Original Article

Acute Effects of *Ruta graveolens L.* on Sperm Parameters and DNA Integrity in Rats

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Abstract

Background: Increase in world population is one of the serious and threatening issues in this century. Therefore, it is vitally important to find safe and effective contraceptive methods, especially for men which already have few choices in this regard. Medicinal plants that were used for contraception in ancient times could be good sources of investigation in this filed. *Ruta graveolens L.* is one the plants introduced in the Iranian traditional medicine as an oral male contraception to be used before intercourse. In this study we tried to investigate the probable effects of the plant on the spermatozoa of male rats.

Methods: *Ruta graveolens L.* aqueous extract (5 *g/kg*) was administered orally to five groups of male rats and sperm motility was checked after half, one, two, four and six hours later. Moreover, one group of rats served as the control group. Subsequently, viability of cells (Eosin-Nigrosin staining), morphological changes (Diff-Quick staining), DNA status (acridine orange dye) and serum testosterone levels were assessed in the treated groups which had significant immotile spermatozoa. For statistical analysis, Student's t-test and one-way ANOVA with Tukey's post-hoc test were employed for comparison between groups.

Results: A significant reduction in sperm motility was seen one hour after administration of the extract in the case groups compared to the controls (36% *vs.* 68.15%, respectively, $p < 0.01$). The motility gradually increased afterwards, and by 6 hours, it was the same as the control group (65.43% and 68.15%, respectively). No significant changes were seen in viability, morphology or DNA structure of spermatozoa in each group. Testosterone levels did not show any significant changes in the treated groups when compared with the controls.

Conclusion: Since a significant temporary immobility of spermatozoa without any adverse effects on other sperm characteristics occurred upon the administration of *Ruta graveolens L.* aqueous extract, it seems that this plant might have the potential to be used for the suggested male contraception.

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Received: Oct. 22, 2011
Accepted: Jan. 16, 2012

Keywords: Male contraception, Rat, *Ruta graveolens L.*, Sperm function assay, Spermatozoa, Iranian traditional medicine.
To cite this article: Halvaei I, Sadeghipour Roodsari HR, Naghibi Harat Zh. Acute Effects of *Ruta graveolens L.* on Sperm Parameters and DNA Integrity in Rats. J Reprod Infertil. 2012;13(1):33-38.

Introduction

Despite current methods of contraception, world population is over seven billion now, and 75 million more people are added to the figure annually. It is estimated that the world population will double by 2050 (1–2).

In many countries about 50 percent of pregnancies are unplanned (3) and delinquency in contraception has lead to abortion in half of the all pregnancies; therefore, safer and more effective contraceptive methods are needed. Male contra-

ceptive techniques account for only 14 percent of all contraceptive methods, and despite presentation of chemical, hormonal, surgical and immuneological methods (4), only two safe choices are practical: using condoms and vasectomy.

Medicinal plants are one of the important sources of new agents in current investigations. According to the world's folk and traditional medicine some plants including Gossypium herbaceum (5), Azadirachta indica (6–7), Tripterygium wilfordii (8), Echiveria gibbiflora (9–10), Allium sativum (11), Carica papaya (12), Alstonia macrophylla (13), Ricinus communis (14), Achyranthes aspera and Stephania hernandifolia (15) have been tested for their male contraceptive effects. In the Iranian Traditional Medicine (ITM), *Ruta graveolens L.* (RGL) is a plant administered for many systemic and local diseases (16). This plant was also prescribed orally for men as a contraceptive agent just before intercourse (17, 18). To our knowledge, this kind of RGL usage is unique to the Iranian Traditional Medicine.

Modern experiments have shown some effects exerted by RGL on male reproductive system and spermatozoa. A two-month oral administration of RGL aqueous extract in male albino rats suppressed Testosterone levels, decreased sexual and aggressive behaviors, reduced sperm motility and count (19). Sperm count reductions due to RGL have been reported in other studies (19–20). Naghibi et al., previously reported the immediate immobilizing effect of RGL aqueous extract on human spermatozoa in vitro conditions (21). The same effects have also been reported by other members of the family *Rutacea* (21–23). Considering the consumption of RGL tea immediately before intercourse as mentioned in ITM and modern findings about its effect on sperm parameters, we designed this study to find out the immediate effects of RGL on rat spermatozoa.

There seemsed to be no other study indicating the effects of RGL on both sperm parameters and DNA integrity of spermatozoa in the literature.

Methods

Plant: Dried RGL herb was purchased from Zard-Band Herbal Medicine Factory. The plant was cultured in botanic farm of the factory in Zard-band village, north of Tehran, with these geometrical characteristics: Latitude 35 ° 47 north, longitude 51 ° 37 east, altitude 1548 *m* from sea. In the last 10 years, the maximum temperature has been 39 ℃, and the minimum -6.8 ℃ and the average humidity has been 489.2 *mm* in this area. The plant was harvested before the seeds were fully developed (June 2009) and dried in dark room with free ventilation. The plant was approved in the herbarium of Pharmacognosy Department of the Faculty of Pharmacy affiliated to Shahid Beheshti University of Medical Sciences by a Botanist with herbarium number 066.

Preparation of the plant extract: *Ruta graveolens L.* herb (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 into a water bath for evaporation. The brown jelly extract was kept in -4 ℃ for further experiments.

Experimental animals: Adult Wistar male rats (42) were divided into six groups (n=7), the first group serving as the control group. *Ruta graveolens L.* aqueous extract (5 *g/kg*) was administered orally to all test groups and sperm motility was checked after half, one, two, four and six hours (groups 2 to 6). The rats were kept in standard conditions (light/dark cycles: 12/12 *hr* per day, temperature: 22–25 ℃, humidity: 40%–50%) and received laboratory rat chow and water ad libitum. All experimental procedures were consistent with the guidelines of Ethical Committee of Tehran University of Medical Sciences about research on animals and humans.

Effect on sperm motility: According to Opdyke (24), the RGL lethal dose for rats is more than 5 *g/kg*. Hence, we considered this dose as the maximum safe dose and fed it to rats to evaluate if the recommended safe dose had any contraceptive effects. Each group received 5 *g/kg* RGL aqueous extract orally by intra-gastric gavage and were checked for sperm parameters after half, one, two, four, six hours. There control group received only the vehicle (distilled water). To determine sperm count and motility, one centimeter of cauda epididymis was resected and minced in 1 *ml* of 37 ℃ normal saline solution. Ten microliter (μl) of a mixed sample was placed on a pre-warmed slide and a minimum of 10 fields were viewed and 200 spermatozoa were assessed by high power microscopy (400× magnifications). Motility was assessed according to the WHO classification system (25). Briefly, the criteria were as follows: grade a: rapidly progressive spermatozoa, grade b: slowly progressive spermatozoa, grade c: no pro-

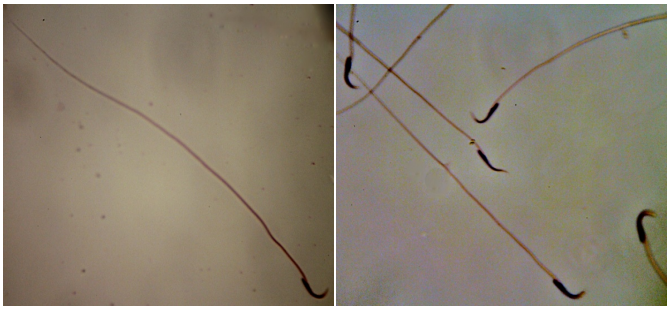

Figure 1. Morphology of rat spermatozoa (1000× magnification)

gressive motility, grade d: immotile spermatozoa. The group which showed the highest significant level compared to the control animals in this test was selected for more evaluations.

Assessment of sperm viability: Sperm viability was asses, using eosin-nigrosin staining test (25). Briefly, 25 *µl* of dye and the same amount of sample were mixed for 30 seconds. Then a smear was provided by a 15 *µl* droplet of the mixture. After drying the smear in room temperature, the slides were examined under a light microscope (1000× magnification). Live spermatozoa were unstained (white) but dead cells were stained red. After counting at least 200 sperm cells, the percentage of dead and alive spermatozoa was calculated.

Assessment of sperm morphology: Morphology assessment was done by Diff-Quick staining method (25). After mincing one centimeter of cauda epididymis in 1 *ml* of 37 ℃ normal saline solution, one drop was placed on microscope slide to prepare a smear and fixed for 15 seconds in fixative solution (1.8 *mg/l* triarylmethane in methyl alcohol) and stained in solution No. 1 (1 *g/l* xanthene in sodium azide-preserved buffer) for 10 seconds and then in solution No. 2 (1.25 *g*/l thiazine dye mixture, 0.625 *g/l* Azure A and 0.625 *g/l* methylene blue in buffer) for 5 seconds and then was dried in room temperature. After mounting the slides, they were seen by a light microscope (1000×magnification) (Figure 1). The slides were assessed for any abnormality in tail, neck or head.

Evaluation of DNA integrity: The spermatozoa of treated rats and the control group were stained by acridine orange dye according to the modified method of Tejada et al. (26). Briefly, a smear was prepared from a 15 *µl* sample and then fixed in a 1:1 (*v/v*) ethanol–acetone solution for 30 *min* at 4 ℃. After drying at room temperature, the slides were placed in a dark jar containing acridine orange dye for 5 *min* in the same temperature. After washing the slides with distilled water, they were immediately viewed and 200 cells were counted in each slide under 1000-fold magnification using a florescent microscope in 470 *nm* florescent beams. Red to yellow-stained cells indicated on a denatured chromatin with single-stranded DNA were considered abnormal while the green ones on an intact chromatin with double-stranded DNA were considered as normal spermatozoa DNA fragmentation index was calculated by dividing the abnormal to the total (normal and abnormal) spermatozoa and reported as percentage.

Assessment of testosterone level: Blood testosterone level was assessed in the controls and the 1 hour test groups. Blood sample was taken directly from heart 1 hour after gavage, under anesthesia. Hormonal analysis was done by ELIZA method using LIAISON® testosterone (310410) assay kit (DiaSorin Inc., USA).

Statistical analysis: One-way ANOVA and Tukey's post-hoc tests were employed for compareson between groups. Student's t-test was applied for statistical comparisons between two groups. The significance level (p-value) was considered smaller than 0.05.

Results

Effects on sperm motility: Thirty minutes after oral gavage of RGL extract in male rats, motility of the spermatozoa was reduced and one hour after gavage the percentile of immotile sperm increased significantly compared to the controls (64% and 31.85%, respectively; p <0.01). The motility gradually increased afterwards, and by 6 hours, it was the same as controls (65.43% and 68.15%, respectively). Thereafter, the immobilizing effect

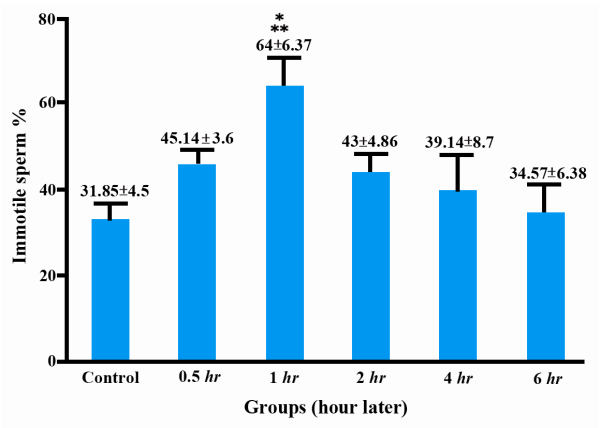

Figure 2. Sperm immobility percentile after intra-gastric gavage Ruta graveolens L. aqueous extract in different intervals. Data are shown as means±SEM, *p <0.05 compared to the 6-hour group, **p <0.01 compared to the controls

Table 1. Comparison of sperm parameters and DNA fragmentation index (DFI) in control and one hour later groups

	Control	RGL	P-value
Immotile spermatozoa %	31.85±4.5	64±6.37	< 0.01
Viability (alive) %	74.28±2.72	65.85±3.8	NS
Morphology (normal) %	66.28±3.18	72.71±4.26	NS
DFI %	10.42±1.55	12±1.39	NS

Data are shown as means±SEM, NS: not significant, RGL: *Ruta graveolens L.*

was reversed and no group was different from the control group (Figure 2). The group which showed the most significant difference in motility was called the "one-hour group" and was used for subsequent assessments. Regarding the progressive and non-progressive motility, although the data showed a trend to decrease both parameters after administration of RGL till one hour and a trend to increase both parameters 1–6 hour after the intervention, but the differences were statistically insignificant for both parameters between different groups (For progressive motility, 48.1±4.3, 25±6.4, 36.3±6.5 and for non-progressive motility, 20.05±4.6, 11±6.5 and 19.13±6.5 for control, one-hour and six-hour groups, respectively).

Effects on sperm viability: Although a reduction about 9% in viability of spermatozoa was seen one hour after RGL gavage compared to the control group, but it was no statistically significant (65.85±3.8 and 74.28±2.72, respectively) (Table 1).

Effects on sperm morphology: Diff-Quick staining revealed no significant differences in normal morphology of spermatozoa in the one-hour and control groups (72.71%±4.26 and 66.28%±3.18, respectively) (Table 1).

Effect on testosterone level: Measurement of blood testosterone level showed that RGL at the dose of 5 *g/kg* had no adverse effects on serum testosterone levels in the treated rats and the reduction was not statistically significant compared to the control group (475±80 and 507.5±79 *ng/ml*, respectively).

Effect on sperm DNA integrity: Acridine orange staining technique did not show any significant difference for abnormal DNA status between RGL treated (one-hour) and control groups (12±1.39 and 10.42±1.55, respectively) (Table 1).

Discussion

In this study, the acute in vivo effect of *Ruta graveolens L.* aqueous extract was evaluated in male rats. With a single highest safe dose of RGL reported in rats to be 5 *gr/kg*, motility of sperm decreased significantly one hour after oral feeding and no changes in morphology, viability, DNA integrity or testosterone levels were seen. The immobilization effect was transient and after 6 hours it was significantly different from the first-hour group, which indicates the reversal of RGL effect.

According to Khouri and El-Akawi, when *Ruta aqueous* extract was fed orally to male albino rats at a dose of 500 *mg/kg* body weight for 60 days a significant decrease in the weight of reproductive organs, cauda epididymis and testicular ducts and a reduction in sperm motility and density were noticed. In addition, spermatogenesis in seminiferous tubules and number of spermatocytes, spermatids and testicular cell populations were diminished. Serum hormonal assay indicated a decrease in testosterone and FSH levels in treated rats (19). Compared to our study, they used much smaller amounts of the extract per dose, although for a longer time, but reported motility reduction as we did. They suggested that all these effects were due to the extract's effect on hypothalamic-gonadal axis and FSH reduction. Since in our study, the acute effect of the extract was examined, the changes in organ weight, sperm density or histopathology did not happen. Hence, RGL's immediate sperm motility reduction could have other reason(s).

In two other studies, daily intra-peritoneal injections of ethanolic or aqueous RGL extract at different doses for 20 days and one week, caused reductions in sperm motility but no exact mechanisms were mentioned (20, 27). Another study by Rahim et al. indicated a reduction in sperm motility after chronic administration of alcoholic extract of RGL (28). The authors proposed that due to the androgenic suppressive effects of the alcoholic extract of RGL, microenvironment changes cauda epididymis might had occurred leading to the inhibitory effects on metabolic pathways. In the current study, since no changes in androgen levels were detected, the mechanism for reduction of motility does not seem to be androgen dependent.

Immediate immobilizing effect of 100 *mg/ml* RGL aqueous extract on human sperm in vitro was also reported previously (21). Likewise, no changes in sperm viability, morphology changes in DNA integrity of human spermatozoa were reported (21).

The immediate immobilizing effect of RGL on human sperm in vitro suggests some ionic current

blockade induced by RGL extract. As Harat et al. reviewed (21), effects of *Ruta graveolens L.* on some inonic channels is one probable mechanism. We hypothesized that this acute effect could be due to ionic channel blockage (*e.g.* K channels) of reservoir spermatozoa in epididymis and vas deferens. Although further studies in this era is necessary for better conclusion. Other members of *Rutacea* family have the same immediate effects on spermatozoa. Immobilization of frog sperm by *Ruta chalepensis L.* (23) and human sperm by citrus lemon have also been reported previously (22). A part of these immediate in vitro effects could be explained by acidic pH.

According to the Iranian traditional medicine, consuming a decoction of RGL (about one cup) before intercourse, it could act as a contraceptive (18).

As we found acute immobilization of sperm does not have a detrimental effect on sperm viability and recovery, zona penetration assay or mating test is suggested to evaluate the fertilizing potential of sperm cells after recovery from immobility.

Conclusion

Considering all the reported findings and the reversible immobilizing effect of the extract on sperm observed in the current study, it seems that RGL has some immediate effects on spermatozoa motility with an unknown mechanism, which should be further investigated to find an acceptable reason for a possible new and safe male contraceptive agent.

Acknowledgement

The authors highly appreciate Dr. Bijan Naghibi Harat's great help in editing the article. Also we thank Dr. Alireza Talebi for reading and considering the manuscript. The authors also appreciated the under takings of Mr. Mohammad Kamalinejad, a botanist, at the herbarium of Shahid Beheshti University of Medical Sciences.

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