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·*Original Article*·

Biophysical mechanism-mediated time-dependent effect on sperm of human and monkey vas implanted polyelectrolyte contraceptive

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Abstract

Aim: To determine the short and long-term morphological effects on sperm as induced by intra-vas alteration of pH and electrical charge. **Methods:** Desired biophysical influences were obtained by injection of reversible inhibition of sperm under guidance (RISUG) into the lumen of the vas deferens of human subjects and the monkey. RISUG is a polyelectrolyte hydrogel complex of styrene maleic anhydride (SMA) and dimethyl sulfoxide (DMSO) which generates an electrostatic charge and also lowers in a near space of pH domain. The morphology of sperm was examined by light microscopy, scanning and transmission electron microscopy. Human study enabled semen collection by masturbation as early as 3 h after injection and studies extended up to 6 months. In the monkey, on vas excision after RISUG implantation, sperm characteristics were examined in serial sections. **Results:** Semenology in clinical studies and histological data of the monkey showed a time-sequenced sperm plasma membrane, tail mitochondria and nuclear decondensation alterations in sperm structural components, which beared marked similarity to changes in the sperm head and tail during capacitation and entry into the ovum. **Conclusion:** The findings provide a means of causing such changes in the sperm that inhibit the fertilizing ability before the nucleus is affected. Therefore achieving non-obstructive vas-based contraception, without genotoxic or teratogenic effects caused by infertile sperm passing into the semen, is feasible. *(Asian J Androl 2007 Mar; 9: 221–227)*

Keywords: reversible inhibition of sperm under guidance; vas deferens; electrical charge; sperm; electron microscopy; acrosome; mitochondria; chromatin decondensation

1 Introduction

Sperm morphology and function are known to be affected with systemic administration of chemical agents, but effects following injection into the vas deferens remain undefined. Limited available data pertains to a reduction in sperm count brought about by the blocking action of passive lumen occluding agents such as silicone rubber and polyurethane. Consequences of partially occluding intra-vas bioactive compounds require further investigation for insight into long-term close interaction of a motile gamete with a bioactive foreign body. *In vitro* preparations are not sustainable because of sperm

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protein denaturation and bacterial growth and, hence, do not provide the necessary information. With polyelectrolytic intra-vas bioactive compound, a very special condition prevails. The motile sperm, possessing a negative electrical surface charge, is exposed to the electrical charge and acidifying pH of the polyelectrolyte. A consistent result will have a bearing on fertility control and possibly on infertility management.

The polyelectrolyte styrene maleic anhydride (SMA) complexed with dimethyl sulfoxide (DMSO), given the name 'reversible inhibition of sperm under guidance' (RISUG), was selected on account of its electrical charge and pH lowering action on hydration and biocompatibility.

Earlier studies have shown that over a period of 72 h after injection three changes occur. First, the drug forms a precipitate inside the lumen, which appears as flakes seen under scanning electron microscopy [1]. The flaky character of the precipitate forms a maze of passages along which sperms can pass brushing the flakes. Second, the precipitate develops a surface electrical charge. Microelectrophoresis shows that small aggregates of hydrated RISUG have different electrical charges. There is a surface mosaic of positive and negative charges with the positive dominating. Concurrently the pH in the vicinity of the precipitate falls. Hence, the sperms passing along the passages in between the precipitate flakes are subjected to an electrical charge stress and pH stress. Third, the vas peristalsis propels some of the precipitate along the vas lumen towards the ejaculatory duct end. In time the precipitate swells by interaction with water molecules in the vas lumen. Microprojections of the flakes are formed and these invaginate into the vas mucosal folds thereby helping to provide a retentive force against evacuation by vas deferens peristalsis. Thus, a stable bioactive implant is formed along a length of the vas deferens, which may extend beyond the internal inguinal ring. In this manner, RISUG transforms from a drug without intrinsic electrical charge into a medical device — a stable implant that has an electrical charge.

DMSO is strongly alkaline and hygroscopic. A part of the styrene maleic anhydride is inevitably converted to styrene maleic acid, which considerably neutralizes the alkaline pH of DMSO. This action reduces the tissue reactivity of DMSO. However, because the sulphur moiety of DMSO is highly reactive it interacts with the etheric oxygen (-O-) of SMA, thereby forming an SMA-DMSO complex and dimethyl maleic anhydride. Positively charged amino groups of intra-vas fluid proteins are replaced by negative carboxyl groups resulting in an increase in the negative charges per amino group. Depending on the steric conformation of the SMA-DMSO-amino acid complexes, a mosaic of positive and negative charge domains occur and subject moving sperm to charge oscillations. The charge oscillations occur because morphological deformations during sperm movement lead to alterations in the domain spacing, which is alternating [2]. Polyelectrolytes by their charge interactions can affect enzyme-substrate distribution and reaction rates [3] and create domains in lipid bilayer membranes [4]. Sperms have a very distinctive lipid composition and alterations in the lipid structures are an integral part of the capacitation process prior to fertilization. The SMA-DMSO-amino acid complex probably affects sperm surface enzymes and lipid domains thereby increasing membrane fluidity and destabilizing sperm membranes. This happens in spite of the luminal pH being rendered acidic by the RISUG, a condition known to stabilize sperms [5]. Membrane breakdown produces acrosomal enzyme acrosin and hyaluronidase release and loss of fertilizing ability.

Safety, contraceptive efficacy and reversibility of a single intra-vas injection of SMA-DMSO combination has been assessed in animal models [6] and clinical studies [7]. The present report draws, in part, upon data obtained from clinical trial subjects of investigations that were not a part of this clinical trial.

2 Materials and methods

The present analysis draws upon information from studies on volunteer men and monkeys. The reasons behind considering humans and monkeys are:

1. Effects on spermatozoa soon after intra-vas injection of RISUG cannot be obtained from the monkey study because electro-ejaculation of the monkey to collect semen a short time after injection, which is done under general anesthesia, is not possible. Even after recovery from the anesthesia monkeys in our laboratory take several days to respond to electro-ejaculation attempts. In contrast, men who are injected under a local scrotal anesthesia have no difficulty in giving semen sample by masturbation 3 h after the injection.

2. Data on changes in the spermatozoa during transit in the vas deferens cannot be obtained in the human because medical ethics does not permit taking out an entire length of

the vas deferens in the human for experimental studies which is a procedure that could be done on the monkey.

3. The low dose administration effects and effects after 6 months could have been obtained both in the human and the monkey. Here the human data has been considered because in the human semen samples can be obtained with regularity whereas the rhesus monkeys in our studies cannot be ejaculated in the summer months.

2.1 Humans

Group I consisted of three subjects and semen was collected 3 h after RISUG injection. Group II consisted of six subjects who were administered a sub-therapeutic dose of 80 μL of RISUG into each vas deferens. Borderline fertility control is manifested but there were spermatozoa present in the ejaculate. Semen samples by masturbation were taken serially beginning 14 days or more after injection. Samples were considered for the analysis 3 months after the injection. Group III consisted of 12 subjects. These subjects were the volunteers in Phase II and Phase III clinical trials, who were administered the standard therapeutic dose of 120 μL of RISUG into each vas deferens. Semen samples by masturbation were taken serially beginning 14 days or more after injection.

All male volunteers were adults of age < 40 years, in good health and had 'proven fertility', with at least two living children, and had no history of having failed to induce conception despite unprotected intercourse over a period of 6 months. Prior to the RISUG injection, at least one semen sample was obtained by masturbation, with no specific period of abstinence, had good semen quality with: (1) count of > 20 million/mL, (2) > 50% spermatozoa having normal morphology; and (3) > 50% motile spermatozoa.

Semen was collected on masturbation and processed according to WHO Guidelines. Sperms were examined under light microscopy (LM) after HE staining, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In Group II subjects, who had intact sperms seen by a Triple Stain Technique (TST) [8], the acrosome reaction was determined. For TST the sperm cells were first stained with 1% Trypan blue and then smeared and fixed (3% glutaraldehyde) on a glass slide. The cells were then stained with 0.8% Bismark brown and 0.8% Rose Bengal at 40ºC and 24ºC respectively. After mounting the slides were examined with light microscopy.

2.2 Monkeys

Group I consisted of one adult (proven fertile) rhesus monkey with bodyweight above 7 kg. The entire length of the vas deferens was taken surgically from the scrotal segment to the ampulla exposed bilaterally. On one side knots were placed around the vas at intervals of 3 cm to isolate sections. Thereafter the entire length of the vas deferens was excised and transverse serial sections were stained with HE stain and examined under light microscope. The vas deferens on the other side was excised without placing knots and was frozen. Serial sections were taken to determine the diameter of the vas lumen along the length of the vas deferens.

Group II consisted of two adult rhesus monkeys, also proven fertile and with bodyweights above 7 kg. Into each vas deferens 120 μL of RISUG was injected. Six weeks post-injection semen samples were collected twice at intervals of 1 week by electro-ejaculation adopting penile stimulation. At 8 weeks post-injection the entire vas deferens was surgically exposed and knots placed around the vas deferens at intervals of 3 cm. The vas deferens was excised and transverse serial sections were stained with HE stain and examined under light microscope.

The polyelectrolyte drug RISUG is in the form of a sterile viscous liquid when in the delivery syringe, which has a special design to generate high-injection pressure. As mentioned, the styrene maleic anhydride component has a covalent bond linkage with the dimethyl sulfoxide. Molecular weight is high, typically of the order of 70 000 and hence the viscosity is high. To inject the drug by means of the blade of a sharp forceps a puncture is made in the midline of the scrotum midway between the penoscrotal junction and the testicular pole. First, the left vas is delivered through the puncture hole. It is very important to avoid damage to the vas deferens blood vessels and nerves because damage alters peristalsis and subsequent distribution of the drug. A 23-gauge needle is used for injection in the volunteer men as the optimum choice between flow of the viscous drug and minimal damage to the vas vessels and nerves. For monkeys, because the size of the vas is smaller, the injection site is often chosen more distal to the epididymal end of the vas above the external inguinal ring. All injections are delivered with the needle pointing distally and maintaining a compression of the proximal vas deferens so that there is no backflow of the drug towards the epididymis. Also it is important to maintain the compression on the vas for a

Table 1. Sperm parameters before and after injection, evaluated through scanning electron micrograph (SEM), $n = 50$. $^{b}P < 0.05$, $^{c}P < 0.05$, compared with before injection.

	Before injection	After injection
Head length (μm)	6.20 ± 1.27	5.70 ± 1.62[c]
Acrosomal Region (μm)	2.90 ± 0.48	2.13 ± 0.97[c]
Ratio (H/A)	2.10 ± 0.21	2.72 ± 0.40[b]
Head surface area (μm^2)	30.04 ± 5.87	21.6 ± 6.65[b]

Table 2. Nature of sperm observed before and after injection (n = 50).

	Before injection	After injection
Normal (%)	74.75 ± 2.21	39.75 ± 1.70
B category (%)	24.25 ± 1.85	38.50 ± 2.08
C category (%)	0	21.75 ± 2.14

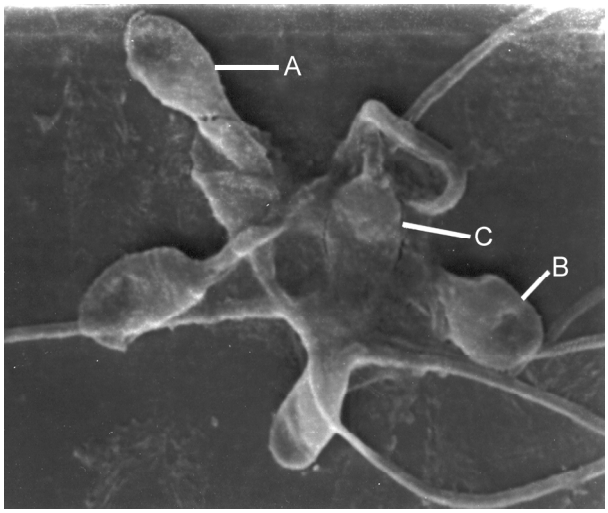

Figure 1. A scanning electron micrograph (SEM) of the semen of a human male subject taken 3 h after the injection. The cluster shows sperms with (A) anteriorly tapering head, (B) more polymer affected and deformed head, and (C) ruptured head surrounded with SMA polymer. Tails are near normal.

period of 2 min after the injection and withdrawal of the needle to ensure that some drug-intraluminal vas fluid interaction takes place and the drug becomes a gel with no backward flow.

3 Results

During pretreatment, the sperm typically had an anterior tapering head, which in HE staining had a light staining anterior part representing the acrosomal cap. A well-marked dark-stained region in the posterior two-thirds of the head existed. The tails were long with gradual curvature changes. The anterior two-thirds of the tail had a curve with the radius of curvature being one-third or more of the length of the tail. The morphology changed after exposure to RISUG.

3.1 Human subject Group I

Fifty percent of 3-h post-treatment sample sperm had an enlarged head with the anterior region of the head broader than the posterior region. The size of the light-staining zone reduced relative to the dark-staining region. Figure 1 is a SEM of the semen of a subject taken 3 h after the injection showing grades of affected sperms and polymer. Results are summarized in Tables 1 and 2. In Figure 1, sperm A is near normal, sperm B has a minimally affected head and sperm C has a markedly altered head structure. All sperms have near normal tails. TEM of semen samples from this group show in 50% of sperms only a patchy loss of plasma membrane with intact acrosomes. Twenty percent of sperms were significantly affected by the RISUG. Heads were enlarged and deformed with loss of the plasma membrane and the acrosome. Chromatin decondensation was present but chromatin was not quite in the granular form.

3.2 Human subject Group II

Morphological examination of sperms showed that the majority had undergone acrosome reaction. TEM shows an effect on acrosome, plasma membrane and the nucleus (Figure 2). Many sperms had a marked loss of acrosome and chromatin in granular form. A detailed acrosome reaction study on four subjects by triple stain technique showed that 65% of sperms had undergone acrosome reaction [9]. The ratio between dead acrosome-reacted sperm to live acrosome-reacted sperm varied widely from subject to subject. Generally, there are more dead sperms seen amongst the acrosome reacted sperm than the acrosome unreacted sperms in the same semen sample. The head may be quite deformed or even disrupted. Tails seem to be more resistant to RISUG. Approximately 80% of the sperms did have the tail but the electronmicrographic images showed that the tails too were af-

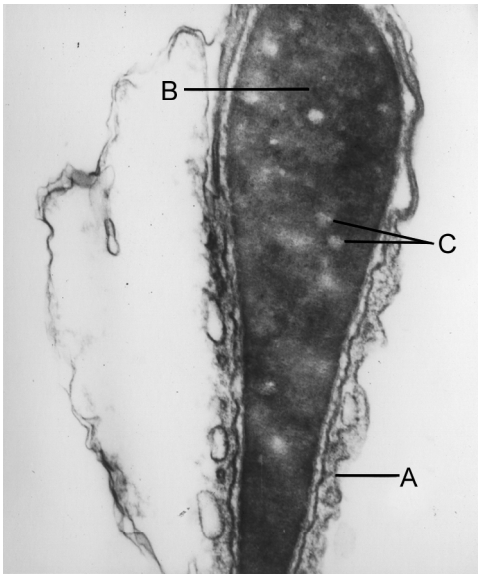

Figure 2. A 6-month human postinjection transmission electron micrograph (TEM). (A): shows losses in the plasma membrane with disturbance to the acrosome; (B): nuclear changes in the form of chromatin decondensation; (C): dispersion of the nuclear vacuoles.

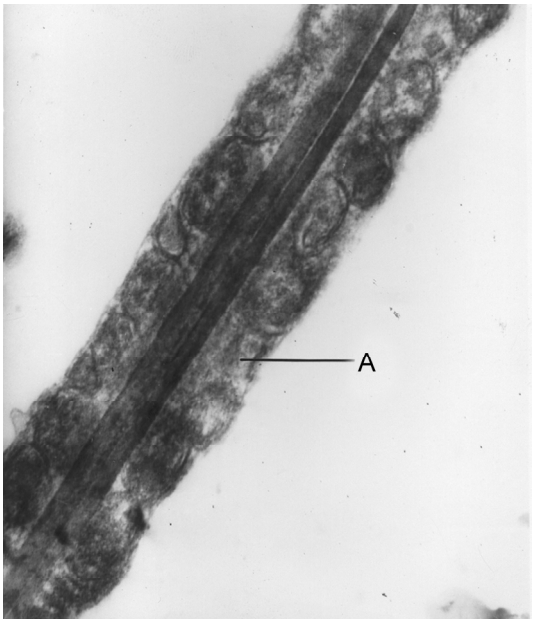

Figure 3. Transmission electron micrograph (TEM) of a mitochondria-affected human sperm tail (A). Central tubule is relatively intact. Dynein arms are often disturbed.

fected (Figure 3). Mitochondrial gyri were disturbed and the transverse view of the tail exhibited changes in the microtubular structure. Head-tail detachment was not prevalent as noted in light microscopic and SEM examinations.

3.3 Human subject Group III

Within 6 weeks of the injection no morphologically intact sperms were present in the ejaculate. Structures that may be recognized as breakdown products of sperms were seen. In all these structures no long tail was present. A few sperms with swollen and partially ruptured heads with what appeared to be stubs of the tail were present. Significantly, instead of head-tail detachment there was a shortening of the tail. The portion of the tail which was lost was not seen as a separate piece.

3.4 Monkey Group I

The sperm morphology seen in the proximal and distal segments of the vas deferens was the same.

3.5 Monkey Group II

In the semen 6 weeks post-injection there was evidence of marked sperm destruction without a total vas deferens lumen obstruction. That is, the semen had only sperm breakdown products. The important observation is that the sperms in the proximal segment of the vas deferens retain the tails. Sperms in the distal segment generally appeared to have very short tails. These forms were not mature sperm precursors. Furthermore, distally, structures like spherically deformed heads were seen without any remnants of the tail.

4 Discussion

Observations on Group I humans correlates well with biophysical study results that it takes approximately 72 h for full hydrolytic conversion and swelling of RISUG to occur. In the short period of 3-h post-injection the reactions are incomplete and invaginations into mucosal folds are not sufficient to develop a retentive action to oppose evacuation caused by vas deferens peristalsis. The ejaculation process is known to enhance peristalsis and segmental contractions in the vas deferens. Hence the 3-h post-injection semen sample contains small aggregates of precipitated RISUG evacuated by the peristaltic action.

Moreover the drug spread from scrotal vas deferens injection site by peristalsis is only partial and distal segments are virtually devoid of RISUG. In the short period of exposure of spermatozoa to RISUG and spermatozoa in the distal segments of the vas not being exposed to RISUG varied degrees of effects on sperm occurs, giving normal and partially affected spermatozoa. All of the above mentioned changes characterize the effects of short-term exposure to RISUG.

In previous studies on monkey, by inserting micro pH probes into the lumen of the vas deferens it has been observed that the pH lowering effect after RISUG injection gradually develops over a week. Therefore, for the 3-h post-injection sample the effects may be attributed more to the electrical charge of the RISUG.

The vas deferens lumen diameter is approximately 0.6 mm with considerable space occupied by RISUG. Sperms move in close proximity to RISUG with polymer charge–sperm charge interaction. Concomitant with transmembrane ion transport variation, water transport is also affected, leading to edema and acrosomal rupture. Acrosomal compounds, acrosin and hyaluronidase, are liberated as can be extrapolated from observations on *in vitro* exposure of sperms to RISUG done in our laboratory. These enzymes are autolytic agents and further act on the sperm thus changing sperm morphology.

RISUG charge also affects sperm chromatin, which normally has well compacted DNA. Normal sperms have protamines as alpha helices. Lying in the major grooves of DNA, protamines neutralize the negatively charged phosphate backbone and enable DNA duplexes to pack tightly together. The charge action of SMA probably inhibits the negative charge neutralization leading to unpacking of the DNA, chromatin decondensation and swelling of the sperm head. Chromatin decondensation is known to be associated with subfertility and infertility [10]. The contraceptive action of RISUG administration is, in part, accounted for by this factor.

The outcome of Group II men differs from the earlier group even though both have low intra-vas deferens RISUG content. In Group II, by vas peristalsis, the drug spreads almost to the entire length of the vas deferens. Also the polymer undergoes complete hydrolytic action with greater manifestation of electrical charge and pH lowering. Effects are significant on account of longer sperm-drug interaction with a more active form of the drug. There are fewer head-tail detachments as compared to that observed on exposure to primary amines such as ethylamine and anionic detergents like sodium dodecyl sulphate [11]. However, there is greater effect on the acrosome and nucleus. The difference indicates that the mechanism of action of RISUG is different to that of primary amines, detergents and sulphydryl reagents. This finding also explains why primary amines, detergents and sulphydryl agents do not serve as an intra-vas contraceptive.

The sperm character in the Group III men has a presentation that differs from the head-tail separation occurring under the *in vitro* action of agents such as primary amines in sperms. Group III sperm appearance can better be described as "dissolution" of the tail with the terminal segments of the tail being more susceptible to the dissolution effect. A possible explanation is that the tail is more negatively charged than the head and so plasma membrane of the tail is more resistant to charge mediated damages than the head. But once the tail membrane does get affected, mitochondria and microtubule-associated protein breakdown rapidly follows. However, the nucleus, with its large DNA content, is more resistant to dissolution because of stabilization by nuclear disulphide bonds (S-S) and neutralization of negatively charged phosphate backbone by the alpha helices of protamines lying in the major grooves of DNA.

Drug action progresses with increased exposure time and path length of sperm-polymer contact. Acrosomal structures are affected first followed by effects on the sperm nucleus. Subsequently tail mitochondrial gyri are disturbed and then there is dissolution of the terminal segments of the tail. The sperm structure seen in serial sections of the monkey vas deferens confirm that the action on the sperm increases as the sperms travel from the proximal to the distal segment of the vas deferens containing the RISUG implant. These results correlate with necroasthenoteratozoospermic changes in ejaculated sperm several days following contraceptive drug injection in the monkey [12].

Overall results presented here show a timed set of actions on sperms mediated by a bioactive compound. Some of the effects such as swollen rounded sperm heads and short tails are known to exist in disease states but are not reported in relation to the actions of synthetic compounds that affect sperms. The disease states which are associated with these specific sperm abnormalities are also linked to low fertility. Therefore, inducing these changes is a means of contraception and this phenomena is further demonstrated by the fertility control in the sub-

jects of the study. Also, the findings suggest that sustained intra-epididymal and intra-vas low pH conditions together with electrical charge abnormalities may be a combination of factors that can produce sperm abnormalities in the diseased state. Therefore, correction of pH and electrical charge levels by intra-epidiymal and intra-vas deferens injection of therapeutic compounds may be a means for managing specific types of infertility.

For the purpose of looking into broader implications of the study to male contraceptive technology it is pertinent to consider a different field, which is sperm-ovum interaction. There is a set of marked similarities between the effects on the sperm following intra-vas deferens exposure to the contraceptive polymer with the events immediately prior and following entry of the sperm into the ovum [13]. Changes in the sperm acrosome in the vicinity of the ovum parallels that occurring near the polymer. In any *in vitro* sperm preparation there is clustering of sperms. A transverse section of polymer injected in the rat and monkey vas deferens, however, does not show any clustering. Sperm heads are seen quite distinct in between zones of the polymer. It is as if once a sperm enters a zone between polymer masses the entry of other sperms is prevented somewhat akin to the prevention of polyspermy. Within the ovum the sequence of removal of the sperm nuclear envelope, nuclear decondensation and removal of the tail are closely matched by the occurrences on exposure of the sperm to the polymer within the vas deferens. These observations suggest a possibility that some of the sperm-ovum interactions are charge-mediated phenomena that can be affected even by a non-biological polyelectrolyte. As the sperm membranes are damaged first with a consequent loss of sperm fertility, a non-obstructive contraception can be achieved without the risk of damaged sperms passing into the ejaculated sperm causing teratogenic effects. This is because before the nucleus is affected the sperms have been rendered infertile. Non-obstructive vas contraception avoids many of the adverse effects of epididymal and testicular pressure rise following vas blockage by vasectomy and plugs.

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