Asian Journal of Andrology (2017) 19, 389–395
www.asiaandro.com; www.ajandrology.com

Open Access

ORIGINAL ARTICLE

Sperm Biology

Contraception with RISUG® and functional reversal through DMSO and $NaHCO_3$ in male rabbits

Abdul S Ansari, Ayesha Badar, Krithika Balasubramanian, Nirmal K Lohiya

The study aimed to evaluate reversal of short- and long-term vas occlusion with reversible inhibition of sperm under guidance (RISUG) using dimethyl sulfoxide (DMSO) and sodium bicarbonate ($NaHCO_3$) in male rabbits (*Oryctolagus cuniculus*). Animals were divided into seven groups containing five animals each. Fortnightly, semen analysis revealed that sperm concentration and output steadily declined after vas occlusion and complete azoospermia was attained at 30–60 days postinjection. Spermatozoa reappeared at 60–75 days of reversal and normozoospermia was noticed between 135 days and 150 days in the reversal groups. All spermatozoa were found nonmotile prior to azoospermia and a gradual recovery in sperm motility was observed between 105 days and 135 days of reversal. A significant decline in viability of sperms was noticed during vas occlusion up to 30–60 days which recovered at 60–75 days postreversal and normalized by 75–105 days in the reversal groups. A significant enhancement in the sperm abnormalities was recorded in all vas occluded animals as well as those in initial periods of reversal. Other parameters, namely, semen volume, ejaculation time, pH, color, and consistency, remained unaltered during all phases of the study. Fertility test, at the intervals of 15 days, demonstrated that animals exhibited complete sterility during the entire period of vas occlusion. A gradual recovery in fertility was observed with the appearance of spermatozoa following vas occlusion reversal and 100% fertility was observed following 135–150 days of reversal. F_1 progeny of reversed animals was found normal. The results suggest that reversal with DMSO or $NaHCO_3$ is feasible, with normal progeny, following short- and long-term contraception.

Asian Journal of Andrology (2017) **19**, 389–395; doi: 10.4103/1008-682X.185000; published online: 2 September 2016

Keywords: contraception; dimethyl sulfoxide; rabbit; reversal; reversible inhibition of sperm under guidance; sodium bicarbonate

INTRODUCTION

In the last few decades, a significant leap has been made in the development of efficient contraceptive methods to achieve fertility regulation. One such advancement is the development of an injectable male contraceptive, reversible inhibition of sperm under guidance (RISUG®)[1–3] as an alternative to vasectomy, to provide safe and long-term contraception in men. RISUG, a polymeric gel consisting of styrene maleic anhydride (SMA) dissolved in dimethyl sulfoxide (DMSO), is a nonhormonal and nontoxic method of contraception with the advantage of being a nonscalpel method and ability to attain easy reversibility.[1,4–6] When injected into the vas deferens, it blocks the sperms that traverse through and destroys them by lowering the surrounding pH and creating charge disturbances in the sperm membrane.[4,7]

Contraceptive efficacy of RISUG has been tested and demonstrated in several experimental models such as rats, rabbits, and langur monkeys. Subsequently, Phase I and Phase II clinical trials have been successfully completed.[8,9] RISUG is presently under Phase III clinical trials across multiple centers in India.[10,11]

Following the successful demonstration of RISUG as an effective contraceptive, studies have been carried out in experimental models to evaluate its functional reversal.[6,12–14] In langur monkeys, noninvasive reversal was achieved by percutaneous squeezing of the vas deferens, along with synchronized application of electrical stimulation, suprapubic percussion, and prerectal digital massage of the vas segments.[6,15] This method of reversal, however, may not be feasible in humans due to different anatomical location of the vas deferens.

Previous studies have shown that at higher pH, RISUG tends to dissolve since its components become unstable. Thus, RISUG can be unbound from the walls of the vas deferens by flushing it out with alkaline solution/solvents such as DMSO and sodium bicarbonate ($NaHCO_3$). Hence, reversal studies have been attempted in rats using DMSO and $NaHCO_3$.[12,16] The efficacy of DMSO and $NaHCO_3$ has been tested and their effects on spermatozoa and the surrounding tissues have been studied.[12–14] These short-term studies on experimental models have reported that reversal of vas occlusion using both these agents results in reversal of azoospermia and restoration of complete fertility. Long-term reversal of vas occlusion in male rats using DMSO and $NaHCO_3$ has been reported recently.[16] This is the first report of functional reversal in rabbit model following short- and long-term RISUG-induced vas occlusion.

MATERIALS AND METHODS

Drug

RISUG® is a copolymer synthesized through gamma irradiation of the monomers, styrene, and maleic anhydride, dissolved in the

Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur - 302 004, Rajasthan, India.
Correspondence: Dr. NK Lohiya (lohiyank@gmail.com)
Received: 31 December 2015; Revised: 22 April 2016; Accepted: 20 May 2016

solvent vehicle DMSO in 1:2 ratio. It was kindly contributed by Prof. Sujoy K Guha, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India.

Animals

Thirty-five New Zealand adult male rabbits (*Oryctolagus cuniculus*), 6–8 months old, weighing 1.5–2.0 kg, were used in the present investigation. The animals were maintained in the Departmental Experimental Animal Facility with 12:12 h light and dark schedule in individual metallic cages (size 16 inch × 18 inch × 15 inch). The temperature in animal house during the study period was maintained at 23 ± 2°C and the relative humidity ranged between 40% and 70%. The animals were fed with rabbit pellet diet (Ashirwad Industries Limited, Chandigarh, India) and water *ad libitum*. The animals were maintained under perfect veterinary supervision in accordance with the guidelines of the regulation of scientific experiments on animals.[17] The experiment protocol was approved by the Institutional Animal Ethics Committee (IAEC).

Experimental design

Rabbits were allocated equally into following seven groups containing five animals in each. Group I: sham-operated control; Group II: vas occlusion with RISUG for 90 days; Group III: vas occlusion with RISUG for 90 days and reversal with DMSO; Group IV: vas occlusion with RISUG for 90 days and reversal with 5% $NaHCO_3$; Group V: vas occlusion with RISUG for 360 days; Group VI: vas occlusion with RISUG for 360 days and reversal with DMSO; and Group VII: vas occlusion with RISUG for 360 days and reversal with 5% $NaHCO_3$.

Vas occlusion

Rabbits of Groups II–VII were subjected to bilateral vas occlusion under sodium thiopentone anesthesia (25 mg kg^{-1} body weight of intravenous injection; THIOSOL Sodium: Neon Laboratories Ltd., Mumbai, Maharashtra, India). A single median incision was made just above the scrotum to locate the vas of inguinal region. The 5–7 μl of the therapeutic dose of RISUG was injected into each vas deferens at initial segment of the vas by a microsyringe. The solidification of RISUG was catalyzed by application of cotton wetted with normal saline and solidified on contact with fluid medium in the vas. The syringe was slowly withdrawn. The vas was placed in its original position and the incision was closed by catgut suture in the inner layer and by silken suture outside. Animals of Group I served as sham-operated control. The vasa of Group I animals were exposed in the similar manner; however, no drug was injected. Postoperative care included antibiotic Ceftriaxone (Cefoat, A to Z Pharmaceuticals Ltd., Surat, Gujarat, India) and anti-inflammatory drug Meloxicam (Melonex, Intas Pharmaceuticals Ltd., Ahmedabad, Gujarat, India), injected in the gluteal muscles. The functional success of vas occlusion was confirmed by semen analysis at intervals of 15 days.

Vas occlusion reversal

Reversal of vas occlusion was performed after 90 days and 360 days of vas occlusion under sodium thiopentone anesthesia. The vas was exposed in a similar way as that of occlusion and injected bilaterally with 250–500 μl of DMSO to dissolve RISUG in Groups III and VI. Likewise, 500–700 μl of 5% $NaHCO_3$ was injected to dissolve the copolymer in Groups IV and VII. The vas was returned to its original position and the incision was closed. Postoperative care was provided as described previously. The success of reversal was assessed after every 15 days semen analysis.

Parameters

The animals of sham-operated control, vas occlusion, and vas occlusion reversal were evaluated for the following parameters.

Seminal characteristics

Semen samples were collected by artificial vagina[18] at an interval of 15 days in all the groups of animals and evaluated for macroscopic (volume, pH, color and consistency) as well as microscopic characteristics (sperm concentration, output, motility, viability, and abnormality) according to the World Health Organization (WHO) manual.[19]

Morphology of ejaculated spermatozoa

Ejaculated spermatozoa of all the groups were processed for light microscopy by Papanicolaou staining method.[19]

Scanning electron microscopy (SEM)

Ejaculated spermatozoa of all the experimental groups were washed twice with phosphate buffer and pelleted at 1500 rpm for 15 min. The sperm pellets were fixed in 2.5% glutaraldehyde for 30 min and washed thrice in phosphate buffer followed by distilled water. A thin film of spermatozoa was smeared on a clean glass slide, air dried, mounted on SEM stub with silver paint, sputter coated at 350 Å with gold and observed under scanning electron microscope (EVO 18, Carl Zeiss, Oberkochen, Germany).

Fertility test

Periodical fertility tests were carried out every 15 days in all experimental groups by cohabitating the male with proven fertile female at 1:2 ratio. Copulation was observed and successful mating was confirmed by wooing sound of the male followed by his falling off the female either backward or in sideways direction. The females used for fertility test were caged separately.

Female rabbits of full term delivery

Fifty percent of pregnant females mated with sham-operated and vas occluded reversed animals were allowed to complete the full term of pregnancy and their record of litters, namely, litter size, body weight, body length, and sex ratio, was maintained.

Female rabbits of cesarean

While the remaining 50% of the pregnant females were undergone cesarean on day 28 under an overdose of intravenous injection of sodium thiopentone. Uterine horns and ovaries were removed, freed from fat and adherent tissues. Weight of gravid uterus, placenta, and ovary was observed. Nongravid uteri were subjected to ammonium sulfide staining for confirmation of nonpregnant status.[20] A number of corpora lutea, implantations, and resorptions were observed. The fetuses were removed by opening uterus and were placed in sequential manner in 0.9% saline solution. All dead and live fetuses were counted and their body weight, length, and sex ratio were studied.

Fertility

On account of the above-mentioned process, the total number delivered females and total number of live and dead litters were recorded to calculate percent of fertility of male rabbits in each group.[21]

Statistical analysis

Values are represented as mean ± standard deviation (s.d.). The mean values were compared by statistical comparisons between sham-operated control group and vas occlusion and/or reversal

groups for evaluation of significant changes by One-way Analysis of Variance test (ANOVA). The difference between means was analyzed by Holm-Sidak multiple comparison using the statistical software SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Seminal characteristics

Semen pH, color, and consistency

The semen pH (7.3–7.6), color (white), and consistency (normal) did not show any marked changes in all the groups following vas occlusion and reversal (data not shown).

Semen volume

Semen volume during preinjection in all groups was observed between 0.57 ± 0.08 ml and 0.59 ± 0.09 ml. Following vas occlusion and reversal, semen volume was found between 0.39 ± 0.07 ml and 0.62 ± 0.05 ml (Group I); 0.37 ± 0.18 ml and 0.57 ± 0.08 ml (Group II); 0.35 ± 0.18 ml and 0.52 ± 0.05 ml (Group III); 0.40 ± 0.08 ml and 0.60 ± 0.07 ml (Group IV); 0.46 ± 0.05 ml and 0.56 ± 0.11 ml (Group V); 0.40 ± 0.14 ml and 0.62 ± 0.05 ml (Group VI); and 0.44 ± 0.05 ml and 0.59 ± 0.11 ml (Group VII), respectively. No significant alterations in semen volume were observed in Groups II–VII when compared with sham-operated control.

Sperm concentration and output

Sperm concentration and output during preinjection periods was recorded between $167.25 \times 10^6 \pm 29.86 \times 10^6$ ml^{-1} and $185.10 \times 10^6 \pm 31.27 \times 10^6$ ml^{-1}, and $96.54 \times 10^6 \pm 16.63 \times 10^6$ per ejaculate and $104.85 \times 10^6 \pm 14.21 \times 10^6$ per ejaculate, respectively. The sperm concentration and output gradually decreased following vas occlusion in Groups II–VII. Complete azoospermia was noticed at 60 days in Group II, 45 days in Groups III, VI, and VII; and 30 days in Groups IV and V, respectively. Preceding reversal, animals showed azoospermia in first 3–4 ejaculations followed by oligozoospermia at 60 days in Groups III ($19.80 \times 10^6 \pm 10.60 \times 10^6$ ml^{-1}), IV ($43.06 \times 10^6 \pm 11.06 \times 10^6$ ml^{-1}), and VII ($14.02 \times 10^6 \pm 9.74 \times 10^6$ ml^{-1}), and 75 days in Group VI ($51.16 \times 10^6 \pm 15.86 \times 10^6$ ml^{-1}), respectively (**Table 1**). After 9–10 ejaculations, animals showed normozoospermia at 135 days in Groups III ($121.52 \times 10^6 \pm 12.37 \times 10^6$ ml^{-1}) and VII ($164.52 \times 10^6 \pm 5.63 \times 10^6$ ml^{-1}), and 150 days in Groups IV ($157.81 \times 10^6 \pm 10.82 \times 10^6$ ml^{-1}) and VI ($166.61 \times 10^6 \pm 13.36 \times 10^6$ ml^{-1}), respectively, comparable to control group. Normal sperm output was gained by the animals following reversal at 120 days in Groups III ($50.83 \times 10^6 \pm 8.37 \times 10^6$ per ejaculate), IV ($54.29 \times 10^6 \pm 8.32 \times 10^6$ per ejaculate), VI ($72.60 \times 10^6 \pm 15.80 \times 10^6$ per ejaculate), and VII ($54.56 \times 10^6 \pm 10.00 \times 10^6$ per ejaculate) subsequently, e.g., proportionate to control group.

Sperm motility

The sperm motility (presented as percentage) during preinjection period in all experimental groups was found between 51.60 ± 10.20 and 62.87 ± 14.58. A complete inhibition of sperm motility was observed following vas occlusion in all experimental groups. Following reversal, Groups III and VII recovered motility at 135 days, e.g., 54.20 ± 3.87 and 57.20 ± 5.71, respectively, whereas Groups IV and VI attained normal motility at 150 days, e.g., 54.56 ± 9.89 and 55.40 ± 3.44 subsequently (**Table 2**).

Sperm viability

The sperm viability (presented as percentage) during preinjection period ranged from 65.02 ± 15.33 to 78.05 ± 9.70. A steep declination in sperm viability was observed prior to attainment of azoospermia in Groups II (13.56 ± 4.61), III (15.80 ± 1.93), IV (15.95 ± 4.96), V (5.18 ± 2.58), VI (14.18 ± 3.72), and VII (9.88 ± 2.97), respectively. Normal viability was resumed after reversal at 105 days in Groups III (59.20 ± 6.97) and IV (63.20 ± 7.19), 120 days in Group VI (61.38 ± 7.99), and 75 days in Group VII (61.67 ± 7.34), respectively.

Sperm abnormality

The sperm abnormality (presented as percentage) during preinjection period in all experimental groups ranged between 21.77 ± 7.11 and 24.86 ± 5.74. The sperm abnormality detected at 15 days of vas occlusion was 26.79 ± 5.05 enhanced to 51.84 ± 6.73 at 45 days in all groups. Following 60–90 days of reversal, the sperm abnormality ranged from 29.50 ± 3.72 to 36.13 ± 3.22 in all groups. After few ejaculations, animals of reversal groups resumed normal morphology, e.g., comparable to control.

Sperm morphology

Semen samples were analyzed for sperm morphology by SEM. The SEM observations of spermatozoa of Group I showed normal morphology with typical oval-shaped head, slender mid-piece, and long tail (**Figure 1a**). During vas occlusion, prior to azoospermia and initial periods when spermatozoa reappeared during reversal various deformities, namely, acrosome damage, elongated head, head-tail separation, damaged, bulging and bent mid-piece, super-coiling in tail, and disrupted plasma membrane, were observed (**Figure 1b–1f**). Following 150 days of reversal period, the sperm morphology was comparable to control.

Fertility test

Female rabbits of full term delivery

The pregnancy record of females of full-term delivery mated with

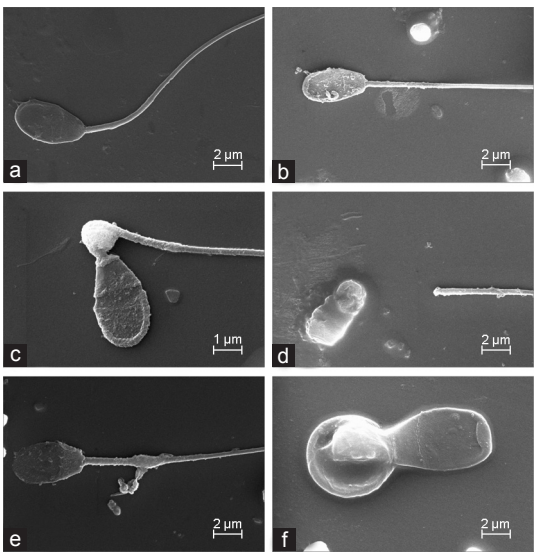

Figure 1: (**a**) Ejaculated sperm of Group I (sham-operated control) animals showing normal morphology with intact head, midpiece, and tail through scanning electron microscope. (**b–f**) Various types of abnormalities in ejaculated spermatozoa during initial period of vas occlusion in Groups II–IV animals showing damaged acrosome, bent and damaged neck, head tail separation, deformed midpiece, super-coiled tail and disrupted membrane (×40 000).

Table 1: Changes in sperm concentration ($\times 10^6$ ml^{-1}) of male rabbits, prior to, and at various intervals following intravasal injection with RISUG and reversal with DMSO/$NaHCO_3$

Mating schedule	*Group I*	*Group II*	*Group III*	*Group IV*	*Group V*	*Group VI*	*Group VII*
Preinjection	176.27±23.98	173.60±37.66	176.20±36.41	171.93±28.04	180.13±46.87	167.25±29.86	185.10±31.27
Vas occlusion (day)							
15	164.80±20.19	79.70±9.71***	67.00±26.54***	26.31±17.34***	7.36±5.39***	45.80±7.01***	35.20±11.71***
30	149.00±23.19	33.90±16.36***	20.04±6.26***	0.00	0.00	26.34±8.90***	5.01±3.86***
45	167.80±12.01	7.80±4.51***	0.00	0.00	0.00	0.00	0.00
60	172.20±9.60	0.00	0.00	0.00	0.00	0.00	0.00
75	141.40±12.12	0.00	0.00	0.00	0.00	0.00	0.00
90	143.00±24.00	0.00	0.00	0.00	0.00	0.00	0.00
105–360#	146.80±17.70	0.00	0.00	0.00	0.00	0.00	0.00
Reversal (day)							
15	164.80±16.13		0.00	0.00		0.00	0.00
30	155.40±15.39		0.00	0.00		0.00	0.00
45	160.20±18.30		0.00	0.00		0.00	0.00
60	164.80±13.19		19.80±10.60***	43.06±11.06***		0.00	14.02±9.74***
75	165.20±12.82		28.18±13.05***	45.44±14.45***		51.16±15.86***	22.51±14.89***
90	146.80±20.41		34.22±7.97***	43.05±11.22***		71.81±10.25***	42.13±19.66***
105	152.40±14.06		67.32±12.54***	93.17±12.54***		91.16±7.12***	95.83±9.85***
120	146.80±18.02		108.43±4.16**	121.44±13.35		124.83±19.76	114.94±12.89*
135	168.40±17.37		121.52±12.37	141.20±16.85*		142.19±12.00*	164.52±5.63
150	164.40±11.57		165.41±14.12	157.81±10.82		166.61±13.36	179.15±21.61

Values are expressed as mean±s.d. Group I: sham-operated control; Group II: vas occlusion with RISUG for 90 days; Group III: vas occlusion with RISUG for 90 days and reversal with DMSO; Group IV: vas occlusion with RISUG for 90 days and reversal with 5% $NaHCO_3$; Group V: vas occlusion with RISUG for 360 days; Group VI: vas occlusion with RISUG for 360 days and reversal with DMSO; Group VII: vas occlusion with RISUG for 360 days and reversal with 5% $NaHCO_3$. *P<0.05; **P<0.01; ***P<0.001; #The value of sperm concentration ($\times 10^6$ ml^{-1}) of Group I animals from 105–360 days at the interval of 15 days fluctuates in the given range. RISUG: reversible inhibition of sperm under guidance; DMSO: dimethyl sulfoxide; s.d.: standard deviation; $NaHCO_3$: sodium bicarbonate

Table 2: Changes in sperm motility (%) of male rabbits, prior to, and at various intervals following intravasal injection with RISUG and reversal with DMSO/$NaHCO_3$

Mating schedule	*Group I*	*Group II*	*Group III*	*Group IV*	*Group V*	*Group VI*	*Group VII*
Preinjection	62.87±14.58	51.60±10.20	60.53±11.40	56.00±11.78	57.40±9.84	57.20±10.83	58.87±10.16
Vas occlusion (day)							
15	58.20±19.92	0.00	0.00	0.00	0.00	0.00	0.00
30	48.20±6.72	0.00	0.00	-	-	0.00	0.00
45	61.60±14.35	0.00	-	-	-	-	-
60	68.40±14.57	-	-	-	-	-	-
75	66.40±15.60	-	-	-	-	-	-
90	75.20±10.78	-	-	-	-	-	-
105–360#	67.00±10.98						
Reversal (day)							
15	59.80±9.93		-	-		-	-
30	61.80±12.43		-	-		-	-
45	59.20±8.61		-	-		-	-
60	64.00±7.24		0.00	0.00		-	0.00
75	66.00±5.40		0.00	0.00		0.00	7.20±2.79***
90	65.20±6.21		14.40±5.99***	0.00		29.60±12.60***	29.40±8.71***
105	56.80±11.72		30.80±5.84***	32.81±11.44**		37.00±4.86*	45.20±9.15
120	58.00±8.37		41.80±6.97**	46.46±6.15		46.60±4.88	45.60±7.23
135	56.40±5.39		54.20±3.87	48.43±7.70		45.80±2.86	57.20±5.71
150	68.80±8.03		49.80±10.25	54.56±9.89		55.40±3.44	56.00±6.63

Values are expressed as mean±s.d. Group I: sham-operated control; Group II: vas occlusion with RISUG for 90 days; Group III: vas occlusion with RISUG for 90 days and reversal with DMSO; Group IV: vas occlusion with RISUG for 90 days and reversal with 5% $NaHCO_3$; Group V: vas occlusion with RISUG for 360 days; Group VI: vas occlusion with RISUG for 360 days and reversal with DMSO; Group VII: vas occlusion with RISUG for 360 days and reversal with 5% $NaHCO_3$. *P<0.05; **P<0.01; ***P<0.001. #The value of sperm motility (%) of Group I animals from 105–360 days at the interval of 15 days fluctuates in the given range. RISUG: reversible inhibition of sperm under guidance; DMSO: dimethyl sulfoxide; s.d.: standard deviation; $NaHCO_3$: sodium bicarbonate

male rabbits following reversal with DMSO (Groups III and VI) and $NaHCO_3$ (Groups IV and VII) did not reveal any change in litter size, body weight and width, and sex ratio of litters when compared with sham-operated control.

Female rabbits of cesarean

Similarly, implantation records in cesarean females following reversal with DMSO and $NaHCO_3$ also revealed no changes in weights of gravid uterus, placenta, ovary, and number of corpora lutea, implantations,

resorptions, body weight, length, and sex ratio of fetuses as compared to sham-operated control.

Fertility

Group I showed 100% fertility throughout the study period. The animals of vas occlusion and reversal groups (II–VII) showed 100% of fertility during preinjection whereas 0% of fertility indicated by sterile mating was observed during vas occlusion and initial periods of reversal. Following reversal, fertility was restored at 90 days (Groups III, VI, and VII) and 105 days (Group IV), respectively (**Table 3**).

DISCUSSION

Many vas-based contraceptives have been attempted to achieve fertility regulation with several limitations of surgical intervention/complications as well as lack of reversibility. RISUG is proposed to be successful in terms of safety and efficacy without significant adverse effects.[22] The present work was aimed to evaluate, in rabbits, short- and long-term reversal of RISUG-induced vas occlusion using DMSO and $NaHCO_3$. The results revealed complete restoration of fertility in male rabbits as evidenced by presence of spermatozoa and normal sperm functional characteristics following reversal. The success of reproductive function retrieval was further confirmed from the pregnancy record of full-term and cesarean females mated with these sterility-reversed male rabbits which were comparable to control.

The preliminary study evaluated teratogenic potential of RISUG in males without affecting pregnant rabbits and their respective F_1 generation at different dose regimen.[23] Similarly, removal of SMA from the vas deferens of rats by the injection of DMSO was demonstrated by Sethi *et al.*[24] in 1990. This short-term study showed restoration of normal mucosal epithelium of the vas deferens, which was compromised during vas occlusion, by 4 weeks following flushing out of RISUG with DMSO thus demonstrating its potential as an effective reversal agent. However, this study presented the reversibility status only at an anatomical level. Functional success of RISUG-induced vas occlusion reversal was evaluated by Lohiya *et al.*[13] by studying the sperm characteristics and teratological observations of the F_1 progenies of the reversed rats. Changes in sperm morphology such as bent/detached tail, acrosomal damage, and other aberrations in sperm head that were observed during vas occlusion and initial periods of reversal gradually returned to normal with 100% restoration of fertility after 90 days of reversal with DMSO. Teratological studies showed the F_1 progeny to be normal and comparable to control which further highlighted DMSO's role as a reversal agent. In another study on reversal, Koul *et al.*[12] reported successful restoration of fertility, following vas occlusion, by flushing of the vas deferens with 10% $NaHCO_3$ by observing the presence of spermatozoa in the vaginal smear and pregnancy in female rats. The principle behind this method of flushing out of RISUG is that SMA is unstable in a basic environment. Hence, $NaHCO_3$ serves as one of the ideal candidates for removal of the SMA bound to the vas deferens.[12] In case of DMSO, since it is a solvent in which SMA dissolves to form a polymeric gel, injection of this chemical would result in dissolution of the polymer from the walls of the vas deferens, subsequently resulting in the appearance of the spermatozoa in the ejaculate. Moreover, genotoxicity studies on rats showed that RISUG-induced vas occlusion and its reversal using DMSO and 5% $NaHCO_3$ were safe and did not cause any genotoxicity in the leukocytes and the testis of these rats thereby confirming the safety of this method of contraception and reversal.[14]

As a further advancement of the short-term studies, evaluation of reversal efficacy have been assessed for a longer duration, e.g., 360 days, in RISUG-injected animals. Ansari *et al.*[16] recently reported achievement of long-term reversal of vas occlusion in male rats using DMSO and $NaHCO_3$. Periodical assessment of

Table 3: Fertility record (percent of fertility) of male rabbits, prior to, and at various intervals following intravasal injection with RISUG and reversal with DMSO/$NaHCO_3$ (male mated with females in 1:2 ratio)

Mating schedule	*Group I*	*Group II*	*Group III*	*Group IV*	*Group V*	*Group VI*	*Group VII*
Preinjection	100	100	100	100	100	100	100
Vas occlusion (day)							
15	100	0	0	0	0	0	0
30	100	0	0	0	0	0	0
45	100	0	0	0	0	0	0
60	100	0	0	0	0	0	0
75	100	0	0	0	0	0	0
90	100	0	0	0	0	0	0
105–360	100				0	0	0
Reversal (day)							
15	100		0	0		0	0
30	100		0	0		0	0
45	100		0	0		0	0
60	100		0	0		0	0
75	100		0	0		0	10
90	100		20	0		60	50
105	100		60	60		70	90
120	100		80	90		90	90
135	100		100	90		90	100
150	100		100	100		100	100

Group I: sham-operated control; Group II: vas occlusion with RISUG for 90 days; Group III: vas occlusion with RISUG for 90 days and reversal with DMSO; Group IV: vas occlusion with RISUG for 90 days and reversal with 5% $NaHCO_3$; Group V: vas occlusion with RISUG for 360 days; Group VI: vas occlusion with RISUG for 360 days and reversal with DMSO; Group VII: vas occlusion with RISUG for 360 days and reversal with 5% $NaHCO_3$. RISUG: reversible inhibition of sperm under guidance; DMSO: dimethyl sulfoxide; $NaHCO_3$: sodium bicarbonate

fertility status, pregnancy record, sperm characteristics, teratology of the F_1 generation, and histological studies indicated that while 100% restoration of reproductive function could be achieved using both solvents, complete reversal with 5% $NaHCO_3$ was earlier (e.g., in 30 days) than with DMSO infusion, wherein 100% of fertility was restored in 45 days. Histological studies of the vas deferens indicated complete regeneration of the vas epithelium, thereby indicating reliability of this methodology of contraception reversal.

In the present study, on rabbits, azoospermia was observed 30–60 days following RISUG injection and the sperm began to reappear around 60–75 days postreversal with DMSO and 5% $NaHCO_3$. Sperm motility and viability began to recover gradually following reversal too. While sperm motility was restored between 105 days and 135 days in reversal groups, sperm viability improved and normalized between 75 days and 105 days in both reversal groups too. In addition, 100% fertility was attained in both groups between 90 days and 105 days. Further, pregnancy record of both full-term and cesarean females mated with male rabbits following reversal was comparable to control and the F_1 progeny of reversed animals were found to be normal. However, in contrast to the reversal studies carried out on rats, in rabbits, recovery of sperm characteristics and fertility were observed to be longer. This delayed reversibility could be attributed to the anatomy and physiology of the experimental model used.

Reversal of RISUG-induced contraception has also been attempted in langur monkeys *Presbytis entellus entellus*, albeit, in a different way as compared to rats.[6] Noninvasive reversal in langur monkeys was achieved by palpitation, percutaneous electrical stimulation and squeezing of the vas deferens, and forced vibratory movements, suprapubic percussion, and prerectal digital massage of the vas segments. This mechanism propels the SMA toward the urethra resulting in normospermia with normal sperm motility and viability and subsequently its fertilizing ability. Revival of sperm characteristics and fertilizing ability were confirmed using sperm functional tests and biochemical analysis.[6] Electron microscopic studies of the vas deferens revealed repair of the ultrastructural changes that occurred during vas occlusion, beginning from 90 days postreversal. Restoration of normalcy in these tissues, as evidenced by presence of prominent plasma membrane, nucleus, and cytoplasmic organelles, was observed after 150 days of reversal.[25] This method was suggested to be a faster and a noninvasive approach to achieve functional reversal of vas occlusion in monkeys.

However, the shortcoming of this method of reversal is that it may not be feasible in humans due to the fact that their vas deferens is difficult to palpate reliably proximal to the scrotum. Hence, reversal of RISUG-induced vas occlusion using DMSO and $NaHCO_3$ is being developed as a reliable, efficient and a safe method. The drug regulatory agency (DCGI) has conveyed its approval for clinical trials.

CONCLUSION

The results of this rabbit study have demonstrated that reversal of long-term RISUG-induced vas occlusion using DMSO and $NaHCO_3$ is feasible, effective, and safe, thus providing better option for reversible male contraception for men, on successful completion of clinical trials.

AUTHOR CONTRIBUTIONS

NKL and ASA conceived and designed the study; AB conducted the experiment and analyzed the data; ASA and KB drafted the manuscript; and NKL finally approved the version to be published. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

ACKNOWLEDGMENTS

The study was supported by the Indian Council of Medical Research, New Delhi. The infrastructural facilities provided by the Head of the Department are gratefully acknowledged. The authors are thankful to Prof. Sujoy K Guha, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India, for providing RISUG. The ultrastructural studies were carried out at the electron microscope facility of the University Science Instrumentation Centre, University of Rajasthan, Jaipur, Rajasthan, India.

REFERENCES

1 Guha SK. Injectable contraceptives for the male. *Exp Opin Invest Drugs* 1995; 4: 537–43.
2 Guha SK. Contraceptive for Use by a Male. US Patent 5488075; 1996.
3 Sharma RS, Rajanna A, Singh BK, Mathur AK, Mukherjee AK. Current status of development of RISUG: an intravasal injectable male contraceptive. In: Sharma RS, Rajanna A, Rajalakshmi M, editors. Recent Advances and Challenges in Reproductive Health Research. New Delhi: ICMR; 2008. p. 135–49.
4 Lohiya NK, Manivannan B, Mishra PK. Ultrastructural changes in the spermatozoa of langur monkeys, *Presbytis entellus entellus* after vas occlusion with styrene maleic anhydride. *Contraception* 1998; 57: 125–32.
5 Lohiya NK, Manivannan B, Mishra PK, Pathak N. Non-invasive reversible vas occlusion with styrene maleic anhydride: a possible alternative to vasectomy. In: Puri CP, Van Look PF, editors. Sexual and Reproductive Health. New Delhi: New Age International (P) Ltd.; 2001. p. 249–68.
6 Lohiya NK, Manivannan B, Mishra PK, Pathak N, Balasubramanian SP. Intravasal contraception with styrene maleic anhydride and its non-invasive reversal in langur monkeys (*Presbytis entellus entellus*). *Contraception* 1998; 58: 119–28.
7 Guha SK. Biophysical mechanism-mediated time-dependent effect on sperm of human and monkey vas implanted polyelectrolyte contraceptive. *Asian J Androl* 2007; 9: 221–7.
8 Guha SK, Singh G, Anand S, Ansari S, Kumar S, *et al*. Phase I clinical trial of an injectable contraceptive for the male. *Contraception* 1993; 48: 367–75.
9 Guha SK, Singh G, Ansari S, Kumar S, Srivastava A, *et al*. Phase II clinical trial of a vas deferens injectable contraceptive for the male. *Contraception* 1997; 56: 245–50.
10 Chaki SP, Das HC, Misro MM. A short-term evaluation of semen and accessory sex gland function in phase III trial subjects receiving intravasal contraceptive RISUG. *Contraception* 2003; 67: 73–8.
11 Lohiya NK, Alam I, Hussain M, Khan SR, Ansari AS. RISUG: an intravasal injectable male contraceptive. *Indian J Med Res* 2014; 140: 63–72.
12 Koul V, Srivastava A, Guha SK. Reversibility with sodium bicarbonate of styrene maleic anhydride, an intravasal injectable contraceptive, in male rats. *Contraception* 1998; 58: 227–31.
13 Lohiya NK, Suthar R, Khandelwal A, Goyal S, Ansari AS, *et al*. Sperm characteristics and teratology in rats following vas occlusion with RISUG and its reversal. *Int J Androl* 2010; 33: 198–206.
14 Ansari AS, Alam I, Hussain M, Khan SR, Lohiya NK. Evaluation of genotoxicity in leucocytes and testis following intra-vasal contraception with RISUG and its reversal by DMSO and $NaHCO_3$ in Wistar albino rats. *Reprod Toxicol* 2013; 36: 53–9.
15 Lohiya NK, Manivannan B, Mishra PK. Repeated vas occlusion and non-invasive reversal with styrene maleic anhydride for male contraception in langur monkeys. *Int J Androl* 2000; 23: 36–42.
16 Ansari AS, Hussain M, Khan SR, Lohiya NK. Relative suitability of DMSO and $NaHCO_3$ for reversal of RISUG® induced long-term contraception. *Andrology* 2016; 4: 306–13.
17 CPCSEA. Guidelines on the regulation of scientific experiments on animals. New Delhi: Ministry of Environment and Forests, CPCSEA Standard Operating Procedures for Institutional Animals Ethics Committee (IAEC); 2010.
18 Walton A. Improvement in the design of an artificial vagina for the rabbit. *J Physiol* 1958; 143: 26–8.

19 WHO. WHO Laboratory Manual for Examination and Processing of Human Semen. 5th ed. Geneva: WHO Press; 2010.
20 Kopf R, Lorenz D, Salewski E. The effect of thalidomide on the fertility of rats in reproduction experiments over two generations. *Naunyn Schmeidebergs Arch Exp Pathol Pharmakol* 1964; 247: 121–35.
21 Derelanko MJ, Auletta CS. Handbook of Toxicology. 3rd ed. Boca Raton, FL: CRC Press; 2014. p. 355–9.
22 Lohiya NK, Manivannan B, Mishra PK, Pathak N. Vas deferens, a site of male contraception: an overview. *Asian J Androl* 2001; 3: 87–95.
23 Sethi N, Srivastava RK, Singh RK. Male mediated teratogenic potential evaluation of new antifertility compound SMA in rabbit (*Oryctolagus cuniculus*). *Contraception* 1990; 42: 215–23.
24 Sethi N, Srivastava RK, Singh RK. Histological changes in the vas deferens of rats after injection of a new male antifertility agent 'SMA' and its reversibility. *Contraception* 1990; 41: 333–9.
25 Manivannan B, Mishra PK, Lohiya NK. Ultrastructural changes in the vas deferens of langur monkeys *Presbytis entellus entellus* after vas occlusion with styrene maleic anhydride and after its reversal. *Contraception* 1999; 59: 137–44.