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Effect of Aqueous Extract of *Ruta graveolens* on Spermatogenesis of Adult Rats

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Abstract: In this study the effect of aqueous extract of RG on spermatogenesis of rat has been investigated. Animals were allocated into three groups as: (1) control which did not receive anything, (2) vehicle which received only normal saline (the same volume as 3rd group according to the weight) and (3) experiment which received *ruta* extract (300 mg kg^{-1} intraperitoneally once a day for 50 days). A day after last injection the animals were deeply anesthetized and dissected. The right testes were extruded and fixed for histological studies. For statistical analysis ANOVA and Tukey as a post hoc. test were used. There was a significant decrease in the number of spermatogonia ($p<0.01$), primary spermatocyte ($p<0.05$), spermatid ($p<0.05$) and lydig cells ($p<0.01$) in experimental group as compared to control and vehicle. Also there were a significant increase in thickness of tunica albugina ($p<0.01$) and decrease in seminiferous tubule diameter ($p<0.05$) in experimental group compared to control and vehicle. So, 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.

Key words: Spermatogenesis, *Ruta graveolens*, spermatogonia, spermatocyte, spermatid, male contraceptive, rat

INTRODUCTION

The population control is 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 include: Oral Contraceptive Pills (OCPs), hormonal injections (1 and 3-month shots), hormonal implants, Intra Uterine Device (IUD) and tubal ligation (Williams, 1966). 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 (Bone *et al*., 2001). 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 (Waites, 2003; Rahim *et al*., 2009). One of the non-surgical and non-hormonal methods in male contraception includes using chemicals extracted from different plants. *Ruta graveolens* (RG), called Sodab (in Persian), from Rutaceae family is one of these plants. The plant is a small evergreen subshrub 2-3 feet (0.6-0.9 m) tall, founded mostly in southern Europe and northern Africa as well as, Peru (Lyma), Brazil, India and Iran (De-Freitas *et al*., 2005; Gutierrez-Pajares *et al*., 2003). The small rectangular leaves are dissected deeply and the stem is fully bifurcated. The small yellowish flowers bloom during spring and summer (Tabib, 1958). 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 (Zargari, 1990). *Ruta graveolens* has many proved properties; its flavonids have antimicrobial properties (Ojala *et al*., 2000) and the antifungal effect is proved (Oliva *et al*., 2003, Meepagala *et al*., 2005). The plant also shows anti-inflammatory (Raghav *et al*., 2006) and antihypertensive effects (Chiu and Fung, 1997). 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 1986-1999 in Uruguay, the most cases were pertained to RG (Ciganda and Laborde, 2003). This plant has been traditionally used as an agent for induction abortion and menstruation in many countries (Ciganda and Laborde, 2003). In Iranian folk medicine Sodab has been used for female and male contraception. Studies about the effect of this plant on spermatogenesis are few. Diawaraa *et al*. (2001) proved that 8 methoxy psuralen (one of the ingredients of Sodab) results in a weight gain in the testicle and epididym.

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Khouri and El-Akawi (2005) reported that oral administration of aqueous extract of RG with the dose of 500 mg kg^{-1} 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. Ahmadi (2005) 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. Previous studies have shown that the extract can zdecrease sperm count and motility in adult rats . They point out there is a need for further experiments in this field (Sailani and Moeini, 2007; Rahim *et al*., 2010).

Harat *et al*. (2008) proved that the aqueous extract of RG can immobilize human sperms *in vitro*. As there has not been any study on administration of aqueous extract of whole plant on testicular tissue of adult animal, we decided to accomplish this entity in current study.

MATERIALS AND METHODS

This experimental study was performed in the Physiology Research Center of Ahvaz Jundishapur University of Medical Sciences (AJUMS) from March 2009 to May 2010. In this experimental study 30 male Wistar albino adult rats weighing 200±20 g with proved fertility were used. The animals divided to 3 equal groups randomly and kept on standard food pellet (obtained from Pars Company, Iran) and tap water *ad libitum* at animal house in Jundishapur University of Medical Sciences, Ahvaz, Iran. The animal room was on a normal light period and a temperature of 23±2°C. 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. One hundred gram of grinded plant (whole parts) was mixed with 1000 cc distilled water and heated. The green extract was purified, then concentrated by vacuum evaporator and kept in refrigerator. According to the pilot study, the LD_{50} was determined 620 mg kg^{-1} and subsequently the sub LD_{50} was determined 310 mg kg^{-1}. The rats were randomly divided into 3 groups of 10, as below:

- **Control group:** There was no injection. The animals were kept in conditions similar to the other groups
- **Vehicle group:** This group received one injection (i.p.) of normal saline every day for 50 days (the same volume as third group according to their weight)
- **Experiment group:** this group received one injection (i.p.) of aqueous extract of *RG* (whole plant) every day for 50 days with the dose of 300 mg kg^{-1}

After 50 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 and 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 camera were used for measuring seminiferous tubule diameter and thickness of tunica albugina.

Statistical analysis: ANOVA and Tukey as a post hoc. test were used. Differences between the means were considered to be significant when $p<0.05$ was achieved.

RESULTS

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

- **A:** Comparing the number of spermatogonia in different groups, after 50 days of daily RG aqueous extract injection

The number of spermatogonia (Mean±SD) in vehicle, control and experiment groups was 21.88 (±1.87), 23.55 (±2.56) and 10.13 (±3.22), respectively.

According to Table 1, there was no statistical difference between vehicle and control groups ($p = 0.09$), but statistical differences between vehicle and experiment groups ($p = 0.001$).

- **B:** Comparing the number of primary spermatocytes in different groups, after 40 days of daily RG aqueous extract injection

Table 1: Comparing the number (Mean±SD) of spermatogonia, primary spermatocytes and spermatid cells in three groups, after 50 days of daily RG aqueous extract injection

Group of study variable	Spermatogonia	Primary spermatocytes	Spermatids
	(Mean±SD)		
Vehicle	21.88±1.87	17.85±0.91	126.78±5.56
Control	23.55±2.56	18.73±0.69	137.71±5.88
Experiment	10.13±3.22*	12.04±1.53*	75.33±3.71*

*Statistical difference to vehicle group ($p<0.05$)

Table 2: Comparing the number (Mean±SD) of lydig cells, seminiferous tubule diameter and the thickness of *Tunica albugina* in three groups, after 40 days of daily RG aqueous extract injection

Group of study variable	Lydig cells	Seminiferous tubule diameter (μm)	The thickness of tunica albugina (μm)
	----------(Mean±SD)----------		
Vehicle	43.51±1.60	35.12±3.5	4.65±0.66
Control	39.71±1.75	36.16±5.73	5.35±0.44
Experiment	29.87±2.70*	20.30±2.36*	8.03±0.38*

*Statistical difference to vehicle group ($p<0.05$)

The number of primary spermatocytes (Mean±SD) in vehicle, control and experiment groups was 17.85 (±0.91), 18.73 (±0.69) and 12.04 (±1.53), 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 ($p = 0.01$).

- **C:** Comparing the number of spermatids in different groups, after 50 days of daily RG aqueous extract injection

The number of spermatids (Mean±SD) in vehicle, control and experiment groups was 126.78 (±5.56), 137.71 (±5.88) and 75.33 (±3.71), 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 43.51 (±1.60), 39.71 (±1.75) and 29.87 (±2.70), respectively.

According to Table 2, there was no statistical difference between vehicle and control groups ($p = 0.06$), but statistical differences between vehicle and experiment groups ($p = 0.006$).

- **B:** Comparing the seminiferous tubule diameter (μm) in different groups, after 40 days of daily RG aqueous extract injection.

The diameter of seminiferous tubule (Mean±SD) (μm) in vehicle, control and experiment groups was 35.12 (±3.5), 36.16 (±5.73) and 20.30 (±2.36), 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 ($p = 0.001$).

- **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 4.65 (±0.66), 5.35 (±0.44) and 8.03 (±0.38), 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 ($p = 0.001$).

DISCUSSION

In this experiment it was shown that injection of Sodab aqueous extract (300 mg kg^{-1}), for 50 days decreases the number of spermatogonia, primary spermatocytes, spermatids, lydig cells and seminiferous tubule diameter significantly. It can also result in significant increase in the thickness of tunica albugina. In an experiment the aqueous extract of upper parts of RG was injected (280 mg kg^{-1}every day, for 1 week). This resulted in a decrease in spermatogonia A and primary spermatocytes, but not in spermatids, spermatozoids and lydig cells (Ahmadi, 2005). 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). Rethy *et al.* (2007) 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 (Rethy *et al.*, 2007). 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 (Petrunkina *et al.*, 2001; Ramesh and Pugalendi, 2006). So, this extract may also decrease spermatogenesis

cell lines via inhibiting DNA duplication and apoptosis consequently. In conclusion regarding to the present study, injection the aqueous extract of *Ruta graveolence* in a period of 50 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. So, the plant *Ruta graveolence* has a potential to be a male contraceptive agent.

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