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Histological Evidence of Male Potent Reproductive Sites by Iranian Botanical Azadiracta Indica (Neem) Seed Extract

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Abstract: From a public health perspective, the need for contraception has never been greater. Although the existing male-specific methods are safe and effective, increasing male options for fertility control could improve family planning. For new male contraceptive methods to have an impact, they must be acceptable to both men and women, as well as effective. It is necessary to use biologically active botanical substances or fertility-regulating agents of plant origin which are ecofriendly. There is no documented evidence referring to the male antifertility of Azadirchta Indica (neem) botanical seed's extract. The epididymis and vas deferens are the sites which can be exploited for male contraception without undue side effects, it was therefore of interest to investigate the effect of alcoholic extract of old and tender AI (neem) seed as an efficient and competent male contraceptive on mouse epididymis. In this experimental case control study sixty adult healthy mice divided into two groups of 40 as the control and 20 treated ones. The treated group was administered by Iranian Botanical Azadirachta Indica seed alcoholic extract, cultivated at Dashteh Moghan (Ardabil province). The seeds of shade-dried matured drupes of A. indica were powdered and the fraction 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) that specified the oral route would be the most feasible rout and standard mode of administration of a plant extract. The target organs, epididymis and vas deferens histology and histocytometric of the two groups were compared. The 50 mg /kg body weight/day showed no significant change in epididymal sperm motility.as compare to the control. Therefore the dose was changed to 100 mg/kg B.W./day for 15 days. Histoarchitecure of epididymis and vas deferens of treated mice were affected after treatment involving disorganization of epididymal and vas deferens epithelium, pycnosis of cell nuclei and clumping of sperm. The mechanism of action of seed extract was probably via altering the hormone levels and causing androgen deprived effect to target organ. This alters vas deferens and epididymal milieu and affects the spermatozoa. It is evident that extract has potential as an antifertility agent.

Key words: Azadirachta Indica, male contraception, male antifertility, epididymis, vas deferens, alcoholic extract, contraceptive agent, mice, Iran

INTRODUCTION

The options available to men for fertility control are much more limited compared to those for women. The male reproductive system, particularly the process of spermatogenesis, sperm maturation and transport and also the sperm-egg interaction are so complex that it has not so far been possible to find an effective intervention that can be converted into a product. Continued efforts over the past three decades to develop additional methods of male contraception have made some significant contributions in the field. However, there is still no method available in the field of male contraception that satisfies the essential criteria of safety, efficacy, economy and complete reversibility. As pointed out[1], the currently available reversible methods are unreliable and the reliable methods are not reversible. However, a number of clinical trials substantiate a view that it is indeed possible to have a male contraceptive that meets all the essential criteria in the near future[2-7].

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[8]. This may help elevate the socio-economic status of any country.

Neem or Margosa i.e. 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

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esteemed tree and has been closely associated with the socio-cultural and religious aspects of Indian life science ancient time. This tree has been so important and invaluable that from its various names, its worth can be guessed[9,10]. 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[11]. Various studies have been reported on the safety evaluation of different parts of neem as well as its various biologically active products[12-15]. 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 potential reproductive sites of mouse to exploit new male contraceptives.

The epididymis and vas deferens are the sites which can be exploited for male contraception without undue side effects, the maturation of mammalian spermatozoa is acquired in the epididymis, where spermatozoa gain their motility and fertilizing capacity.The epididymis play active roles 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[16,17]. Although several epididymis-specific secretory proteins have been identified, little is known about the sperm maturation events in the epididymis[18].

The only effective technique available for male contraception is vasectomy, being practiced world wide, despite that it is a permanent surgical procedure and its successful reversal is not assured[19,20].

Although the transport of spermatozoa from the testis towards the ejaculatory duct could be altered or arrested at the level of the testis, epididymis or vas deferens. Among the options available for the regulation of male fertility, disruption of sperm transport in the vas deferens is an attractive one, the vas deferens is the site where intervention would be possible with minimal hormonal and systemic interference and may give better scope for reversal.

The present study was design to reveiw the histological alteration of the issues related to various potential sites of contraception by Iranian cultivated AI alcoholic seed extract.

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.

Fig. 1: Tree of AI cultivated at Dashteh Moghan district of Ardabil province, Iran botanical garden

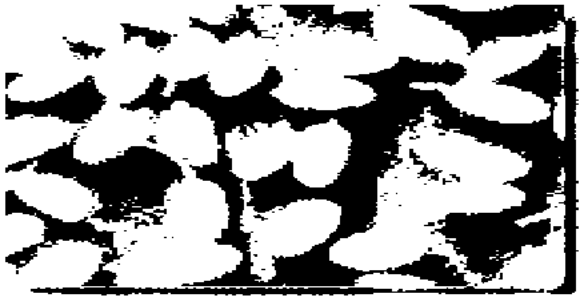

Fig. 2: Fresh seeds of AI cultivated at Dashteh Moghan district of Ardabil province, Iran botanical garden

Fresh seeds of AI (Fig. 1) cultivated at Dashteh Moghan district of Ardabil province, Iran botanical garden (Fig. 2) were used.

The extract was prepared according to WHO protocol CG-04 for the preparation of an alcoholic extract[21]. In brief, the seeds were shed-dried, powdered and extracted with 95% ethanol (v/v) at 55-60°C for 3 h. The solvent was distilled off under reduced pressure and the resulting mass was dried under vacuum and kept at.-24°C until use.

Sixty colony bred, healthy adult male and female albino mice of Swiss strain, weighing between 25 and 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 h light 12 hr dark regimen. Animals were fed with pelleted 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 15 mL kg^{-1} B.W. 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 kg^{-1}

b.wt./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 100 mg kg^{-1} b.wt./day(4 mg/0.2mL of distilled water/mouse); oral for15 days.

The 50 mg kg^{-1} body weight/day showed no significant change in epididymal sperm motility.as compare to the control. Therefore the dose was changed to 100 mg kg^{-1} B.W. /day for 15 days.

A suspension of the extract was made everyday in sterile distilled water (100 mg mL^{-1}) prior to administration. The required extract was administered orally with a glass syringe fitted with a feeding needle.

The animals were orally administered a daily above mentioned dose 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°C and exposed to 12 to 14 hrs day light.

Schedule of sacrification: After 24 hrs from the last dosing, the animals were weighed and sacrificed under ether anaesthesia.

Histology: Epididymides and vas deferens from control and treaded mice were fixed in alcoholic Bouin's fixative and embedded in paraffin. Serial longitudinal sections through the medial aspect of the organ were cut at 10 μm and stained with hematoxylin and eosin.

Male mice were sacrifised by an overdose of diethylether then vas deferens and epididymides were removed, weighed. Organs were fixed by immersion in alcoholic Bouin's fixative for 24 h and dehydrated through graded alcohols before the entire organ was embedded in paraffin. Serial longitudinal sections through the medial aspect of the organ, incorporating the caput and cauda regions and vas deferens were cut[22].

Scanning electron microscopy: 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[23]. This modification involved impregnation of sperm with 1% tannic acid, which serves as a mordant, as noted above. 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.

RESULTS

The 50 mg kg^{-1} body weight/day showed no significant change in epididymal sperm motility.as compare to the control. Therefore the dose was changed to 100 mg kg^{-1} B.W. /day for 15 days.

Scanning electron microscopy : Scanning Electron Microscopic (SEM) photographs showing in the morphology of normal and 15 days AI seed extract treated mice spermatozoa from the cauda epididymis Fig.3 and 4 represent SEM of normal cauda epididymis.

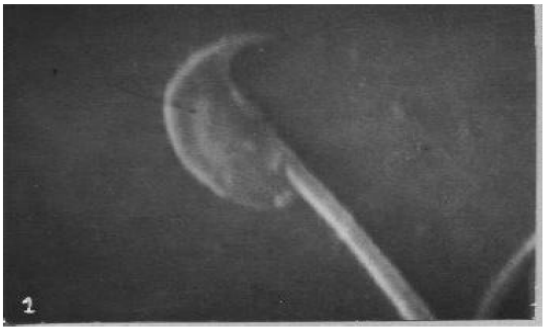

Fig. 3: SEM photograph cauda epididymis sperm of of normal fertile, adult mouse showing the scimitar shaped head. X 2750

Fig. 4: SEM photograph of mid-piece region of control mouse cauda epididymis spermatozoa showing the typical buldge towards the rear end of the mid piece. X 2500

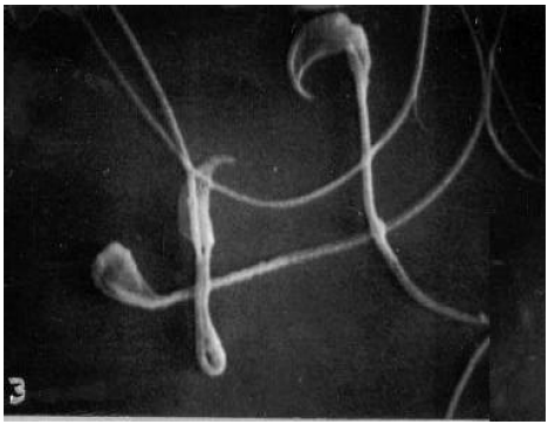

Fig. 5: SEM photograph of cauda epididymis spermatozoa of 15 days AI seed extract treated mice, showing abnormal head and bent mid piece regions. X 1000

Fig. 6: SEM photograph cauda epididymis spermatozoa of 15 days AI seed extract treated mice showing abnormal head region.X 4125

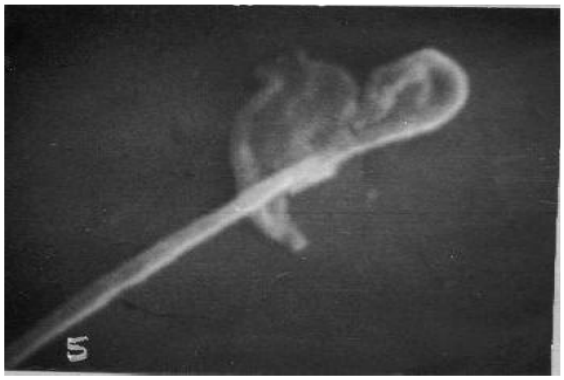

Fig.7: SEM photograph cauda epididymis spermatozoa of 15 days AI seed extract treated mice showing decapitated head and bent mid piece region. X 2500

Normal fertile, adult mouse showing the scimitar shaped head (Fig.3) and mid-piece region of control mouse cauda epididymis spermatozoa showing the typical buldge towards the rear end of the mid piece (Fig.5).

Figure 5, 6 and 7(X 1000, X 4125, X 2500) display SEM photograph of cauda epididymis spermatozoa of 15 days AI seed extract treated mice, showing abnormal head and bent mid piece regions, abnormal head region, decapitated head and bent mid piece region.

Histology: Histoarchitecure of epididymis and vas deferens of treated mice were affected. Afeter treatment, disorganization of epithelium, pycnosis of cell nuclei and clumping of the sperms in epididymis and vas deferens were observed.

The caput epididymis of control animal consists of many tubules with pseudostratified epithelium having sterocillia. In between the tubules is the intertubular connective tissue fibres. The lumen of the tubules contain sperm bundles (Fig. 8). The histology of the caput epididymis in 15 days treated animals showed disorganization of epithelium as compared to the control ones. The tubular epithelium cell nuclei were also pycnotic (Fig. 9).

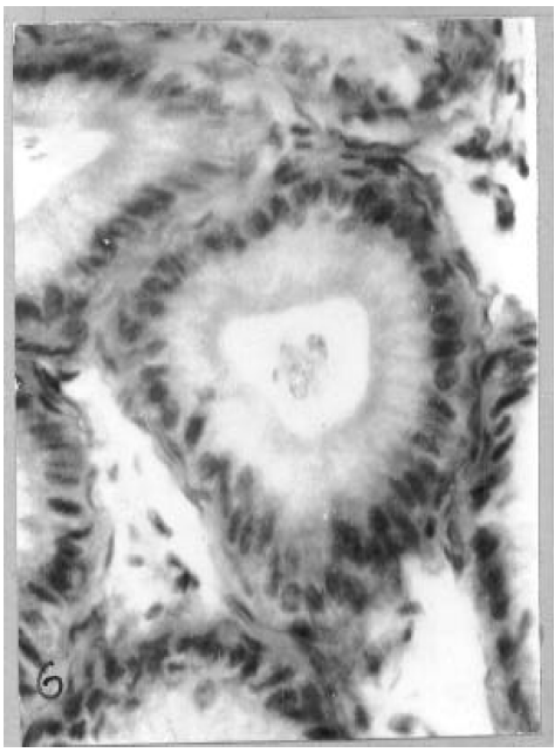

Fig. 8: T.S. of caput epididymis of control mouse showing tubules With pseudostratified epithelium having stereocilia and Sperm bundles. X 600

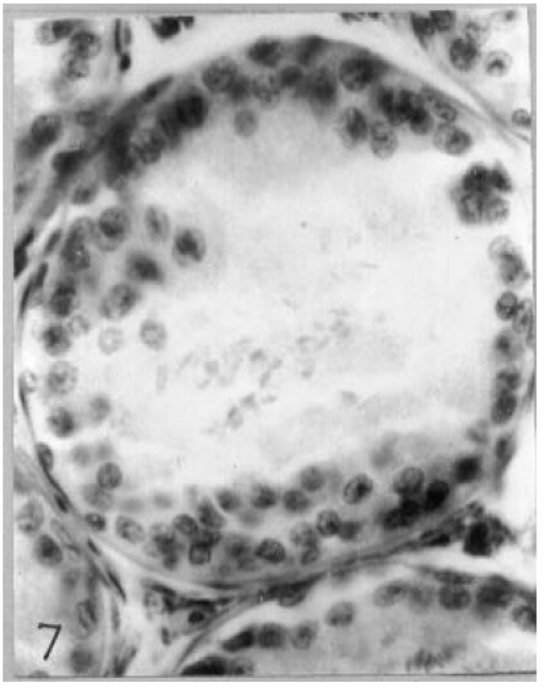

Fig. 9: T.S. of caput epididymis of AI seed extract treated mouse. Pycnosis of epithelial nuclei and disorganization of epithelium. X 600

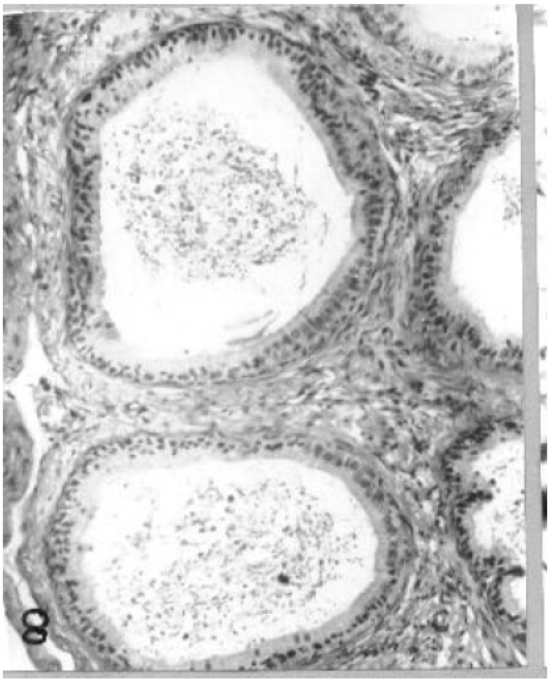

Fig. 10: T.S. of cauda epididymis of control mouse showing Typical pseudostratified epithelium having stereocilia X 120

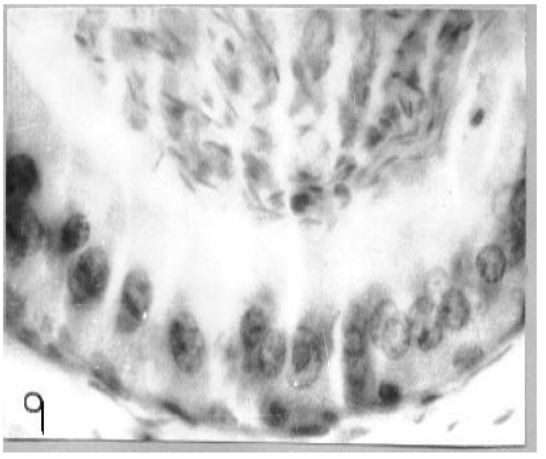

Fig. 11: T.S. of cauda epididymis of AI seed extract treated mouse.X 700

The histology features of the cauda epididymis were not affected in treated mice, except that there occured a slight decrease in the tubular diameter as compared to control (Fig. 10 and 11).

The proximal vas deferens of control animal consists of 3 muscle layers. Viz., middle circular and inner longitudinal layers.The lamina proporia is present in between inner longitudinal muscle layer and pseudostratified epithelium and stereocilia. The lumen is round with sperm bundles.

Histological characteristics of proximal vas deferens were not altered in15 days treated mice in comparison to control (Table 1).

The distal vas deferens of control animal has similar structure to that of proximal region. But, the epithelium layer is folded so as to form a star shaped lumen and the lamina propria is more prominent (Fig. 12).

Histological characteristics of distal vas deferens in 15 days treated animals showed pycnosis of epithelial cell nucei and clumping of sperms as compared to control (Fig. 13).

DISCUSSION

Lack of male involvement and lack of widely acceptable reversible male methods are separate but interlinked issues. The first issue can be addressed immediately by a change in programme focus, in taking men into account, in gaining their support for their partner's decision to use a method and in encouraging them to use a male method. The lack of acceptable reversible methods for men can only be addressed through a commitment to research[24].

Table I: Histocytometric data(µm) of caput, cauda epidisymis and vas deferens of control and Azadirechta Indica seed extract treated mice

Tissue	Histocytometric data	Control	Azadirechta Indica seedextract treated mice
Caput Epididymis	Td[a]	140.2±2.0	136.8±1.7
	ECH[b]	30.9±2.15	29.37±1.09
Cauda Epididymis	TD	192.1±5.01	190.00±3.72
	ECH	6.25±0.8	16.02±0.8
Vas deferens			
Proximal	ECH	29.00±2.01	28.5±1,83
Distal	ECH	45.04±2.26	44.82±2.07

a= Tubular diameter. b= Epithelial cell height

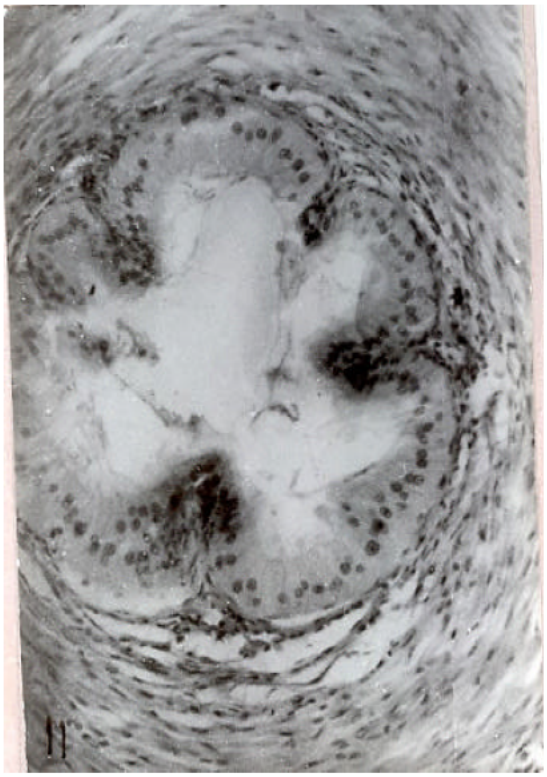

Fig. 12: The proximal vas deferens of control animal consists of 3 muscle layers. Viz., middle circular and inner longitudinal layers.The lamina proporia is present in between inner longitudinal muscle layer and pseudostratified epithelium and stereocilia. The lumen is round with sperm bundles

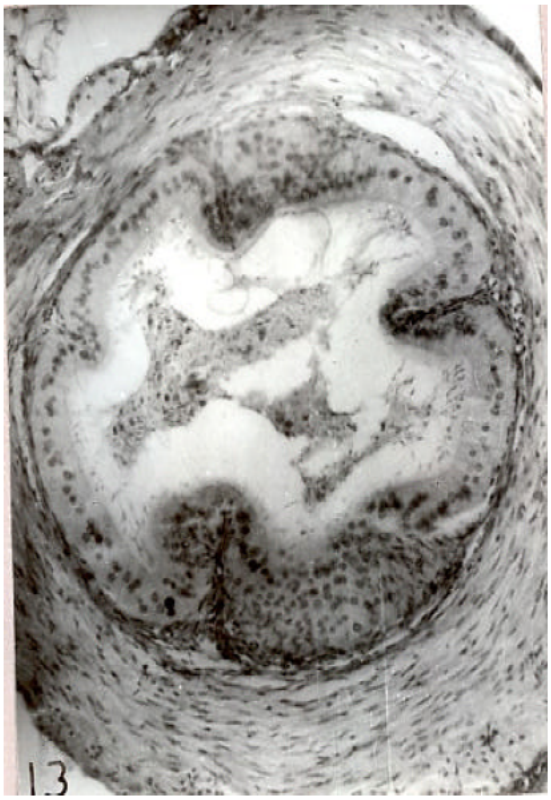

Fig. 13: Histological characteristics of distal vas deferens in 15 days treated animals showed pycnosis of epithelial cell nucei and clumping of sperms

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. In a large survey of attitudes of married men in rural area, the majority had no concept of family spacing, and had not taken any initiative to improve their knowledge or acceptance of condoms or vasectomy[25].Among the options available for the regulation of male fertility, disruption of sperm transport in the vas deferens or epididymis is an attractive one. Our study is directed toward understanding the histological alteration of the epididymis and vas deferens to develop novel strategies for male contraception based on posttesticular sperm maturation in the epididymis and vas deferens.

Recently many laboratories are engaged in developing a male contraceptive from plants[26]. 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. Recent extensive efforts have been made to study the antifertility drugs from plants[41-43]. 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.

The importance of the neem tree has been recognized by US National Academy of Sciences[27], which published a report in 1992 entitled 'Neem-a tree for solving global problems'.For thousands of years the beneficial properties of Neem (Azadirachta indica A. Juss).

The present study took into consideration the structure of reproductive organs of adult male mouse. The effect on sperm morphology and histological studied in particular. All the parameters were assayed accoreding to WHO Protocos MB-50 and CG-04[28,29], 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 1) which indicates that the extract did not promote body weight gain causing obesity and/or water and electrolyte retention. Our findings are partially in accordance with the investigations of Mali P.C. *et al.*,[30] that reaveld neither doses of colocynthis root they used (50. 100 and 200 mg kg^{-1} B.W./day) altered body weight of the animals and Chinoy N.J.and M.Geeta Rang[31] whostudied the effects of cartica Papaya seed extract on the Physiology of the Vas deferens of albino rats.This is significant since Na^+/K^+ balance is important for maintaining the microenvironment particularly of epididymis for sperm maturation[31].

According to khan P.K.[32], the leaf extract of neem was evaluated the extract was found to induce structural and numerical change. 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, where in SEM photographs showd 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 in 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 has potential as antifertility agent, particularly as spermicidal agent. According to khan P.K.[32], the leaf extract of neem was evaluated the extract was found to induce structural and numerical change that corrobating present study.

Among the options available for the regulation of male fertility, disruption of sperm transport in the vas deferens is an attractive one. Histoarchitecure of epididymis and vas deferens of treated mice by AI seed extract were affected. Afeter treatment involving,disorganization of epithelium, pycnosis of cell nuclei and clumping of the sperms in epididymis and vas deferens were observed in this study.

Upadhyay SN[33] reported that male Wistar rats of proven fertility were given a single dose (50 microliters) of neem oil in the lumen of the vas deferens on each side; all males treated with neem oil remained infertile throughout the 8 months of observation period. Epididymal and vas histology were normal without any inflammatory changes or obstruction.The intra-vas administration of neem oil resulted in a block of spermatogenesis without affecting testosterone production; the seminiferous tubules, although reduced in diameter, appeared normal and contained mostly early spermatogenic cells which partly corroborates our morphological observations.

Among the options available for the regulation of male fertility, disruption of sperm transport in the vas deferens is an attractive one.

It is known that the accessory sex organ viz.epididymis and vas deferens are androgen dependent target organs and manifest differential sensitivity to androgens for maintenance of their structure and function[31].It is also known that any change in circulating androgens would affect the internal microenvironment of epididymis and thereby lead to alteration in sperm motility and metabolism[31]. In the present study therefore all the changes observed in histology of epididymal and vas deferens as well as sperm morphology would be the result of probable androgen deprivation effect to these organs.

Vasectomy is the only available method of male contraception adopted world wide in family welfare programmes. However, some of the concerns, particularly the need for surgical intervention and the enhanced anti-sperm antibodies that may cause failure of reversibility, even after skilled vasovasostom, require serious consideration[34]. An ideal intravasal device used for male contraception should be easy to insert, flexible, prevent sperm passage and be capable of easy removal to restore vas patency and so permit the return of fertility. Initial attempts have been made to solve the problems associated with the reversal of vasectomy[35].

A number of substances can cause permanent sterilization by injection. Since 1964, researchers have tested at least 26 different combinations of chemicals in the vas[36-38]. The only two requirements for the chemical are that it be nontoxic and be a sclerosing agent, an agent which will produce enough scarring of the vas wall to block the vas as was observed in the AD alcoholic seed extract of the present study which followed that.

Mason[39] American researcher was experimenting with intra-epididymal (rather than intra-vas) injections of

pH-neutral zinc rather than sclerosing agents, but until this research would be more accepted most attention focused on vas-based injections. Hence further investigations are warranted and being pursued.

CONCLUSIONS

Azadirachta indica therefore is thought to have the effect on the transport of sperm into the epididymis with simultaneous maturation of spermatozoa, suspension of sperm in seminal plasma during ejaculation or penetration of ovum by spermatozoa. The mechanism of action of seed extract was probably via altering the hormone levels and causing androgen deprived effect to target organ. This alters epididymal milieu and affects the spermatozoa. It is concluded that this option is likely to meet the need for long-term reversible male contraception with minimal invasive intervention in the near future. Further studies are called for understanding the exact mechanism.

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