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# Antifertility effect of Iranian n*eem* seed alcoholic extract on epididymal sperm of mice

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## Abstract

**Background:** About 90% of the world's contraceptive users are women. This gender-based usage has occurred due to the emphasis of family planning programs and contraception research. Condom, vasectomy and withdrawal are the only male contraception devices available with less assurance for men. For new male contraceptive to have an impact, they must be acceptable to both men and women, as well as effective. A hormonal method will likely come to the market within the next few years. It is necessary to use biologically active botanical substances or fertility-regulating agents of plant origin which are ecofriendly.

**Objectives:** The epididymis is a site which can be exploited for male contraception without undue side effects. It was therefore of interest to investigate the effect of biologically active botanical ecofriendly plants such as Azadirchta Indica (neem) seed alcoholic extract as an efficient and competent male contraceptive on male mouse epididymis.

**Materials and Methods:** In this experimental case control study sixty adult healthy mice divided into two groups of 40 as the control and 20 as the treated group. The treated group was administered by Iranian Botanical Azadirachta Indica seed alcoholic extract, cultivated at Dashteh Moghan (Ardabil province). The seeds was extracted with ethanol then administered first 50 mg/kg body weight /day then 100 mg/kg body weight/day orally for 15 days, following WHO guide lines (MB-50). The target organ, epididymis parameters viz. sperm motility, sperm count fertility rate, Scanning Electron Microscopic (SEM) morphology of spermatozoa and ATPase activity of epididymis of the two groups were compared.

**Results:** The 50 mg/kg body weight (BW)/day showed no significant change in epididymal sperm motility, as compare to the control. Therefore the dose was changed to 100 mg/kg BW/day for 15 days. The body and organ weights (epididymis) of the treated animals were not significantly changed as compare to control group ($p>0.05$). The treatment brought about a significant reduction in fertility rate when normal cycling female mice were mated with treated males ($p<0.001$). Decline in ATPase activity in caput and cauda epididymis was observed ($p<0.001$). SEM photographs showed spermatozoa with abnormal head and bent mid-piece region.

**Conclusion:** Decrease in ATPase activity could be attributed to androgen dependent parameters. However, the fertility rate was also significantly reduced which can be due to the decrease in cauda epididymal sperm motility and their morphological abnormalities. Since the effect on epididymal sperm motility and morphology was manifested in short period of 15 days, it is evident that the extract has potential as an antifertility agent. As this extract do not cause change in the body and organ weight, it is likely that no effect occurred on electrolyte balance.

***Key words:*** *Azadirachta Indica, Male contraception, Epididymis, Contraceptive agent*

## Introduction

Fertility control is an issue of global and national public health concern. Current methods of contraception result in an unacceptable rate of unintended pregnancies. Approximately 50% of all pregnancies are unintended at conception; 50% of those occur in the 94% of sexually active couples who report using some method of contraception (1). The only male-specific contraceptive methods

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currently available are withdrawal, condoms, and vasectomy. As concerns regarding side effects and convenience of these existing methods prevent their universal acceptance (2,3), the development of additional male methods of fertility control can provide tremendous social and public health benefits. Because methods that require infrequent administration have the lowest typical (user) failure rates (4), considerable research has focused on a hormonal male contraceptive, analogous to the female pill. Contraceptive vaccines, and inhibitors of spermatogenesis and sperm motility, provide a potential for nonhormonal male contraceptives. Any advantage of new strategies must be weighed against the potential for increased rates of transmission of pathogens such as HIV if male condom use is reduced. It has, therefore, become necessary to use biologically active botanical substances or fertility-regulating agents of plant origin which are ecofriendly in approach and interfere with the natural patterns of reproduction (5). The seed oil of Azadirachta indica A. Juss (neem) is used in traditional medicine for its antidiabetic, spermicidal, antifertility, antibacterial, and wound healing properties. The study was undertaken to investigate the quantitative aspects of follicular development in cyclic female albino rats after oral administration of polar (PF) and non-polar (NPF) fractions of A. indica seed extract at 3 and 6 mg/kg body weight-1 day-1 and Melia azedarach Linn. (dharek) seed extract at 24 mg/kg body weight-1 day-1 for 18 days, the extracts also significantly reduced ($p=0.05$) the total number of normal follicles in the neem (6).

The epididymis plays an active role in sperm development, and sperm maturation is dependent on the unique luminal environment of the epididymis, including specific proteins synthesized and secreted by the epididymal epithelium (7,8). Although several epididymis-specific secretory proteins have been identified, little is known about the sperm maturation events in the epididymis (9). Our aim has been to understand epididymal function further to develop novel strategies for nonhormonal male contraception.

Azadirachta Indica (AI) A.Juss, Family-melieceause, is the most versatile tree ever found, which is a native of Indian subcontinent. It is a highly esteemed tree and has been closely associated with the socio-cultural and religious aspects of Indian's life since ancient time. This tree has been so important and invaluable that from its various names, its worth can be guessed (10,11). The Sanskrit name of neem is "aristha" which means "Warder of evil and pestilence". African calls it is "muarubaini" meaning "forty uses or forty cures". However, the Persians have given the most appropriate name Azad-dirakht-Hind which literally means free tree of Indian and from which perhaps its latinised botanical name Azadirachta Indica came (12).

The contraceptive effect of neem leaf extract has been demonstrated in female rats (13). Intrauterine administration of neem oil in rat results in high contraceptive efficacy (14) and Purified neem seed extract (Praneem) has also been demonstrated to abrogate pregnancy in both baboons and bonnet monkeys, when administered orally (15). A direct spermicidal activity of neem oil occurs in vitro and in vivo (16,17). In rats, intra-vas administration of neem oil results in blocked spermatogenesis without affecting testosterone production, sexual behavior, or antisperm antibody production (18). No pregnancies resulted from mating these rats, but reversibility was not addressed.

There is no documented evidence referring to the male antifertility of AI seeds particularly cultivated in botanical garden. It was therefore of interest to evaluate the effective concentration of Alcoholic extracted of botanical AI seeds on epididymis of mouse to exploit new male contraceptives, thus; the present study is an attempt to investigate the effects of *A. indica* seed extracts on reproduction of albino male mouse.

## Materials and Methods

The present experimental case contro study is an attempt to investigate the effects of A. Indica seed extracts on reproduction of albino male mice. Fresh seeds of AI cultivated at Dashteh Moghan district of Ardabil province, Iran botanical garden were used.

The extract was prepared according to WHO protocol CG-04 for the preparation of an alcoholic extract (19). In brief, the seeds were shed-dried, powdered and extracted with 95% ethanol (v/v) at 55– 60°C for 3 hr. The solvent was distilled off under reduced pressure and the resulting mass was dried under vacuum and kept at -24ºc until use.

Sixty colony bred, healthy adult male and female albino mice of Swiss strain, weighing between 25and 35 g were used. Animals were housed in polypropylene cages, measuring 430 x 270 x 150 mm, under controlled environmental conditions with provision of a 12 hr light 12 hr dark regimen. Animals were fed with pelleted

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standard mice feed. Water was provided ad libitum.

**Group A–** Animals in this group were given vehicle (normal saline or sterile distilled water) alone orally for 15 days in the dose of 15ml/kg BW to serve as vehicle-treated control.

**Group B–** Animals in this group were treated with Iranian Botanical Azadirachta Indica seed alcoholic extract, cultivated at Dashteh Moghan district, Ardabil province (95% EtOH) extract at the dose of 50mg/kgBW/day(4 mg/0.2ml of distilled water/mouse); oral for15 days.

**Group C-** Animals in this group were treated with Iranian Botanical Azadirachta Indica seed alcoholic extract, cultivated at Dashteh Moghan district,Ardabil province (95% EtOH) extract at the dose of 100mg/kgBWt./day(4 mg/0.2ml of distilled water/mouse); oral for15 days.

The 50 mg/kg BW/day showed no significant change in epididymal sperm motility, as compare to the control. Therefore the dose was changed to 100 mg/kg BW/day for 15 days.

A suspension of the extract was made everyday in sterile distilled water (100 mg/ml) prior to administration. The required extract was administered orally with a glass syringe fitted with a feeding needle.

The animals were orally administered with above mentioned dose daily for 15 days. The activity of the plant's seed extracts on male reproductive function was examined, following its administration to animals of proven fertility.

Both groups were maintained on standard air conditioned animal house at a temperature of 25±2$^{o}$C and exposed to 12 to 14 hours day light.

***Schedule of sacrification***

After 24 hr from the last dosing, the animals were weighed and sacrificed under ether anaesthesia.

***Fertility test***

Ten of the treated animals were subsequently mated with normal cycling females. Prior to mating the females were isolated for one month to rule out pre-existing pregnancy.

Normal cycling females were cohabitated with the treated males on 8$^{th}$ day in the ratio of 2:1 in one cage. The vaginal smears were checked the next morning to observe the presence of sperm which indicated positive mating. This day was considered day zero of pregnancy. Then these females were separated and maintained on normal diet for 16 days after which they were autopsized. The number of implantation sites in each utrine horn and the number of corpora lutes were counted (20).

The treated males were weighed and autopsied. The caput and cauda epididymides were carefully dissected out and weighed to the nearest mg and utilized for ATPase activity which was assayed following the methods of suspension.

***Sperm motility and density***

For sperm motility and density, known amount of cauda epididymis was teased in 0.2 ml of physiological saline. Within 5 min of sacrifice, one drop of evenly mixed sample was applied to a glass slide under a cover glass. The percent motility was determined by counting both motile and immotile spermatozoa per unit area (20). After this, cauda epididymal sperm density (count) was determined by routine procedures and expressed as million/mm$^3$ of suspension (20).

***Tissue biochemistry***

Adenosine Triphosphatease (ATPase) activity in sperm suspention was assayed following previously described method (21), based on hydrolysis of substrate ATP into ADP and inorganic phosphate (Pi). The Pi formed at the end of incubation, was assayed to determine the rate of reaction.

For preparation of sperm suspention, substrate buffer (3 mM), ATP disodium salt in tris HCl buffer, $MgCl_2$ (3mM), NaCl (150 mM) and KCl (30 mM) were added to test sample one by one followed by epididymis homogenate and tris sucrose buffer, incubated at 37$^0$C for 30 min. After incubation 10% TCA was added and mixed well and centrifuged at 3000 rpm for 15 min. The clear supernatantwas used for Pi determination.

The enzyme activity was expressed as µmolor Pi released/hour for 100 mg of fresh tissue weight.

***Scanning Electron Microscopy (SEM)***

SEM of sperm of normal and treated mice were also considered. Freshly isolated active sperm were fixed in cold 2.5% glutaraldehyde buffered with 0.01 M phosphate buffer for 30 min. Following a buffer wash, the cells were postfixed in buffered 1% osmium tetroxide for 30 min, immersed in 1% tannic acid for 30 min, and dehydrated in a graded ethanol series. Sperm were either filtered, using 0.45-µm filter paper, following glutaraldehyde fixation or settled onto polished aluminum stubs following ethanol dehydration.The cells were dried using a modification of the hexamethyldisalazane technique (22). This modification involved impregnation of sperm with 1% tannic acid, which

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serves as a mordant. Sperm treated with the tannic acid were less likely to appear collapsed or crenate. Specimens were coated with gold and examined with an AMRay 18201 scanning electron microscope.

***Statistical analysis***

Data are expressed as mean ± SE and analysed for statistical significance by using Student's t-test.

## Results

Administrate of 50 mg/kg BW/day AI extract showed no significant change in epididymal sperm motility of treated group as compare to the control. Therefore the dose was changed to 100 mg/kg BW/day for 15 days.

***Body and organ weights***

Azadirachta Indica alcoholic seed extract regimen did not altered body weight of the animals as compared to the control group. The weight of cauda and caput epididymis were not changed in treaed group when compared with control group animals (Table I).

***Sperm motility and count***

Epididymal sperm motility was significantly diminished in Azadirachta Indica alcoholic seed extract treated group. Antifertility effects of Azadirachta Indica did not produced a significant reduction on cauda epididymal sperm density (Table I).

***Fertility***

Reduction in the fertility rate was observed in treated groups. The fertility rate was 97% positive in the control animals. However, after treatment for 15 days, it was only 17% positive which was significantly lower in comparison to control ($p<0.001$) (Table I).

***Tissue biochemistry***

Table I present the adenosine triphosphate activity (ATPase) in the caput and cauda epididymis of normal and treated animals. The activity of the enzyme was significantly decrease in treated animals in comparison to control group ($p<0.001$) (Table I).

**Table I.** Body and organ weights after 15 days treatment with A. Indica (95% EtOH) seed extract in male mice. Cauda epididymal sperm motility and density. Fertility of male mice in control and 100 mg/kg b.wt/day groups treated groups with A. Indica ( 95% EtOH) seed extract for 15 days when mated with females (male : female ratio, 1 : 2).

| **Treated groups** | **F.R.(%) Ave.No. Of imp. sites** | **Epididymal Sperm count ($10^6$/ml)** | **Cauda epidim. Sperm Motility (%)** | **ATPase ctivity of epimidymis (µmoles of Pi liberate/hour for 100mg tissue wt)** | |
|---|---|---|---|---|---|
| | | | | **Caput** | **Cauda** |
| Group A (control) | 97% +Ve (7-8) | 53.18±0.95 | 70.39±1.33 | 26.79±0.6 | 15.97±0.86 |
| Group B (treated with AI extract) | 17% +Ve[a] (0-2) | 50.74±1.13[b] | 20.67±1.51[c] | 25.22±1.9[d] | 7.65±1.7[e] |

*Data are expressed as mean ± SE*
*F.R.: fertility rate*
*[a]p<0.001 , [b]p>0.05 , [c]p<0.001 , [d]p<0.001 [e]p<0.001.*

***Scanning Electron Microscopy***

Scanning electron microscopic (SEM) photographs was performed for showing the morphology of normal and 15 days AI seed extract treated mice spermatozoa from the cauda epididymis. Figure 1 & 2 represent SEM of normal cauda epididymis. Normal fertile, adult mouse showed the scimitar shaped head (Figure 1) and mid-piece region of control mouse cauda epididymis spermatozoa showing the typical buldge towards the rear end of the mid piece (Fig.2).

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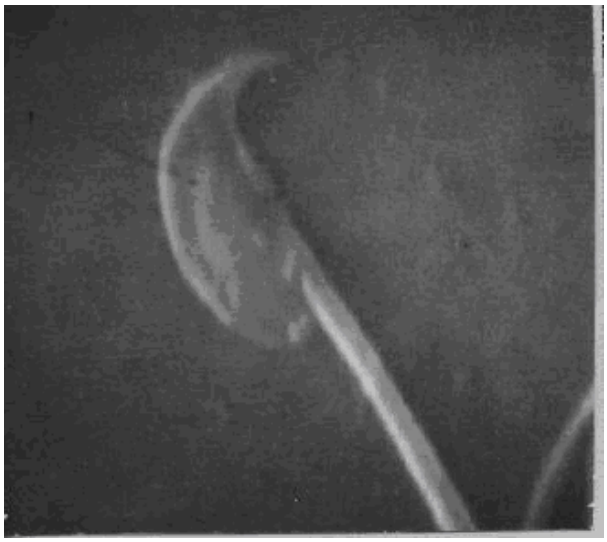


**Figure 1.** SEM photograph cauda epididymis sperm of of normal fertile, adult mouse showing the scimitar shaped head. X 2750

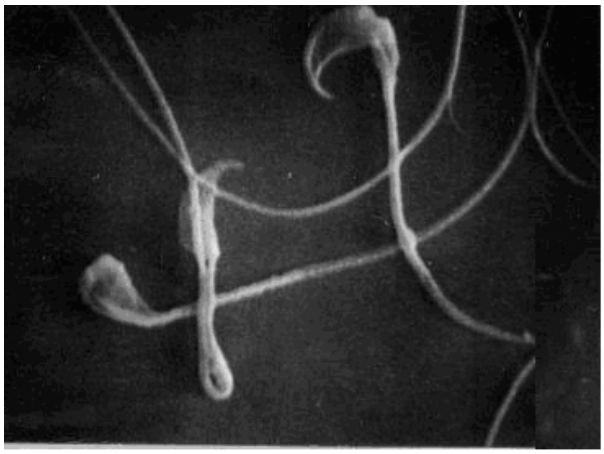


**Figure 2.** SEM photograph of cauda epididymis spermatozoa of 15 days AI seed extract treated mice, showing abnormal head and bent mid piece regions. X 1000.

## Discussion

Existing methods of male contraception are safe, effective, and probably underutilized. For much of the developing world, a lack of basic education and access to health care services limits male involvement in family planning. For new male contraceptive methods to have an impact on the worldwide problem of unintended pregnancies, they must be acceptable to both men and women, and result in an increase in overall efficacy of use. In a multicenter study designed to assess attitudes toward a male hormonal method (23) the acceptability of such methods varied between groups, but the majority welcomed a new hormonal method of contraception, and 44% to 83% stated that they would use a male contraceptive pill. Men in this study preferred an oral method over an injectable or implant system. These results suggest that potential acceptability of a male pill is high. In a study of men's attitudes, well-being, and sexual activity during a 1-year testosterone contraception trial (23), more than 65% of participants stated that the method improved their feeling of freedom and control over reproduction, and 68% reported enhanced sexual activity. The method was rated “as expected” or “better than other methods” by the great majority of participants. Although most aspects of the men's health and sexual lives did not change, the frequency and quality of intercourse was significantly higher during the efficacy phase compared with the recovery phase. The authors theorize that the decline during the recovery phase may be explained by a pharmacologic down-regulation of androgen receptors. Any emerging strategy that decreases the use of male condoms may have the unintended consequence of increasing the rate of transmission of infectious diseases, including HIV.

The epididymis is a site which can be exploited for male contraception without undue side effects. It has been identified as the site where the essential posttesticular sperm maturation and storage occurs. Nevertheless, little is known about the process of sperm maturation and factors affecting it. Understanding the physiology of the epididymis could provide new possibilities for infertility treatment on one hand, and could suggest new strategies for the development of a new nonhormonal male contraceptive on the other. Our studies are directed toward understanding the physiology of the epididymis to develop novel strategies for male contraception based on inhibition of posttesticular sperm maturation in the epididymis.

However, new methods of male contraception become available, family planning and public health experts must cautiously evaluate the impact of these new technologies before recommending them.

Recently many laboratories are engaged in developing a male contraceptive from plants (24). Plants products as contraceptives will be more acceptable for economic reasons in terms of self-reliance and the possible practicability for a male pill approach in countries where population pressure is high.

Studies on the effects of plant products on male reproductive system and fertility are comparatively few and far fetched. From a public health perspective, the head for contraception has never been greater.

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The importance of the neem tree has been recognized by US National Academy of Sciences (25), which published a report in 1992 entitled 'Neem – a tree for solving global problem's.

Biswas *et al* (2002) have reviewed the biological activities of some of the neem compounds, pharmacological actions of the neem extracts, clinical study and plausible medicinal applications of neem along with their safety evaluation (26).

The present study took into consideration the structure and physiology of reproductive organs and fertility rate of adult male mouse. The effect on sperm morphology, motility, density and fertilizing ability were studied in particular. All the parameters were assayed accoreding to WHO Protocols MB-50 & CG-04 (19, 20), which specifically test the antifertility effect of plant product in potents.

The obtained results revealed that the extract did not affect body and organ weights of the epididymis (table I) which indicates that the extract did not promote body weight gain causing obesity and/or water and electrolyte retention, this is significant since $Na^+/K^+$ balance is important for maintaining the microenvironment particularly of epididymis for sperm maturation. Our findings are partially in accordance with the investigations of Chinoy and Geeta Rang (27).

Deshpande, Mendulkar and Sadre (1980) reported that control mice showed 100% fertility rate. Treated group in their study received freshly prepared, water extract of crushed green leaves of Azadirachta Indica in the dose of 1 ml per mouse orally every day for one month (28). The number of pregnancies as well as the litter size were significantly reduced in Azadirachta Indica treated animals which will be corrobating the present observations that showed a significant decrease of fertility rate (<83%) when normal cycling female mice were mated with AI extract treated males. Although mating had occurred, the number of implantation sites were markedly reduced from 7 to 2 (table I). The sex behaviour and libido was not affected by treatment. The reduction in fertility could be attributed to decrease in cauda epididymal sperm motility and their morphological abnormalities, where in SEM photographs showed spermatozoa with abnormal head and bent midpiece region. A large number of spermatozoa were also found to be decapitated. Most of the spermatozoa were lacking the progressive motility and were sluggishly motile or stationary with movement of tail indicating that AI seed extract interfered with sperm motility. Since the effect on epididymal sperm motility and morphology was manifested in short period of 15 days, it is evident that the extract has potential as antifertility agent, particularly as spermicidal agent. Khan and Awasthy (2003), evaluated the leaf extract of neem. The extract was found to induce structural and numerical changes (29). A significant increase in frequency of sperms with abnormal head morphology and the decrease in mean sperm count were also observed. This spermatotoxic effect of the neem extract corroborates our morphological observations.

There was no change observed in sperm count because the total spermatogenesis time is 34.5 days in mouse and the treatment was only for fifteen days. Continuation of treatment for 40 days or more would enable one to study the effect of extract on spermatogenesis if any. An alternative approach to Vasectomy for long-term male contraception following single application of AI of neem oil in the lumen of the vas-deferfns product having immunomodulatory properties was described by Upadhyay, Dhawan and Talwar (1993). They reported males Wistar rats treated with neem oil remained infertile throughout 8 months of observation period when epididymal history was normal without any inflammatory changes or obstruction which further support this view (18).

The present study also elucidates the fact that seeds of AI could be used as a male contraceptive without having any toxic effect on vital organs such as liver and kidney by parallel manifestation of toxicity test.

ATP is the source of energy for sperm motility. ATP is hydrolaysed by ATPase activity. Rahman, Siddiqui and Jamil (1999) reported sub-chronic effect of neem based pesticide on ATPase in rat brain (30). Their reports indicated that ATPase were potently inhibited by Vepacide an active ingredient from neem seed oil but no report was regarding the activity semen enzymes. More studies should be performed to evaluate the effect of neem extract on the activity of semen enzymes. The activity of this enzyme in caput and cauda epididymis of treated mice was significantly reduced (tableI) that corrobating Rahman study.

Sharma *et al* (1996) have also demonestrated that neem oil extract has an antifertility, anti-implantation and abortifacient properties (31).

They use NIM-76, a fraction isolated from neem oil. This fraction kills all human sperm in vitro in under 20 seconds at a concentration of 25mg/ml.With increases in NIM-76 concentration, they observed linear decrease in percentages of

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motile as well as progressively motile sperm with time. In our study we observed a significant decrease of epididymal sperm motility (table I) which is matched with this study.

## Conclusion

Active botanical substances can be used as fertility-regulating agents. Azadirachta Indica was thought to have the effect on the transport of sperm into the epididymis; a site which can be exploited for male contraception without undue side effects were tested in vitro on mice. The extract alters epididymal milieu and affects the morphology of spermatozoa. Therefore Azadirchta Indica (neem) seed might be encounter as an efficient and competent male contraceptive on mouse epididymis, but further studies are called for understanding the exact mechanism.

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