Original research

Effects of the alcoholic extract of *Ruta graveolens* on spermatogenesis and sex hormones in immature Balb/C mice

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Received: July 2016; Accepted: November 2016

Abstract: ***Background:*** No special information has been reported about anti-fertility effect of *Ruta graveolens*. The present study aimed to investigate the effects of *Ruta graveolens* alcoholic extract on fertility of male mice and its contraceptive effects.

Methods: 30 immature male Balb/C mice were allocated to three groups of intact control, vehicle, and *Ruta graveolens* treatment that received Ruta extract. A single sub-LD50 300 mg/kg dose of alcoholic extract of the plant was injected intraperitoneally, every day for a week. A month after the last injection, the animals were deeply anesthetized and dissected. Blood was collected intracardially for hormonal assay. The testes were extruded, weighed and then fixed for histological studies.

Results: Administration of 300 mg/kg *Ruta graveolens* showed no significant changes in weight of testis, but induced a significant decrease in number of type A spermatogonia (df: 2, 27; F=6.51; p=0.005) and number of spermatid cells (df: 2, 27; F=4.28; p=0.02) compared to control. Four weeks after injection of *Ruta graveolens* serum, testosterone level (df: 2, 27; F=3.43; p=0.047) significantly decreased compared to control animals. However, there were no significant changes in serum follicle stimulating hormone (df: 2, 27; F=3.34; p=0.051) and luteal hormone (df: 2, 27; F=0.15; p=0.87) levels.

Conclusion: The results indicated that alcoholic extract of *Ruta graveolens* diminishes the activity of male reproductive system by reducing spermatogonia and spermatids, but has no effect on serum level of follicle stimulating hormone and luteal hormone, and might be a useful substance for birth control; however, further studies are suggested.

Keyword: Spermatogenesis; Spermatogenesis; Ruta; Contraceptive Agents; Mice, Inbred BALB C

Cite this article as: Nasirinezhad F, Ahmadi A, Abrishamifar SM. Effects of the alcoholic extract of *Ruta graveolens* on spermatogenesis and sex hormones in immature Balb/C mice. J Med Physiol. 2016; 1(2):78-83.

1. Introduction

Studying the male genital system is important due to its relation with wanted and unwanted pregnancies. In the past 50 years, various hormone and non-hormone compounds have been identified that cause infertility in men (1), but because of their side effects or reduction of libido using these compounds has not been focused on. An ideal anti-androgen compound must be harmless and efficient but should not reduce the libido and moreover, it should be able to prevent unwelcome pregnancies by affecting spermatogenesis (2).

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Nowadays, traditional medicine is spreading and using herbal extracts instead of chemical ones is the centre of attention because they have fewer side effects. Rutin, a glycoside flavonoid compound, is one of the most critical factors of infertility, which seems to play the same role as the estrogen and progesterone in mammals (3). The Rutase family contains more than 1600 species, all of which grow particularly in temperate zones. In this family, *Ruta graveolens* is a static plant with 30-80 cm height and thick and fleshy opaque coloured leaves and yellow male-female flowers, which flowers in May and December (4, 5).

Dictamine, psoralen, arborinine, and graveoline are some of the compounds present in this plant and its alcoholic extract, which prevent platelet aggregation or have anti-tumour effects (6). The *Ruta graveolens* oil contain methyl nonyl ketone, which causes abortion (7).

Furanocoumarins are among the secondary metabolites of this plant and psoralen is its linear type (non-branch), which changes into 8-metoxy psoralen (methoxsalen) due to hydrolysis. Methoxsalen can react with DNA after being activated with the light (8).

The effects and uses of this plant include: anti-implantation and anti-fertilization effects(9), mutagenic effects (10), anti-cancer effects (11), blocking potassium channels in nerve fibres (12), reacting with calmodulin and changing protein kinase C and inhibiting cell growth (11), utilizing homeopathy in curing patients with cancer (13), anti-bacterial effect (14, 15), curing dermal diseases like Vitiligo (16), analgesic and anti-inflammatory effects (17), and healing effects on atherosclerosis (18), hepatic disorders (19), respiratory infections like allergy and asthma (20), and anti-androgenic activity (21). Yet, no special information has been reported about anti-fertility property of *Ruta graveolens*. According to the entries mentioned in the current study, we have endeavoured to investigate the effects of intraperitoneal injection of *Ruta graveolens* alcoholic extract on testicular tissue and serum levels of testosterone, follicle stimulating hormone (FSH) and luteal hormone (LH).

2. Method

2.1. Animal

In this experimental study, 30 immature 4-5 weeks-old Balb/c mice were randomly divided into 3 groups (10 animals per group) of intact control (no treatment), vehicle treated (olive oil without plant extract), and *Ruta graveolens* treated animals (administration of 300 mg/kg of *Ruta graveolens* alcoholic extract). The animals were purchased from the Experimental Study and Research Centre of Iran University of Medical Sciences and kept in standard conditions (light/dark cycles: 12/12 hr per day, temperature: 22–25 ºC, humidity: 40%-50%) and received laboratory mice chow and water ad libitum. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care found in Ethical Committee of Iran University of Medical Sciences. All experimental procedures were consistent with the guidelines regarding research on animals and humans. Special mice food was prepared for the animals, obtained from Pars livestock and Poultry Company, which was available to them along with urban purified water in special drinkers.

2.2. Preparation of the plant extract

Aerial parts of *Ruta graveolens* herb were obtained from Tehran University Garden and were utilized after systematic confirmation. The plant (100 gr) was immersed in 200 ml of distilled water, put on stirrer for 48 hours in room temperature and a dark place, and filtered through a paper filter. Afterwards, the container was placed in a water bath for evaporation. The brown jelly-like extract was kept in -4ºC for further experiments.

2.3. The treatment procedure

After confirming LD50 (600mg/kg), 300 mg/kg of the extract solved in olive oil was intraperitoneally injected in *Ruta graveolens* treatment group every day for one week. Vehicle group received olive oil without plant extract. The control group were kept in the same place, but received no injection. The mice's weight was measured at the beginning and the end of the experiment.

Four weeks after the last injection, the animals were deeply anesthetized via intraperitoneal injection of mixture of ketamine (60 mg/kg; Sigma Aldrich, Germany) and xylazine (8 mg/kg; Sigma Aldrich, Germany). Then bloodletting was done from the left ventricle and the blood serum was kept in the fridge at -20°c after centrifugation (5 minutes, 1500rpm) in order to measure the hormone levels via radioimmunoassay method, employing commercial diagnostic kits (Vidas, BioMetrieux, France) according to the manufacturer's guideline.

2.4. Histological evaluation

Simultaneously, testicles were removed, immediately weighed, and then fixed in 10% formalin for histological evaluations. Sections (5 micrometers thick) were prepared and stained with Ehrlich's haematoxylin and eosin (22). 20 sections were selected from each animal and the number of A and B spermatogonial cells, primary spermatocytes, spermatids, spermatozoids and Leydig cells of seminiferous tubes and the thickness of testis white sheath were investigated using hemocytometer lamel and light microscope. For determining the volumetric density of components in the testicular tissue, based on morphologic studies, the diameter of white sheath was measured and the parietal and free cells were enumerated using the dissector technique. For this purpose, the sections belonging to each group were studied using optical microscope. By putting a graded square behind the optical lens of the microscope, a specific unit was designed for the microscopic fields, then by moving the sample under the microscope, sampling from a field was done in space of every 4 fields.

2.5. Statistical analysis

Data were analysed by SPSS version 16.0 software. Mean and standard error of mean (SE) were calculated. One-way ANOVA and Bonferroni post-hoc were used to assess significant differences between studied groups. $P<0.05$ was considered as significant. Percentage of weight gain was calculated based on the following formula:

$$Percentage\ of\ weight\ gain = \frac{final\ weight}{initial\ weight} \times 100$$

For assessing the effect of administering *Ruta graveolens* extract on testis weight, relative weight of testis was calculated for each mouse based on the following formula:

$$Related\ weight\ of\ testis\ = \frac{testis\ weight}{body\ weight} \times 100$$

3. Result

Percentage of weight gain in control, vehicle, and *Ruta graveolens* treated groups were 144.02±7.67%, 96.91±7.32%, and 92.25±14.79%. Percentage of weight gain in vehicle (p=0.01) and *Ruta graveolens* treated (p=0.005) animals were lower than control group (df: 2, 27; F=7.43; p=0.003). Since the difference between vehicle and *Ruta graveolens* treatment groups was not significant (p>0.99) it seems that administration of olive oil is associated with lower body weight compared to control (vehicle effect). However, relative weight of testis had no noticeable differences between groups (df: 2, 27; F=1.35; p=0.28).

Injection of *Ruta graveolens* caused a significant reduction in the number of type A spermatogonia compared to vehicle (p=0.005) and control (p=0.049) groups (df: 2, 27; F=6.51; p=0.005). However, this treatment had no effect on the number of type B spermatogonia compared to the control (p=0.43). Administration of olive oil (vehicle; p=0.01) significantly increased the number of spermatogonial B cells compared to control (df: 2, 27; F=4.14; p=0.01). In addition, *Ruta graveolens* decreased the number of spermatids (df: 2, 27; F=4.28; p=0.02) compared to control and vehicle animals (p=0.03).

Four weeks after injection of *Ruta graveolens,* serum testosterone level (df: 2, 27; F=3.43; p=0.047) had significantly decreased compared to control animals (p=0.043). However, there were no significant changes in serum FSH (df: 2, 27; F=3.34; p=0.051) and LH (df: 2, 27; F=0.15; p=0.87) levels.

Intraperitoneal administration of *Ruta graveolens* had no effect on the number of primary spermatocytes (df: 2, 27; F=2.38; p=0.11), number of spermatozoa (df: 2, 27; F=0.16; p=0.85), thickness of tunica albuginea (df: 2, 27; F=2.32; p=0.12), number of Sertoli cells (df: 2, 27; F=0.17; p=0.84), number of Leydig cells (df: 2, 27; F=0.43; p=0.66), and diameter of seminiferous tubules (df: 2, 27; F=1.44; p=0.26) (Table 1).

Histological investigation of the testicular sections showed apoptotic cells, cells with picnose nucleus, spermatozoids and a small number of spermatids in the centre of the tube in the animals injected with *Ruta graveolens* extract (Figures 1-3).

4. Discussion

In the current research study, efforts have been made to assess the effect of *Ruta graveolens* alcoholic extract on the male genital system. Sperm reduction, changes in

Table 1: Mean and standard error of fertility variables in studied animals

Time	Control	Vehicle	*Ruta graveolens* treated	p
Percentage of body weight gain	144.02±7.67	96.91±7.32#	92.25±14.79#	0.005*
Relative weight testis (%)	0.28±0.05	0.35±0.01	0.29±0.03	0.28
Number of spermatogonium A	2.9±0.43	3.4±0.4	1.6±0.23#	0.005*
Number of spermatogonium B	18.1±1.93	27.1±2.38##	22.35±1.56#	0.01*
Number of primary spermatocyte	21.9±1.79	22.3±1.72	17.85±1.22	0.11
Number of spermatid	46.3±4.29	43.5±5.07	29.0±4.05#	0.02*
Number of spermatozoa	24.05±3.18	25.80±4.06	23.25±2.38	0.85
Thickness of tunica albuginea (µm)	5.65±0.37	6.55±0.60	7.30±0.62	0.12
Number of Sertoli cells	2.55±0.35	2.30±0.25	2.45±0.30	0.84
Number of Leydig cells	39.70±3.23	37.20±3.63	41.65±3.13	0.66
Diameter of seminiferous tubules (µm)	85.85±3.57	78.95±4.51	78.30±1.85	0.26
Serum FSH level (U/L)	0.15±0.022	0.16±0.02	0.26±0.05	0.051
Serum LH level (U/L)	0.13±0.21	0.23±0.02	0.16±0.04	0.87
Serum testosterone level (ng/ml)	1.016±0.19	0.77±0.05	0.51±0.13#	0.047*

*, significant difference between the studied groups based on one-way ANOVA.
#, significant difference with the control group at level of p<0.05.
##, significant difference with the control group at level of p<0.01.

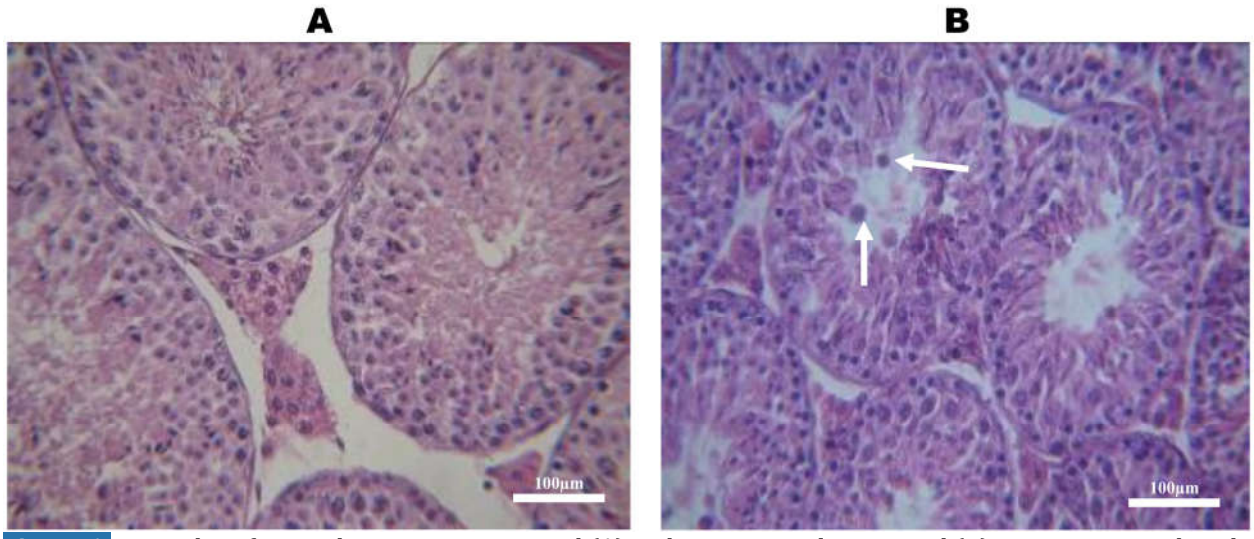

Figure 1: Examples of testicular sections in control (A) and *Ruta graveolens* treated (B) groups picnosed nucleuses (arrows) are obvious at the tube's centre (×400).

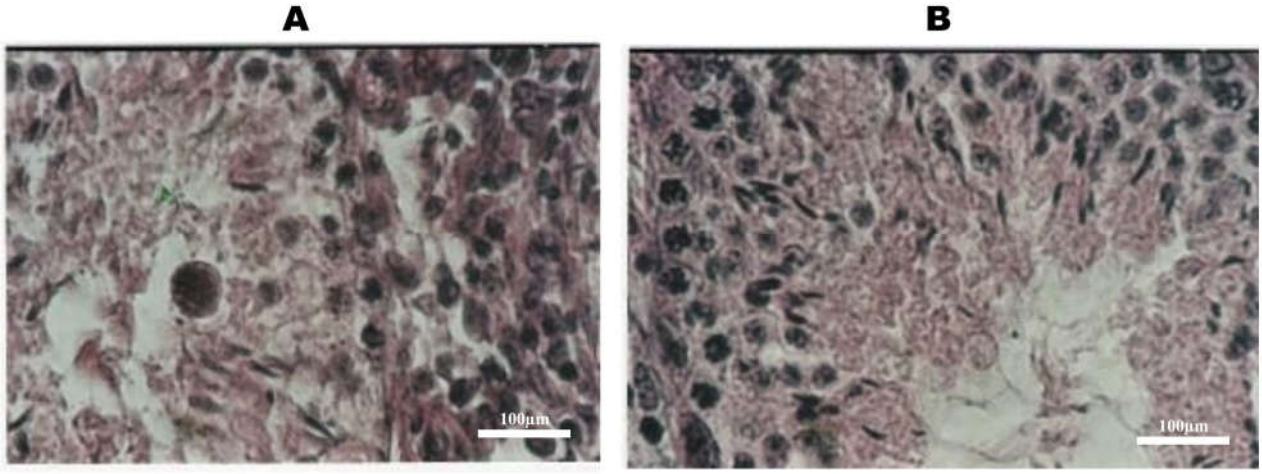

Figure 2: A part of testicular tissue's transversal section in control (A) and *Ruta graveolens* treated group (B) groups. Picnosed nucleus and few spermatids are seen in the *Ruta graveolens* treated group (×400).

sperm production and disorders in androgen production are side effects of this extract.

The reduction in the number of spermatogonia and spermatids in this study indicates that there are factors in the extract, which can interfere with cellular division (23). The alkaloids of the alcoholic extract effects (4), which have anti-tumour effect, might impact the cells in spermatogenesis. These components can form covalent bonds with deoxyribonucleic acid (DNA) after being activated with light and at higher densities, lead to DNA compactness and eventually DNA sedimentation (8, 24). On the other hand, arborinine alkaloid of Ruta leads to inhibition of DNA synthesis (25) as well as sterilization. Dictamine is another Ruta alkaloid that prevents mutagenesis of the compounds by inactivating p450 cytochrome enzyme. It is possible that this feature is because of other compounds' competition with furanocoumarins in the DNA location (8). Flavonoids, acridones and furanocoumarins are alkaloids presenting a wide range of *Ruta graveolens* activities. Gutierrez et al. in 2003 mentioned that these alkaloids, especially flavonoids, lead to inhibition of cell division and cell growth by suppressing tyrosine and protein kinase via making changes in protein kinase C and reacting with calmodulin (11).

It has been mentioned that Ruta extract reduces cell division and prevents increase in the number of cells (26). Quercetin and flavonoids are other compounds of the extract that are non-steroidal anti-inflammatory drugs (NSAIDs) according to an existing report, and lead to increased cell death in cancerous cells in rats' intestines. Quercetin inhibits cyclooxygenase activity. An acceptable hypothesis is that NSAIDs and flavonoids increase

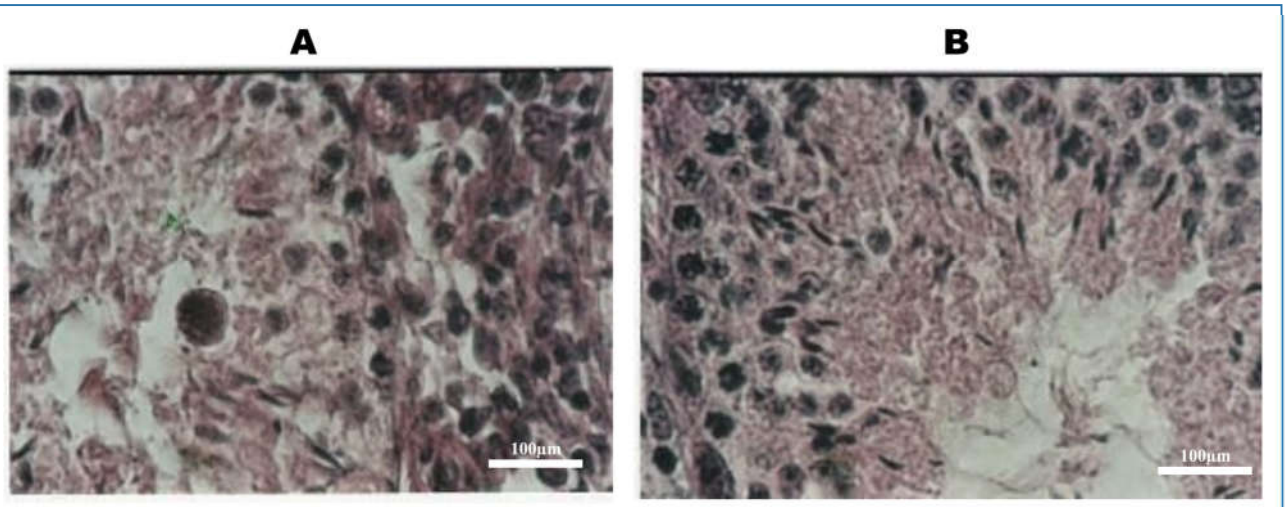

Figure 3: A part of testicular tissue's transversal section in control (A) and *Ruta graveolens* treated group (B). Disorganization in the tube, penetration of the spermatogonial cells into its central part and few spermatids are seen (×400).

the apoptotic process (27). Therefore, according to the above entries, Methoxsalen and psoralen affect the cell division process and DNA replication, and can result in disorders in spermatogenesis and subsequently lead to cellular apoptosis.

Review of Diawaraa et al. in 2001 about the effect of psoralens and methoxsalen (bergapten) on the function of Wistar rats' genital system, has suggested that oral use of these compounds does not affect the weight of seminal vesicle, prostate, epididymis and the hypophysis (25). In the present study, the weight of the testis and the diameter of seminiferous tubes have not changed significantly, which is consistent with Diawaraa et al's (2001) hypothesis.

Sertoli cells, unlike spermatogonial cells, gradually lose the ability of dividing after birth. These cells take part in the differentiation of spermatids by releasing glial neurotropic factors (26). Thus, the decrease in Sertoli cells' quantity in this research leads to their dysfunction. Histologic assays, observing undistinguished spermatids in the central parts of the tube, and reduction of spermatozoids confirm the above-mentioned hypothesis.

Decrease in serum testosterone level and cells involved in spermatogenesis process leads to increase in LH and FSH hormones by negative feedback regulation (28). In the present study, the serum level of LH and FSH did not change. Therefore, it seems that the alcoholic extract does not affect the hypophysis-gonad axis and the process of releasing these hormones from the hypophysis is normal (29).

Finally, the significant reduction of body weight in the animals injected with Ruta extract can be the result of the effects of the herbal extract on the slow body growth process. Since the difference between vehicle and *Ruta graveolens* treatment groups was not significant it seems that administration of olive oil is associated with lower body weight, not Ruta extract.

5. Conclusion:

According to the results obtained from this research, it seems that *Ruta graveolens* extract leads to decrease in spermatogonia and spermatids; however, it does not exert an influence on serum level of LH and FSH. It is suggested to carry out more studies in this field to reveal the exact mechanism of *Ruta graveolens* affecting male fertility.

6. Acknowledgment

Hereby, we thank Ms. Mehrnoosh Yazdanbakhsh for English editing the article.

7. Conflict of interest

None.

8. Funding source

This study was supported by a grant from Iran University of medical sciences. (Grant number: 92-13-20497).

9. Author contribution

All authors passed four criteria for authorship contribution based on recommendations of the International Committee of Medical Journal Editor.

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