Effect of Neem oil on the structure and function of the mature female albino rat ovaries

Efeitos do óleo de Nim (Neem) na estrutura e função de ovários de ratas albinas adultas

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ABSTRACT

Objective: The study was undertaken to observe the effect of Neem oil treatment on the fertility, microscopic structure of ovary and the associated changes in the serum levels of female reproductive hormones in mature female albino rats. Methods: The animals were divided in different groups as A1 = treated females at low dose (0.6 ml of Neem oil/animal), A2 = treated females at high dose (1.2 ml of Neem oil/animal), A3 = controls for group A1 (corresponding dose of peanut oil) and A4 = controls for A2 (corresponding dose of peanut oil). Animals were kept under observation for a period of six weeks. At the end of this period animals were anesthetized, blood was removed by cardiac puncture and sacrificed. Ovaries were removed and fixed in 10% formol saline for microscopy and methanol for high-performance liquid chromatography purpose. Results: Microscopic sections of the ovaries have revealed decrease in the number of mature ovarian follicles. Significant changes in the levels of associated reproductive hormones and presence of higher concentrations of active Neem components in the gonads amongst the treated female rats have also been shown in this study. Conclusions: From these findings it can be concluded that Neem oil has a dose depended anti-fertility potential in the female albino rats.

Keywords: Azadirachta indica/therapeutic use; Ovary; Fertility/drug effects; Ovarian follicle/growth & development; Rats

INTRODUCTION

Neem, *Azadirachta indica A. Juss* is widely prevalent and highly esteemed wonder tree of Indian subcontinent(1), and belongs to the family Meliaceae. *Azadirachta indica*, commonly known as Neem, is found in tropical and subtropical areas of the world. The leaves and oil have antifertility effect, while seed oil (40%) is insecticidal. The gum discharged by the stem act as stimulant. The tree is regarded as “Village Dispensary” in India as reported in a book called “Neem – a tree for solving global problems” (US National Academy of Sciences)(2). Naqvi(3) has reviewed the pharmacological importance of *Azadirachta indica*, while Biswas et al.(4) have reviewed biological activities of Neem. More than 135 compounds

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have been isolated from different parts of Neem leaf extract protects liver from damage, and it has been used in treating diabetes. Neem leaf extract significantly inhibits viral replication and induces in vitro production IL-1 interferon. It acts as a first line of defense against HIV infection. It has antifertility and immuno stimulating and immuno modulating potential.

In this study, it was evaluated the fertility effect of Neem oil treatment on the microscopic structure of the ovaries, its effect on serum concentration of female reproductive hormones and the quantitative analysis of the active Neem components in ovarian tissue by using high-performance liquid chromatography (HPLC) method.

METHODS

The present study included 48 mature female albino rats of weight between 150 to 200 g. The animals were obtained from the Animal House of Baqai Medical University in Karachi. The strain of albino rats was obtained from Jinnah Post-graduate Medical Institute Karachi where it was originally obtained from Charles River Laboratory in Brooklyn, Massachusetts, USA and was crossbred. The animals were kept in an experimental room for one week prior to the commencement of study, for acclimatization to experimental conditions with 12 hours light and dark cycle. The animals were fed at laboratory chow and water ad libitum (Chart 1).

Twelve animals were administered with low dose, 0.6 ml/animal of Neem oil and were assigned as group A1. Another 12 animals were administered with high dose, 1.2 ml/animal of Neem oil and were assigned as group A2. The corresponding number of animals was administered with the corresponding dose of peanut oil and were assigned as group A3 and A4. They acted as Control Groups for A1 and A2, respectively. All animals were administered with single oral dose. The animals were kept under observation for a period of six weeks.

At the end of the experiment, the animals were anesthetized by deep ether anesthesia, blood was removed from the heart by cardiac puncture, for hormonal estimation and then, they were sacrificed. One of the ovaries from each animal was removed and fixed in 10% formal saline, for microscopy, and the other ovary was removed and placed in methanol and water, 1:1 composition for HPLC purpose.

MICROSCOOPY

Ovaries which were fixed in formal saline blocked in paraffin wax cut horizontally at 5 µ thickness and stained with hemotoxyline and eosin. Stained horizontal sections were observed under light microscope for microscopic changes.

HORMONAL ANALYSIS

Blood removed from the animals by intracardiac method was centrifuged at 2,000 rpm (revolution per minute) to separate the serum for the measurements of FSH, LH, progesterone and estradiol.

The quantitative determination of hormones was done by using enzyme-linked-immunosorbsent assay (ELISA). The kits used for this purpose were provided as follows:
- for progesterone and luteinizing hormone: By Equipar Srl via G.Ferrari 21 /N-21047. Saronno (va), Italy.
- for estradiol: By Biocheck, Inc, 323 vintage park Dr.Forster City, CA 94404.
- for FSH: By Pishtazteb Diagnostics – No. 1,855, 13th Alley, Simaye Iran.

QUANTITATIVE ANALYSIS OF NEEM COMPOUNDS BY HPLC METHOD

Cleaning procedure of samples for HPLC

For determination of residual components of Neem oil, the ovaries were grounded in Teflon Pyrex tissue at 500 rpm, by speed control homogenizer. Then, the Neem oil components were subjected to the following procedures:

a) Extraction of Neem oil

Neem oil may bind with fat present in tissues. Therefore, extraction by using Rotavapor was necessary.

**Soxhlation method:** for the extraction of Neem compounds from samples, Holden and Marsden method was used. A known quantity of samples (1 g sample) was macerated with Na₂SO₄ (anhydrous sodium sulphate) and transferred into a thimble made of filter paper. The thimble was then placed in the extractor which was fitted to the bolt head flask containing 170 ml of n-hexane, which was then fitted with condenser connected to the tap...
water for cooling. The apparatus was then placed on a water bath. The process of extraction was carried out for three hours during which all the fat contents were extracted with the solvent. Better recoveries were noticed by this method. The fat extracted solvent later on was reduced to about 1 ml in rotavapor. For complete recovery of Neem compounds, the column chromatography (Sorption) was employed and the material was passed three to four times through the columns of alumina(21) and silica(21).

b) Quantitative analysis of Neem compounds by HPLC method

HPLC has been used for the separation of compounds by using a packed column (Zorbax™ NH2), a polar bound phase with particle size of 7 µm in diameter. The columns were packed to uniform bed density by using a high pressure slurry loading techniques. This column was used with fractionated n-hexane as a mobile phase with a flow rate of 0.5 ml/minute. An ultraviolet detector was used at a wavelength of 250 nm, pressure 200 kg/cm² and absorbance 0.32 with chart speed 2.5 mm/min, for the detection of peaks of Neem compounds. The purified samples of different concentrations were made and 10 µL of the samples were injected by special chromatographic syringe, in the HPLC apparatus, attached with a chart recorder on the basis of retention time (RT) with the standards peaks. The area of each peak was calculated to quantify the detection of different compound residues in the samples.

Statistical analysis of results

In the present study the data were subjected to the t Student test for statistical analysis.

RESULTS

Female reproductive hormones

At low doses

- Follicle stimulating hormone
  Mean serum follicle stimulating hormone (FSH) concentration in orally treated female rats at low dose was 64.11 ± 1.37 (Iu/l), while the FSH concentration in untreated/control rats was 40.36 ± 4.45 (Iu/l). The FSH level in treated females at low dose are 58% higher and statistically highly significant (p < 0.001) when compared to control females of this group.

- Leutinizing hormone
  Mean serum leutinizing hormone (LH) concentration in orally treated female rats at low dose was 42.88 ± 1.62 (Iu/l), while the LH concentration in untreated/control rats was 43.90 ± 1.02 (Iu/l). The LH level in treated females at low dose are 7% lower and statistically non-significant (p < 0.05) when compared to control females of this group.

- Estradiol
  Mean serum estradiol concentration in orally treated female rats at low dose was 24.89 ± 4.33 (pg/ml), while the estradiol concentration in control rats was 39.03 ± 2.24 (pg/ml). The estradiol level in treated females at low dose are 59% lower and statistically significant (p < 0.05) when compared to control females of this group.

- Progesterone
  Mean serum progesterone concentration in orally treated female rats was 29.92 ± 3.73 (ng/ml), while the progesterone concentration in control rats was 37.64 ± 1.02 (ng/ml). The progesterone level in treated females at low dose are 51% lower and statistically significant (p < 0.05) when compared to control females of this group.

At high doses

- Follicle stimulating hormone
  Mean serum follicle stimulating hormone (FSH) concentration in orally treated female rats at high dose was 53.81 ± 1.06 (Iu/l), while the FSH concentration in control rats was 37.20 ± 1.46 (Iu/l). The FSH level in treated females at high dose is about 44.81% higher and statistically highly significant (p < 0.001) when compared to control females of this group.

- Leutinizing hormone
  Mean serum leutinizing hormone (LH) concentration in orally treated female rats at high dose was 28.37 ± 1.48 (Iu/l), while the LH concentration in control rats was 17.25 ± 1.56 (Iu/l). The LH level in treated females at high dose are 64% higher and statistically highly significant (p < 0.001) when compared to control females of this group.

- Estradiol
  Mean serum estradiol concentration in orally treated female rats at high dose was 37.99 ± 1.26 (pg/ml), while the estradiol concentration in control rats was 55.64 ± 1.79 (pg/ml). The estradiol level in treated females at high dose is about 31.77% lesser and statistically highly significant (p < 0.001) when compared to untreated females of this group.
• Progesterone
  Mean serum progesterone concentration in orally treated female rats at high dose was 20.95 ± 3.48 (ng/ml), while the progesterone concentration in control rats was 33.63 ± 2.68 (ng/ml). The progesterone level in treated females at high dose is about 37.70% lesser and statistically highly significant (p < 0.001) when compared to control females of this group.

Number of follicles/section of the female ovaries

At low dose
• Primary follicles
  The mean number of primary follicles at low dose treated animals was 5.5 ± 0.43, while the number of primary follicles in control animals was 8.08 ± 0.33 when studied under the light microscope.

• Secondary follicles
  The mean number of secondary follicles was 3.41 ± 0.28 at low dose treated animals while the number of secondary follicles in control animals was 5.41 ± 0.39 when studied under the light microscope.

• Mature follicles
  The mean number of mature follicles at low dose treated animals was 1.58 ± 0.14 while the number of mature follicles in control animals was 3.91 ± 0.357 when studied under the light microscope. This difference in the number of mature follicles is statistically highly significant (p < 0.001).

At high dose
• Primary follicles
  The mean number of primary follicles at high dose treated animals was 3.5 ± 0.99, while the number of primary follicles in control animals was 8.08 ± 0.33 when studied under the light microscope.

• Secondary follicles
  The mean number of secondary follicles was 2.41 ± 0.66 at high dose treated animals while the number of secondary follicles in control animals was 5.41 ± 0.39 when studied under the light microscope.

• Mature follicles
  The mean number of mature follicles at high dose treated animals was 0.00 ± 0.00, while the number of mature follicles in control animals was 3.91 ± 0.357 when studied under the light microscope. This difference in the number of mature follicles is statistically significant (p < 0.05).

EFFECTS ON HISTOLOGY OF THE FEMALE OVARY

The histological features of the ovaries of control rats presented with normal features as evidenced by the presence of all types of follicles (Figure 1), normal vascularity, compact stroma and intact germinal epithelium. High dose treatment has severely affected the ovarian structure, large number of developing follicle, as well as mature follicle have undergone atresia since the histological sections does not show any mature follicle (Figure 2). In some of the developing follicles only the fluid is present and possibly the maturing ovum has been degenerated (Figure 3). The cortical stroma still seems to be compact with intact germinal epithelium. Primary and secondary follicles are shown to be decreased in number and no mature follicle has been shown by any of the animals treated at high doses (Figure 2).
At the low dose treatment, however, the histological features are not as much affected as by the high dose treatment. Ovarian sections as this dose have shown all types of follicle including primary, secondary and mature (Figure 4), though the number of these follicles is less than the numbers shown in the histological sections of control rats (Figure 1). The vascularity and stromal organization and germinal epithelium at this dose show to be not affected at all, since the sections do not show any changes.

### HPLC

HPLC was done at the end of study by removing the ovaries. HPLC chromatographic recordings have shown consistent peaks for two compounds present in Neem oil (Figures 5, 6, 7). The compounds are Azadirachtin and Azadirachitin. Standard peaks recorded for these compounds were observed at the following concentrations:

- azadirachtin peak recorded at 7 ± 0.6 min. with 1.99 µg /10 µL concentration per sample;
- azadirachitin peak recorded at 9 ± 0.7 min with 8.57 µg/ 10 µL concentration per sample.
At low dose treatment

- Peak for Azadirachtin: 0.8 µg / 10 µL concentration per sample.
- Peak for Azadirachtinin: 2.2 µg / 10 µL concentration per sample.

At high dose treatment

- Peak for Azadirachtin - 15.3 µg / 10 µL concentration per sample.
- Peak for Azadirachtinin - 1.1 µg / 10 µL concentration per sample.

DISCUSSION

FSH is higher in the treated female rats at both doses while the other female reproductive hormones including, LH, estradiol and progesterone levels are lower in treated rats as compared to control rats. About 58 and 44% rise in the level of FSH; in the treated female rats at low and high dose group reflects the fact that the Neem oil has interfered with maturation and growth of follicles in the ovary which has also been shown by the microscopic sections of the ovary in which the number of follicles decrease as they step towards maturation (Figures 2 and 4).

This inhibitory effect of Neem oil on follicles has probably resulted in the release of excess FSH from the anterior pituitary through negative feedback effect, where decreasing estrogen levels from the ovary result in excess release of FSH. The levels of the (other gonadotrophins) LH, estradiol and progesterone are higher in control rats as compared to the treated animals. This is in agreement with the normal reproductive physiological mechanisms.

The essential prerequisite in the process of ovulation is a complex sequence of hormonal events. These include a timed preovulatory rise of threshold levels of estradiol followed by an ovulatory LH peak and subsequent rise in progesterone. It should be emphasized that estradiol and progesterone concentrations vary during different phases of the estrous cycle. In the present study, it was measured the hormone concentration at the end of the study’s respective phase of estrous cycle like Barragán et al., which may contribute to the difference in hormone concentration results amongst the treated and control animals.

During the initial development, the ovarian follicular cells produce estrogen and during the later stage under the influence of LH they produce progesterone but some estrogen, since the maturation of follicle have been affected by Neem oil at both doses, therefore the levels of estradiol and progesterone are lower in treated animals. Lower preovulatory LH was possible due to THE low levels of preovulatory estradiol, and failure of ovulation was a secondary consequence to this altered hormonal milieu.

Upadhay and Kaushic, after intrauterine Neem treatment in Wistar rats have reported no effect on serum progesterone levels. In other similar study, they have noted a degenerative effect of Neem on embryos while administered in the uterine horns of Wistar rats. These findings bring to the conclusion that Neem treatment in Wistar rats has no adverse or toxic systemic effect. However, in the present study the Neem oil has been administered orally and the HPLC was done at the end of study.

Failure of presence of mature ovum (Figure 3) in the presence of higher levels of FSH and unaffected or higher levels of LH in the present study may happen because of the local inhibitory effect of Neem oil on the ovarian tissue. This has been supported by the presence of a very high concentration of Neem Compound Azadirachtin and Azadirchtinin in the ovarian tissue as shown by the HPLC findings (Figures 6 and 7) in the present study. These compounds have shown a dose dependent relationship, confirming a sterility potential of Neem oil in female albino rats.

Mukerjee et al., Talwar et al. and Mukerjee et al., in their studies have also reported a contraceptive effect during early post implantation period as they observed the complete resorption of embryos in Wistar rats after oral administration of the Nim-76, a pure active fraction of Neem seeds. According to the abovementioned authors, this early post implantation
contraceptive effect is possibly because of the activation of cell mediated immune reaction as they have reported activation of T-lymphocytes as well as phagocytic cells along with elevation in gamma interferon. They did not measure the levels of reproductive hormones and they have not observed the changes in microscopic features of the gonads, as well as the residual levels of Neem oil compounds which have been estimated in the present study.

CONCLUSIONS
The present study concludes that the Neem oil has an antifertility potential and this effect was evident in female albino rats at both doses used in this study.

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