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CHANGE IN SPERM ULTRA-STRUCTURE OF (*MACACA MULATTA)* MONKEYS AFTER VAS OCCLUSION WITH RISUG AND ITS NON-INVASIVE REVERSAL

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ABSTRACT

Birth control, also known as contraception and fertility control, is methods or devices used to prevent pregnancy. Although different contraception methods work in different ways, contraception generally prevents sperm from reaching and fertilizing an egg which is how a pregnancy starts. Birth control methods have been used since ancient times, but effective and safe methods only became available in the 20th century. This is followed by a number of hormonal contraceptives including oral pills, patches, vaginal rings, and injections. Less effective methods include barriers such as condoms, diaphragms and contraceptive sponge and fertility awareness methods. The most effective methods of birth control are sterilization by means of vasectomy in males and tubal ligation in females is not usually reversible. RISUG (Reversible inhibition of sperm under guidance) is a non-hormonal male contraceptive and it could be reversed easily. In male monkeys pretreated vas occluded with RISUG has been attempted at the level of semen physical parameters, semen biochemistry & ultra-structure of sperm and its non-invasive reversal procedure has been assessed in all injected monkeys after vas occlusion. The result revealed that following non-invasive reversal narmospermia obtained after three successive ejaculations in all the animals and did not show any remarkable changes in semen physical & biochemical parameters as well as Ultra- structure of sperm. Therefore the result shows that the RISUG is effective for male contraceptive as well as reversed through non-invasive procedure and has no any adverse effects.

Key Words: RISUG, Male Contraception, Vas occlusion, Non-Invasive reversal: Monkey.

INTRODUCTION

A Number of investigations to block the lumen of the vas deferens using defferent chemicals and solid plugs have been carried out [1-3] Many Studies show that men want access to better contraceptives. In a recent study of British men, 80% placed a hypothetical male pill as one of their top three contraceptive choices [4]. Another study found that over 60% of men in Germany, Spain, Brazil and Mexico were willing to use a new method of male contraception [5]. Long acting and reversible fertility control still remains a challenge for the scientific community. Various contraceptive methods, including the use of hormone medications, intrauterine contraceptive devices, barrier contraception, periods of abstaining from sex, and surgery have been developed over the last few decades to prevent fertilization. Yet contraception failure and user dissatisfaction is not decreasin [6]. The disadvantages of the two most common male contraceptive methods are not trivial: vasectomies are not readily reversible, and condoms have a high typical use failure rate. Condoms account for an additional 13% of contraceptive use in developed countries [7]. Vasectomy is the first vas-based surgical male contraceptive method and the only effective technique known as the most simple, popular and readily available form of voluntary family planning for men [8]. However the acceptance of vasectomy has been confined mostly to a small number of people and its global acceptance rate is only 10% [9, 10]. In India, only 2% of reproductive age males rely on

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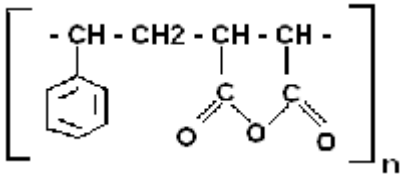
vasectomy for birth control, mainly because it is a surgical intervention and of the false apprehensions that the procedure reduces sexual potency [11]. Contraindications to vasectomy include scrotal pathology, haematoma, allergy to local anaesthesia, genito-urinary or groin infections and sperm granulomas [12, 13]. A limitation of the vasectomy is that its reversibility cannot be possible and therefore need for another method of vas occlusion with higher success rate of reversibility. Among the several reversible vas-occlusive procedures, Intravasal injectable contraceptive using RISUG is an injectable compound that partially blocks the vas deferentia (tubes that carry sperm), providing effective contraception for up to 10 years per dose. It is effective immediately. This contraceptive has completed Phase I and II clinical trials in India [14, 15]. It has been in Phase III clinical trials in India since 2002 [16]. RISUG injected in vas deferens no-scalpel procedure, is considered to be a batter alternative to vasectomy [17] Reversible inhibition of sperm under guidance (RISUG) is an injectable male contraceptive of polyelectrolytic nature possessing antimicrobial properties [18], has two contraceptive effects: partial blockage of the vasa deferentia and disruption of the sperm that pass through it. Researchers postulate that RISUG ruptures the membranes by stressing their ion exchange mechanisms [19] Sperm that are present in the ejaculate after RISUG has been administered have broken cell membranes [20].

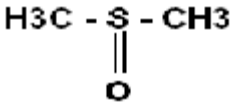
MATERIALS AND METHODS

Drug

RISUG (Reversible Inhibition of Sperm under Guidance) is composed of a copolymer of styrene and maleic anhydride (1:1) ratio. The styrene maleic anhydride complex with Dimethyl sulphoxide (DMSO) in (1:2) ratio.



(Styrene Maleic Anhydride)



(Styrene Maleic Anhydride)

Animal

Seven healthy adult male rhesus monkeys of *Macaca mulatta* strain, weighting 9 to 11 kg were purchased from Central Animal Facility (CAF), A.I.I.M.S. after approval from Institutional Animal Ethics Committee (IAEC) of All India Institute of Medical Sciences. The animals were housed individually in the Iron cages. All the animals were subjected to pretreatment sampling at least 10 days intervals before subjecting them to treatment. The semen samples were collected by electro stimulations for this study.

Vas Occlusion

For the RISUG Injection a small incision was made close to the external inguinal segment and the vas deferens was exposed from the spermatic cord. 20 µl RISUG was injected into the lumen of the vas, after injection the spermatic cord sheath was closed with catgut suture. Before following this process animals were anesthetized with ketamine hydrochloride (NEON Laboratories Ltd., Mumbai) at 25 mg/kg body wt. This procedure was performed bilaterally. Post operative care was provided with antibiotics and anti-inflammatory drugs and all the animals had uneventful postoperative recovery.

Noninvasive reversal

Non-invasive reversal procedure was performed by the method of Lohia *et al*., [19] In 5 of 7 vas occluded monkeys under mild anesthesia using ketamine aimed to propel the RISUG from the vas deferens to ejaculatory ducts. In this technique involved palpation, percutaneous squeezing of vas deferens at the scrotal and inguinal segment, electrical stimulation and viberation through viberator at the inguinal segment, supra-pubic percussion and per-rectal digital massage, a finger massage to the ampullary segment of the vas deferens in successive steps. The procedures were repeated 3-4 times.

Parameters

a)Semen analysis

Semen volume, pH, color**,** liquefaction time, sperm concentration, motility and sperm morphology were recorded according to the WHO [21] laboratory manual for the examination of human semen and sperm-cervical Mucus Interaction.

b) Seminal Plasma biochemistry

Seminal plasma free of spermatozoa, obtained after centrifugation was used for the biochemical analysis of Acid phosphatase Fructose & Glycerylphosphoryl Choline (GPC).

c) Ultra-structure of sperm

For ultrastructural studies, the spermatozoa were separated by centrifugation, washed with phosphate buffer (0.1 M; pH 7.2) and collect pellets by centrifugation. The pellets were immediately fixed in 2.5% glutaraldehyde buffer for 30 min and washed three times in phosphate buffer. For SEM, a thin film of spermatozoa was smeared on an SEM stub with silver paint, air dried, sputter coated with gold and observed under a scanning EM (Model Leo 435 VP). For TEM, the sperm pellet was post fixed in 1% OsO4 for 30 min, washed, dehydrated in acetone,

embedded in low viscosity spur media and polymerized at 60 °C for 48 h. The ultrathin sections were stained with uranyl acetate and lead citrate and observed under Philips TEM (Model Morgagni 268D).

RESULT:

There was no any morbidity observed in the animal after non-invasive reversal.

Semen analysis

After non-invasive reversal semen physical parameters did not show any considerable changes from those of pre-treatment samples. Non-invasive reversal influence, spermatozoa appeared in the semen with low sperm counts which steadily augment and improved subsequent ejaculations, and normospermia was obtained after third ejaculation. Sperm motility of the animals, from first ejaculation to third ejaculations illustrates gradually improvement following the reversal. Normal Viability of the spermatozoa was obtained in 4 of 7 animals through sperm viability test by Nigrosin-eosin method. Partial viability and dead spermatozoa were observed in remaining 2 animals. In all animals sperm viability normal like pretreatment was attained subsequent to third ejaculation. (Table 1-3)

Seminal plasma Biochemistry

In pre-treated semen samples Acid phosphatase, Fructose LDH, GPC respectively they slight turn down later than vas occluded. All these parameters illustrated regular improvement following non-invasive reversal and they arrive at pretreatment level after fourth to fifth reversal (Table-1-3).

Ultra-structure of spermatozoa

In pre treated monkeys semen showed normal sperm (Fig 1) after vas occlusion with RISUG azoospermia were found (Fig2) and after reversal normospermia was obtained following first three ejaculations, prior to these ejaculations oligospermia was observed. The ultra-structure of first three ejaculated spermatozoa, scanning electron microscopy (SEM) exposed severe coiling and rupture of tail (Fig3). Observation by transmission electron microscopy (TEM) in pre treated monkeys shows normal sperm head (Fig 4) after vas occlusion, azoosperma showed with ruptured tail (Fig 5) and in non-invasive reversal samples revealed spoiled membrane in the sperm head, degeneration of mitochondrial sheath However the normal morphology of the spermatozoa was observed followed by third to fourth subsequent ejaculations. (Fig 6)

Table 1. Pre-injection Semen Examination

Animal No:-	**M-02**			**M-04**			**M-05**			**M-07**			**M-08**		
Sample No.:-	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
1.Volume of semen*	1.2948	0.9088	1.3754	0.2786	0.3892	0.298	0.2337	0.2078	0.3542	1.2151	1.0404	1.1319	1.0489	1.0611	1.0011
2.Sperm mili/ml**	325	290	296	352	348	380	258	270	282	395	420	295	640	345	325
3.Sperm Motility	85	80	80	80	70	85	80	85	85	80	70	80	70	75	70
4.Sperm Viability%	90	90	85	85	75	90	85	90	90	95	88	95	94	88	90
5. Sperm Morphology	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**	**N**
Seminal Plasma Biochemistry Examination															
1. Fructose	13	12.5	12.8	11	12.25	11.54	11	11.2	12.25	11.5	10.9	11.2	11	12.2	11.5
2.Acid phosphatase	54.52	53.25	52.85	50.2	50.75	49.8	49	51.25	50.8	52.5	49	53.5	49.2	48	48.2
3.LDH	27.52	26.8	27	25.5	24.2	24.5	22.25	24	23.5	24.9	25.5	25	24	22.8	23.5
4.GPC	2	1.9	2	1.62	1.55	1.7	1.65	1.72	1.6	1.8	1.95	2.1	1.9	1.8	1.9
***Volume = in ml ; **Sperms count in million per millilitre, A = Abnormal sperms: N = Normal, Occ = Occassional, C = Curve**															

Table 2. Post-injection Semen Examination

1.Volume of semen*	0.0162	1.295	1.375	0.2543	0.3452	0.443	0.291	1.0186	0.271	0.271	0.374	0.32	0.2073	0.293	0.2891
2.Sperm mili/ml**	2	Occ	0	3	Occ	Occ	3	Occ	Occ	5	Occ	Occ	10	Occ	Occ
3.Sperm Motility	33	0	0	15	0	0	3	0	0	10	0	0	5	0	0
4.Sperm Viability%	55	0	0	55	0	0	40	0	0	50	0	0	45	0	0
5.Sperm Morphology	**A**	**C**	**0**	**A**	**C**	**0**	**A**	**C**	**0**	**A**	**C**	**0**	**A**	**C**	**0**
Seminal Plasma Biochemistry Examination															
1.Fructose	9.5	11	10.25	10.5	10	9.5	11.8	10.5	10	11.2	11	11.5	11.2	10.2	9.5
2.Acid phosphatase	38	41	38.75	40.5	38	37	39	37	37	40	39	38.5	39.5	39	38
3.LDH	22	24	22.8	20	18	17	18.5	18	17.5	21.2	19.5	18	18	17.5	17
4.GPC	0.5	0.39	0.4	0.4	0.45	0.5	0.45	0.48	0.35	0.5	0.38	0.42	0.4	0.38	0.32

Table 3. Post-reversal Semen Examination

1.Volume of semen*	0.4652	0.8642	1.3105	0.2022	0.2025	0.283	1.0225	0.856	1.036	1.008	0.8253	0.9024	1.0232	0.8062	0.8256
2.Sperm mili/ml**	25	95.5	150.5	18.8	86.2	130	29	100.5	135	22	98	135	25	95.5	145
3.Sperm Motility	45	50	75	20	40	70	25	50	60	30	40	40	30	40	50
4.Sperm Viability%	65	60	95	50	65	85	55	65	65	45	55	50	40	45	55
5.Sperm Morphology	coil + N	N	N	coil+ N	N	N	Coil+N	N	N	Coil+N	**N**	**N**	Coil+N	**N**	**N**
Seminal Plasma Biochemistry Examination															
1.Fructose	11	12.5	13.5	10	11.5	12.5	9.5	11	12	10.9	11	11.7	10.8	11.2	11.5
2.Acid phosphatase	52.1	53.5	54	48.1	49.8	51.1	49.5	50.3	47.5	48.7	49.2	49.5	48.8	49	49.2
3.LDH	24	26	26.5	22.1	23.3	24.8	22.5	23.5	25.5	22.5	22.7	23.7	21.5	21.8	23.5
4.GPC	1.6	1.8	1.9	1.5	1.6	1.8	1.4	1.6	1.8	1.3	1.5	1.9	1.2	1.5	1.7

Fig 1. Pre Injection Normal Sperm (SEM)

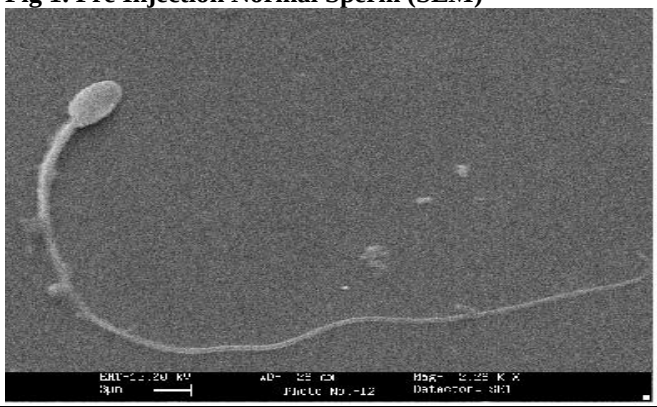


Fig 2. Post Injection Azoospermia (SEM)

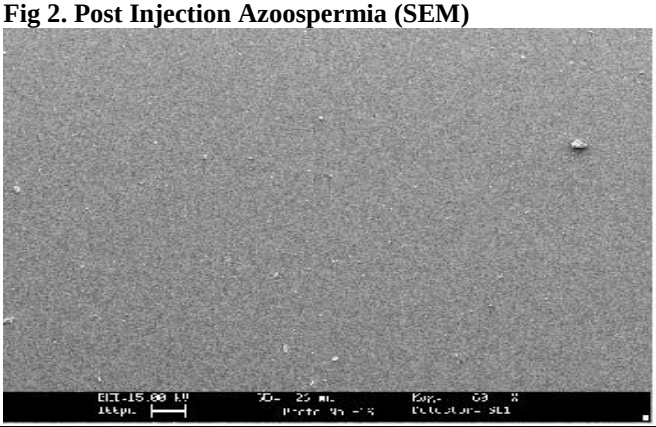


Fig 3. Post Reversal Coiled tail sperm (SEM)

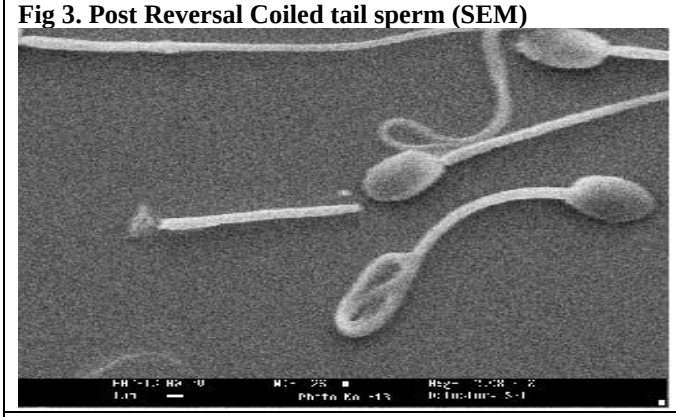


Fig 4. Pre Injection Normal Sperm Head (TEM)

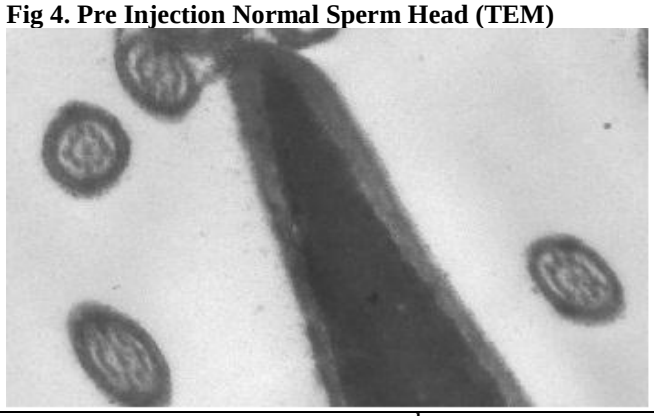


Fig 5. Post Injection Azoospermia (TEM)

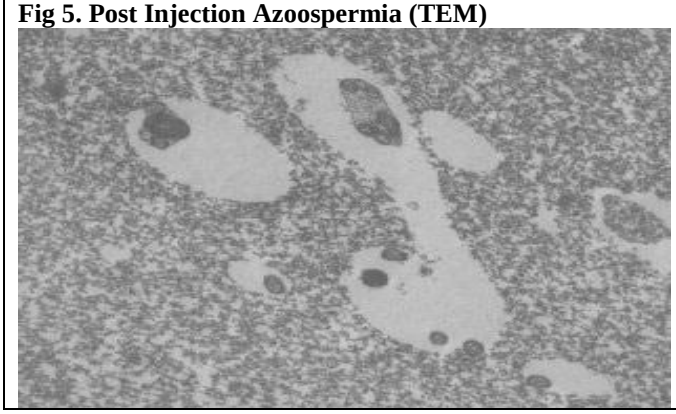
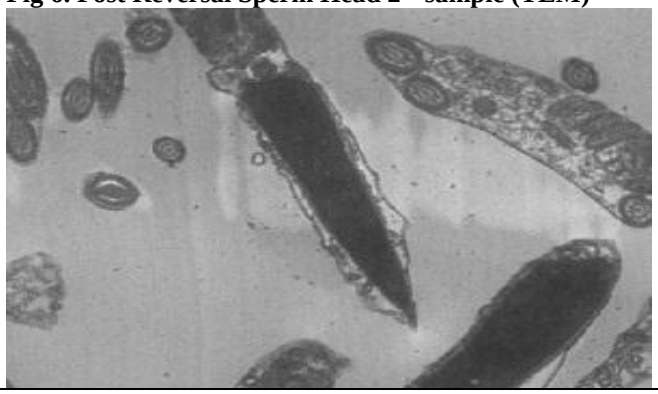


Fig 6. Post Reversal Sperm Head 2nd sample (TEM)



DISCUSSION

Vas occlusion by RISUG is a recent and effective procedure, Styrene maleic anhydride (SMA) is a hydrophilic polymer used in RISUG which origin an initial swelling at the site of administration. The mechanism of action of RISUG may be endorsed in part to a total or partial blockage and also the charge related effect of the polymer, which cause the majority of sperm to disintegrate. A few of those that escapes such an effect were found to be morphologically abnormal and thus they incapable of fertilization [22]. Vas occlusion with SMA has been proven to be the one that meets all the essential criteria of a male contraceptive [23]. Structural changes of the seminal vesicle and prostate in langurs following vas occlusion with SMA and after non-invasive reversal at the level of histology and ultra-structure along with the seminal plasma markers of these organs, Studied by Manivannan [24]. Azoospermia resulted after vas occlusion and continued until the non-invasive reversal; spermatozoa of the initial ejaculations were immotile with damages in the acrosome and mid- piece, indicating that the spermatozoa are incapable of penetrating the eggs [19]. Although fructose, acid phosphatase in the seminal plasma showed a reduction following vas occlusion, it could not be related to the morphology of seminal vesicle and prostate [24]. Scanning electron microscopy (SEM) revealed severe coiling of tail, rupture of acrosomal envelope, and bent midpiece associated with damaged mitochondrial sheath. Observations by transmission electron microscopy (TEM) revealed vacuolization in the nucleus, membrane damage in the acrosome, loss of segmented columns, and numeric aberrations in the centriole of the neck, as well as degeneration of mitochondrial sheath and axoneme in the midpiece, and absence of outer plasma membrane in the midpiece and tail indicate that the necrospermic status of the spermatozoa during initial ejaculations may offer instant sterility after vas occlusion with SMA [25]. Spermatozoa had recovered normal morphology and sperm function tests revealed that the vas morphology also regained a normal pseudostratifed columnar epithelium containing basal and principal cells suggest that the SMA-based spacing technique for male contraception could be extrapolated to the human by use of no-scalpel injection and non-invasive reversal [26]. Ultra-structural changes in the vas deferens of langur monkeys after vas occlusion with styrene maleic anhydride (SMA) and after noninvasive reversal, the vas epithelium regained a state of normalcy as evidenced by prominent plasma membrane, nucleus, cytoplasmic organelles, and stereocilia [24]. Vas occlusion with RISU and its non-invasive reversal do not damage the accessory reproductive organs [24]. Ultra-structure of the testes after vas occlusion revealed vacuolization in the cytoplasm of Sertoli cells and degenerative features in the membranes of the spermato-cytes and spermatids in the affected seminiferous tubules. The sub- cellular features of the normal tubules were similar to those of controls [27]. Ultra- structure of spermatozoa by SEM & TEM indicates that the spermatozoa achieve fertilizing skill after reversal. The semen biochemistry shows that reversal procedure does not affect the reproductive gland. The reduction of LDH and GPC in the seminal plasma in vas occluded animal and their reversal to normalcy, following reversal in the present investigation could be mainly due to obstruction in the vas deferens as these are the components mainly of testicular and epididymal origin [28]. The present investigation express that the reversal of vas occluded by RISUG through a non-invasive approach is feasible even after reversal and restoration to normalcy of morphological and physical characteristics of spermatozoa this makes RISUG globally more adequate than any other vas based method.

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