.

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