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Sterility and Abortive Effects of the Commercial Neem (*Azadirachta indica* A. Juss.) Extract NeemAzal- T/S[®] on Female Rat (*Rattus norvegicus*)

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Abstract: *Azadirachta indica* A.Juss. (neem) is an important plant species with reported reversible infertility and spontaneous abortive properties. NeemAzal-T/S[®] 1% was fed to 3 experimental groups by stomach intubation (5, 15, 25 mg/kg) for 6 days and the control group given tap water. Hematological parameters were determined on days 4 and 9 of the experiment. On day 10, animals were studied for histopathological changes of uterus. The rest were tested for fertility. On day 7 of the pregnancy, the experimental groups were fed with 3 doses of NeemAzal-T/S[®] and tap water (control) for 6 days. There were significant increase in the hematocrit and hemoglobin levels in all groups and RBC count in the first and second group on day 9 in the treated animals compared with the control. There was a significant decrease in the progesterone level only in the second group on day 9. Reproduction occurred after 40 and 75 days in the first and the second group, respectively. In the third group, half of the animals died and reproduction occurred after 3 months in the rest. Histopathological results of the uterus indicated papilloma in the endometrium, proliferation in surface layer and secretory cells with circular nucleus, and clear cytoplasm, which increased in the animals treated with higher doses. Based on the results of this study, it appears that NeemAzal-T/S[®] could be used as a natural and comparatively safe infertility agent to control harmful rodents.

Key Words: Neem extract, sterility, abortion, histopathology, hematology, progesterone

Introduction

Rodents are among the most important agricultural pests and a number of studies have been conducted to examine suitable control measures to minimize their damage. Many synthetic chemical compounds, especially zinc phosphide - a very dangerous chemical, have been used to control them. Due to their reckless use, these compounds are risky and dangerous and could cause food, soil, air, and surface- and underground-water pollution. Moreover, residue of these pesticides may have harmful effects on insects and other non-target organisms and also could bring about an increase in the resistance of the pests to these pesticides, which ultimately result in their outbreak (Singleton et al., 1999). Since there is a great concern about the pollution and toxic effects of synthetic pesticides to the

environment all over the world, there is an effort to replace them with safer and less toxic pesticides that are environmentally friendly such as biopesticides. One such candidate is the use of plant extracts or essential oils to protect the crops against pests and diseases. Meliaceae is a plant family whose trees have numerous useful characteristics such as medicinal and pesticidal properties. One candidate of this family, *Azadirachta indica* (neem), possesses these characteristics and has been used widely in ayurvedic medicine in India (Randhawa and Parmar, 1993). This tree is also called Wonder Tree in most parts of the world because of its numerous properties.

Neem compounds have been known for many years and have shown to have antiviral (Gogati and Marathe, 1989), antibacterial (Singh and Sastry, 1997), antifungal (Kher and Chaurasia, 1997), antiinflammatory, and

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antipyretic properties. Polysaccharides from aqueous bark extracts of this tree have also been shown to possess antitemporal and interferon inducing properties as well (Fujiwara et al., 1982). Numerous studies on experimental animals have shown that neem seed extracts have transient and reversible antifertility and abortive effects (Bardhan et al., 1991, Mukherjee et al., 1999). Antifertility effects of neem oil were observed by Lal et al. (1986). Female contraceptive tablets from neem extracts are extensively used in India at present. The objective of this study was to determine the effective dose of neem seed extract for sterility and abortive effects on female rodent pests.

Materials and Methods

Infertility test: Twenty-four female 4-5-month-old (150-200 g) Wistar rats were used in this study. They were randomly selected and divided into 4 groups of 6 rats each. The first group was kept as the control group whereas the remaining 3 groups served as the experimental animal group. The animals were housed in transparent polythene cages with a stainless steel wire line ceiling. The room temperature in which the animals were housed was about 25 °C and a light/dark cycle of 12:12 h was maintained. Animals were fed with standard commercial diet (Brook Bond, Lipton) and given tap water ad libitum. The animals were kept in this condition for a week to become acclimatized to the laboratory conditions prior to any experimental manipulation. The control group was given plain tap water. The experimental groups were fed with NeemAzal-T/S® 1% extract (5 mg/kg, 15 mg/kg, and 25 mg/kg) by stomach intubation for 6 days. The animals received the doses as follows:

Control group: 0.5 ml plain tap water

Group I: 0.1 ml NeemAzal-T/S® + 0.4 ml tap water

Group II: 0.3 ml NeemAzal-T/S® + 0.2 ml tap water

Group III: 0.5 ml NeemAzal-T/S®

Hematological parameters and progesterone levels were determined on days 4 and 9. For hematological studies, blood samples were collected in small vials containing 1 drop of 2% EDTA to avoid coagulation and then the parameters WBC (White Blood Cell) count, RBC

(Red Blood Cell) count, Hb (Hemoglobin) level, MCHC (Mean corpuscular Hemoglobin Concentration), and hematocrit and lymphocyte count were determined by use of cell counter. The other fraction of the blood samples was collected in small vials without EDTA to facilitate coagulation and to obtain serum. The serum was frozen at -20 °C and, at the end of the experiments, progesterone levels were determined by a diagnostic kit (DSL-10-3900) and an ELISA reader. On day 10, 2 animals from each group were sacrificed and dissected out for histopathological studies of uterus. On day 15, the experimental animals were placed with fertile male rats to determine the delay in reproduction, which may be caused by NeemAzal-T/S®. All the animals were weighed on days 1, 3, 5, 8, and 10 of the experiment (Semler et al. 1992).

Statistical analysis of the data was performed by SPSS software using 2-way ANOVA test. In case of any differences observed between the groups, Newman-Keul's test followed.

Abortive test: At the beginning of the experiment, 30 female and 10 male rats coupled with each other (3 females and 1 male rat in each case). Vaginal smears were taken on the following day. Smears were taken by a pipette, put on a slide, and observed under a light microscope. This was done in order to monitor the estrous cycle and any presence of sperm in the smear was considered as the first day of pregnancy. Twenty females were selected by this way and divided into 4 subgroups, each containing 5 rats, under the similar laboratory conditions as stated earlier. No experiment was performed on these animals for 6 days. On day 7 of pregnancy (day 8 of the experiment), animals were fed by stomach intubation as follows:

Control group: 0.5 ml plain tap water

Group I: 0.1 ml NeemAzal-T/S® + 0.4 ml water

Group II: 0.3 ml NeemAzal-T/S® + 0.2 ml water

Group III: 0.5 ml NeemAzal-T/S®

All animals were fed for 6 days. At the end of the experiment, 2 animals from each group were sacrificed and dissected out for gross morphology and histopathological changes in the uterus.

Results

Effect of NeemAzal-T/S[®] on hematological parameters

Statistical analysis showed no significant differences in the hematological parameters on day 4 of the experiment (first blood sampling day) (Table 1). On day 9 of the experiment (second blood sampling day), hematocrit and hemoglobin levels showed significant increase in all 3 experimental groups compared with the control group. RBC count showed significant increase in the first and the second group. Other parameters showed no significant

differences between the experimental and control groups (Table 2).

Effect of NeemAzal-T/S[®] on progesterone level

Statistical analysis of the serum showed no significant differences in the progesterone level between the experimental and control groups on day 4 of the experiment. On day 9 of the experiment, progesterone level showed significant decrease in the second experimental group (15 mg/kg NeemAzal-T/S[®]) compared with the control group ($P < 0.01$) (Figure 1a).

Table 1. Hematological parameters of the female rats exposed to various doses of NeemAzal-T/S[®] (day 4 of the experiment- first sampling).

Hematological Factors	Control	Experimental Groups		
		5 mg/kg	15 mg/kg	25 mg/kg
WBC (M/mm ³)	6.48 ± 0.69	7.97 ± 1.32	8.99 ± 1.66	5.31 ± 0.37
RBC (M/mm ³)	7.15 ± 0.17	7.59 ± 0.22	7.70 ± 0.16	8.45 ± 0.70
Hb (g/dl)	14.37 ± 0.19	15.77 ± 0.42	16.02 ± 0.19	18.52 ± 2.03
MCV (fl)	55.72 ± 1.05	55.75 ± 0.70	54.98 ± 1.16	54.76 ± 0.86
MCH (Pg)	20.13 ± 0.43	20.77 ± 0.85	20.80 ± 0.27	21.76 ± 0.96
MCHC (g/dl)	36.08 ± 0.25	37.27 ± 0.46	37.86 ± 0.46	37.12 ± 0.32
Hct (%)	39.45 ± 0.75	40.27 ± 0.74	40.30 ± 0.85	40.50 ± 0.86
Lym (%)	80.17 ± 1.51	93.67 ± 0.65	86.22 ± 5.39	77.24 ± 7.03

N = 6

Table 2. Hematological parameters of the female rats exposed to various doses of NeemAzal-T/S[®] (day 9 of the experiment- second sampling).

Hematological Factors	Control	Experimental Groups		
		5 mg/kg	15 mg/kg	25 mg/kg
WBC (M/mm ³)	5.61 ± 0.80	7.74 ± 1.46	10.74 ± 1.62	7.55 ± 2.23
RBC (M/mm ³)	6.51 ± 0.26	7.76 ± 0.11**	7.22 ± 0.24*	7.28 ± 0.11
Hb (g/dl)	13.38 ± 0.34	17.03 ± 0.48***	15.26 ± 0.28**	14.80 ± 0.40*
MCV (fl)	55.24 ± 1.32	58.77 ± 1.01	55.90 ± 1.31	55.83 ± 1.99
MCH (Pg)	20.54 ± 0.41	21.90 ± 0.40	21.22 ± 0.57	20.63 ± 0.37
MCHC (g/dl)	37.10 ± 0.16	37.23 ± 0.03	37.86 ± 0.75	36.93 ± 0.67
Hct (%)	36.02 ± 0.90	45.73 ± 1.28**	40.26 ± 0.78***	40.60 ± 1.04*
Lym (%)	77.38 ± 3.97	81.63 ± 9.36	86.28 ± 3.28	89.43 ± 0.88

N = 6, *P < 0.05, **P < 0.01, ***P < 0.001

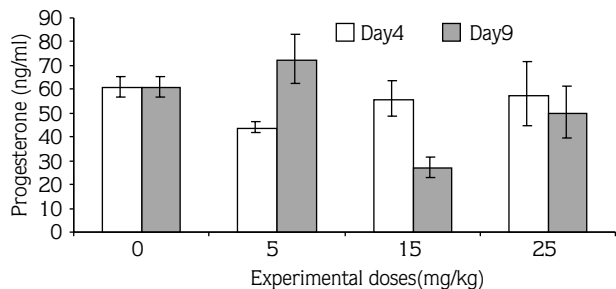


Figure 1a. Progesterone levels in serum of female rats exposed to various doses of NeemAzal-T/S[®].

Effect of NeemAzal - T/S[®] on animal weight

Body weight of the third group was significantly lower compared to the control group on day 8 of the experiment ($P < 0.05$). There were no significant differences in the weight of the other groups compared to the control group (Table 3).

Effect of NeemAzal-T/S[®] on uterus

In the control animals, the transverse section of the uterus shows normal endometrial lining and connective tissue and clear cytoplasm and secretory cells are normal with circular nucleus at the bottom.

Endometrial abnormalities, including edema in the endometrial connective tissue, proliferation in surface layer secretory cells with circular nucleus at the bottom and clear cytoplasm and papilloma in endometrium, were observed. Figures 1b and 1c show the transverse section of functional layer of endometrium in the first group.

Proliferation in fibrocytes and connective tissue cords with mononuclear cells are seen in the chorion. Figures 2a and 2b show surface layer of endometrium in the second group. Cell proliferation and cells with circular nucleus and clear cytoplasm are seen. Endometrial section of the third group shows cell proliferation in the connective tissue leading to the formation of papilloma (Figure 3a). Figure 3b shows these changes with higher magnification and cell proliferation with secretory cells.

Effect of NeemAzal-T/S[®] on the gross morphology of the uterus

Figure 4 shows a nonpregnant uterus. Connective tissue layer shows pink color with thin blood vessels in it and a layer of fat tissue without blood vessels surrounding it. Figure 5 shows a 2-week pregnant uterus (control group) with a few embryos in both of its branches. Abundant blood vessels made it appear brown in color. In the first experimental group, which was treated with 5 mg/kg NeemAzal-T/S[®] for 6 days, there were 2 embryos in one of the branches of the uterus and zero embryo in the other branch. The abundance of blood vessels was not as much as the control group. The thickness of the fat layer and the length and width of the branches are decreased compared to control (Figure 6). Figure 7 shows the uterus of the second group where the thickness of the fat layer was also decreased with some blood vessels surrounding it. Some rudiments or debris were seen in each branch. In the uterus of the third group, changes in connective tissue were observed along with the dilation of the blood vessels in the branches (Figure 8).

Table 3. Weight of the female rats exposed to various doses of NeemAzal-T/S[®] (g).

Day	Control	Experimental Groups		
		5 mg/kg	15 mg/kg	25 mg/kg
Day 1	173.17 ± 8.93	167.00 ± 7.24	173.40 ± 10.50	179.20 ± 5.75
Day 3	180.67 ± 9.23	168.50 ± 6.75	166.40 ± 11.53	170.60 ± 6.55
Day 5	180.83 ± 10.80	164.00 ± 5.97	166.60 ± 11.30	153.00 ± 7.43
Day 8	183.33 ± 12.19	160.00 ± 5.76	167.00 ± 13.24	154.00 ± 9.34*
Day 10	181.50 ± 13.42	160.67 ± 7.84	180.00 ± 10.66	174.25 ± 8.52

N = 6, *P < 0.05

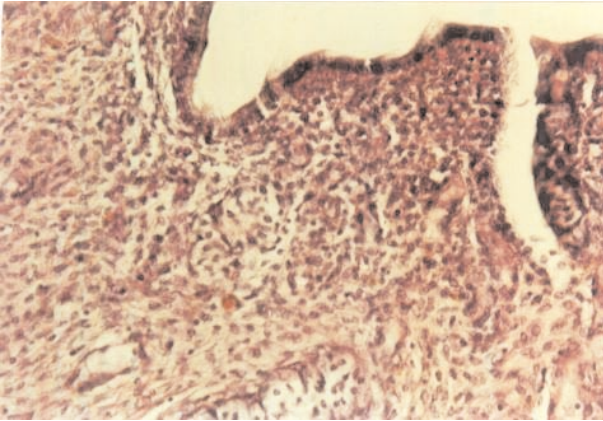


Figure 1b. T.S. of the endometrium of the first experimental group (5 mg/kg NeemAzal-T/S®) showing connective tissue cords with mononuclear cells in the chorion. (HE × 20)

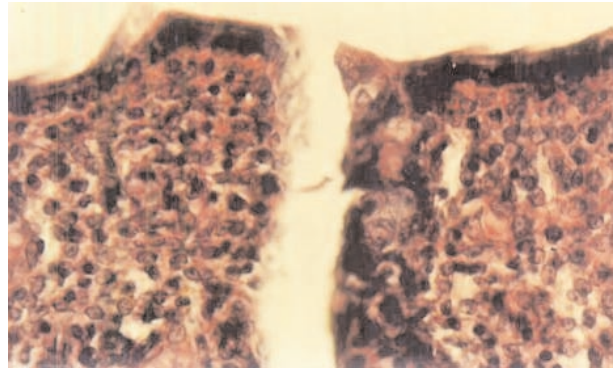


Figure 1c. T.S. of the endometrium of the first experimental group (5 mg/kg NeemAzal-T/S®) showing connective tissue cords with mononuclear cells in the chorion. (HE × 40)

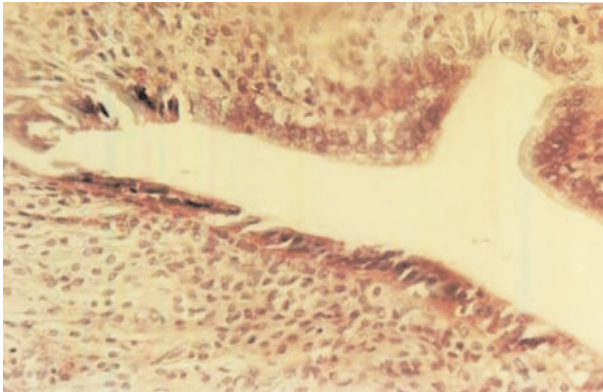


Figure 2a. T.S. of the endometrium of the second experimental group (15 mg/kg NeemAzal-T/S®) showing cell proliferation and cells with circular nucleus and clear cytoplasm. (HE × 20)

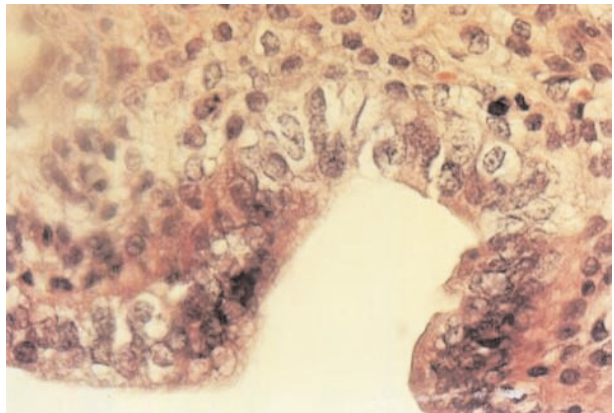


Figure 2b. T.S. of the endometrium of the second experimental group (15 mg/kg NeemAzal-T/S®) showing cell proliferation and cells with circular nucleus and clear cytoplasm. (HE × 40)

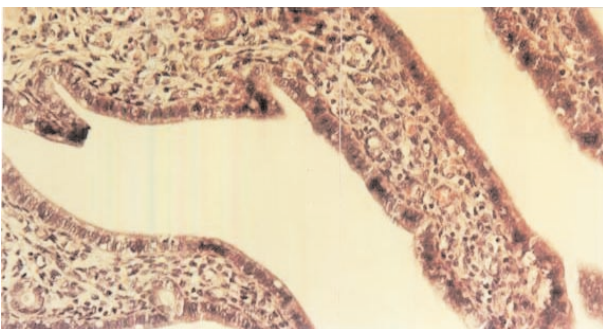


Figure 3a. T.S. of the endometrium of the third experimental group (25 mg/kg NeemAzal-T/S®) showing cell proliferation in connective tissue leading to papilloma formation. (HE × 20)

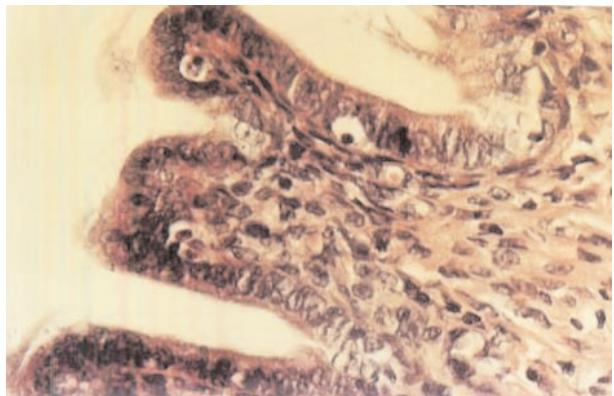


Figure 3b. T.S. of the endometrium of the third experimental group (25 mg/kg NeemAzal-T/S®) showing cell proliferation in connective tissue leading to papilloma formation. (HE × 40)



Figure 4. A nonpregnant uterus in the control group with pink color and thin blood vessels. A layer of fat tissue without blood vessels surrounds it.



Figure 5. A 2-week-old pregnant uterus in the control group with normal fetuses in both the branches of it and abundant blood vessels, which make it look brown in color.



Figure 6. A 2-week-old pregnant uterus in the first experimental group (5 mg/kg NeemAzal-T/S[®]) with 2 fetuses in just one of the branches.



Figure 7. A 2-week-old mated uterus in the second experimental group (15 mg/kg NeemAzal-T/S[®]) with no fetuses in either branches.

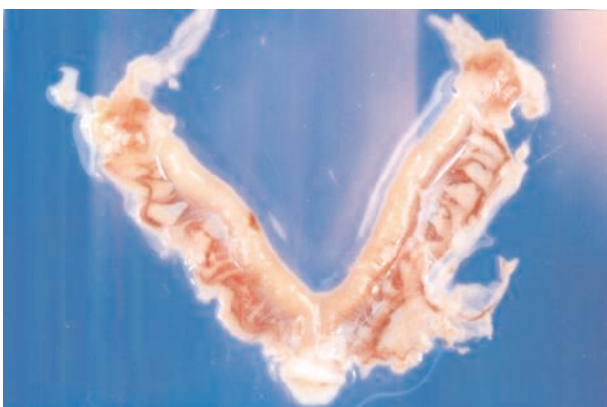


Figure 8. A 2-week-old mated uterus in the third experimental group (25 mg/kg NeemAzal-T/S[®]) with heavily dilated blood vessels and no fetuses in either branch.

Effect of NeemAzal-T/S[®] on delay for pregnancy

The mean delay for pregnancy in the first, second, and third experimental group were 40, 75, and 90 days, respectively, whilst in the control group, pregnancy and reproduction were seen after 23 days (normal reproduction cycle in rats).

Discussion

In this research the effect of NeemAzal-T/S[®] was studied using hematological parameters, progesterone levels, and body weights in female rats treated with different doses of NeemAzal-T/S[®] for 6 days. There were no significant differences observed in the body weight of

the experimental and control groups (Table 3). Parshad et al. (1994) also found no significant difference in body weight between neem treated rats and control rats during the 10-week experimental period. This is also in agreement with Raizada et al. (2001) who reported the absolute body weight of 90-day-azadirachtin-treated male and female rats were comparable to the controls and no significant differences were found in their values.

In this study hematological parameters and progesterone level were determined on days 4 and 9 of the experiment. On day 4, there were no significant differences in hematological parameters (Table 1) and progesterone levels (Figure 1a) between the experimental and control groups. However, on day 9 of the experiment, hematocrit and hemoglobin levels in all 3 experimental groups and RBC count in the first and second group showed significant changes (Table 2). Progesterone level showed a significant decrease in the second experimental group compared with the control group (Figure 1a). Parshad et al. (1994) reported significant increase in PCV, RBC, and WBC counts in rats treated with aqueous extract for 10 weeks. Talwar et al. (1997) found that the oral administration of purified extracts of neem caused no significant alteration in blood chemistry and hematological factors of rats and bonnet monkeys. In a previous study, Talwar et al. (1995) showed that intrauterine administration of purified neem seed oil in rats did not produce any abnormality in progesterone level and hematological parameters, such as Hemoglobin level, neutrophil, lymphocyte, monocyte, and eosinophil counts (Talwar et al., 1997). Raizada et al. (2001) reported no significant changes in Hb, RBC, WBC, and differential leukocyte counts when animals were exposed to azadirachtin for 90 days.

In the present study, the results of gross morphology and histopathology showed some abnormalities and these changes were increased by higher NeemAzal-T/S[®] doses. Papilloma was seen in the endometrium of the third experimental group. Other abnormalities included cell proliferation in the connective tissue of the endometrium, proliferation of secretory cells with circular nucleus and clear cytoplasm. Histopathological studies of the uterus by Upadhyay et al. (1994) showed no changes in the endometrium of bonnet monkeys treated by intrauterine neem application.

Anti-implantation effect of neem oil may be attributed to its anti-estrogenic potential (Rair et al., 1988). In mice

and rats, estrogen is an indispensable hormone for nidation. There is a surge for estrogen on day 4 or 5 after fertilization, which is essential for sensitization of the uterus for induction of decidual cell reaction (Finn, 1965). After absorbance, neem oil reaches the endometrium via the general circulation and exerts its antifertility action. The antiestrogenic effect of neem oil renders the uterus hostile to the decidual stimuli of the blastocyst, making it unfavorable for nidation or continuation of pregnancy (Rair et al., 1988). However, no direct experimental evidence for antihormonal effect of neem oil was provided. In fact, Prakash et al. (1988) reported that neem oil does not have any estrogenic, antiestrogenic, or progestational activity. Furthermore, a single intrauterine neem treatment leads to a long-term preimplantation block in fertility (Kaushic and Upadhyay, 1995). This action obviously cannot be due to any direct action of neem oil since the oil is cleared from the uterine lumen within 3-4 days, whereas the block of fertility lasts for 3-4 months in rats (Upadhyay et al., 1994) and 7-12 months in bonnet monkeys (Upadhyay et al., 1994). In addition, intrauterine neem treatment does not alter the ability of the uterine endometrium for decidualization and, therefore, confirms that the antifertility effect of neem is not mediated by antihormonal action of neem oil (Kaushic and Upadhyay, 1995). Moreover, circulating progesterone levels and histological examination of the uterus do not indicate any antihormonal activity of neem oil. The antifertility effect is due to the degeneration of preimplantation embryo. Neem oil possesses immunomodulatory properties. It enhances phagocytic activity and antigen presenting ability of macrophages and also induces the production of interferon gamma and leukocyte accumulation in the treatment area, leading to embryonic damage and finally infertility (Kaushic and Upadhyay, 1995).

The mechanism of action of the active fraction of neem seed extract is partially understood (Mukherjee et al., 1999). Neem extracts have strong immunomodulatory properties (Labadie et al., 1989). Evidence has been gathered to show that immunological mechanisms play a role in the maintenance of pregnancy. Cytokines secreted by T-helper cells, such as gamma interferon and TNF alpha, have detrimental effects on fetal survival, where IL-3 and GM-CSF help in gestation (Chaouat et al., 1990). Oral administration of neem seed extracts caused an increase in the weight of mesenteric lymph nodes. Flow cytometry analysis showed a transient increase in CD4 and more particularly in the CD8 type cells in both the

mesenteric lymph nodes and in the spleen. These changes caused a higher level of TNF- α and IFN- δ in the serum and termination of pregnancy (Talwar et al., 1997).

Considering the results, when no hormonal changes due to neem extract was observed in this study, it is suggested that the infertility or abortive effects of the NeemAzal-T/S® could be due to its effect on the immune system. It could also be speculated that there was a direct effect of the extract on the uterus by blocking the receptor for progesterone. Therefore it is concluded that doses of

15 and 25 mg/kg of NeemAzal-T/S could bring about a delay of 75 and 90 days in reproduction, respectively. This delay could control the outbreak of the population of rodent pest by blocking 2 to 3 reproduction cycles by a single dose. Considering its cost:benefit ratio and suitably formulated (rodent baits) neem seed extract could be used as an antifertility agent to control harmful agricultural rodents. By this new method, the control of the deleterious rodents could be achieved without using harmful pesticides.

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