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Study of Spermatogenesis in Wistar Adult Rats Administrated to Long Term of *Ruta graveolens*

Mahshid Bazrafkan [1]; Aligholi Sobhani [2,*]

[1]Department of Anatomy, School of Medicine, Alborz University of Medical Sciences, Karaj, IR Iran
[2]Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran

*Corresponding author: Aligholi Sobhani, Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran. Tel: +98-9123266638, E-mail: sobhania@tums.ac.ir

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Background: In Iranian folk medicine *Ruta graveolens* has been used for female and male contraceptive. There are few studies about the effect of this plant on spermatogenesis.
Objectives: In this study the effect of long term administration of aqueous extract of RG on spermatogenesis has been investigated.
Materials and Methods: Animals were allocated into 1) control: which did not receive anything, 2) vehicle which received only normal saline and 3) experiment: which received *Ruta* extract (300 mg/kg administered by gavage once a day for 100 days). A day after last gavage all the individuals were killed by euthanasia. The right testes and epididymis were extruded. The sperm motility was assessed and classified as progressive, no progressive.
Results: There was a significant decrease in the number of spermatogonia ($P < 0.01$), primary spermatocyte ($P < 0.05$) and spermatid ($P < 0.05$) in experimental group as compared to control and vehicle. As shown in Table 3 the sperm count ± SD in 1 gram of epididymis was 2597.5 ± 172.39 in vehicle, 2671.8 ± 38.57 in control groups and 607.4 ± 520.19 in experimental group. Therefore group 3 has a significant lower sperm count in comparison with other groups ($P < 0.05$). Sperm with progressive motility was 55.25 ± 2.81 in vehicle, 53.42±1.82 in control group and 11.16 ± 2.17 in experimental group. Statistical analysis show that rats in experimental group have a significant lower sperm motility in comparison with other groups ($P < 0.05$). There was no difference between other groups ($P > 0.05$).The fertilization capacity of sperm of rats in experimental group was significantly lower than other groups ($P > 0.05$).
Conclusions: It is concluded that the aqueous extract of *Ruta graveolens* diminishes the reproductive system activity and might be a useful substance for birth control process.

Keywords: Male Contraceptive; Sperm; Fertilization

1. Background

The population control is the practice of artificially altering the rate of growth of a human population and nowadays, a major problem in some countries. Health care professionals attempt to achieve this goal by using different and possibly new contraception methods. Most of these methods are related to women and may use one or more of the following practices although there are other methods as well: contraceptive pills (OCPs), hormonal injections (1 and 3-month shots), hormonal implants, intra uterine device (IUD), and tubal ligation, medical abortion, emigration, decreasing immigration. The method (s) chosen can be strongly influenced by the religious and cultural beliefs of community members. Fortunately, along with development in male genital physiology knowledge, this belief that women are responsible in contraception is gradually changing. So there are ongoing researches to finding new contraception methods working on men (1, 2). "The male contraception" is now a subject of interest for research throughout the world and WHO collaborates with countries and international agencies who are dealing with this issue (3, 4). One of the non-surgical and non-hormonal methods in male contraception includes using chemicals extracted from different plants. *Ruta graveolens* (RG) is one of the 565 species from 125 families of medicinal plants used traditionally in various geographical regions that are considered as sources of drugs from which a great number of substances have been discovered (4). This plant is a small evergreen subshrub 2-3 ft (0.6-0.9 m) tall, founded mostly in southern Europe and northern Africa as well as, Peru (Lyma), Brazil, India, and Iran (5, 6). The small rectangular leaves are dissected deeply, and the stem is fully bifurcated. The small yellowish flowers bloom during spring and summer. Flowers arranged as clusters and have 4 petals other than the central flower which has 5. The fruit is capsulated and is covered by round shaped nodules on the surface of capsul (7). *Ruta graveolens* has many proved properties; its flavonids have antimicrobial properties (8) and the antifungal effect is proved (9, 10). The plant also shows anti-inflammatory (11), antihyper-

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tensive effects (12). This plant currently used as a flavoring agent, insect repellent, toothache and earache relief, intestinal vermifuge and as an antidote for toxins such as snake and scorpion venoms (13-17). The RG properties of female contraception and abortion are reported in Brazil, India, Peru, and Mexico. It was reported that, among 86 cases of abortion due to 3 different plants in the period of 1986999 in Uruguay, the most cases were pertained to RG (18). This plant has been traditionally used as an agent for induction abortion and menstruation in many countries (18). In Iranian folk medicine "Sodab" has been used for female and male contraception. Studies about the effect of this plant on spermatogenesis are few (19) Proved that 8-methoxy psuralen (one of the ingredients of Sodab) results in a weight gain in the testicle and epididym (20). Reported that oral administration of aqueous extract of RG with the dose of 500mg/kg for 60 days can decrease the weight of genital organs and sperm motility in rat. They also claimed this extract can change sexual behavior including decrement in mating and sexual functions in male adult rats (21). Showed that the aqueous extract of upper ground parts of RG in immature rats can decrease the activity of genital organs and probably can be used as an agent for contraception (22). Proved that the aqueous extract of RG can immobilize human sperms in vitro. Previous study had shown that injection of aqueous extract (300 mg/kg), for 50 days (equal of one period of spermatogenesis) decreases the number of spermatogonia, primary spermatocytes, spermatids, lydig cells and seminiferous tubule diameter significantly and as same as sperm count and motility (23). As our best knowledge there is not any report about effect of long term administration of aqueous extract of RG on spermatogenesis.

2. Objectives

The study aimed to evaluate the effect of long term administration of the *Ruta graveolens* L. on the count, motility and in vitro fertilization capacity of Wistar rat's sperm.

3. Materials and Methods

This experimental study was performed in the physiology research center of Ahvaz Jundishapur University of Medical Sciences (AJUMS) from April 2009 to August 2010.In this experimental study male Wistar adult rats weighing 200 ± 20g with proved fertility were used. All animals were housed individually per cage under a 12-h light/dark cycle, 20 ± 2 °C temperature and 60-65% humidity-controlled room with food and water ad libitum.

3.1. Preparation of Extracts

The plant was obtained from medicinal plant research institute of jahad-e-daneshgahi, Tehran University of Medical Sciences, Tehran, Iran and was used after systematic confirmation.100 gram of grinded plant (whole parts) was mixed with 1000cc distilled water and heated. The green extract was purified and concentrated by vacuum evaporator. These extracts were stored in the dark, at room temperature, in a desiccator with silica gel for further use.

3.2. Experimental Animals

According to the pilot study, the LD50 was determined 620 mg/kg and subsequently the subLD 50 was determined 310 mg/kg. The rats were randomly divided into 3 groups of 10, as a: Control group; there was no injection. The animals were kept in conditions similar to the other groups, b: Vehicle group; this group received of normal saline every day for 100 days (the same volume as third group according to their weight), c: Experiment group; this group received of aqueous extract of RG (whole plant) every day for 100 days by gavage with the dose of 300 mg/kg. After 100 days the animals were sacrificed by chloroform, and the right testes were removed and kept in Buen fixative. After processing each testis was divided to three parts and then each part was sliced serially and parallel each 5 μm. 10th, 20th, and 30th slices of each part (9 slices for each rat) were then stained by H&E method and observed by means of a light microscope. Spermatogonia, primary spermatocytes, spermatids, and lydig cells were counted in 9 slices, and then the mean of these 9 was calculated for each rat.seminiferous tubule diameter and thickness of tunica albugina were measured in 9 slices (randomly 3 seminiferous tubules in each slice) and then the mean of these 9 was calculated for each rat. Stereo investigator Motic software along with a motic Image plus 2.0 cameras were used for measuring seminiferous tubule diameter and thickness of tunica albugina.

3.3. Sperm Motility Analysis

Sperm motility of three groups was determined using a Makler chamber. All counts were performed at 37°C in T6 media. The sperm motility was assessed and classified as progressive, no progressive. Initial sperm motility was manually assessed by a single individual in duplicate for each sample by evaluating 100 sperms. Total motility was defined as any movement of the sperm head, and progressive motility was defined as the count of those spermatozoa that moved in a forward direction (24).

3.4. Measurements of Sperm Count

Sperms were collected from the right epididymis of each rat by flushing with the same volume (about 8 mL) of T6 medium. Collected samples were centrifuged at 100 g for 2 minutes, and the precipitate portion was resuspended in 10 mL of fresh T6 medium. A fraction of suspension (100 μL) was mixed with an equal volume of 1% Trypan blue in the same medium, and numbers of sperms were counted in four chambers of hemocytomere slide (25). The sperm number was expressed per milliliter of suspension.

3.5. Oocyte Collection

Adult female wistar rats that were between 10 to 12 weeks old were administered intraperitoneally with 10 IU Pregnant Mare Serum Gonadotropine (PMSG) for superovulation; this was followed 46-48 hours later by the intraperitoneal administration of 10 IU Human Chorionic Gonadotropine (HCG). Rats were killed 124 hours after hCG injection by cervical dislocation method. After disinfection with 70% alcohol and opening the abdomen wall, the Y shaped uterus, ovaries and oviducts were identified. The oviducts were excised as follows: clamping cornuas, dissecting the peritoneum and fat between ovary and tube and then cutting the fallopian tube from the proximal end and cumulus-oocyte complexes were collected in T6 medium. The granulosa cells of oocytes were removed by pipetting in T6 medium containing 80IU/mL hyaluronidase and mature oocytes obtained and randomly divided into three groups (26).

3.6. In vitro Fertilization

In vitro fertilization was carried out in drops of T6 medium plus 5 mg/mL BSA under mineral oil. A pre-incubated capacitated sperm suspension of different groups as mentioned above was gently added to the freshly ovulated ova which divided in three groups to give a final motile sperm concentration on 100000 mL. The combined sperm-oocyte suspension was incubated for 4-6 hour. The ova were then washed through several changes of medium and finally incubated in drops of T6 + 5 mg/mL BSA under mineral oil. Fertilization was assessed by recording the number of 2 cell embryos 24-26 hours after completion of fertilization in vitro (27).

3.7. Statistical Analysis

All the reported values except fertilization rate were reported as means ± Standard Deviation (SD). Fertilization rate reported as percentage (%).For comparison of data between two groups, the Student t-test was carried out to detect any significant difference .Differences between two groups were considered to be significant when $P < 0.05$ was achieved.

4. Results

There was significant difference ($P < 0.05$) between experiment group and control/vehicle regarding to the number of spermatogonia, primary spermatocytes, spermatids, seminiferous tubule diameter and thickness of tunica albugina.

A: comparing the number of spermatogonia in different groups, after 100 days of daily RG aqueous extract injection.

The number of spermatogonia (mean ± SD) in vehicle, control, and experiment groups was 21.66 ± 0.87, 22.25 ± 1.22 and 15.73 ± 2.21 respectively. According to Table 1, there was no statistical difference between vehicle and control groups ($P = 0.07$), but statistical differences between vehicle and experiment groups ($P = 0.01$).

B: comparing the number of primary spermatocytes in different groups, after 100 days of daily RG aqueous extract injection.

The number of primary spermatocytes (mean ± SD) in vehicle, control, and experiment groups was 17.78 ± 2.07, 18.49 ± 1.30 and 12.07 ± 1.33 respectively. According to Table 1, there was no statistical difference between vehicle and control groups ($P = 0.08$), but statistical differences between vehicle and experiment groups ($P = 0.02$).

C: comparing the number of spermatids in different groups, after 100 days of daily RG aqueous extract injection.

The number of spermatids (mean ± SD) in vehicle, control, and experiment groups was 140.77 ± 5.87, 139.66 ± 3.60 and 85.33 ± 4.01 respectively. According to Table 1, there was no statistical difference between vehicle and control groups ($P = 0.07$), but statistical differences between vehicle and experiment groups ($P = 0.01$).

A: comparing the number of lydig cells in different groups, after 50 days of daily RG aqueous extract injection.

The number of lydig cells (mean ± SD) of in vehicle, control, and experiment groups was 38.41 ± 1.30, 37.37 ± 1.56 and 39.33 ± 1.75 respectively.

According to Table 2, difference between vehicle and control groups ($P = 0.09$), and vehicle and experiment groups ($P = 0.09$) were not significant.

B: comparing the seminiferous tubule diameter (μm) in different groups, after 100 days of daily RG aqueous extract injection.

The diameter of seminiferous tubule (mean ± SD) (μm) in vehicle, control, and experiment groups was 33.17 ± 1.77, 34.25 ± 2.05 and 22.59 ± 3.13 respectively.

According to Table 2, there was no statistical difference between vehicle and control groups ($P = 0.09$), but statistical differences between vehicle and experiment groups ($P = 0.03$) was significant.

C: comparing the thickness of tunica albugina (μm) in different groups, after 50 days of daily RG aqueous extract injection.

The thickness of tunica albugina (mean ± SD) (μm) in vehicle, control, and experiment groups was 4.30 ± 0.11, 4.73 ± 0.15 and 7.98 ± 0.67 respectively. According to Table 2, there was no statistical difference between vehicle and control groups ($P = 0.08$), but statistical differences between vehicle and experiment groups ($P = 0.04$).

As shown in Table 3 the sperm count ± SD in 1 gram of epididymis was 2597.5 ± 172.39 in vehicle, 2671.8 ± 38.57 in control groups and 607.4 ± 520.19 in experimental group. Therefore experiment group has a significant lower sperm count in comparison with other groups ($P < 0.05$). Sperm with progressive motility was 55.25 ± 2.81 in vehicle, 53.42 ± 1.82 in control group and 11.16 ± 2.17 in experimental group. Statistical analysis shows that rats in experimental group have significant lower sperm

Table 1. Comparing the Number of Spermatogonia, Primary Spermatocytes and Spermatid Cells in Three Groups, After 100 Days of Daily RG Aqueous Extract Administration [a]

Group of Study Variable	Spermatogonia	Primary Spermatocytes	Spermatids
Vehicle	21.66 ± 0.87	17.78 ± 2.07	140.77 ± 5.87
Control	22.25 ± 1.22	18.49 ± 1.30	139.66 ± 3.60
Experiment	15.73 ± 2.21 [b]	12.07 ± 1.33 [b]	85.33 ± 4.01 [b]

[a] Data are presented as mean ± SD.
[b] Statistical Difference to Vehicle Group (P < 0.05).

Table 2. Comparing the Number of lydig Cells, Seminiferous Tubule Diameter and the Thickness of Tunica Albugina in Three Groups, After 40 Days of Daily RG Aqueous Extract Administration [a]

Group of Study Variable	Lydig Cells	Seminiferous Tubule Diameter, μm	The Thickness of Tunica Albugina, μm
Vehicle	38.41 ± 1.30	33.17 ± 1.77	4.30 ± 0.11
Control	37.37 ± 1.56	34.25 ± 2.05	4.73 ± 0.15
Experiment	39.33 ± 1.75	22.59 ± 3.13 [b]	7.98 ± 0.67 [b]

[a] Data are presented as mean ± SD.
[b] Statistical Difference to Vehicle Group (P < 0.05).

Table 3. Sperm Count in 1 Gram of Epididymis, Different Type of Motility and in-Vitro Fertilization Capacity of Sperm in in Three Groups, After 100 Days of Daily RG Aqueous Extract Administration [a]

Group of Study Variable	Sperm Count, 10^6/1g of Epididymis	Progress Motility	Non-Progress Motility	Immotile Sperm	Fertilization Capacity
Vehicle	2597.5 ± 172.39	55.25 ± 2.81	25.20 ± 3.19	19.55 ± 5.61	83.3
Control	2671.8 ± 38.57	53.42 ± 1.82	26.25 ± 2.90	20.33 ± 4.17	79.6
Experiment	607.4 ± 520.19 [b]	11.16 ± 2.17 [b]	54.39 ± 1.66 [b]	34.45 ± 5.23 [b]	3.3 [b]

[a] Data are presented as mean ± SD and precent.
[b] Statistical difference to vehicle group (P < 0.05)

motility in comparison with other groups (P < 0.05). There was no difference between other groups (P > 0.05). The fertilization capacity of sperm of rats in experimental group was significantly lower than other groups (P < 0.05).

5. Discussion

The world outrageous grows of population lead up to the development of various methods to prevent pregnancy. Nowadays the men accept their role to attend in preventing pregnancy methods. Some of these methods are non-hormonal which may pertain to plants. *Ruta graveolens* (RG) is one of these plants. This plant which grows in some countries has been used as a contraceptive agent in ancient medicine in both sexes. In this experiment it was shown that administration of aqueous extract (300 mg/kg), for 100 days decreases the number of spermatogonia, primary spermatocytes, spermatids and seminiferous tubule diameter significantly. It can also result in significant increase in the thickness of tunica albugina. Previously (23) had shown that injection of aqueous extract (300 mg/kg), for 50 days (equal of one period of spermatogenesis) caused decreases in number of spermatogonia, primary spermatocytes, spermatids, lydig cells and seminiferous tubule diameter. Our data in this study is reinforced the previous study of (23). In an experiment the aqueous extract of upper parts of RG was injected (280 mg/kg every day, for 1 week). This resulted in a decrease in spermatogonia A and primary spermatocytes, but not in spermatids, spermatozoids and lydig cells (21). This is different from the current study which uses a similar dose. It seems the reasons are 1) the short period administration of RG extract and 2) obtaining the extract only from upper parts of the plant, not the whole plant. This shows that the material which interferes in spermatogenesis does not exist in the upper parts or is less concentrated in upper parts compared to other parts of the plant. So, in the short period study mentioned above when only the upper parts are used, there is no complete inhibition on normal sperm development and consequently less effect on main cells responsible for spermatogenesis, specially spermatids, spermatozoids, and lydig cells. Albeit, the time period is important item too, and for this reason in this experiment the period of injection was chosen close to the spermatogenesis time period of rat (50 days), to determine the effect of long period administration. As the extract injection could decrease spermatogenesis cell lines, it seems its ingredients can

prevent cell division (especially myosis) (28). Showed that furanocoridons presented in RG (xanthoxin and bergapten) which are classified as alkaloids, induce apoptosis in cancer cells and this can explain the reduction of spermatogenesis cell lines in the current study. It means these cells are affected by apoptotic processes too (28). In two different studies it has been shown that RG contains metoxalen, cuersetin, and flavonoids which can inhibit DNA duplication, cell proliferation and stimulate apoptosis (29, 30). So this extract may also decrease spermatogenesis cell lines via inhibiting DNA duplication and apoptosis consequently. In conclusion regarding to the present study, administration the aqueous extract of *Ruta graveolence* in a period of 100 days brings about structural changes in testicular tissue and decrement in spermatogenesis cell lines. Increment in dosage, injection period and injection frequency, and usage of whole plant-obtained extract can affect more. Another data of this study regard to sperm parameter like sperm count, sperm motility and fertilization capacity. In this study we observed that the administration of *Ruta graveolence* for 100 days promoted a decreased the epididymal sperm count and fertilization capacity. This finding is in agreement with the previous studies (20, 23, 31). It is well known that the function of accessory reproductive organs and also process of spermatogenesis are androgen dependent (32) so the alcohol extract of *Ruta graveolens* may act directly or indirectly on the pituitary gland secretary function and cause decrease in the androgen. In this study we observed that, the progress motility of sperm in treated rats significantly decreased in compare another groups. This manifestation may due to an alteration in the microenvironment in the cauda epididymis, which also had a inhibition action on the metabolism of the treated rats as a result of androgen inhibitory effect of the alcoholic extract of *Ruta graveolens* (20). The reduced fertilization capacity of treated rats is probably due to a decrease in the number and progress motility of sperm. As a conclusion, the *Ruta graveolens* can be suggested as agent against male fertility but the exact mechanism of action is not understood yet so more experimental study shall be done to reveal its effect as a male contraceptive plant.

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Authors' Contributions

Mahshid Bazrafkan carried out part of experiment, concept and design the study; Aligholi Sobhani cooperated in carrying out the experiment, analysis of data and manuscript preparation.

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