

Preclinical Toxicity Studies of RISUGadv as A New Molecule for the Prevention and Management of Prostate Cancer

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ABSTRACT: The aim of this study was to assess the toxic effect of the newly developed contraceptive RISUGadv in male Charles Foster rats. Young and healthy male rats of Charles Foster strain were employed in the study. They were randomly assigned to two groups, control and treated, each consisting of fifteen animals. The contraceptive DMSO and RISUGadv was injected surgically in the vas deferens of the anesthetized control and treated group rats, respectively and observed for a period of 14 days. Initial and final body weights and food/water consumption of the animals were recorded. The haematological and biochemical parameters were analyzed. At the end of the study all the animals were sacrificed and necropsied, the organ weight was taken and their histopathological slides were prepared for microscopic examination. Results on body weight, food and water consumption, haematology, biochemistry, absolute and relative organ weights did not show any significant change and were well within the limit of normalcy. General health check-up, mortality, gross and microscopic examination of organs and tissues also did not reveal any sign of toxicity. From the toxicity point of view this newly developed injectable contraceptive RISUGadv does not have any adverse effect and is safe to use.

KEYWORDS: RISUGadv, Male contraceptive agent, Toxicity profile.

I. INTRODUCTION

RISUGadv, an advanced form of male contraceptive drug RISUG being combination of styrene maleic anhydride (SMA) and dimethyl sulphoxide (DMSO) have been developed by Prof. S.K. Guha and his team at I.I.T. Kharagpur, India. Previous researches had confirmed its spermicidal activity [1], teratogenic safety [2] and its non-toxicity [3]. When injected into the vas deferens, the positive and the negative charges on the polymer surface causes a surface charge imbalance on the sperm membrane system and significant damage to its content leading to its destabilization and eventually the surface of sperm burst; thus making it immotile and incapable to fertilize an egg [4,5,6,7]. Significant damage to the midpiece region, clustering of mitochondria, its fusion with the head region and topological alterations in the flagellar and midpiece region of RISUG-treated spermatozoa has also been observed using atomic force microscopy (AFM) [7,8]. RISUGadv is an advancement of the contraceptive RISUG and consist of a co-polymer styrene maleic anhydride (SMA), magnetic particles: iron oxide, electrically conductive particles: copper dissolved in 99% pure dimethyl sulphoxide (DMSO) [9]. It is long time effective, non-invasively reversible and controllable, antimicrobial as anti HIV and prostate cancer preventive contraceptive method [10, 11]. Copper's intrinsic high electrical conductivity makes the overall compound electrically conductive and displaces zinc from the sperm membrane which accounts in decreased motility of spermatozoa and lowers the fertilizing potential of the sperm [12, 13, 14]. RISUGadv has also been proposed as HIV inhibiting vas deferens implant when delivered in below contraceptive threshold dosage. On being injected into the lumen of the vas deferens, the polymer produces styrene maleic acid which lowers pH, induces electrochemical action leading to a stable electrical charge generation and

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releases mandelic acid which induces acrosome reaction in sperms and subsequently releases hyaluronidase and inactivates sperm [10]. Specifically in the case of cancer, RISUG works by generating a 'nano-drug' locally that attacks mutant cells. Aquaporins (Aqp) helps in the transportation of water or some other molecules (like RISUG) with water from vas deferens to prostate [15, 16]. Aqp 7 and 9 are widely present on the epidermal region of vas deferens and prostate. Along with osmotic gradient, a change in physico-chemical factors of drug molecules inside the system might lead to better passage through aquaporins in several regions of male reproductive tract [16, 17]. Moreover, one time administration into the lumen of the vas gives long term action. All these properties of RISUGadv fulfill the needs for HIV clearance of semen [10, 18]. The major advantage of this method is that the drug can be flushed out whenever required. The objective of the present study is toxicity evaluation of this newly developed contraceptive drug in male rats after injecting into vas deferens.

II. MATERIALS AND METHODS

Animals: Thirty young and healthy male rats of Charles Foster strain of 160-180g body weight were issued by the Division Laboratory Animals, CDRI, Lucknow and were acclimatized for 7 days to the laboratory conditions. Throughout the study, a sequence of 12 hour cycles of light and darkness was maintained in the room with the help of artificial lighting. The animals were fed with laboratory pellet diet and water *ad lib*. The ethical approval was given by CSIR-CDRI Institutional Animal Ethics Committee (IAEC).

Chemicals: RISUGadv was supplied by Dr. Sujoy Kumar Guha, Professor of Biomedical Engineering at the School of Medical Science and Technology, Indian Institute of Technology Kharagpur, India. It was synthesized by dispersing iron oxide nanomicro-sized particles and copper nanomicro-sized particles into SMA and DMSO under a dry XL grade nitrogen atmosphere and characterized for particle distribution, particle size measurement and transmittance peaks. All other chemicals used in this study were of analytical grade and purchased from Sigma Aldrich Chemical Company (India).

Experimental Design: Apparently healthy and disease free male rats were employed in the study. The rats were randomly divided into two groups, containing fifteen rats each and subjected to bilateral vas occlusion under ether anaesthesia. The vas deferens was exposed by a single median incision surgically. Group I rats injected with 0.01 ml DMSO in the vas deferens, served as control. Group II rats were taken as treated group and were injected with RISUGadv. The vas deferens of rats was 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 the antibiotic drug Terramycin (Pfizer Ltd, Bombay) was injected intramuscularly to each rat for 5 consecutive days. Animals were kept under watch to observe gross behavioral changes (if any) for a period of 14 days. At the end of the study, all the rats belonging to either group were necropsied for organ weight and histology.

Body weight record: Body weight of each rat of both the group was recorded at start and end of the study.

Food and water Record: Monitoring 24-hour food and water consumption of the animals per group was done at the beginning and end of the study by giving a measured quantity of pellet diet and water followed by estimation of the amounts remaining at the end of 24 hours. Average food and water consumption per animal was calculated for each group.

Haematology: At the start and end of the experiment, blood samples were taken by snipping the tail tip of each rat and various haematological parameters (haemoglobin, total RBC count, total leucocyte count, packed cell volume or haematocrit, mean corpuscular volume, mean corpuscular haemoglobin concentration and platelet count) were analyzed with the help of MS-9 automatic haematology analyzer from M/S Melet Schloesing Ltd France. Leishman stained peripheral blood smears were screened for performing a differential leucocyte counts or DLC (polymorph, lymphocyte,

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monocytes, eosinophil and basophil), for studying the morphology of cellular constituents of blood and for detecting the presence of any abnormal cells.

Blood biochemistry: Serum was separated from the blood drawn by cardiac puncture of each anaesthetized rat just before sacrifice and general metabolic functions (glucose, cholesterol, triglycerides, total protein, albumin), liver functions (alkaline amino transferase, aspartate amino transferase, alkaline phosphate, bilirubin) and kidney functions (blood urea nitrogen, creatinine), calcium and phosphorus were estimated by a fully automated random access Chemistry Analyser (Beckman, Synchron CX5) using standard kits supplied by M/S Beckman, USA:

Organ weights: All the rats belonging to the different groups were sacrificed by exsanguination under light ether anesthesia. Absolute organ weights of adrenal, brain, gonads, heart, kidney, liver, lung and spleen were recorded on a chemical balance and their relative organ weights (g/100g b.wt.) were also calculated. The organs and tissues were preserved in 10% formalin till they were processed for paraffin embedding and sectioning.

Histology: Representative pieces from all the organs and tissues were processed for paraffin embedding. Four to six micron thick sections were cut, and staining by haematoxylin and eosin was done according to standard methods for microscopic examination.

III. RESULTS

General health check-up and 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 rats.

Body weight record: There was a uniform and comparable gain in body weight among the treated and control groups of animals, as compared to initial values (Table 1).

Food and water consumption record: Initial and final monitoring by measurement of the initial and leftover water and solid diet (pellets) in the containers revealed irregular variations in the average 24-hour water and food intake of animals in the control and treated groups. There was no evidence of any effect of treatment with the test material (Table 1).

Table 1: Body weights, average food intake and average water intake in control and RISUGadv-treated rats after 14 days (n=10 number of animals, Mean \pm S.D.).

Group No.	Body weights(g)		Average food intake (g)		Average water intake (ml)	
	Initial	Final	Initial	Final	Initial	Final
Group I	178.40 \pm 15.53	230.13 \pm 42.85	14.705 \pm 2.15	17.282 \pm 2.34	21.2 \pm 1.5	23.00 \pm 1.15
Group II	178.87 \pm 14.91	247.47 \pm 39.02	13.015 \pm 1.45	14.165 \pm 2.24	17.75 \pm 3.4	17.46 \pm 4.54

Haematology: Compared to the respective control values, there were no variations in the values of the hematological parameters (Hgb, T-RBC, Hct, MCV, MCHC, TLC, DLC and platelet) of the treated group rats and were well within the limit of normalcy (Table 2 and 3).

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Table 2: Initial haematology in control (Group I) and RISUGadv-treated (Group II) rats.

Group No.		Red Blood Cells					White Blood Cells					Platelets ($\times 10^3/\text{mm}^3$)	
		Hgb (g%)	T-RBC ($\times 10^6/\text{mm}^3$)	Hct (%)	MCV (micro n ³)	MCHC (g%)	TLC ($\times 10^3/\text{mm}^3$)	Differential Leucocyte Count (%)					
								P	L	M	E		B
Group I	Mean	12.91	7.24	42.35	58.47	30.50	13.54	10.13	82.47	3.73	2.80	0.60	279.40
	\pm S.D.	0.93	0.61	3.09	2.04	1.62	4.12	6.74	2.35	2.17	0.49	0.49	61.97
Group II	Mean	13.25	7.25	42.47	58.27	31.27	13.59	14.80	82.47	3.20	3.20	0.53	325.67
	\pm S.D.	0.86	0.51	2.12	3.08	2.13	3.70	18.59	9.32	2.37	2.86	0.50	85.50

Table 3: Final haematology in control (Group I) and RISUGadv-treated (Group II) rats.

Group No.		Red Blood Cells					White Blood Cells					Platelets ($\times 10^3/\text{mm}^3$)	
		Hgb (g%)	T-RBC ($\times 10^6/\text{mm}^3$)	Hct (%)	MCV (micro n ³)	MCHC (g%)	TLC ($\times 10^3/\text{mm}^3$)	Differential Leucocyte Count (%)					
								P	L	M	E		B
Group I	Mean	13.52	7.21	42.19	58.60	31.13	15.05	15.59	82.40	2.20	3.0	0.53	514.27
	\pm S.D.	0.61	0.33	2.13	2.35	3.93	2.95	6.89	7.27	0.75	1.97	0.50	65.03
Group II	Mean	11.79	6.63	39.98	60.33	29.65	16.85	25.34	76.53	2.40	5.60	0.93	415.67
	\pm S.D.	0.56	0.61	3.70	2.82	1.76	4.31	15.66	6.27	0.80	1.85	0.25	52.83

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.

Blood biochemistry: Irregular variation were seen in terminal blood biochemical values but were well within the limits of normalcy and comparable among the treated and control animals (Table 4).

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Table 4: Terminal Biochemistry in control (Group I) and RISUGadv-treated (Group II) rats.

Group No.		General Metabolic Functions					Liver Functions				Kidney Functions			
		GLU (mg/dl)	CHOL (mg/dl)	TG (mg/dl)	TP (mg/dl)	ALB (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)	TBIL (mg/dl)	BUN (mg/dl)	CRTN (mg/dl)	Ca (mg/dl)	P (mg/dl)
Group I	Mean	44.03	72.67	60.85	6.57	3.38	57.88	146.91	577.7	0.22	25.92	0.62	10.41	10.26
	±S.D.	16.69	19.90	19.04	1.85	0.14	11.81	19.28	34.52	0.04	5.59	0.07	0.68	1.20
Group II	Mean	44.31	76.19	77.30	6.99	3.28	70.58	135.94	532.59	0.19	21.69	0.59	11.00	11.39
	±S.D.	21.81	11.86	18.16	0.41	0.14	13.99	17.48	37.38	0.04	2.82	0.05	2.87	1.06

Absolute and relative organ weights: Mean values of absolute (g) and relative (g/100g b. wt.) organ weights of adrenal, brain, gonads, heart, kidney, liver, lung and spleen showed no significant variations and remained within the limits of normalcy in comparison to the control animals (Table 5 and 6).

Table 5: Absolute Organ Weights (g) in control (Group I) and RISUGadv-treated (Group II) rats.

Group No.		Adrenal		Brain	Gonads		Heart	Kidney		Liver	Lungs	Spleen
		Rt	Lt		Rt	Lt		Rt	Lt			
Group I	Mean	0.020	0.022	1.638	1.361	1.284	0.816	0.790	0.789	7.950	1.564	0.697
	±S.D.	0.004	0.004	0.273	0.311	0.323	0.165	0.151	0.156	1.661	0.283	0.151
Group II	Mean	0.009	0.007	0.683	0.480	0.489	0.362	0.359	0.347	3.539	0.684	0.232
	±S.D.	0.009	0.010	0.735	0.619	0.600	0.367	0.344	0.340	3.436	0.687	0.310

Rt=Right, Lt=Left

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Table 6: Relative Organ Weights (%) of control (Group I) and RISUGadv-treated (Group II) rats.

Group No.		Adrenal		Brain	Gonads		Heart	Kidney		Liver	Lungs	Spleen
		Rt	Lt		Rt	Lt		Rt	Lt			
Group I	Mean	0.012	0.009	0.010	0.735	0.619	0.600	0.367	0.344	0.340	3.436	0.687
	±S.D.	0.002	0.002	0.002	0.088	0.161	0.183	0.065	0.035	0.033	0.578	0.104
Group II	Mean	0.011	0.009	0.009	0.721	0.608	0.601	0.343	0.325	0.326	3.234	0.650
	±S.D.	0.001	0.001	0.001	0.058	0.065	0.079	0.030	0.021	0.020	0.333	0.131

Rt=Right, Lt=Left

Histopathological examination: The microscopic examination of any slides of the above mentioned organs and tissues (viz. Brain, heart, liver, lung, kidney, adrenal and spleen) did not reveal any pathological change in treated as compared to control group of animals. Also, no adverse effect was recorded at the site of insertion and adjacent areas of the vas deferens.

IV. DISCUSSION

Sperm morphology is a significant factor for fertilization and pregnancy, especially the anterior part of the sperm i.e. acrosome and any change in it can cause impairment in the gamete interaction⁶. Thus, any morphological change in the sperm cell is one of the most important aspects while developing a contraceptive [19]. There are three important key enzymes – 5'-nucleotidase (5'-NT), hyaluronidase and proacrosin-acrosin system which facilitate sperm-oocyte interaction. On treatment with RISUGadv, it causes excessive loss of plasma membrane-associated enzyme 5'-NT (82%) and hyaluronidase (90%) from the acrosomal membrane and inactivation of the active form of acrosin which results in complete degeneration of the acrosomal membrane that indicates RISUG is an effective contraceptive [6]. 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) [9]. The new implant device RISUGadv gives better spermicidal action than RISUG, detectable by X-ray and magnetic imaging and its *in vivo* distribution can be controlled outside the body with the application of an external pulsed magnetic field (PMF) [20]. The modification in the form of RISUG to RISUGadv 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 further the contraceptive efficacy of the compound [14]. 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 RISUGadv, no such hazard exists as the magnetic nanoparticles present in it are coated with a non-toxic polymer styrene maleic anhydride [9]. When RISUGadv 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 [14]. Though, the gonad weight analysis and sperm anomaly assay proved *in vivo* safety and effectiveness [21], 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 [21,22].

V. CONCLUSION

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The data obtained in the present study on acute toxicity profile of RISUGadv indicate that RISUGadv is safe and therefore, opens a pathway to use it as an effective contraceptive and prevention of prostate cancer drug in future.

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