International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 5 (1): 1-12, 2009
ISSN 1811-7775

Spermicidal Action of Styrene Maleic Anhydride Polyelectrolyte in Combination with Magnetic and Electrically Conductive Particles

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Abstract: The aim of this study is to evaluate impact of a new polymeric contraceptive SMA-Fe_3O_4-Cu-DMSO called Smart RISUG (acronym for Smart Reversible Inhibition of Sperm under Guidance), a colloidal suspension of styrene maleic anhydride co-polymer (SMA), nano-micro iron oxide (Fe_3O_4) and copper powder (Cu) (one milligram) dissolved in dimethylsulphoxide (DMSO) (1:30); on Albino rat's sperm solution (one milliliter). Experiments to assess the morphology and viability of control as well as treated sperm cells were performed by using microscopic techniques like High Resolution Transmission Electron Microscopy (HRTEM), Field Emission Scanning Electron Microscopy (FESEM), Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM)-X ray microanalysis, phase contrast microscopy and Fluorescent Activated Cell Sorting (FACS). Treated cells indicate uniform adhesion of Smart RISUG particles to the sperm cell membrane, topological alteration, decrease in the cell count, reduced cell motility and viability, increased sperm abnormality and complete cell inactivation in about 72 h. This study suggests use of smart RISUG as a potential non-invasively reversible male/female contraceptive in future.

Key words: SMA-Fe_3O_4-Cu-DMSO, Smart RISUG, treated sperm cells, AFM, FESEM, sperm inactivation, contraception

INTRODUCTION

Current pandemic of exploding population is asking for an urgent need to develop new potential contraceptives. Even today male contraception has rare options. In a systemic effort to avoid hormonal method of contraception, a new male contraceptive Smart RISUG (Smart Reversible Inhibition of Sperm under Guidance) has been developed by this research group, that consists of a co-polymer Styrene Maleic Anhydride (SMA), magnetic particles iron oxide (8-12%), electrically conductive particle copper (3-8%) together dissolved in 99.9% pure dimethylsulphoxide (DMSO). It has been a continuous endeavour of present team to develop long-time effective, non-invasively reversible, non-invasively controllable, antimicrobial particularly anti-HIV and prostate cancer preventive contraception methods for male and/female that is expected to provide a valuable addition to the unmet reproductive health need (Guha, 2005; Sharma *et al*., 2003; Lohiya *et al*., 2000).

Sperm morphology is regarded as a significant prognostic factor for fertilization and pregnancy (Franken, 1998). While developing any contraceptive, morphological changes in the sperm cell remains one of most important aspect (Cheng and Mruk, 2002). In view of the unequivocal role played by spermatozoa in the reproductive biology there has been an active interest in studies related to spermatozoa (Mann *et al*., 1982). Paumgartten *et al*. (1998) reported continuous exposure of male rats to β-myrcene for 91 days prior to mating and during the mating period does not impair male fertility. In past, sperm production in mice was found to be inhibited by anticancer drug Annexin V, when injected into the seminiferous tubules (Maeda *et al*., 2002). The anticancer drug 5-fluorouracil exerted toxic effects on testis of wistar rats by germinal epithelial sloughing, tubular atrophy and generation of multinucleated cells. This antimetabolite found to decrease the sperm count in a dose and time-dependant manner (D'souza, 2003). Drug carboplatin was found to be more genotoxic than Cisplastin by using sperm abnormality assay as one of the parameters

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(Vijayalaxmi and D'Souza, 2004). An organophosphorous pesticide methyl parathion (MP) could not affect fertility parameters in rats though is known to affect human fertility at same dose (Narayana *et al*., 2005). *In vitro* and *in vivo* pharmacological activities of oral male pill (S) -3-(4-chloro-3-fluorophenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethylphenyl) propionamide (C-6) was proved to decrease the serum testosterone concentration in rat after two weeks (Chen *et al*., 2005).

One of the studies was made on effects of *Alstonia boonei* stem bark on albino rats in which sperm count, motility and viability was found to be effected (Raji *et al*., 2005). Drastic reduction of sperm count in male rats was reported by *Ricinus communis* (Linn.) plant by significant decrease in the concentration of fructose (Sandhyakumary *et al*., 2003). Detergent based vaginal contraceptive, aryl phosphate derivatives of bromo-methoxy-azidothymidine was reported to have dual-function spermicidal and anti HIV action due to presence of bromo-methoxy functional group (D'Cruz *et al*., 1998). D'Cruz *et al.* (2002) proved cyclohexenyl pyridyl NNIs, especially N-[2-(1-cyclohexenyl) ethyl] N'-[2-(5-bromopyridyl)]-thiourea in combination with the urea analog, a non-detergent type spermicide to act as a fertility control agent for woman. Spermicidal activity of synthetic antimicrobial peptide dermaseptins (DS1 and DS4) depended on the dose and presence of chelating agents (Zairi, 2005). But the past research available in literature does not indicate use of similar multifunctional composition like SMA-Fe_3O_4-Cu-DMSO developed and tested for its spermicidal action.

Earlier study by researchers and clinicians associated with present RISUG team gives reports on the characteristic of sperm cells obtained from subjects (rat, rabbit, monkey) injected with novel male contraceptive molecule RISUG (Reversible Inhibition of Sperm Under Guidance) that is presently undergoing extended Phase III clinical trials in India (Chaki *et al*., 2003) after successfully completing Phase I (Misro *et al*., 1979; Guha *et al*., 1993) and Phase II (Guha *et al*., 1997) clinical trials in India. Experiments conducted on albino rats have also proved spermicidal activity of RISUG, the male injectable antifertility agent. RISUG was evaluated safe and free from toxicity parameters in rats (Sethi *et al*., 1989). According to inventor of RISUG (Sujoy K. Guha), when co-polymer of styrene and maleic anhydride dissolved in DMSO was injected into the vas deferens of rats the morphological changes detected were confined to the mucosa (Verma *et al*., 1981). The SMA agent was also proved to cause no teratological action in rats (Sethi *et al*., 1992). After injection, RISUG lowers the pH sufficiently to kill the spermatozoa passing through (Misro *et al*., 1979). As sperm come into contact with the polymer, the combination of positive and negative charges on the polymer surface causes the surface of the sperm to burst (Sharma *et al*., 2001; Chaudhury *et al*., 2004). Sperm are thus immotile and unable to fertilize an egg whereas azoospermia was achieved as early as five days after injection (Guha *et al*., 1997). Phase II clinical trials of the contraceptive clearly indicated that the absence of living sperm in the ejaculate of RISUG injected subjects is not to be interpreted as a total obstruction of the vas deferens as obtained with vasectomy.

Smart RISUG is an advancement of the male contraceptive RISUG wherein the presence of Styrene Maleic Anhydride (SMA) specially prepared for contraception itself speaks undoubtable long-term efficacy of the molecule. Use of magnetic iron oxides here exploit two major advantages; their low toxicity to human beings (Marchal *et al*., 1989) and the possibility to use their outstanding properties by allowing the high magnetization of the iron oxide to target drugs or antibodies to a specific cell through an external magnetic field (Lubbe *et al*., 1996). In addition, incubation with copper wire in semen or cervical mucus has been found to significantly reduce the subcellular levels of both sodium and potassium in spermatozoa but does not affect the ratio between these two elements. The copper metal used here also displaces zinc from the sperm head region, possibly replacing it by copper. This may account for the decreased motility of spermatozoa in contact with copper ions (Maynard, 1975). Roblero *et al.* (1996) also reported decrease of sperm motility in the presence of copper.

Vast literature search does not indicate use of styrene maleic anhydride-iron oxide-copper-dimethylsulphoxide composite (SMA-Fe_3O_4-Cu-DMSO) for biomedical purpose like fertility control. The main purpose behind this new contraceptive development was non-invasive drug imaging and control of distribution of the compound from outside when placed in the male/female reproductive tube. After drug development, the objectives of present study were to evaluate effects of the Smart RISUG composite on albino rat's sperm cell *in vitro* and *in vivo*. It is expected that employing High Resolution Transmission Electron Microscopy (HRTEM), Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM)-X ray microanalysis, Field Emission Scanning Electron Microscopy (FESEM), phase contrast microscopy and Fluorescent Activated Cell Sorting (FACS) etc., both for the control and treated sperm cells would help to elucidate the spermicidal action of the Smart RISUG and to find new possibilities of non-invasive fertility control with help of the external magnetic field.

MATERIALS AND METHODS

Chemicals: Iron oxide powder Fe_3O_4 (Eisen (III) oxid, Pulver <5 μ, 98%) was purchased from Sigma-Aldrich Chemie GmbH, Germany. Cat.; 31, 006-9. Styrene Maleic Anhydride (SMA) provided especially for this study from the RISUG® Pilot Plant, Indian Institute of Technology Kharagpur. Copper powder-625 mesh, APS 1-1.5 μ, 99% (metals basis) obtained from Alfa Aesar, Shore Road, Heysham, Lancaster. DMSO (Dimethyl sulphoxide D2650, HYBRI-MAX®, hygroscopic, FW 78.13) was supplied from SIGMA-ALDRICH Pvt. Ltd., Singapore. Embryo tested and sterile filtered Tyrode's solution, acidic Cat.; T1788 was purchased from Sigma-Aldrich Chemie GmbH, Germany. Glutaraldehyde solution 2.5% was purchased from Merck CAS No. 111-30-8. Phosphate Buffer Saline (PBS) was purchased from Merck, Darmstadt, Germany. Ethanol, absolute CH_3 CH_2 OH (A.R., M.W. 46.080) was purchased from Hong Yang Chemical Corporation, China. Osmic acid 2% solution w/v for microscopy was provided from ISO 9001-2000 certified CDH (P) Ltd. New Delhi, India. Araldite 506 epoxy resin, Viscosity (25°C): 500-700 cps, weight per epoxide: 172-185 was also obtained from Sigma Aldrich., St.Louis, USA. Uranyl acetate and Lead (II) citrate Trihydrate for electron microscopy, Fluka grade, Mr1053.83 was supplied from Sigma Aldrich laboratory UK. Giemsa's stain CAS No. 51811-82-6, Merck Specialties Pvt. Ltd. Worli, Mumbai was used for microscopy. Eosin yellowish (C.I.No. 45380), Merck, Mumbai was used for staining. Propidium iodide (PI) solution 1.0 MG/ML, P4864, FW 668.4 for fluorescent staining purpose was also purchased from Sigma-Aldrich, Inc., USA.

Synthesis of smart RISUG: RISUG was synthesized as per the protocol given by the inventor mentioned in USP 5,488,075 (Guha, 1996) in several batches during December 2006 to March 2008 after gamma irradiation dosimetry calibration studies in July-November 2006. Purified styrene and maleic anhydride were mixed in 1:1 ratio. Styrene was added to maleic anhydride properly dissolved in ethyl acetate and the solution was purged vigourously for 5 minutes with N_2 gas before placing the stoppered bottle into gamma irradiation chamber. The styrene maleic anhydride polymerization was done at 0.32 Gy sec^{-1} at 37°C with a total dosage of 2 Gy. Precipitation with petroleum ether was immediately followed after cooling the solution. Further rigorous purification processes were done to remove monomers and other impurities by soxhlet distillation, drying, washing, final precipitation, drying and thus SMA obtained was powdered to be stored till dissolution. The SMA-Fe_3O_4-Cu-DMSO named Smart RISUG was synthesized in several batches in January 2007 to July 2008 for the present study by adding 10% (w/v) iron oxide nano-micro particles ($<5\times10^{-6}$ m), 5% Cu powder and SMA together into the dimethyl sulphoxide (1:30) under the nitrogen atmosphere. Thereafter, the solution obtained was continuously stirred for 48 h at 35°C following the method mentioned in Canada patent 2367414 (Guha, 2006).

Collection and treatment of epididymal sperm cell: Reproductively mature male albino rats (0.15-0.240 kg) were housed in wire mesh cages under standard conditions (temperature 25-29°C, 12 h light and 12 h darkness cycles) and fed artificial standard diet and water *ad libitium* (under supervision of animal ethical committee, Indian Institute of Technology Kharagpur). After anaesthetizing the animal with ketamine (40-80 mg kg^{-1}) about 10-2 m stream of distal cauda epididymal fluid was expelled from the cauda into 3×10^{-3} L of 0.1 M PBS (pH 7.4) in a 35 mL polystyrene culture disk. The epididymal tissue was removed and the dish kept in incubator for ten minutes. Sperm samples were then diluted to about 106 spermatozoa mL^{-1}. The viable cells were collected by swim-up technique. For *in vitro* efficacy assays sperm cells having motility >70%, count 15-20$\times10^6$ cells mL^{-1} were incubated in glucose free modified Tyrode's solution (m-TALP) medium with the RISUG or Smart RISUG precipitate as per the case for the desired time of treatment.

High resolution transmission electron microscopy (HRTEM): Every 1 mL rat sperm sample was treated with 0.01×10^{-3} litre of DMSO, 120×10^{-6} L RISUG, 120×10^{-6} L Smart RISUG, respectively. Primary fixation was done with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post fixed in 1% osmium tetroxide and embedded in Epon. Post polymerization at 60°C, ultrasections ($80\text{-}100\times10^{-9}$ m) were contrasted with uranyl acetate and lead citrate and samples were observed at X 14,000 magnification using a JEM 2100 electron microscope, Oxford Instruments, Oxfordshire, UK providing high magnifications, bright field imaging, Selected Area Electron Diffraction (SAED).

Scanning electron microscopy (SEM)-X-ray microanalysis: For SEM-X-ray microanalysis observations, immediately after collection both control as well as treated cells exposed *in vivo* with Smart RISUG for 72 h; washed in physiological medium to remove any mucus etc., fixed with 2.5% glutaraldehyde in 0.1M PBS. Post washing and alcohol drying cells were smeared and fixed on sterile glass slides, dried, sputter coated in a vacuum with electrically conductive layer of gold and

observed under a JEOL JSM-5800 scanning microscope, OXFORD ISIS-300, made in England.

Field Emission Scanning Electron Microscopy (FESEM): For FESEM the sample preparation procedure was similar like SEM except that treatment with the drug was *in vitro*. FESEM was performed with Carl Zeiss SMT-Nanotechnology Systems Division, model 7426, ISO 9001 certified Oxford Instruments England giving resolution at 5.9 KeV, BIAS-500V wherein accelerating voltage range is 0.5 to 30 kV.

Atomic Force Microscopy (AFM): Albino rat's control as well as *in vivo* treated sperm cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for half an hour at 37°C. After proper washing with deionized distilled water, cells were immobilized on 0.01% Poly-l-lysine coated slides prior to analysis by non-contact mode AFM (Thermo Microscopes, CP Research Model and Sunnyvale, CA, USA). Samples were analyzed for changes by tapping mode AFM on the outer surface of spermatozoa after treatment with Smart RISUG. A 50 μm long silicon cantilever, with a force constant of 17.2 N m^{-1} was vibrated near its resonance frequency of 320 kHz. The deviation of the vibration frequency from resonance depends on the tip-sample preparation and forms the data set providing the topography of the sperm cell.

Phase contrast microscopy: For cell count, motility observation, viability assay and sperm anomaly assays the inverted microscope CKX41 (Olympus Optical Co., Shizuoka and Tokyo, Japan) equipped with a 40X objective for negative phase contrast available in this biomedical research centre was used and images were obtained with a Steves Digicams C-5060 wide zoom camera attached to it. Epididymal sperm cells were collected, minced, filtered and stained with 1% aqueous eosin Y. An aliquot was taken in leukocyte pipette, diluted and discharged into Neubaur chamber. Cell count per milliliter was calculated by multiplying the average count per square with the dilution factor × 10^4. The sperm motility and viability was assessed by calculating motile and viable spermatozoa per unit area and expressed as Mean±SEM.

Sperm anomaly assay: Post 35 days of drug treatment sperm cells were collected for sperm abnormality assay because germ cells which are exposed at late spermatogonial stage to the chemical, would reach the cauda epididymis after undergoing a series of changes during the course of development to give rise to sperms (Wyrobek and Bruce, 1975). After sacrificing the animal, sperm cells were stained with 1% aqueous eosin for about 20×60 sec. An aliquot (0.05 mL) from the sperm suspension (1 mL) was diluted 40 times (1:40) with PBS and mixed thoroughly. A drop of sperm suspension was smeared on a clean slide and scored 2000 sperms per animal for sperm abnormalities with the help of the hemocytometer.

Gonad weight changes: Initial and final testis weights of animals were recorded after 1, 2, 4 and 12 week. For each timing there were three groups of animal (each having eight albino rats) injected with 0.01×10^{-3} L of DMSO, 120×10^{-6} L RISUG and 120×10^{6} L Smart RISUG, respectively. Animals were sacrificed under ketamine anaesthesia after completion of respective treatment duration. The testis were removed and weighed. The mean value ± SEM of eight animals from each group were taken ($p<0.05$).

Fluorescence Activated Cell Sorting (FACS): In one of the experiments to test the *in vivo* efficacy of Smart RISUG, cell viability assay was done with FACS Calibur system, Becton and Dickinson, NJ, USA with fluorescent stain propidium iodide (PI). Immediately after collection cells were washed in 0.1 M PBS, pH 7.4 to be followed by staining with PI. Only dead cells could be stained as per this protocol. Thus viability count of the sperm cell after exposure with the new compound for different timing could be assessed.

Statistical analysis: The test of significance between the data of the experimental and control series are expressed as mean±SEM determined by the student's t-test according to Fisher and Yates.

RESULTS

In vitro efficacy of Smart RISUG

Mechanism of entry of Smart RISUG particles into the spermatozoa: High resolution transmission electron microscopy throws more light on the mechanism of entry of Smart RISUG particles into the sperm plasma membrane. Firstly, HRTEM study compared the shape and size of the SMA-Fe_3O_4-Cu-DMSO (Smart RISUG) composite particles that were found to be in the range of 50×10^{-9} to 150×10^{-9} m size suitable for drug delivery (Fig. 1a, b). Figure 1b showed uniform particle attachment to the sperm cell surface. Smart RISUG particles were transported into sperm cell by attachment to the plasma membrane or nearby surface of the sperm membrane (Fig. 1b, c). It appeared that particles were not endocytosed by the sperm cells rather they attached or just punctured the site of entry and thus inactivated the sperm cells. Figure 1d shows complete sperm cell damage.

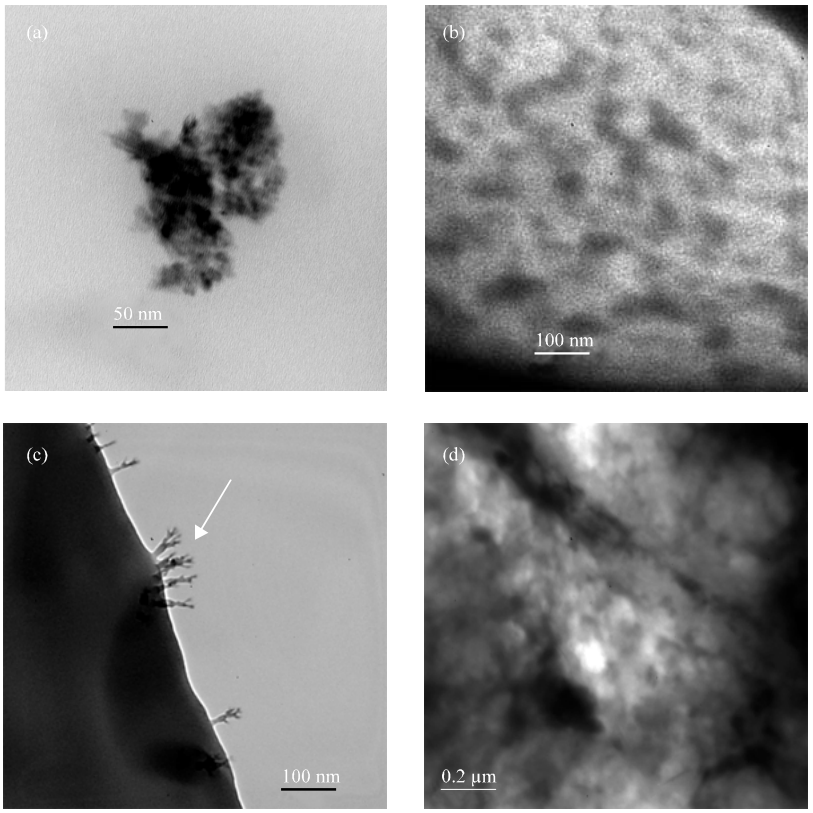

Fig. 1: HR TEM of (a) 50-150×10^{-9} m sized Smart RISUG particles, (b) Surface of rat sperm membrane showing uniform drug particle attachment, (c) Mechanism of entry (white arrows) of Fe_3O_4-Cu-SMA-DMSO (Smart RISUG) particles into sperm cell membrane and (d) Damaged sperm cell after treatment with Smart RISUG

Sperm cell disintegration after treatment with Smart RISUG: Above efficacy notion is further supported with FESEM used for obtaining high quality, low voltage images with negligible electrical charging of samples. Figure 2a observed positive control cells whereas Fig. 2b showed control cells treated with 0.01×10^{-3} L DMSO for 30×60 sec. Sperm head treated with RISUG for same timing. Figure 2c showed post-treatment blabbing and damage at the acrosomal region while Fig. 2d observed complete disintegration of sperm head membrane as well as curved middle piece and damaged tail region after 120×60 sec of RISUG treatment. On the other hand, after initial 30×60 sec effects of Smart RISUG treatment on sperm cells (Fig. 2e) sperm head looked quite disintegrated and acrosomal region appears affected with the drug. Subsequently, after 120×60 sec of treatment with Smart RISUG (Fig. 2f) sperm cell appeared completely squeezed out of matrix, proteins and enzymes vital for fertilization. Figure 2f further explained complete disintegration of sperm head as well as cell flattening within two hours of Smart RISUG treatment of the albino rat's sperm cells.

***In vivo* efficacy of Smart RISUG**

Topological alteration after treatment with smart RISUG: AFM image of untreated rat sperm cells (Fig. 3a) gave evidence of smooth surface topography throughout the length of the spermatozoa. A cluster of sperm cells with prominent head were observed in Fig. 3a. The cell clustering is similar as shown with FESEM. In contrast, sperm cells treated with Smart RISUG (Fig. 3b) gave disintegrated surface topology with a complete loss of smoothness. The three-dimensional AFM image of the same showed evidence of leaching out of enzymes *hyaluronidase* and *acrosin* from the acrosomal region. It also showed curved tail and damage to the middle piece. A significant reduction in the cell height had been observed for treated sperm cells with AFM. An interesting observation here was the pattern of attachment of Smart RISUG particles to sperm cell throughout sperm cell membrane complimenting HRTEM observation.

Sperm cell count, motility and viability: As evident from data in Table 1, sperm cells on treatment with RISUG and Smart RISUG for 30×60 sec caused 27.36 and 29.66%

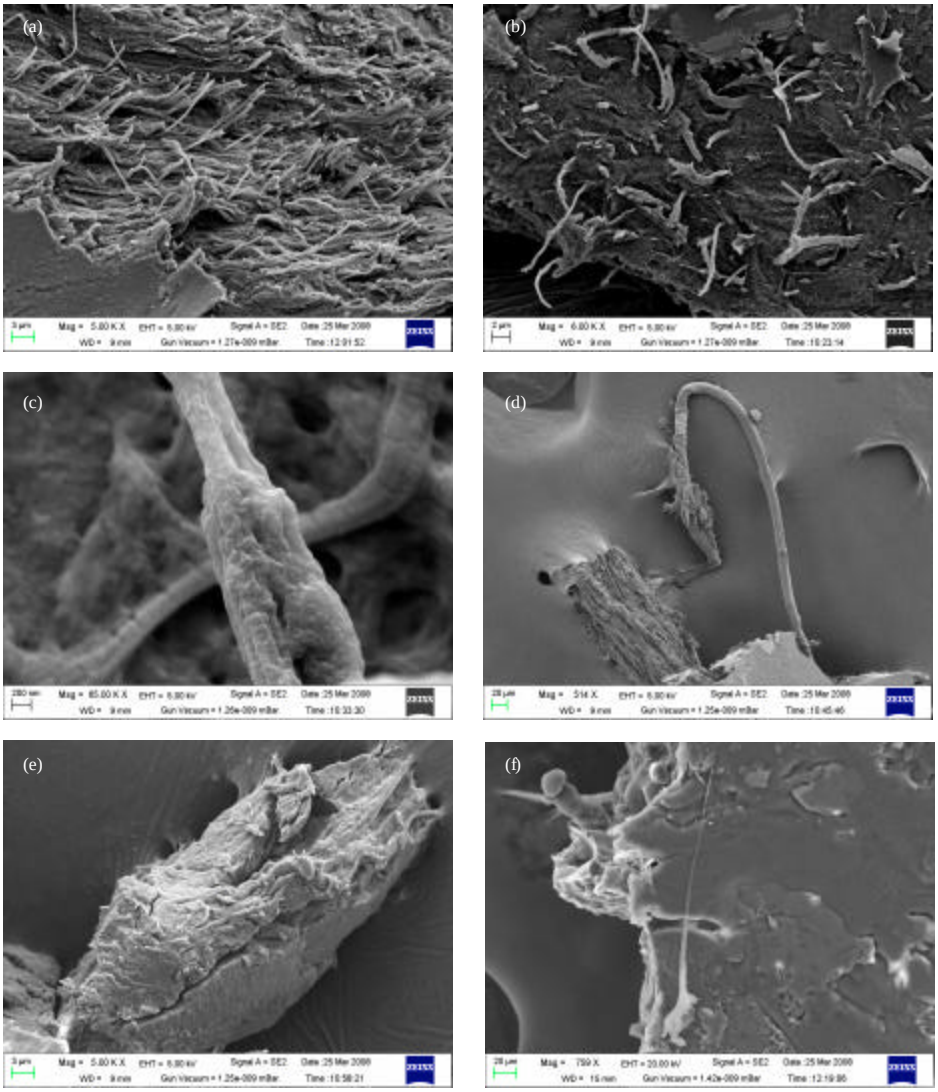

Fig. 2: Field Emission Scanning Electron Micrographs (FESEM) of (a) Untreated sperm cell, (b) DMSO treated cells (control), (c) RISUG-treated sperm cell (30×60 sec) with topographical changes in acrosomal region, (d) RISUG-treated cells (120×60 sec) with curved middle piece and tail damage, (e) Smart RISUG treated (30× 60 sec) cell with disintegrated apical region and some damage to the neck region and (f) Smart RISUG treated (120×60 sec) cells showing disintegrated head region and cell appearing to be squeezed out of key enzymes

Table 1: Effect of smart RISUG exposure on the sperm cell count, motility (%), viability (%) and sperm anomaly (%) (n = 8)

Treatments	Time of exposure (sec)	Epididymal sperm count ($\times 10^6$ mL^{-1}) ±SEM	Sperm motility (%) ±SEM	Sperm viability (%)	Abnormal sperms (%) ±SEM
PBS	30×60	25.22±1.10	68.37±3.97	98.68	1.06±0.50
DMSO	30×60	10.83±2.12	40.22±9.19	91.14	6.33±0.45
R	30×60	18.32±5.25	55.50±4.66	92.58	4.14±0.20
SR	30×60	17.74±0.98	45.12±4.13	93.12	3.52±7.23
R	60×60	17.35±4.75	52.50±3.60	92.32	5.19±0.18
SR	60×60	16.71±1.55	40.56±3.45	93.02	4.37±0.20
R	120×60	15.42±3.56	50.39±4.60	90.12	5.33±0.44
SR	120×60	14.65±3.90	36.92±2.85	90.59	4.45±0.22

PBS: 0.1 M pH 7.4 Phosphate buffer saline, DMSO: Dimethyl sulphoxide, R: RISUG, SR: Smart RISUG. Data presented as Mean±SEM ($p<0.05$)

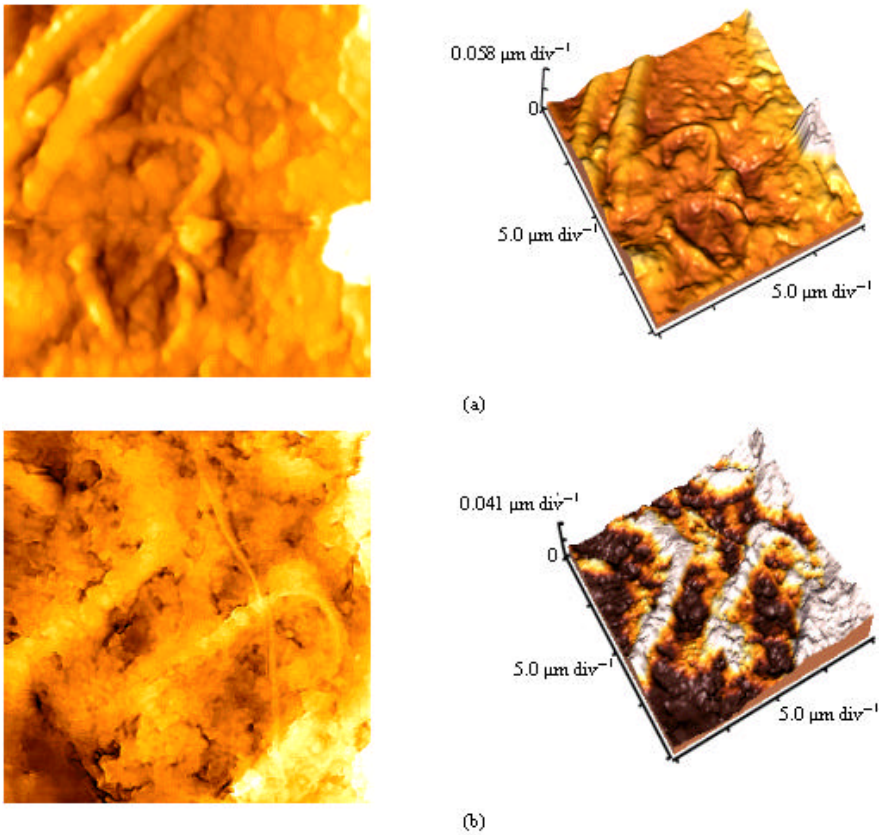

Fig. 3: Atomic Force Microscopy (AFM) images of rat spermatozoa (a) 2D and 3D images of untreated cell showing cluster cells with smooth surface, (b) 2D and 3D images of Fe_3O_4-Cu-SMA-DMSO (Smart RISUG) treated cells showing blabs in plasma membrane, enzyme leaching, shortened height and curved tail

Table 2: *In vivo* 4 weeks exposure effect of Smart RISUG on albino rat's testis weight (n = 8)

		Testis weight (weeks)			
Treatments	Dose (10^{-6}L)	1	2	4	12
C (NS)	120	100.00±5.7	100.00±6.0	100.25±3.20	100.50±5.6
R	120	102.56±1.2	99.00±2.5	99.32±2.70	100.12±3.3
SR	120	101.43±2.3	98.98±2.4	99.05±1.56	99.98±1.9

C: Control, NS: Normal Saline, R: RISUG, SR: Smart RISUG. Data presented as Mean±SEM ($p<0.05$)

decrease in the cell count, respectively. Similarly, on 60×60 sec treatment percentage decreased in count was increased to 31.21 and 33.75%, respectively with two drugs. Further 120×60 sec exposure caused 38.86 and 41.91% decrease in the sperm cell count, respectively.

The most significant effect of Smart RISUG was found on the motility of albino rat's spermatozoa, the most significant feature of a male gamete to perform its reproductive function. In initial half an hour RISUG and Smart RISUG caused approximately 18.80 and 34.01% decrease in the sperm motility, after one hour these drugs caused 23.22 and 41.32% decrease in motility percentage and after two hours of treatment the motility percentage reduced to 26.12 and 47.17%, respectively. Thus we can say that after two hours almost 50% of cells were immotile. Table 2 also explains decreasing viability of rat sperm cells on treatment with RISUG as well as Smart RISUG.

Sperm anomaly assay: Both RISUG and Smart RISUG increased the population of sperm with abnormal shape (Fig. 4, Table 1). Primary abnormalities were small and pyriform heads in sperms treated for 1 h or more. The most common abnormality of the epididymal sperm was curved middle piece on treatment with Smart RISUG after one week of *in vivo* exposure. In comparison to control cells treated with 0.1 M, pH 7.4 PBS *in vivo* RISUG caused increased anomaly about 74.4% in half an hour, 79.58% in one hour and 80.12% in 2 h. Whereas Smart RISUG caused comparatively less increased anomaly about 69.89% in half an hour, 75.75% in 1 h and 76.18% in 2 h.

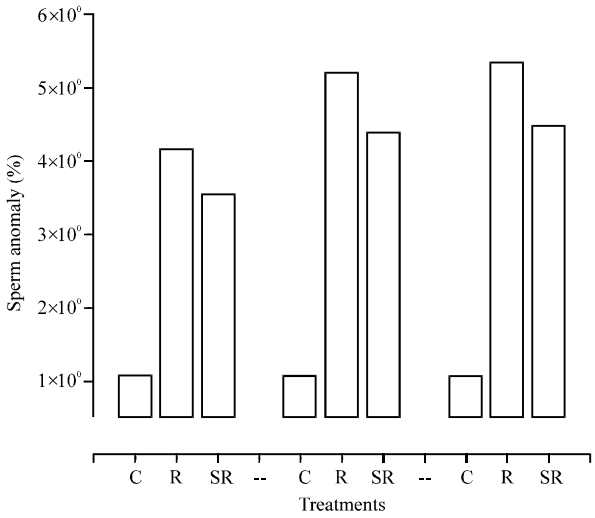

Fig. 4: Sperm anomaly assay. Y-axis represents Increase in the sperm abnormality percentage (%) of treated cells with respect to control cells treated with normal saline only. X-axis representing *in vivo* treatment with C, control; R, RISUG and SR, Smart RISUG in three groups for half an hour , one hour and two hours respectively and Y-axis represents that percentage abnormality ($p<0.05$)

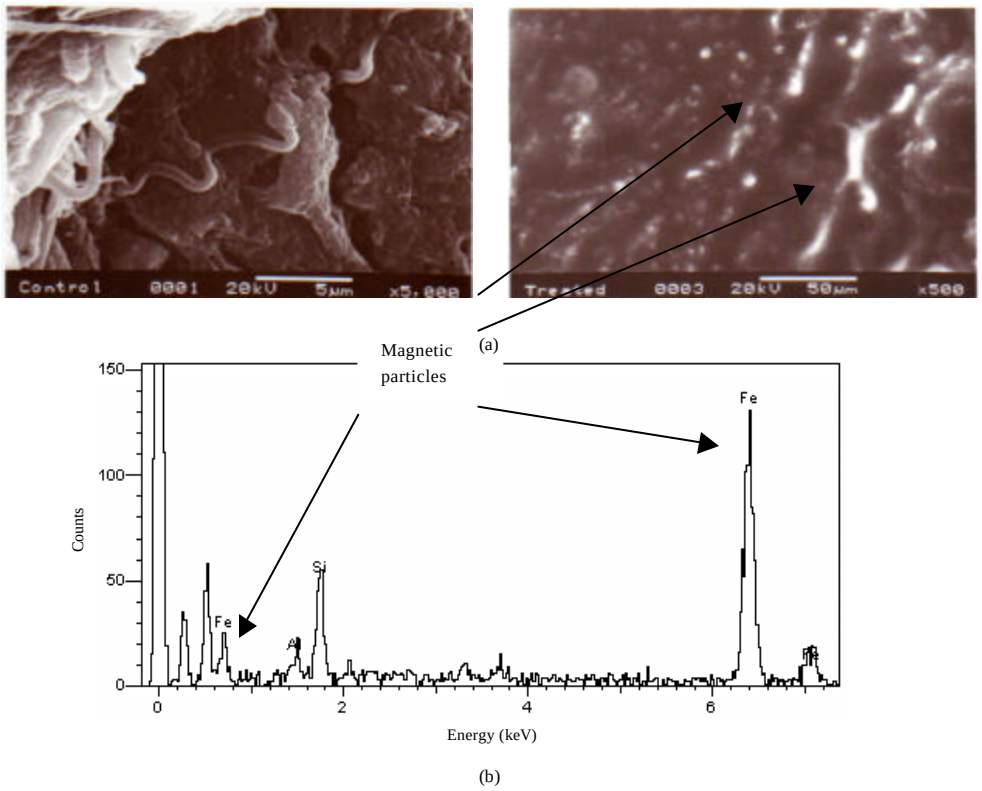

Fig. 5: Scanning Electron microscopy-X ray microanalysis graphs (a) of untreated albino rat's sperm cell (b) of cells exposed to Fe_3O_4-Cu-SMA-DMSO (Smart RISUG) complex for 72 h *in vivo* that shows complete cell damage and the presence of drug particles in treated cells

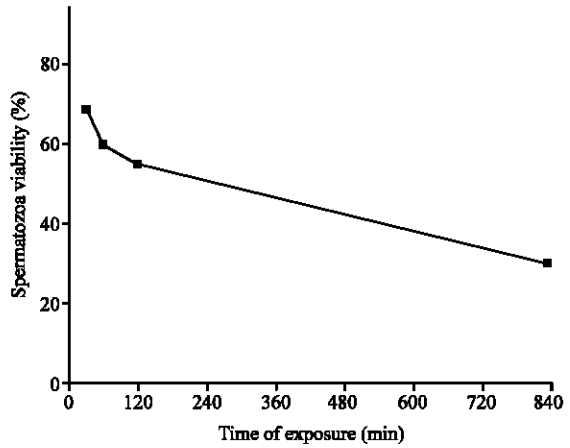

Fig. 6: This graph shows decreasing spermatozoa viability with increasing time of *in vivo* exposure of the spermatozoa to Fe_3O_4-Cu-SMA-DMSO drug (Smart RISUG)

Testis weight changes: Neither RISUG nor Smart RISUG found to have any significant effect on the testis weight of rats treated for one week, two weeks, four weeks and 12 weeks (Table 2). A slight increase in the testis weight in initial 1 week in both cases is restored in second week only and maintained thereafter.

Spermicidal action of the Smart RISUG compound: Prior to Smart RISUG treatment, a magnified control cell is observed in Fig. 5a while complete destruction of the albino rat's spermatozoa within 72 h of *in vivo* exposure to the Smart RISUG composite (Fig. 5b) was observed with aid of Scanning Electron Microscopy (SEM). X-ray microanalysis attached with SEM of treated sperm cells illustrated presence of iron particles in the damaged sperm cells (Fig. 5).

Sperm viability assay: Further efficacy of this new molecule is more evident from sperm cell viability assay conducted with the fluorescent activated cell sorting (FACS) where *in vivo* exposure of male gametes to SMA-Fe_3O_4-Cu-DMSO compound (Smart RISUG) showed gradual increase in the percentage of dead cells marked with Propidium iodide (PI) stain as time of exposure was increased (Fig. 6). Vice-versa, it can be said that the viability of sperm cells decreased significantly with increasing time of exposure to the new contraceptive molecule Smart RISUG.

DISCUSSION

To analyze the spermicidal action of the new male contraceptive Smart RISUG, a comparative study has been performed employing HRTEM, FESEM, AFM, Phase contrast microscopy, SEM-X ray microanalysis and FACS. All microscopic observations are in good agreement with sperm inactivating action of drug Smart RISUG. HRTEM (Fig. 1b, c) shows that nano-micro Smart RISUG particles are uniformly attached to the sperm plasma membrane or nearby it and make its way into the cell spontaneously. Smart RISUG causes membrane rupture of the spermatozoa at the acrosomal region and tail region; former one leads to leaching out of enzymes *acrosin* and *hyaluronidase* thus rendering the spermatozoa infertile and later shows rupture of tail region that obviously leads to loss of the sperm motility due to presence of copper.

Slide preparation for both control and drug treated cells observed with 40 X negative phase contrast microscope illustrates decrease in the cell count, motility percentage and increase in percentage sperm anomaly (Table 1). Post *in vitro* drug treatment, FESEM studies also gives clear evidence of topological alterations in the sperm cell acrosomal region (Fig. 2c-f), loss of plasma membrane integrity (Fig. 2c, e) and flattening of the middle piece (Fig. 2f) that may have caused damage to the powerhouse of cell, the mitochondria on treatment with Smart RISUG.

Explaining *in vivo* efficacy of Smart RISUG, AFM 2D and 3D images for both control as well as treated sperm cells were observed. Significant effects evident by comparison between Fig. 3a, b are destabilization as well as blabbing of the sperm plasma membrane, decrease in the sperm height, leaching out of enzymes from the acrosomal region that certainly cause loss of reproductive function and tail curving that may have caused decreased motility due to presence of copper. Figure 6 is an addition to the viability studies done with light microscopy with help of FACS. It gives quantitative data about the efficacy of the compound within a couple of hours followed by almost complete destruction caused by Smart RISUG investigated with SEM-X ray microanalysis, also evidencing presence of the drug particles inside the treated cells (Fig. 5).

Biocompatible superparamagnetic nanoparticles like magnetite (Fe_3O_4) have been widely used for *in vivo* biomedical applications including MRI contrast enhancement (Lee *et al*., 2005). Drug targeting is another perceived use of superparamagnetic particles. So, in terms of drug delivery magnetic nanoparticles offer the possibility of use of external magnetic fields to obtain better localization than that achieved with non-magnetic particles (Gupta and Wells, 2004). But since magnetic nanoparticles are less easily destroyed or inactivated by cells than many non-magnetic ones there is the disadvantage that persistent particles may cause later cell damage and death. The same considerations apply to

situations where magnetic nanoparticles are being used for generating hyperthermia by the application of external fields. But here, as we are using magnetic micro-nanoparticles coated with a non-toxic polymer SMA, there exist no such hazard and at the same time the proportion of magnetic particles being used in this work is very less about 8-12%.

Smart RISUG was found to elevate the abnormality in the sperm shape. In comparison to untreated cells the RISUG caused about 80% increase in abnormality in two hours while Smart RISUG was found to increase the abnormality upto 76% in two hours, though to some lesser extent in comparison to RISUG (Table 1, Fig. 4). Data in Table 1 shows that neither RISUG nor Smart RISUG has any persisting effect on rat testis weight. Both gonad weight analysis (Table 2) and sperm anomaly assay (Fig. 4) proves *in vivo* safety and efficacy of the new drug, respectively. It is also shown in Table 1 that RISUG causes about 39% decrease and Smart RISUG causes about 42% decreases in the cell count in 2 h. Percentage motility was decreased to 26 and 47% by RISUG and Smart RISUG, respectively in 2 h thus proving that smart RISUG has significant effect on the motility rate of the spermatozoa making it inactive and thus unable to reach female gamete.

It is evident from HRTEM that the Smart RISUG particles 50-150 $\times 10^{-9}$ m (Fig. 1a) are not endocytosed rather they make their way by attaching to the cell membrane (Fig. 1b-c, 4) and puncturing it at the site of entry (Fig. 1d) to disturb the cell membrane integrity and inactivate the cell. Earlier studies on the Smart RISUG characterization and its transport into sperm cell under guidance of external pulsed magnetic field [unpublished data] propose that the route of Smart RISUG delivery would be either by localized injection into the reproductive tube, or non-invasive drug targeting with aid of external magnetic field applied at the pelvic region by keeping the drug on external surface of the target tissue after increasing the skin pore size and then applying external magnetic field. The same may apply for new drug reversibility and control of distribution of the drug inside the reproductive tube. A detailed non-invasive imaging would help to elucidate this point.

In continuation of earlier studies, FESEM analysis gave the clearer image of gradual spermicidal action of Smart RISUG. Comparison of Fig. 2a with Fig. 2c-d shows gradual spermicidal action of RISUG with increasing *in vitro* treatment time. Whereas comparison of Fig. 2a with Fig. 2e-f are also in agreement of the spermicidal action of new drug giving the old drug a new nanotechnological dimension. Also the results of *in vivo* FACS experiments explain significant decrease in the treated sperm cell viability in initial 2 h (Fig. 6) followed by small changes. Results of SEM-X ray microanalysis also says that a minimum of 48-72 h may be required for proper action of the new drug and achieving 100% azoospermia. Moreover, these data clearly indicate that Fe_3O_4-Cu-SMA-DMSO (Smart RISUG) composition have highest spermicidal action within a given span of time (Table 1).

Combination of polymers with nano-microsized solid materials displays novel and often enhanced properties compared to the traditional materials. There are numerous advantages of the use of magnetic and electric micro-nanoparticles. Firstly, it can be localized in desired region by applying local magnetic field gradients. It produces localized hyperthermia when organ loaded with nanoparticles is exposed to electromagnetic radiation. Superparamagnetic particle administered in moderate quantity causes no real hazards. One important advantage for the magnetic nanoparticle is their superparamagnetism that enables their stability and dispersion upon removal of the magnetic field as no residual magnetic force exist between the particles. Superparamagnetic iron oxide nanoparticles are relatively non-toxic when administered intravenously. Since, ancient times copper is known to be antimicrobial and magnetic particles have also been found to be antimicrobial (Williams *et al*., 2006). In addition, the attachment of drugs to magnetic nanoparticles can be used to reduce drug doses and potential side effects to healthy tissues and the costs associated with drug treatment. Surface modified Fe_3O_4 may prove very useful for non-invasive imaging of the contraceptive implant by magnetic resonance imaging, CAT scan, ultrasound etc and make drug delivery as well as reversibility possible with much lower magnetic field gradients.

CONCLUSION

The data in this study clearly indicate that SMA-Fe_3O_4-Cu-DMSO composite named 'Smart RISUG' is a promising fertility control agent as there is a positive correlation between increasing treatment time and its spermicidal activity. Present work clearly reports that these new drug particles are able to inactivate sperm cells and lead to sperm cell disintegration. The proximal end of the sperm cell plays an important role in the fertilization success and any kind of disturbance in the membrane system as observed in HR-TEM, FESEM and AFM analysis causes loss of gamete interaction. Research emphasizes that in all mammalian sperm; destabilization of the plasma membrane leads to vesiculation which subsequently causes leaking of key molecules (Sethi *et al*., 1989). Our nanobiomedical work also indicates that magnetic and electric nano-micro particles can be a promising tool for biomedical applications *in vitro* and *in vivo*. SMA-DMSO composite (named RISUG) has been proved safe in long-term (Sethi *et al*., 1989; Guha *et al*., 1993, 1997). Magnetic

material iron-oxide (Fe_3O_4) added to the RISUG enhances the activity of contraceptive polymer particularly by immobilizing sperm cells, enhancing the drug stability as well as control of distribution of the drug in the reproductive tube and also facilitates non invasive imaging. Electric particle copper itself augments enhanced contraceptive efficacy, reduces sperm motility (Skandhan *et al*., 2005) and increases sperm abnormality and antimicrobial properties of this new generation of RISUG. Smart RISUG particles affect cell integrity, induces topological changes with reduction in the cell count, motility and viability. It was concluded that all these parameters are crucial for reaching to female gamete and successful fertilization. Therefore, this study may open pathway to use Smart RISUG as a potential non-invasively reversible new contraceptive molecule in future.

ACKNOWLEDGMENTS

This study has been supported by Ministry of Health and Family Welfare (MOHFW), Government of India. The authors thank the chairman and members of Institutional Animal Ethics Committee for their understanding and permission given for carrying out the experiments. We also thank Central research facility, Indian Institute of Technology Kharagpur for providing instrumentation facility.

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