WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 12, 847-859. Research Article ISSN 2277– 7105

SAFETY EVALUATION OF NANOTECHNOLOGY BASED BIOMEDICINE- RISUG-M, A MALE CONTRACEPTIVE AGENT IN RATS

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Article Received on
06 August 2017,
Revised on 28 August 2017,
Accepted on 20 Sept. 2017
DOI: 10.20959/wjpr201712-9698

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ABSTRACT

The non-hormonal contraceptive agent, RISUG-M (SMA-Fe_3O_4-Cu-DMSO), consists of a co-polymer styrene maleic anhydride (SMA), magnetic particles: iron oxide (8-12%), electrically conductive particles: copper (3-8%) dissolved in 99% pure dimethylsulphoxide (DMSO). Present study was conducted to assess the toxic effects of the RISUG-M in male Charles Foster rats. Healthy adult rats were randomly divided into two groups (Gr.) consisting 10 animals each. Gr. I. rats were injected with vehicle only (DMSO) in vas deferens served as Control. Rats in G.II were injected with RISUG-M in the vas deferens bilaterally and observed for a period of 14 days. On day 15 of autopsy, blood samples were collected for haematology and biochemical analysis, and body organs (viz. brain, heart, liver, lungs, kidney, adrenal, spleen and gonads) were dissected out, weighed and fixed in formalin for histopathology. Results showed no any significant changes in body weights, organ weights, food and water consumption, hematology, biochemistry of marker enzymes and histopathology of vital organs in RISUG-M treated as compared to control rats. There were no any toxic effects/ mortality observed during entire treatment period. From the toxicity point of view this newly developed injectible contraceptive does not have any adverse effects during 14 days toxicity study.

KEYWORDS: RISUG-M, Male Contraceptive Agent, Toxicity Profile.

INTRODUCTION

The non-hormonal contraceptive, named RISUG (an acronym for Reversible Inhibition of Sperm Under Guidance) has expected to provide a valuable addition to the currently limited options of male contraception.[1] RISUG (Mark sans Pharma, Mumbai, India) consists of a co-polymer styrene maleic anhydride (SMA) dissolved in 99.9% pure dimethylsulphoxide (DMSO) has been developed by Prof. S.K. Guha and his team at I.I.T. Kharagpur.[2] Earlier studies have been shown the reproductive functional success, safety of vas occlusion by RISUG, and its reversal by dimethylsulphoxide (DMSO), followed by multigenerational (F1-F3) teratogenicity studies in rats when RISUG - a co-polymer of styrene maleic anhydride (SMA) dissolved in 0.01 ml DMSO was injected into the lumen of the vas deferens bilaterally at the dose levels of 0.25, 0.50 and 1.00 mg/vas/rat.[3] Previous studies with RISUG have also shown spermicidal activity and its non-toxicity[4,5] and teratogenic safety[6] in rats. Injection of RISUG causes degenerative changes to sperm acrosome and its contents when it comes in contact with the polymer. The positive and the negative charges on the polymer surface causes the surface of sperm burst, making it immotile and incapable to fertilize an egg.[7-9]

RISUG-M is an advancement of the contraceptive agent, RISUG which consists of a co-polymer styrene maleic anhydride (SMA), magnetic particles: iron oxide (8-12%), electrically conductive particles: copper (3-8%) dissolved in 99% pure DMSO.[10] It is long time effective, non-invasively reversible and controllable. It also shows antimicrobial, anti HIV and anti-prostate cancer activity in males.[11,12] Due to large surface-to-volume ratio and magnetic properties, the contraceptive magnetic nanoparticles tend to aggregate and adsorb to plasma proteins.[13] But the use of SMA causes the surface coverage of magnetic particles Fe_3O_4 with the safe and effective polymer significantly increases the stability and ensure proper distribution by eliminating aggregation and adsorption of proteins.[14,15] It has been demonstrated from the well known phenomenon that both the concentration of magnetic particles and the cross – linking density of the ferrogels play a crucial role in the magneto elasticity[16] and its effect is due to change of the electricity of ferromagnetic polymeric compound with magnetization.[17] On applying magnetic field, ferrogels acquires a net magnetic moment due to ordered orientation of particles in the field direction which is reversible and the material reverts to randomized orientation on switching off the external field.[18] Therefore, the use of magnetic iron oxides have two advantages - low toxic to human beings and target drugs or antibodies to a specific cell by applying magnetic field.[19]

Further, copper particles add two functions to the contraceptive drug – its intrinsic high electrical conductivity makes the overall compound electrically conductive which can be reverted to facilitate removal for restoration of fertility. Secondly, it displaces zinc from the sperm membrane and head by itself which accounts in decreased motility of spermatozoa and lowers the fertilizing potential of the sperm.[20-22]

Therefore, the present study was undertaken to evaluate toxic effects on body and organ weights, food and water intake, haematological and biochemical aspects, and histopathology of a new injectable male antifertility agent, RISUG-M, in rats.

METHODS

Chemicals: The test compound RISUG-M, a male antifertility agent was provided by Dr. Sujoy K. Guha, Professor of Biomedical Engineering at the School of Medical Science and Technology, Indian Institute of Technology (IIT), Kharagpur, India. All other chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich Chemical Company (India).

Animals

Total of 40 adult rats (170-180 g body weights) of Charles Faster strain used in this study were obtained from Institute's animal house. Animals were acclimatized for 1 week, maintained in standard laboratory conditions (24±2^{0}C) with 12**:**12 h light and dark cycles in individual polypropylene cages and fed with pelleted standard rat diet (Lipton India Ltd., Bangalore) and water *ad libitum*. Experimental protocol was approved by the 'Institutional Animal Ethical Committee' (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (Approval No. IAEC/2011/140 dated 30-11-2011).

Experimental Design

Healthy and disease-free rats were selected in the present study on the basis of initial health check-up. Adult male rats were divided into two groups (Gr.) consisting of fifteen animals each. Gr. I rats were injected with vehicle (0.01 ml DMSO) only in the lumen of vas deferens, served as Control. Rats in Gr. II were subjected to bilateral vas occlusion under ether anesthesia. The vase were exposed by a single median incision surgically just above the urethral opening as per method described previously.[5] The distal ampullary portion of vas deferens was located beneath the fat bodies having greater diameter of duct where RESUG-M

was injected into the lumen of each vas deferens. The vases of treated rats were placed properly in their original position and the incision was closed by stitching with catgut internally and upper skin incision with nylon thread. Post-operative care was taken by dressing with Neosporin antibiotic powder and merbromin solution (2% w/v) and anti-inflammatory drugs. The antibiotic, Terramycin (Pfizer Ltd, Bombay) was injected intramuscularly to each rat for 5 consecutive days as per method of Sethi et al.[4]

The body weights, food and water consumption were recorded initially (day 0) before the beginning of the experiment and after 14 days post-injection period. On day 15, all the animals were sacrificed and blood samples were collected for biochemical analysis and hematology. Hematological parameters were studied initially and terminally. The body organs (viz. brain, heart, liver, lungs, kidney, adrenal, spleen and gonads) were dissected out freed from connective tissues /blood clots in chilled saline and weights recorded. The tissues from different organs were fixed in 10% formalin for histopathology purpose.

Statistical Analysis

Data were expressed as mean ± S.D. Student's '*t*' test and one-way ANOVA (one factor analysis of variance) was applied for statistical significance and comparisons between control and treated groups of rats. P values < 0.1 and 0.2 were considered as non-significant and p values < 0.05 considered as significant.

RESULTS

General Health Check-Up & Mortality

Animals belonging to control and treated group were generally active and healthy throughout the period of the study. No mortality was seen in either control or treated group of rats. The vas occluded control and RESUG-M- injected animals had adopted the normal behavior within a week of surgery. None of them showed hypo- and hyper- excitability of nervousness during implants, post-operative reversal and handling thereafter.

Food and water consumption

Measurement of the initial water and leftover water and pellets given to the animals, did not show any significant change in the average 24-h water and food intake of animals in treated groups as compared to controls (Figure 1).

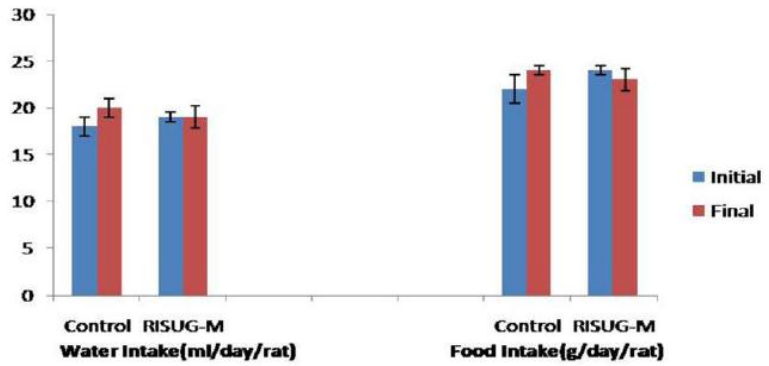
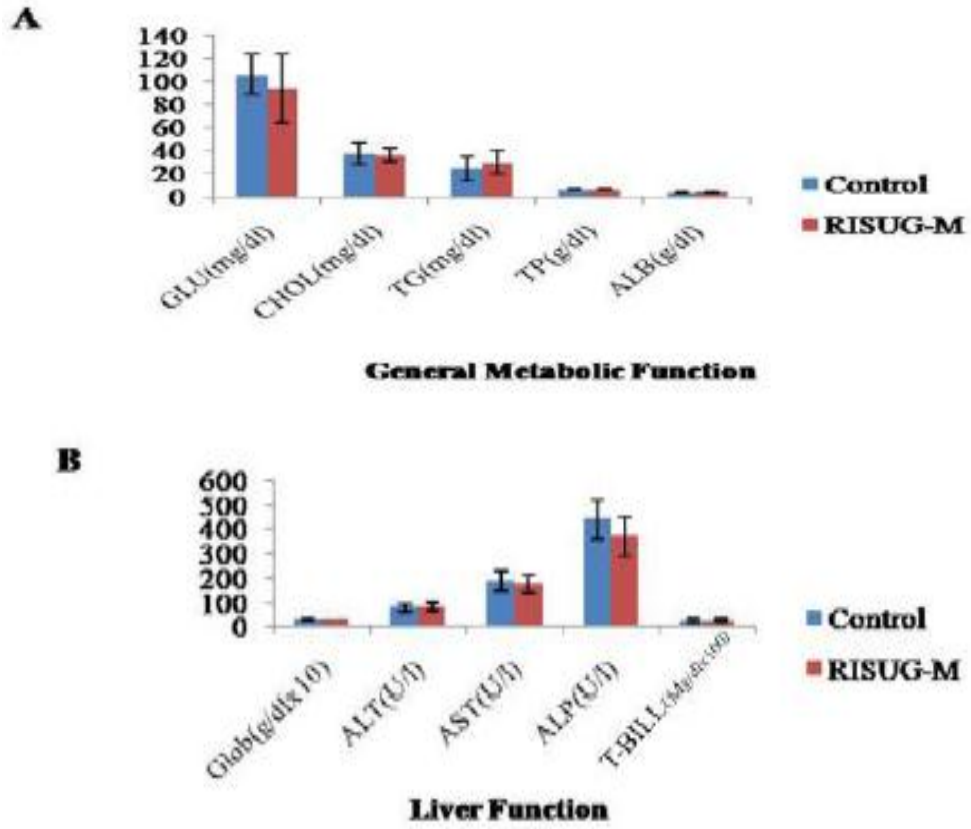


Figure. 1: Showing average water (ml) and food (g) consumption (/day/rat) in control and RISUG-M-treated rats before (day 0) and after 14 days of intravasal injection.

Biochemistry

The biochemical parameters 'marker' for general metabolic functions which included glucose, cholesterol, triglycerides, total protein, albumin and globulin did not show any significant difference in control and RESUG-M injected group of rats. Similarly, the animals from both the groups did not show any significant variation in the biochemical parameters of kidney function (blood urea nitrogen, criatinin, calcium and phosphate) and liver function (globulin, ALT, AST, ALP, T-Bill) (Figure 2A-C).



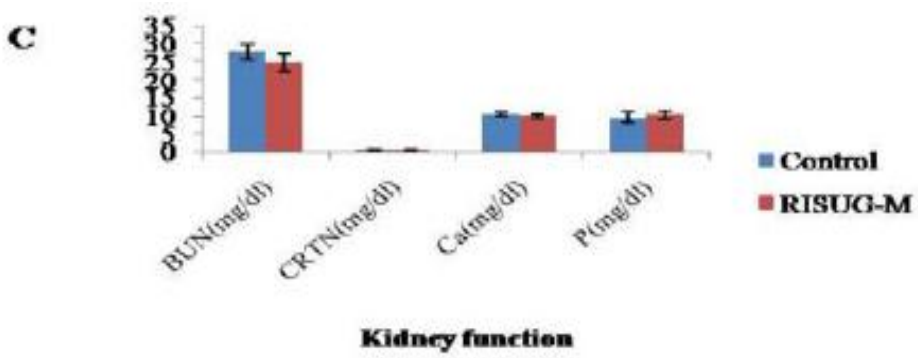
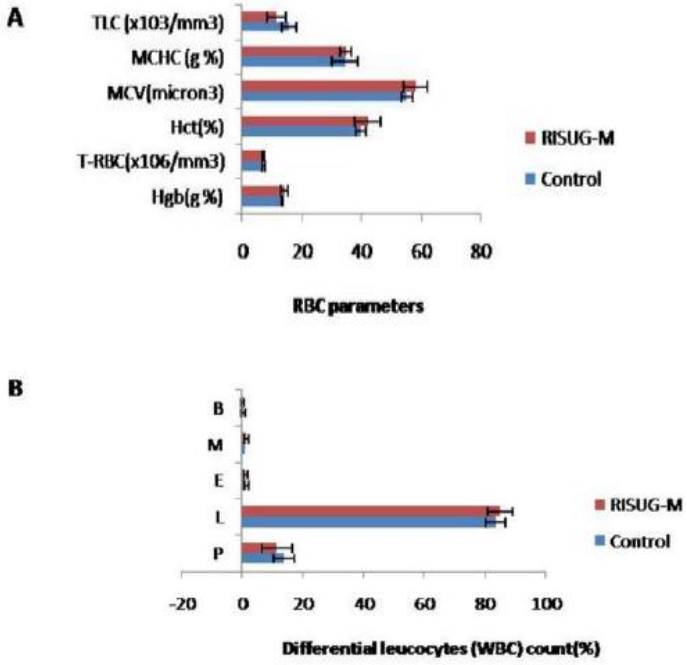


Figure. 2: Showing serum biochemistry (terminal) for general metabolic functions for glucose(GLU), cholesterol(CHOL), triglycerides(TG), total protein(TP) and albumin(ALB) (2A), liver function such as globulin(GLOB), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Total Bilirubin (T-Bill) (2B) and kidney function viz. blood urea nitrogen(BUN), criatinin(CRTN), calcium(Ca) and phosphate(P) (2C) in control and RISUG-M-treated rats.

Haematology

There were no significant changes observed in any of the haematological parameters viz. Hgb(g%), RBC (x106/mm^3), Hct(%), MCV(micron^3), MCHC(g%), TLC(x10^3/mm^3), DLC(%) of polymorphs, lymphocytes, macrophages and eosinophils and platelets (x10^3/mm^3) of treated- as compared to control- groups of animals. All the parameters were well within the limit of normalcy (Figure 3A-C).



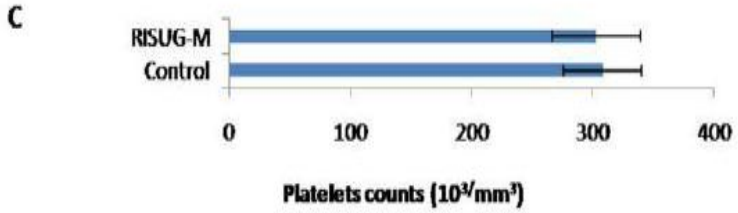


Figure. 3: Haematological parameters of Red blood cells (A) viz. Hgb (g%), RBC (x106/mm^3), Hct (%), MCV (micron^3), MCHC (g%), TLC (x10^3/mm^3), (B) white blood cells (DLC (%) of polymorphs, lymphocytes, macrophages and eosinophils) and (C) platelets (x10^3/mm^3) in control and RISUG-M treated rats. Hgb=Haemoglobin, T-RBC=Total Red Blood Cell, Hct=Haematocrit, MCV=Mean corpuscular volume, MCHC=Mean corpuscular haemoglobin concentration, TLC=Total leucocyte count, DLC=Differential leucocyte count, P=Polymorph, L=Leucocyte, M=Monocyte, E=Eosinophil, B=Basophil.

Body and organ Weights

The data obtained on body and organ weights are represented in Table 1 and 2. Results showed that there was no any significant change (P < 0.1-0.2) in body weights before vas occlusion (day 0) or after 14 days vas occlusion with single injection of RESUG-M and comparable gain in body weights among the animals of both control and treated groups was seen (Table 1). Similarly, the vital organs weights viz. brain, heart, liver, lungs, kidney, adrenal, spleen and gonads, did not show any significant change in its absolute or relative organ weights in RESUG-M-treated as compared to control rats (Table 2).

Table 1. Body and absolute organ weights in control and RESUG-M treated rats after 14 days [a].

Group/ Treatment	Body weights(g)		Absolute Organ weights (g)										
	Initial	Final	Adrenal		Brain	Gonads		Heart	Kidney		Liver	Lungs	Spleen
			Rt	Lt		Rt	Lt		Rt	Lt			
I. Control	152 ±16	182 ±23.5	0.023 ±0.003	0.025 ±0.004	1.686 ±0.167	0.956 ±0.104	0.98 ±0.13	0.615 ±0.075	0.712 ±0.06	0.695 ±0.045	6.987 ±0.642	1.389 ±0.184	0.645 ±0.112
II.RISUG-M	150 ±20	181 ±28	0.025 ±0.005	0.023 ±0.004	1.752 ±0.072	1.024 ±0.094	1.012 ±0.072	0.643 ±0.093	0.709 ±0.059	0.721 ±0.059	7.045 ±0.74	1.329 ±0.164	0.679 ±0.119

Rt- Right, Lt- Left, [a] All values are mean ± SD, (n=15 number of animals).

Table 2. Relative organ weights (gm/Body weight) in control and RISUG-M treated rats after 14 days [a].

Groups/ Treatments	Relative Organ weights (%)										
	Adrenal		Brain	Gonads		Heart	Kidney		Liver	Lungs	Spleen
	Rt	Lt		Rt	Lt		Rt	Lt			
I. Control	0.012 ±0.002	0.011 ±0.001	0.941 ±0.053	0.53 ±0.065	0.527 ±0.07	0.339 ±0.028	0.386 ±0.031	0.389 ±0.032	3.898 ±0.192	0.769 ±0.036	0.353 ±0.025
I. RISUG-M	0.011 ±0.001	0.012 ±0.003	0.965 ±0.093	0.562 ±0.043	0.555 ±0.054	0.358 ±0.093	0.394 ±0.063	0.394 ±0.058	3.883 ±0.263	0.74 ±0.157	0.367 ±0.028

Rt- Right, Lt- Left, [a] All values are mean ± SD, (n=15 number of animals).

Histopathological Examination

The microscopic examination of the histological slides of the vital organs (viz. brain, heart, liver, lungs, kidney, adrenal, spleen and gonads) did not reveal any pathological changes in RESUG-M injected rats as compared to controls (Figure 4).

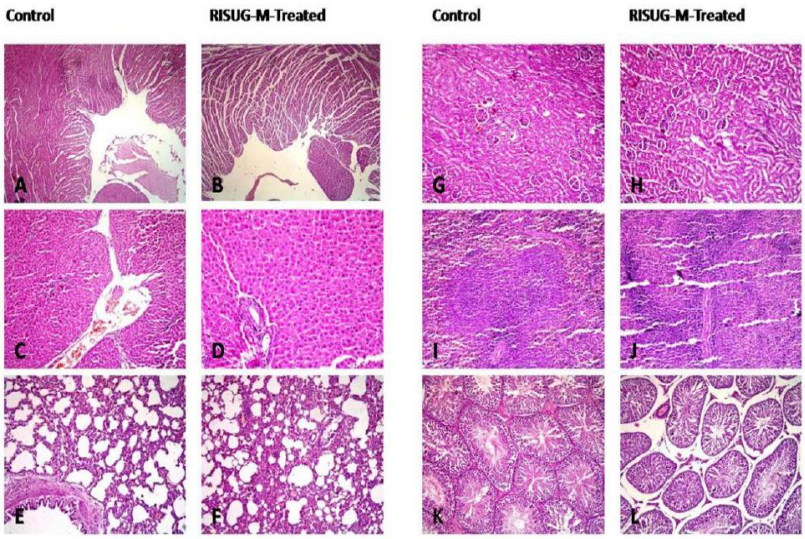


Figure 4. Histology of Heart (A), Liver (C), Lung (E), Kidney (G), Spleen (I) and Testis (K) of Control rats. RISUG-M treatment after 14 days did not show any pathological changes in Heart (B), Liver (D), Lung (F), Kidney (H), Spleen (J) and Testis (L) of treated rats as compared to controls. 100 X magnification for all figures; Haematoxylin-Eosin (H & E) staining.

DISCUSSION

The surgical contraceptive method, 'surgical vasectomy', blocks the passage of spermatozoa permanently and there is no vasectomy reversal in further, which causes epididymal

dysfunction, abnormality in spermatozoa and unable to restore fertility.[23] Recently, chemical occlusion of the vas deferens by RISUG has been considered to be an ideal male contraceptive method that is in Phase III clinical trial after successful completion of phase I and II clinical trials. The polymer SMA can be used to block the lumen of the vas deferens over an extended period of time. In our laboratory, systemic toxicity evaluation of RISUG-injection had been carried out in detail in rat, rabbit and rhesus monkeys previously.[4-6, 24-27] Findings indicated that SMA-injection did not cause any systemic toxicity, male mediated teratogenicity and multigenerational teratogenicity in experimental animals.[3] Results of the present study on acute toxicity profile in rats with RESUG-M, did not show any significant change in gross behavior, food and water intake, haematological parameters and biochemical analysis of marker enzymes of kidney, liver and function and in histoarchitecture of body organs in RESUG-M injected as compared to control rats in after 14 days treatment.

Previous studies have shown that the male gamete-spermatozoa and its morphology play a significant role in fertilization process, especially the anterior part, acrosome which secrets three important key enzymes – 5'-nucleotidase (5'-NT), hyaluronidase and proacrosin-acrosin system which facilitate sperm-oocyte interaction. Any change in it by means of antifertility agents, acrosin/hylronidase inhibitors and spermicidals leads to impairment of gamete interaction and fertilization of ova.[28-30] Thus, any morphological change in the sperm cell is one of the most important aspects while developing a contraceptive.[31] The treatment of RISUG-M causes significant decrease in plasma membrane-associated enzymes, 5'-Nucleotise, hyaluronidase and acrosin from the acrosomal membrane.[28] This was also confirmed by performing a comparative study employing 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).[10] This new implant device RISUG-M have shown better spermicidal action than RISUG as detected by X-ray and magnetic imagining and it's *in vivo* distribution can be controlled outside the body with the application of an external pulsed magnetic field (PMF).[32] The modification in the form of RISUG to RISUG-M does not alter the effect on sperm as low concentration of iron oxide and copper are added. The only difference is that the combination of the iron oxide-copper - PMF enhances the spermicidal action and the contraceptive efficacy of the compound.[10] A disadvantage of magnetic nanoparticles is that they are not easily destroyed or inactivated by the cells and thus the persistent particles may cause cell damage and death. But in the case of

RISUG-M, no such hazard exists as the magnetic nanoparticles present in it are coated with a non-toxic polymer styrene maleic anhydride. When RISUG-M is injected and PMF is applied, the iron oxide enters the sperm membrane forming a mosaic structure enveloped with copper micro strands which creates a large surface of electrical charge interaction for the distribution of sperms and thus enhanced the spermicidal action in a sustained manner.[10, 22, 33] Though, the gonad weight analysis and sperm anomaly assay proved *in vivo* safety and effectiveness of RISUG-M in rats, the drug is further evaluated for any possible sign of toxicity before applying to humans despite the moderate amount of iron oxide is used which does not adversely affect the sperm functionality.[34,35]

The data obtained in this acute (14 days) toxicity study on body and organ weights, gross behavior, food and water consumption, haematology, biochemistry and histopathology, indicate that the RISUG-M (SMA-Fe_3O_4-Cu-DMSO), is safe and can be used further as an effective non-surgical male contraceptive agent in future.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. Sujoy K. Guha for kindly providing the compound, RISUG-M for the above study and the research grant [SSP-207] in the form of collaborative research work to our Institute. CDRI Communication Number: 158/2017/RKS

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

DECLARATION OF CONFLICTING INTERESTS

The Authors declare that there is no conflict of interest.

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