

Studies on the membrane integrity of human sperm treated with a new injectable male contraceptive

K.Chaudhury¹, A.K.Bhattacharyya² and S.K.Guha^{1,3}

¹School of Medical Science and Technology, Indian Institute of Technology, Kharagpur 721302 and ²Department of Biochemistry, University College of Science, University of Calcutta, Calcutta 700019, India

³To whom correspondence should be addressed. E-mail: guha_sk@yahoo.com

BACKGROUND: The aim of this study was to evaluate the integrity of sperm surface characteristics in the presence of a new male contraceptive, RISUG [1 mg styrene maleic anhydride (SMA)/100 µl dimethylsulphoxide (DMSO) in 1 ml sperm solution]. **METHODS:** Progressively motile human sperm were treated *in vitro* with RISUG. The cells were analysed for the release of 5'-nucleotidase (5'-NT) (a plasma membrane marker) using 3 mmol/l 5'-AMP and 3 mmol/l β-glycerophosphate as substrates. Hyaluronidase (an acrosomal membrane marker) was analysed using hyaluronic acid as a substrate. The contents of free and total acrosin, and % proacrosin (all acrosome markers) were assayed using 0.5 mmol/l α-N-benzoyl-L-arginine ethylester (BAEE). **RESULTS:** RISUG caused almost complete disintegration of the plasma membrane leading to significant ($P < 0.0001$) release of 5'-NT into the surrounding media. Complete dissolution of the acrosome with concomitant vesiculation of the membrane system, as judged from the loss of hyaluronidase, was observed. Total acrosin content in the sperm was also reduced to almost 10%, and proacrosin dropped to 13.2% in the presence of RISUG in comparison to 90.2% in control ($P < 0.0001$), indicating dispersion of acrosomal contents. **CONCLUSION:** Under *in vitro* conditions, RISUG, at a concentration of 1 mg SMA dissolved in 100 µl of DMSO, caused significant damage to the acrosome and its contents, indicating loss of functional ability of sperm.

Key words: acrosin/hyaluronidase/male contraceptive/5'-nucleotidase/sperm membrane destabilization

Introduction

There are very few methods available for male contraception even today. In order to address this unmet reproductive health need, rigorous research is being carried out all over the world for the development of safe, acceptable, reversible male contraceptives. Extensive work by our research group over the past two decades has led to the development of an effective, non-toxic, reversible, long-acting contraceptive method for men. This non-hormonal contraceptive, named RISUG (an acronym for Reversible Inhibition of Sperm Under Guidance) is expected to provide a valuable addition to the currently limited options of male contraception (Ananthaswamy, 2002). RISUG consists of a co-polymer styrene maleic anhydride (SMA) dissolved in 99.9% pure dimethylsulphoxide (DMSO) (Guha, 1996). A single therapeutic dose of this contraceptive (60 mg of SMA in 120 µl of DMSO) is injected bilaterally into the vas deferens of the male in a minimally invasive manner, which leads to the disintegration of sperm or causes necrozoospermia.

Koul *et al.*, (1998) have shown that sperm breakdown products as well as whole, but damaged, sperm are present in the ejaculate of RISUG-injected subjects. Contraception is maintained, however, because damaged sperm are incapable of fertilization. Several reports have been made by investi-

gators on the characteristics of sperm cells obtained from subjects injected with this contraceptive. Phase II clinical trials of RISUG clearly indicate that this absence of living sperm is not to be interpreted as a total obstruction of the vas deferens as obtained with vasectomy (Guha *et al.*, 1997). Mishra *et al.*, (2003) have also investigated the status of spermatogenesis and sperm parameters in langur monkeys following long-term vas occlusion with RISUG. The ejaculated sperm were found to be necroasthenoteratozoospermic, suggesting instant sterility. Residues of cells that are seen could be due to disruption of the membrane system (Guha, 1996). An additional advantage of this technique is that it causes a partial blockage of the vas deferens with concomitant flow of functionally inactive cells (Sharma *et al.*, 2001; Chaudhury *et al.*, 2002). RISUG, despite not being tissue adherent, is retained in the folds of the inner wall of the vas deferens for a long period of time. Non-toxicity (Sethi *et al.*, 1990), reversibility (Koul *et al.*, 1998; Guha, 1999) and teratogenic safety on reversal (Sethi *et al.*, 1992) have also been confirmed. Phase I (Guha *et al.*, 1993) and Phase II (Guha *et al.*, 1997) clinical trials have been successfully completed and currently a Phase III multicentre trial is underway.

Surface charge distribution on sperm cells has been studied by staining the fixed cells with colloidal iron hydroxide

particles and then observing under scanning electron microscopy (SEM). Microelectrophoretic mobilities of the sperm cells have also been observed at 25°C in sorbitol phosphate buffer at pH 7.4 under a potential gradient of 2 V/cm and constant current of 80 mA on a Neubauer chamber fitted with two parallel stainless steel electrodes separated by a distance of 1 cm. Subsequently, zeta potential has been calculated (Sharma *et al.*, 1995). A similar study has been carried out with RISUG-treated sperm, which clearly shows that the polyelectrolytic nature of RISUG induces a surface charge imbalance on the human sperm membrane system (Sharma S, Guha SK and Anand S, unpublished data), the integrity of which is essential for sperm–oocyte interaction. However, nothing is known in terms of the leakage with concomitant loss of membrane markers from sperm treated with RISUG. Therefore, in an effort to establish the effect of RISUG on the destabilization of the human sperm membrane system, the present *in vitro* studies have been performed.

Three important key enzymes that facilitate sperm–oocyte interaction are (i) 5'-nucleotidase (5'-NT) which is associated with the anterior part of the plasma membrane and is involved in sperm metabolism (Caporiccio *et al.*, 1992) and appropriate timing of acrosome reaction (Centola *et al.*, 1997); (ii) hyaluronidase that causes dispersion of cumulus cells around the oocyte *in vitro* (Lin *et al.*, 1994); and (iii) the proacrosin–acrosin system which helps the sperm to penetrate through the zona pellucida, the species-specific glycoprotein layer of the oocyte. The presence of 5'-NT in the human male genital tract was shown for the first time by Konrad *et al.* (1998). 5'-NT is localized on the outermost surface of the human sperm (Takayama *et al.*, 2000). The extent of membrane damage may be positively correlated with the amount of 5'-NT released into the sperm's environment. The role of hyaluronidase in fertilization was first discovered from experiments on tubal ova (McClellan and Rowlands, 1942). The cumulus oophorus cells are held together by a hyaluronic acid matrix, and hyaluronidase is known to have an essential role in sperm penetration through that layer by hydrolysis and depolymerization of the hyaluronic acid-containing matrix of the follicular cell layer surrounding the oocyte (Zaneveld and De Jonge, 1991). Experiments with hyaluronidase antibodies and inhibitors provide further evidence for a role of sperm hyaluronidase in the process of fertilization (Dunbar *et al.*, 1976; Reddy *et al.*, 1980). The enzyme is specifically localized in the acrosome of human sperm and may be activated effectively by human seminal proteins (Mandal and Bhattacharyya, 1995). Acrosin, a sperm-specific proteinase (EC 3.4.21.10), is a trypsin-like enzyme mainly responsible for the passage of sperm through the zona pellucida of the ovum (McRorie and Williams, 1974) and is located in the sperm acrosome (Bhattacharyya and Zaneveld, 1982). Most of the acrosin (~93%) is present in human sperm in an inactive zymogen form called proacrosin (Parrish and Polakoski, 1979; Goodpasture *et al.*, 1980). The subject has been reviewed by one of the authors (Bhattacharyya and Kanjilal, 1991) and it has been established that *in vitro* immobilization of sperm causes release of the enzyme (Bhattacharyya and Zaneveld, 1978). An

extensive amount of work has been done by others and ourselves on the quantification of total acrosin, acrosin inhibitors and proacrosin present in human ejaculated sperm (Schill, 1990; Reichart *et al.*, 1993; Chatterjee *et al.*, 1999).

Materials and methods

Chemicals

Hyaluronic acid (from human umbilical cord, potassium salt, grade I), hyaluronidase (from bovine testes, type IV), Triton X-100, *N*-acetylglucosamine, *p*-dimethylaminobenzaldehyde, human serum albumin (fraction V), α -*N*-benzoyl-L-arginine ethylester (BAEE), 5'-adenosine monophosphate (5'-AMP), β -glycerophosphate and benzamidine were purchased from Sigma Chemical Co. (USA). Spectrapor membrane tubings were purchased from Spectrum Medical Industries Inc. (USA). The polymers styrene and maleic anhydride used for the synthesis of RISUG were obtained from Fluka (Switzerland). Other chemicals were of analytical or guaranteed reagent grade and were purchased from BDH (UK) or from E. Merck (Germany).

Collection and treatment of human semen

Fresh ejaculates were collected by masturbation after 3–5 days abstinence from selected proven fertile individuals aged 26–39 years. The samples were allowed to liquefy for 20 min and the sperm were separated by centrifugation at 3000 g for 10 min for the removal of seminal plasma. The sperm pellets were then layered under 2 ml of Biggers–Whitten–Whittingham medium (BWW; Biggers *et al.*, 1971) modified according to Overstreet *et al.* (1980) containing no pyruvate, lactate, dextrose and albumin. The progressively motile cells were separated from the top and were pelleted again at 5000 g for 5 min. The pellet was then washed once in 2 ml of BWW medium and the sperm were then dispersed, divided into two equal volumes (groups A and B) and were kept in a 5% CO₂ incubator at 37°C for 3 h. Group A consisted of the experimental fraction where the sperm were treated with RISUG (1 mg of the polymer styrene maleic anhydride (SMA) dissolved in 100 μ l of DMSO in a volume of 1 ml suspension) *in vitro* for 5 min which was standardized to be the optimal time for significant damage of membranes, at 30°C, and group B served as control samples which received DMSO only.

Synthesis of RISUG

Styrene and maleic anhydride monomer, after rigorous purification, were taken in a 1:1 ratio (20 g styrene and 20 ml maleic anhydride). Ethyl acetate was added to the styrene and maleic anhydride mixture and N₂ gas purged into the glass bottles. Polymerization was done by gamma irradiation (0.3 Gy/s at 37°C with a total dosage of 2.4 Gy) which was followed by precipitation with petroleum ether and soxhlet distillation using 1,2-dichloroethane and distilled water respectively. Monomers were removed meticulously. The SMA obtained was purified, powdered and stored in stoppered sterile glass tubes.

Estimation of 5'- nucleotidase

Washed sperm pellets (after removal of all seminal plasma containing nucleotidases and phosphatases) were taken from both groups A and B and resuspended in 20 mmol/l Tris–HCl saline buffer (pH 7.4) containing 0.55 mmol/l MgCl₂ such that each reaction system contained 10 \times 10⁶ cells/ml. An aliquot of 100 μ l of sperm suspension was added to the buffered substrate solution 3 mmol/l 5'-adenosine monophosphate (5'-AMP) or 3 mmol/l β -glycerophosphate (β -GP) as substrate in 20 mmol/l Tris–HCl saline buffer, pH 7.5 to

make a final assay volume of 0.5 ml and then incubated at 37°C for 30 min with occasional shaking. The reaction was stopped by adding 10% trichloroacetic acid and was kept in ice for 10 min. It was then centrifuged at 10 000 g, the supernatant collected and the amount of inorganic phosphate (Pi) released was measured following the method of Das *et al.* (2002). The activity of 5'-NT was calculated by subtracting the activity of non-specific phosphatases using β -GP as substrate from the activity calculated with 5'-AMP as substrate, and the results are expressed in terms of mg Pi released/h/10⁶ sperm.

Hyaluronidase assay

The treated (group A) pellets, as well as the control cells (group B), were extracted with 1.0 ml of 2% v/v Triton X-100 in 0.1 mol/l acetate buffer with physiological saline (pH 3.8) for 30 min at 37°C. The extract was then centrifuged at 7000 g for 10 min and the supernatant was used for evaluation of hyaluronidase activity. The assay was carried out according to the modified method of Aronson and Davidson (1967). The 500 ml reaction mixture contained 300 mg hyaluronic acid in 150 ml 0.1 mol/l acetate buffer (pH 3.8) containing 0.9% w/v NaCl, 100 ml human sperm extract from RISUG-treated or control (DMSO-only) groups. The volumes were adjusted to 500 ml by 0.1 mol/l acetate buffer (pH 3.8). The reaction was carried out at 37°C for 1 h. The reaction was initiated by addition of the enzyme to substrate preincubated at 37°C for 10 min. This assay system demonstrated linear kinetics up to 105 mIU of hyaluronidase activity. The reaction was stopped by raising the reaction pH from 3.8 to 8.9 by adding 10 ml 4 mol/l NaOH and 100 ml 0.8 mol/l potassium tetraborate solution (pH 9.2). The reaction mixture was then assayed for the release of *N*-acetylglucosamine using the method of Reissig *et al.* (1955) using *N*-acetylglucosamine as the standard. The mixture was kept in a boiling water bath for 3 min, and the cooled mixture was treated with 3 ml 1% (v/v) *p*-dimethylaminobenzaldehyde reagent in glacial acetic acid, containing 1.25% (v/v) 10 mol/l HCl, and incubated at 37°C for 60 min. Absorbance was measured at 585 nm, with respect to relevant blanks. The unit activity of hyaluronidase, expressed in mIU, was defined as the amount of enzyme that caused the release of 1 mmol/l of *N*-acetylglucosamine in 1 h at 37°C (Zaneveld *et al.*, 1973).

Estimation of proacrosin and acrosin

The human sperm pellets, containing 50–75 × 10⁶ cells, from both treated and control groups were washed twice with 10 mmol/l phosphate buffer, pH 7.4, and were separated at 6000 g for 10 min at 0–4°C. The washed sperm were then extracted with 1 ml HCl, pH 3.0 at 4°C in the presence of 50 mmol/l benzamidine. Inert plastic tubes were used since acrosin sticks to glass tubes unless siliconized. Extraction was carried out for 1 h at 4°C with continuous stirring using a magnetic stirrer. The acid-extracted sperm suspension was then centrifuged at 30 000 g for 30 min at 4°C. The supernatant thus obtained contains both proacrosin (precursor) and free acrosin at pH 3. Benzamidine was dialysed out using Spectrapor 3500 cut-off bags with three changes of 1 mmol/l HCl at pH 3.0 in the cold (0–4°C). The content of free acrosin was assayed directly, and the proacrosin (inactive) content was estimated by converting the precursor acrosin to active acrosin on incubation at 37°C for 10 min in 100 ml 50 mmol/l Tris–HCl buffer, pH 8.0 and then assayed by decreasing the pH to 3.0, following the methods of Bhattacharyya *et al.* (1979) and Goodpasture *et al.* (1980). The total acrosin was calculated as free plus activated acrosin. The assay system contained 0.5 mmol/l BAEE and 50 mmol/l CaCl₂ in 50 mmol/l Tris–HCl buffer, pH 8.0, and the change in absorbance was measured at 253 nm at 25°C in a Hitachi U 3210 spectrophotometer. A molar absorbance

Table I. Semen parameters as per World Health Organization (1999) guidelines

Semen parameters	Values (mean ± SD)
Volume (ml)	3.4 ± 1.1
Sperm count (/ml)	116 ± 38 × 10 ⁶
Sperm motility (%)	62 ± 5
Progressive motility (%)	38 ± 5
Normal morphology (%)	64 ± 3

difference of 1150/mol/cm was used to convert change in absorbance to micromoles of hydrolysed BAEE. One milli International Unit (mIU) of acrosin activity was defined as the amount of enzyme which hydrolysed 1 nmol BAEE per minute at pH 8.0 and at 25°C.

Statistical analysis

Data obtained from RISUG-treated cells were compared to that of controls using the Student's *t*-test with the level of significance set at $P < 0.001$.

Results

Semen value parameters (± SD) as per World Health Organization (1999) guidelines are reported in Table I. Table II shows the activity of 5'-NT in terms of inorganic phosphate (Pi) released per hour per 10⁶ cells in the environment outside sperm suspension for the experimental fraction (group A) and control sperm (group B). A significant release ($P < 0.0001$) of the enzyme from the plasma membrane into the solution surrounding the sperm treated with RISUG (1 mg SMA dissolved in 100 ml DMSO) was observed as compared to control sperm (treated only with DMSO). The results showed that only ~7% of hyaluronidase remains associated with the sperm membrane system on treatment with RISUG, and most of the enzyme ($P < 0.0001$) is released into the medium. It is, however, interesting to note that RISUG did not effectively inhibit the enzyme and that the activity could be detected in the medium. In control conditions, the enzyme mostly remained bound to the membrane. Table II also demonstrates the activity of free acrosin, total acrosin, inhibited acrosin and the proacrosin content in both the groups. A significant decrease ($P < 0.0001$) in both free acrosin and total acrosin activity was observed in the sperm treated with the contraceptive RISUG as compared to control sperm. A significantly higher content of acrosin ($P < 0.0001$) was found to exist in the proacrosin form (90.2%) in group B sperm, compared to the small amount of acrosin in the proacrosin form (13.2%) in sperm belonging to group A (treated with RISUG). In control cells, in the absence of SMA and using only DMSO as the solvent, complete activation of proacrosin occurred within 10–15 min.

Discussion

The anterior part of the sperm head plays an important role in the success of fertilization and any disturbance in the membrane system causes impairment in the gamete interaction. It is well documented that in all mammalian sperm, destabilization of the plasma membrane along with

Table II. Release of enzymes from plasma and acrosomal membranes of human sperm following *in vitro* treatment with RISUG^a

Membrane-associated enzymes	Group A (RISUG-treated cells) 10 mg SMA/1 ml DMSO	Group B (controls) DMSO only	P value
5' Nucleotidase ^b (n = 15)			
Release into supernatant	57.2 ± 3.2	19.2 ± 1.6	< 0.0001
Associated with sperm	11.9 ± 2.9	50.9 ± 3.7	< 0.0001
Hyaluronidase ^c (n = 15)			
Released into supernatant	10.3 ± 0.9	1.0 ± 0.5	< 0.0001
Associated with sperm	0.9 ± 0.6	12.4 ± 1.6	< 0.001
Proacrosin-acrosin system (n = 16)			
Free acrosin ^d	5.2 ± 3.1	197.2 ± 14.6	< 0.0001
Total acrosin ^d	105.5 ± 25.2	1275.2 ± 35.2	< 0.0001
Inhibited acrosin activity ^d	13.2 ± 1.2	90.2 ± 1.6	< 0.0001

Values in parentheses are the number of experiments.

^aRISUG: Reversible Inhibition of Sperm Under Guidance [1 mg styrene maleic anhydride (SMA)/100 µl dimethylsulphoxide (DMSO) in 1 ml sperm solution].

^bActivity is expressed in terms of µg inorganic released/h/10⁶ sperm.

^cAmount of enzyme that causes the release of 1 nmol of *N*-acetylglucosamine in 1 h at 37 °C.

^dResults are presented as mIU/min/10⁶ sperm.

the underlying outer acrosomal membrane system leads to vesiculation which subsequently causes leakage of key molecules (Bhattacharyya and Zaneveld, 1982; Zaneveld and De Jonge, 1991). Ultrastructural studies on human sperm have also indicated disintegration of the surface membranes by sperm-inactivating agents (Courtot *et al.*, 1994). Excessive loss of the plasma membrane-associated enzyme, 5'-NT, occurred on treatment with RISUG. In the present study, under *in vitro* conditions, at a concentration of 1 mg SMA dissolved in 100 ml of DMSO the sperm (comprising of 10–12 × 10⁶ motile cells) failed to retain the integrity of the plasma membrane as judged by 82% loss of the marker molecule into the surrounding medium, thereby indicating an effective contraceptive effect of RISUG. However, it remains to be resolved whether the enzyme is released in association with the membrane or in free form.

The role of hyaluronidase in solubilizing the cumulus oophorus and aiding successful attachment to the oocyte has been shown by several workers (Reddy *et al.*, 1980; Zaneveld and De Jonge, 1991) and the inhibition of the enzyme causes blockage of fertilization of mammals (Perreault *et al.*, 1980; Joyce *et al.*, 1986). We have shown previously that this enzyme remains mostly bound to human sperm membranes and can be activated by seminal molecules (glycoproteins) (Mandal and Bhattacharyya, 1995). Almost 90% loss of hyaluronidase from the acrosomal membrane system on treatment with RISUG indicates complete degeneration of the acrosome, possibly owing to vesiculation of plasma membrane with outer acrosomal membrane. Considering the essentially cationic nature of hyaluronidase along with some anionic characteristics (Harrison, 1988), the mechanism of interaction with the polyelectrolyte SMA dissolved in DMSO may be assumed to be some kind of an interaction probably leading to destabilization of the membrane system. The key enzyme for the penetration of the zona pellucida by the sperm is acrosin (Bhattacharyya *et al.*, 1979) and fertilization is prevented if the enzyme is inhibited under *in vitro* conditions. However, evidence indicates that delayed fertilization does occur in mice sperm lacking acrosin protein (Adham

et al., 1997). It was concluded that determination of acrosin may be a useful indicator of the fertility potential in men (Schill, 1990; Reichart *et al.*, 1993). Bartoov *et al.* (1994) established a direct relationship between sperm acrosin, quantitative estimation of enzymatically active non-zymogen (free) acrosin, proacrosin and total acrosin activity in different patients with male factor infertility activity and IVF. Quantification of acrosin, along with its precursor, which mostly remains associated with the inner acrosomal membrane of human sperm, indicates significant inactivation of the active form of the enzyme on treatment with RISUG. The low content of proacrosin on RISUG treatment indicates disruption of the membranes, which, when associated with sperm, show a high content of proacrosin (92.2%). The results are in good agreement with the TEM images showing disintegration and complete vesiculation between plasma and acrosomal membranes on treatment with RISUG (Guha, 1996). Our results clearly indicate the loss of functional competence of human sperm on treatment with the newly developed contraceptive, RISUG. This is of particular importance with regard to the possibility of developing RISUG as a highly effective contraceptive.

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