

In vitro effect of azadirachtin-A, a tetranortriterpinoid of neem seed kernel, on rat spermatozoa

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Abstract: Since, there is only few documented evidence referring to assessment of the clear specific actions of medicinal plant source or active constituents in rodent model, the present study is undertaken to evaluate the effective concentration of technical azadirachtin-A, a tetranortriterpinoid of neem seed kernel, to immobilizes the cauda epididymal spermatozoa within 20 seconds and effectively inhibits sperm motility, viability and plasma membrane integrity in a dose-dependent manner. Spermicidal efficacy was evaluated *in vitro* by a modified Sander–Cramer test using different concentrations. The mode of spermicidal action was evaluated by (a) Sperm morphology and viability tests (b) hypo-osmotic swelling (HOS) tests and (c) transmission electron microscopy. The minimum effective concentration (MEC) of azadirachtin-A for 100 % immobilization of sperm in 20 secs was around 4.50µg/million sperm. No morphological changes were found in the sperm head, midpiece or tail and no viable sperm seen. Further, sperm membrane integrity observations, ultra studies shown that most of most of the principal pieces of treated sperm were without plasma membrane and azadirachtin-A exposed spermatozoa lost their response, by curling of the tail, to hypo osmotic medium in a dose-dependent manner, indicating the impairment of functional integrity of the plasma membrane. The present study therefore appears reasonable that azadirachtin-A provides the sperm-immobilizing effect either directly executing its effects by structural and functional modulation of the plasma membrane or by way of its synergism with blockage of some biochemical pathway like energy utilization.

Keywords— Azadirachtin-A, spermatozoa, MEC, HOS and rat

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Introduction

Contraception is literally the prevention of conception, but generally is taken to mean the prevention of pregnancy (Herfindal and Gourley, 2000). The development of new fertility regulating drug from medicinal plants is an attractive proposition, because from times immemorial humans have relied on plants and their products as sources of drugs and therapeutic agents, although in recent times, synthetic drugs are used extensively in modern medicine. In the last few years, a number of articles have been published on medicinal plants and many of them are reported to possess spermicidal, abortifacient or antiandrogenic activities. Majority of plant-derived spermicides were triterpene saponins of several structural types, flavonoids and phenol compounds (Farnsworth and Waller, 1982). Indian neem tree *Azadirachta indica* (Syn: *Melia azadirach*: Meliaceae) is recognized since long for its unique properties. Articles have been reported that *A. indica* leaves cause impairment of spermatogenesis and exhibit antiandrogenic effect in albino rats (Aladakatti *et al.*, 2001; Aladakatti & Nazeer Ahamed, 2005).

Khillare and Shrivastav (2003) reported that the lyophilized *A.indica* leaves extract is carbohydrate nature with spermicidal activity at effective concentration to immobilize and kill 100% human spermatozoa within 20 seconds. A number of chemical components have been isolated from neem plant and many of the secondary compounds of the neem have been identified, purified and some have been tested for their effects on mammals (Atawodi and Atawodi, 2009).

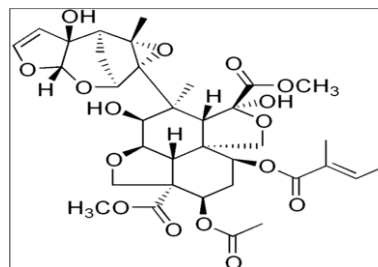


Fig.1: Chemical structure of azadirachtin-A tetranortriterpinoid is an active major ingredient isolated from seed kernel of neem tree (*Azadirachta indica*)

Azadirachtin (Fig.1), a tetranortriterpenoid class of limonoids, found principally from the seeds of the neem tree (*Azadirachta indica* A. Juss), is a potent insect growth regulator, sterilant and anti-feedant (Mordue (Luntz) and Blackwell, 1993). Technical azadirachtin, a mixture of seven structurally related isomers of tetranortriterpinoid have been reviewed on environmental behaviour and biological effects. However, the toxicity of a compound has always become an issue in therapeutic usage; azadirachtin has been demonstrated as a low acute/subchronic toxicant in mammalian species (Raizada *et al.* 2001; Srivastava and Raizada 2001). Instead subcutaneous administration of technical azadirachtin-A exerted detrimental effects at high dose on biochemical parameters of male reproductive functions (Aladakatti *et al.* 2011). Recent study shown that dose dependent pre-administration of azadirachtin-A reduced the hepatocellular necrosis and therefore protect the liver against toxicity caused by CCl₄ (Baligar *et al.*, 2014). A literature survey revealed a few reports with regard to *A.indica* and its chemical composition and biological evaluation, but no investigation has yet been carried out particularly on active principles azadirachtin-A 's fertility-related activity. In this study, we are reported on the sperm immobilization activity of azadirachtin-A in rats and it has been observed that azadirachtin-A effectively inhibits sperm motility, viability and plasma membrane integrity in a dose-dependent manner.

Materials and Methods

Animals

Colony bred healthy adult male albino rats (Wistar strain) weighing 200 g were utilized for experiments. All animals were proven fertility and obtained from the rat colony maintained in the department. They were housed at a temperature of 22 ± 2^o C with 12:12 light and dark cycle. They were maintained on a standard rat pellet diet and water was given *ad libitum*. The animals were acclimatized to the laboratory conditions before conducting experiments and the care of the laborator

the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) regulations. Necessary approval from the Institutional Animal Ethics Committee (IAEC) was obtained before undertaking animal experimentation.

Chemicals

Azadirachtin-A technical 15-17% sample was purchased from the SPIC Ltd., Chennai, India. All other reagents were procured from either Sigma or Hi Media Lab Pvt. Ltd. (Mumbai, India).

Test for spermicidal activity

The spermicidal activity of the azadirachtin-A was determined by following a modified version of the Sander and Cramer test (1941). Different concentrations of azadirachtin-A in physiological saline (0.5, 1.0, 1.5, 2.0, 2.5, 3.0µg10µg /mL) were prepared by serial dilution, and 100 µL of each was mixed with an aliquot of 20 µL of sperm suspension (20 X 10⁶/mL). The mixtures were observed under a phase contrast microscope (X 40) for 20 sec and counted for motile sperm. The sperm that lost complete motility within 20 sec following exposure to azadirachtin-A separately was subsequently tested for motility revival. Two hundred fifty micro liters of Baker's buffer (glucose 3%, Na₂HPO₄ · 2 H₂O 0.31%, NaCl 0.2%, KH₂PO₄ 0.01%) was added to the test solutions (which showed total immobilization), incubated at 37°C for 60 min and observed again for any motility. The minimum concentration of azadirachtin-A which caused 100% immobilization within 20 sec with no revival of motility in the buffer after 60-min incubation at 37°C, was considered to be the minimum effective concentration (MEC).

Assessment of sperm morphology and viability

Sperm morphology and viability tests were done according to WHO (1999), Eliasson & Treichl (1971) and Jeyendran *et al.* (1984). Control and different concentrations of azadirachtin-A treated sperm suspensions were incubated for 30 min at 37°C and are examined separately at 400 X under phase contrast microscope using eosin-Y and Nigrosin staining method to record any change in morphology of the sperm. Similarly, for viability assessment, one drop of treated and control sperm suspensions were mixed with 2 drops of 1% Eosin Y. After 30 s, 3 drops of 10% Nigrosin solution was added and mixed. A drop of the sperm suspension-Eosin-Nigrosin mixture was placed on a clear microscope slide; allowed to dry and observed under X 400 magnification.

Assessment of plasma membrane integrity

Hypo-osmotic swelling (HOS) tests were performed to evaluate the effect of azadirachtin-A on sperm plasma membrane integrity (WHO, 1999). Control and different concentrations azadirachtin-A treated sperm suspensions were exposed to HOS solution (75 mM fructose and 20 mM sodium citrate) for at least 30 min at 37°C. The number of spermatozoa exhibiting characteristic swelling or tail coiling was counted under a phase contrast microscope (X 400).

Ultrastructural study

Preparation of rat spermatozoa for ultra study was performed as described elsewhere (Aladakatti and Nazeer Ahamed, 2005). Briefly, an aliquots of control and different concentrations of azadirachtin-A treated sperm suspension were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4; 0.1 M) for 4 hr at 4°C, washed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4; 0.1 M) for 6 hr. After three successive washings in distilled water, stained *en bloc* in 2% aqueous uranyl acetate for 6 hr, dehydrated in acetone series, infiltrated in epon-araldite mixture for 10 hr and embedded in the same media in a beam capsule. The blocks were cut in LKB Bromma ultramicrotome.

Ultrathin sections were cut at 100-300 Å, mounted on copper grids and stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). The stained sections were scanned in Jeol-TEM 100 C X II electron microscope for ultrastructural observations.

Results

Spermicidal activity

The spermicidal activity of graded doses of azadirachtin-A was studied separately *in vitro* condition using cauda epididymal plasma. The results of Sander- Cramer test showed potent spermicidal activity of this active principle. The minimum effective concentration (MEC) of azadirachtin-A for 100 % immobilization of sperm in 20 secs was around 4.50 µg/million sperm (Fig.2, Table).

Sperm Morphology and Viability

The morphological and viability studies of spermatozoa were done using Eosin-Y and Nigrosin. No morphological changes were found in the sperm head, midpiece or tail compared with untreated spermatozoa. The viability of sperm was studied it was found that 4.50 µg of azadirachtin-A kills 100% of one million sperm within 20secs.

Sperm membrane integrity

Rat spermatozoa showed typical morphological changes when subjected to hypo-osmotic shock. These changes were clearly visible by phase contrast microscopy. In this experiment, more than 95% of the control spermatozoa responded to hypoosmotic solution by curling of the tail, while in azadirachtin-A exposed spermatozoa lost their response to hypoosmotic medium in a dose-dependent manner, indicating the impairment of functional integrity of the plasma membrane (Fig.3).

Ultrastructural studies

Ultrastructural studies showed considerable membrane damage in the azadirachtin-A exposed sperm (at MEC). As compared with the intact plasma membrane of the control spermatozoa (Fig. 4A), the treated sperm exhibited dissolution of the acrosomal cap (Fig. 4C). Transverse sections of mid-piece of control sperm tail and different portions of principal piece showed normal structures of mitochondrial sheath and fibrous sheath., including outer dense fibres, axonemal component, central pair, longitudinal column of the fibrous sheath along with their plasma membrane were well defined (Fig. 4B), however, most of the principal pieces of treated sperm were without plasma membrane except outer dense fibres and axonemal component were normal (Fig. 4D).

Minimum effective concentration (MEC) of azadirachtin-A required to 100 % immobilization of rat sperm in 20 secs					
Sample no	Cauda epididymal sperm count 20 mill/ml	Conc. Grade azadirachtin-A used (µg)	% Motility		MEC(µg)
			Initial	After (20 secs)	
1	20 x 10 ⁶	0.50	80	70	4.50µg/mL
2	20 x 10 ⁶	1.00	80	70	
3	20 x 10 ⁶	1.50	70	60	
4	20 x 10 ⁶	2.00	70	50	
5	20 x 10 ⁶	2.50	70	35	
6	20 x 10 ⁶	3.00	75	20	
7	20 x 10 ⁶	3.50	75	10	
8	20 x 10 ⁶	4.00	80	05	
9	20 x 10 ⁶	4.50	70	00	
10	20 x 10 ⁶	5.00	70	00	

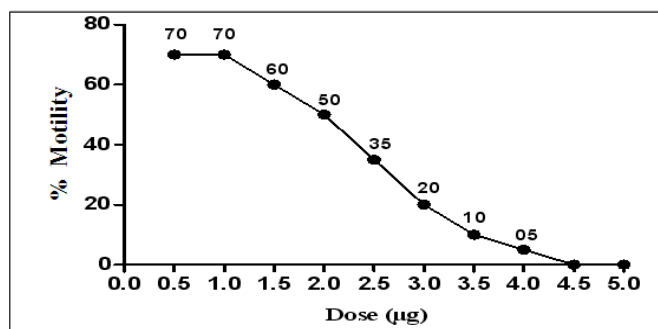


Fig.2. Dose dependent inhibition of sperm motility. Sperm of fertile albino rats were exposed to varying concentrations of azadirachtin-A and motility was observed under phase contrast microscope. Each point represents mean \pm SEM of at least three observations.

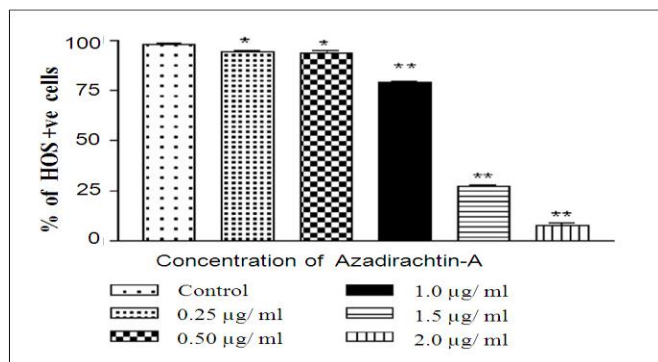


Fig.3. HOS test. Aliquots of control and azadirachtin-A treated (at different doses) sperm suspension were exposed to 1 mL of HOS solution, and the number of HOS-reactive sperm exhibiting typical tail coiling was counted under a phase contrast microscope. Each bar represents mean \pm SEM of five observations. *P<0.05, **P<0.001.

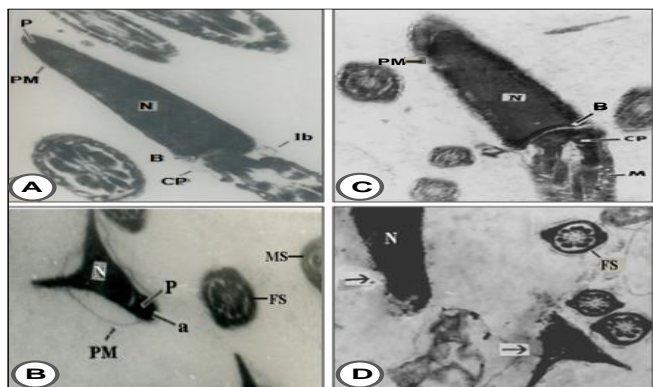


Fig.4. Transmission electron micrographs of sperm samples incubated in the presence (A, B) or absence of azadirachtin-A (C, D) at MEC. Median section through the sperm head and tail of controls showing proper features of nucleus (N) covered with perforatorium (P), acrosome (a), basal plate (B) and connecting piece of tail (CP), mitochondrial sheath (MS) and fibrous sheath (FS) were with plasma membrane [A(X 18,000) & B (X 12,000)]. Azadirachtin-A treated spermatozoa exhibit dissolution of the acrosomal cap (arrows) and Mitochondria (M) are seen, in oblique section, in contact with connecting piece (C X 21,700). T.S. of mid-piece shows normal pattern of mitochondrial sheath (MS) and fibrous sheath (FS) along the length of the structure. However, most of the principal pieces of sperm were without plasma membrane except outer dense fibres and axonemal component were normal and most of the sections of heads and tails have fuzzy material on their surface (D X 18,500).

Discussion

The epididymis provides a favourable milieu for the acquisition of fertilizing ability, motility, storage and survival of spermatozoa. Sperm possess two principal attributes, viz., motility and the fertilizing ability, which are prerequisites for fertilization. Any negative impact on motility would seriously affect the fertilizing ability (Bauer *et al.*, 1974). Plant origin compound azorellanone significantly inhibited several functions that are essential for fertilization, such as sperm motility, sperm-zona binding, sperm acrosome reaction and Ca^{2+} influx, and sperm protease activities. Neem derivatives, viz., sodium nimbin and sodium nimbidinate (Sharma and Saksena, 1959a; Sharma and Saksena, 1959b) have been found to possess weak spermicidal action *in vitro*. Praneem polyherbal cream has shown spermicidal effect at effective concentration to immobilization (100%) of sperm within 20secs (Garg *et al.*, 1993). Kumbar *et al.* (2012) evaluated the effective concentration of nimbolide, an isoprenoid of neem leaf, to immobilize and kill 100% rat spermatozoa and suggested that isoprenoid of leaf is hydrophilic in nature and mix immediately with water as well as body fluids and kill sperm within 20 secs.

In present study, active principle azadirachtin-A, exerted dose-dependent sperm immobilizing effects. The MEC was found to be 4.5µg/ml and such effect was considered spermicidal because no revival of sperm motility was demonstrated after the azadirachtin-A exposed sperm were incubated in Baker's buffer. The spermicidal activity was included to check with different concentration of azadirachtin-A in rats and results revealed this active principle is potent spermicidal, which is demonstrated through dose-dependent study on the effect of sperm motility and further confirmed by viability test. At phase contrast microscopic evaluation, we couldn't see any morphological changes in the sperm head, mid-piece and tail compared control sperm. The spermicidal activity tends to suggest that active principle showed spermicidal effect at the effective concentration to immobilize and kill 100% rat spermatozoa within 20 secs may be due to blockage of some biochemical pathway like energy utilization (Killare and Shrivastav, 2003; Kumbar *et al.*, 2012). Further, we checked this azadirachtin-A as sperm motility inhibitor by using different concentrations and results exhibited the inhibition in motility pattern at lower concentrations. These observations tend to suggest that this compound, in future at effective concentration, may be used in the formulation of vaginal contraceptives. It is interesting to note that use of herbal contraceptives generally did not lead to permanent sterility in rodents as a model, since discontinuation of the treatment allowed a prompt return to normal fertility.

The plasma membrane plays a vital role in the process of sperm migration and fertilization. A number of spermicidal agents are known to execute their effects by structural and functional modulation of the plasma. Since azadirachtin-A was shown to be dose dependent active and present study pointed out that at a concentration of 4.5 µg/ml shows good rat spermatozoa immobilize capacity. Most of plant spermicidal compounds act on the sperm surface, disrupting the plasma membrane. The currently used azadirachtin-A compound acted in a similar manner. It produced disruption of lipids within the sperm membrane, particularly, on the acrosome and mid-piece causing rapid loss of sperm motility (Killare and Shrivastav, 2003; Kumbar *et al.*, 2012). We therefore took interest in examining if the spermicidal effect of azadirachtin-A was mediated by adverse modulation of sperm membrane. In this study we adopted HOS technique and ultrastructural evaluation of rat sperm. Results exhibited the damage to the membrane architecture was evidenced by the significant reduction in sperm viability and tail

curling. A property of the cell membrane was its ability to permit the transport of molecules selectively. This is not only essential for the maintenance of sperm motility, but also for the induction of the acrosome reaction and possibly other key events related to fertilization. Studies supported this study that the general property of the plasma membrane was lost on incubation of motile sperm (Kumara et al., 2007; Dubey et al., 2010; Kumbar et al., 2012).

The ultrastructural evaluation of spermatozoa, however, demonstrated that the effect of azadirachtin-A on functional integrity of sperm membrane was a secondary consequence of structural damage. The picture clearly reflected that membrane architecture of the sperm was lost with dissolution of the outer acrosomal membrane and membrane of sperm mid piece. HOS is a response that reflects the functional integrity of sperm membrane. Following exposure to hypoosmotic environment, intact sperm membrane permits free passage of fluids into the cell to reach osmotic equilibrium. As a result, the sperm volume increases and plasma membrane bulges. Since the plasma membrane around the sperm tail fiber is more loosely attached than that around other parts, sperm tail is particularly susceptible to hypoosmotic exposure and responds by coiling (Jayendran et al., 1984). This characteristic feature was exhibited in >95% of the sperm in the control set, while the azadirachtin-A treated sperm showed no such morphological distortion in the tail part except membrane. This observation suggests that the functional integrity of sperm membrane was lost following exposure to azadirachtin-A.

Though speculative, it therefore appears reasonable that azadirachtin-A bestows the sperm-immobilizing effect either directly executing its effects by structural and functional modulation of the plasma membrane or by way of its synergism with blockage of some biochemical pathway like energy utilization. From the foregoing findings, as well as the commercial availability of azadirachtin-A from neem seed kernel, solubility in saline, coupled with unaltered pH of the medium and effectiveness, the future potential of azadirachtin-A as a local sperm-immobilizing agent appears highly promising. However, further investigation is required to understand the exact mechanism of action by blocking of some biochemical pathway like energy utilization for sperm motility.

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