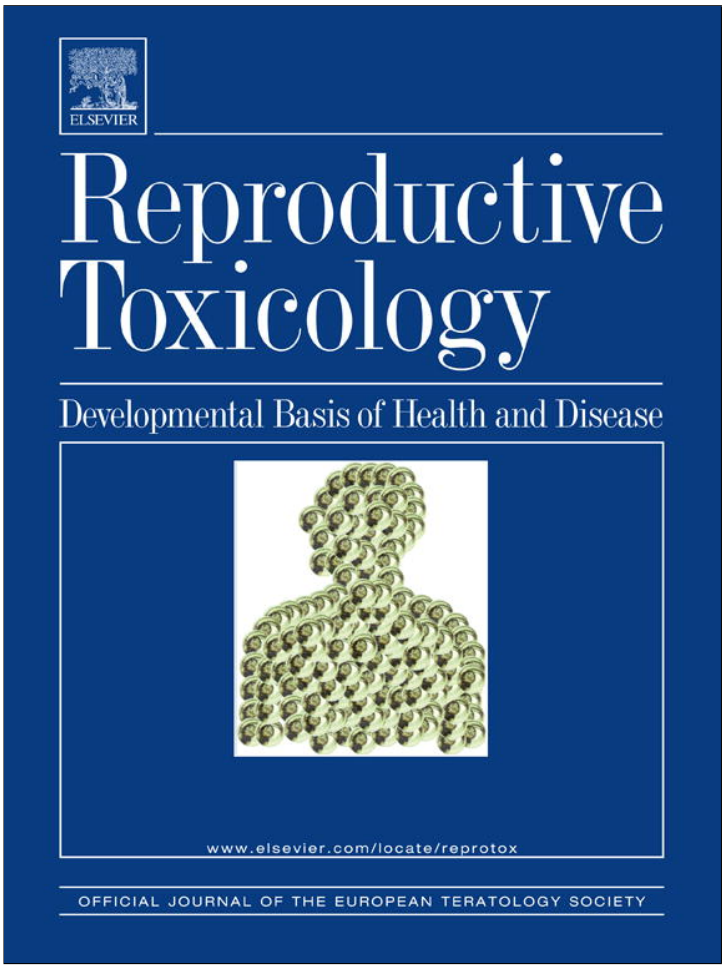

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Evaluation of genotoxicity in leukocytes and testis following intra-vasal contraception with RISUG and its reversal by DMSO and $NaHCO_3$ in Wistar albino rats

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ABSTRACT

Evaluation of genotoxicity of RISUG® – a vas based contraceptive, was carried out in the present study. Animals were allotted into groups of sham operated control, vas occlusion with RISUG (5–7 μl) for 360 days and reversal by DMSO (250–500 μl) and 5% $NaHCO_3$ (500 μl). Blood samples and testis were collected at 360 days of vas occlusion and 90 days of vas occlusion reversal for comet analysis. Hydrogen peroxide induced samples were used as positive control. Olive moment, tail length and percentage DNA in tail were recorded with minimum variation in all groups for both leukocytes and testis. When compared with positive control the variation was highly significant for both 20 μM and 50 μM H_2O_2 ($p < 0.001$). It is concluded that vas occlusion with RISUG at the contraceptive dose regimen is not associated with genotoxicity in leukocytes or the testis of pre- and post-reversal rats.

1. Introduction

Vasectomy accounts for more than 20% of current male contraception [1]. Other vas based techniques such as an intra-vas device, HIFU (high-intensity focused ultrasound) transducer, injectable plug, vas-chip are under consideration [2]. The reversible inhibition of sperm under guidance (RISUG) created a new concept in vas-based contraception methods. Upon injection it coats the wall of lumen and gradually blocks the vas deferens also referred as ductus deferens. Dimethyl sulfoxide (DMSO), a constituent of RISUG, allows penetration of the polymer into the inner wall of vas deferens and provides retention [3]. Vas occlusion with RISUG eventually leads to azoospermia [4]. DMSO and $NaHCO_3$ have been used to reverse the effects of RISUG [5,6], and short-term studies carried out in our laboratory showed functional reversal with DMSO as a solvent [6].

Intravasal contraception with RISUG established safety and efficacy in phase I and phase II clinical trials [7,8], and multicentric phase III clinical trials, are currently ongoing in India. Studies reported that blockage of vas deferens through vasectomy results in production of anti-sperm antibodies [9,10]. Physical stress on the reproductive tract may lead to exposure of sperm antigen, resulting in to autoimmunity through continuous stimulatory inflammation. RISUG might also impose similar stress as it is an occlusive polymer, thus, long-term study of RISUG treatment is essential to confirm whether its components are exposed to leukocytes and if this has genotoxic consequences to germ cells. No reports are currently available in the open literature concerning the potential genotoxicity of vas occlusion with SMA (styrene maleic anhydride)/RISUG and reversal by DMSO/$NaHCO_3$. Here, we used single cell gel electrophoresis (SCGE) or Comet assay to examine DNA damage [11,12] in male Wistar albino rats. A modified SCGE technique was used to assess DNA damage in circulating leukocytes and testis [13,14] following vas occlusion with RISUG and its reversal by DMSO or $NaHCO_3$.

2. Materials and methods

2.1. RISUG

The RISUG (reversible inhibition of sperm under guidance) is a copolymer synthesized by gamma radiation of monomeric styrene and maleic anhydride, which is made ready to inject by dissolving it in requisite amount of DMSO (1:2 ratio). The RISUG used for the present investigation was kindly provided by Prof. S.K. Guha, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India.

2.2. Animals

Adult Wistar albino male rats (*Rattus norvegicus*) (3–4 months, weighing 150–175 g) were used in the present study. The animals were maintained in

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department's experimental animal facility with provisions of 12 h light and dark photoperiod, adjusted temperature of 23 ± 2 °C and relative humidity ranged between 32 and 70% in individual polypropylene cages (size 43 cm × 27 cm × 15 cm). The animals were fed twice a day with pellet diet (Ashirwad Industries, Chandigarh, India) and had water *ad libitum*. Animals were remained healthy throughout the experimental study and did not show sign of any distress. The animals were maintained under perfect veterinary supervision and the experiment was performed according to the guidelines for care and use of animals in scientific research by Indian National Science Academy (INSA) [15]. All animals were euthanized by ether overdose at the scheduled termination of experiment (ether is recognized as an older but common practice). The experimental protocol has the approval of the Institutional Animal Ethical Committee (IAEC).

2.3. Experimental design

Animals were allocated into four groups, containing 10 animals in each. Group I was used as sham operated control ($n = 5$ for 360 days and $n = 5$ for 450 days), group II as vas occlusion with RISUG for 360 days ($n = 10$) and group III as vas occlusion with RISUG for 360 days and reversal by DMSO ($n = 10$) and group IV as vas occlusion with RISUG for 360 days and reversal by $NaHCO_3$ ($n = 10$).

2.4. Vas occlusion

Animals of groups II–IV were subjected to bilateral vas occlusion under ether anesthesia. A single median incision was made to expose the vas of inguinal region and RISUG was injected at a dose of 5–7 μl in both vas deferens toward distal region by a micro-syringe. The polymerization of RISUG was catalyzed by application of normal saline. Once the polymer was solidified with fluid medium *in situ*, the vas was returned to its inguinal position and the incision was closed by catgut suture (for inner layer) and silken suture (for outer layer). The vasa of group I (sham operated control) animals were subject to the surgical protocol without exposure. Post-operative care was taken which included antibiotic and anti-inflammatory drugs, injected in the gluteal muscles. A routine fertility test was conducted at regular intervals by cohabitation of RISUG injected animals with fertile females at 1:2 ratio, to confirm functional success of vas occlusion.

2.5. Vas occlusion reversal

Surgical reversal was performed after 360 days of vas occlusion. The vas deferens were similarly exposed as during vas occlusion and were injected bilaterally with 250–500 μl of DMSO to dissolve the co-polymer in group III. Likewise, 500–700 μl of 5% $NaHCO_3$ was injected to alleviate the RISUG and vas deferens binding in group IV animals. The vas was returned to its original position and the incision was closed similarly as during vas occlusion. Post-operative measures were taken as indicated previously. Confirmation of successful reversal was assessed by periodical fertility test by mating with fertile female (1:2 ratio) rats.

2.6. Sample collection for SCGE analysis

Blood samples were collected under terminal anesthesia in EDTA vials by cardiac puncture and used immediately for SCGE analysis to minimize exposure of samples to ambient oxygen [16]. The reason behind using whole blood directly for SCGE analysis was to minimize any further oxidative damage induced during isolation, preservation and handling of leukocytes [17]. Blood samples were diluted by adding 10 μl of whole blood into 90 μl of phosphate buffered saline (PBS) containing 20 mM EDTA and 5% DMSO to chelate Ca^{2+} and Mg^{2+} and to avoid further oxidative damage by free radicals.

The testes were dissected from adherent tissues, minced and fixed in 2.5% glutaraldehyde overnight. For preparation of single cell suspension, tissues were washed in PBS (Ca^{2+} and Mg^{2+}-free) and trypsinized [18–20]. In brief, pre-warmed (37 °C) 0.25% trypsin (pH 7.4) (Himedia Laboratories Pvt. Ltd., Mumbai, India) was added to 1–2 g of testicular tissue and incubated for 30 min at 37 °C. Following incubation, tissues were mechanically agitated and the process was repeated with fresh trypsin, to achieve complete cell suspension. The resulting cell suspension was thus washed thrice in PBS at 300 rpm for 10 min and resuspended in PBS containing 20 mM EDTA and 5% DMSO. Cell viability was determined by Trypan blue (Himedia Laboratories Pvt. Ltd., Mumbai, India) staining.

The blood and testicular samples collected from sham operated control (360 days ($n = 5$) and 450 days ($n = 5$)) were also used to prepare parallel positive controls. They were exposed to 20 and 50 μM H_2O_2 (Merck Specialties Pvt. Ltd., Mumbai, India) for 35 min at room temperature, exposure was stopped by washing the samples in PBS containing 20 mM EDTA and 5% DMSO.

2.7. Single-cell gel electrophoresis (SCGE) or Comet assay

The SCGE assay was executed under alkaline condition following the procedure of Singh et al. with minor modifications [13,14,21]. Briefly, 0.7% (w/v) high resolution blend (HRB) agarose 3:1 (Amresco, Solon, OH, USA) was prepared and kept at 4 °C, until use. Just prior to the use the HRB agarose was melted in a boiling water bath and kept at 50–60 °C. First layer on partially frosted slide (Microcil Manufacturer, Vyara, India) was coated by 0.7% HRB agarose by placing 80 μl on the clear window and covered by cover slips (Microcil Manufacturer, Vyara, India) and left to settle for 10 min at 4 °C. The second layer was made by adding 10 μl of sample (nearly 10^4 cells) in to 70 μl of 0.7% HRB agarose 3:1 and poured over the first layer and cover slips were placed on the slides and then stored at 4 °C for 10 min. After removing the cover slips, a third layer of 0.7% HRB agarose 3:1 was made over the second layer and the slides were covered with cover slips and finally left to settle for 10 min at 4 °C.

For positive control samples coverslips were removed and slides were submerged in 20 and 50 μM H_2O_2 for 35 min at room temperature, washed in PBS containing 20 mM EDTA and 5% DMSO. Slides were then submerged into pre-chilled (4 °C) alkaline lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% SDS, 1% Triton X-100 and 10% DMSO; pH 10) and kept at 4 °C for 1 h for leukocytes and 4 h for testicular cells. Slides containing testicular cells were additionally treated with 10 mM dithiothreitol (DTT) (Sigma–Aldrich, St. Louis, USA) for 30 min at 4 °C followed by 4 mM lithium diiodosalicylate (LIS) (Sigma–Aldrich, St. Louis, USA) for 90 min at room temperature, to decondense the DNA of spermatocytes. After DTT-LIS treatment slides were submerged into treatment solution (pH 7.4) containing 2.5 M NaCl, 5 mM Tris–HCl, 0.05% SDS and RNase (50 μg/ml) for 2 h at 37 °C.

The slides were washed in pre-chilled distilled water and placed in a horizontal gel electrophoresis chamber. Subsequently, submerged into electrophoresis buffer (300 mM sodium acetate and 100 mM Tris–HCl; pH 13) and left for 20 min at room temperature for alkaline DNA unwinding. Samples were then allowed to electrophoresed for 30 min (24 V (0.7 V/cm) and 330 mA) in dark at room temperature, current was adjusted by increasing or decreasing the buffer level. Further to this the slides were neutralized by 0.4 M Tris–HCl (pH 7.4), allowed to precipitate by absolute ethanol, air dried and stored for subsequent comet analysis.

For comet, staining was carried out by ethidium bromide (Himedia Laboratories Pvt. Ltd., Mumbai, India). Slides were examined at 400× magnification under fluorescent microscope (Lambo America Inc., California, USA) equipped with dual excitation filter. A total of 30 comets were analyzed per sample per animal for the olive moment (summation of tail intensity profile values multiplied by their relative distances to the head center, divided by total comet intensity), tail lengths (head diameter subtracted from comet length) and percentage DNA (total tail intensity divided by total comet intensity and multiplied by 100). Complete analyses of olive moment, tail length and percentage of DNA in the tail were made by the CometScore freeware version 1.5 software (TriTek, Sumerduck, VA, USA).

2.8. Statistical analysis

Results were expressed as mean ± SD. Student's *t*-test and one way analysis of variance (ANOVA) were employed for statistical comparison as and when required. $p < 0.05$ was considered significant. A cohort study was also conducted for analysis of risk factors by association between cause and effect. Pearson's correlation test was applied in order to determine the correlation between test groups.

3. Results

3.1. Leukocytes

The baseline DNA damage parameters are summarized in Table 1. Olive moment, tail length and %DNA in 30 comets/sample/animal were considered to quantify DNA damage in circulating leukocytes of groups I–IV. The baseline DNA damages in leukocytes in all test groups were ranged between 6% and 7%. Parallel positive controls, however, showed significant increase in DNA damage in all parameters ($p < 0.001$). Images of comets appeared in leukocytes are shown in Fig. 1. The length and fluorescent intensity of the tail of comets appeared in hydrogen peroxide induced (20 μM and 50 μM H_2O_2) leukocytes were much greater than comets appeared in group I (360 and 450 days) and groups II–IV.

3.2. Testicular cells

Similar results were observed in testicular cells for olive moment, tail length and %DNA in 30 comets/sample/animal (Table 2). Nevertheless, the percentage damages ranged between 3% and 5%, which was lower than leukocytes. Parallel positive control induced by hydrogen peroxide (20 μM and 50 μM H_2O_2) were found severely damaged when compared to groups I–IV ($p < 0.001$) and ranged from 44% to 61%. Fig. 2 shows limited to no tail in testicular cells of groups I–IV. However, tail length and fluorescent

Table 1
Olive moment, tail length and %DNA (mean ± SD) observed in leukocytes were examined in comets of group I ($n = 5$, 360 and $n = 5$, 450 days), group II ($n = 10$), group III ($n = 10$), group IV ($n = 10$), hydrogen peroxide induced damage H_2O_2 20 μM ($n = 5$, 360 and $n = 5$, 450 days), and H_2O_2 50 μM ($n = 5$, 360 and $n = 5$, 450 days).

	Vas occlusion			Reversal		
	Olive moment (px)	Tail length (px)	%DNA	Olive moment (px)	Tail length (px)	%DNA
Group I	3.48 ± 0.87	12.51 ± 2.21	6.82 ± 1.44	3.83 ± 1.04	13.61 ± 3.23	6.78 ± 1.15
Group II	3.56 ± 1.15	12.46 ± 3.07	6.69 ± 1.51	–	–	–
Group III	–	–	–	3.77 ± 1.67	14.02 ± 5.91	7.04 ± 2.93
Group IV	–	–	–	3.59 ± 1.10	13.72 ± 4.18	6.30 ± 1.90
H_2O_2 20 μM	13.30 ± 0.79*	44.23 ± 0.83*	24.53 ± 2.23*	12.97 ± 0.72*	45.67 ± 1.52*	25.96 ± 2.49*
H_2O_2 50 μM	25.54 ± 1.32*	145.02 ± 2.56*	48.28 ± 2.16*	25.00 ± 1.23*	144.36 ± 2.32*	47.40 ± 2.74*

±SD, standard deviation; px, pixel.
* Significantly different ($p < 0.001$) when compared to test samples.

intensity of the tail of comets appeared in induced samples (20 μM and 50 μM H_2O_2) were significantly higher.

3.3. Comparative analysis of comets

Significant correlations were observed in test groups (I–IV) between percentage of DNA damage in leukocytes and testis (Table 3). Strong negative strength of association was observed between leukocytes and testis of group I (360 and 450 days), group IV and 50 μM H_2O_2 induced positive control ($r = -0.417$, $r = -0.741$, $r = -0.395$ and $r = -0.795$) whereas, group II showed weak correlation ($r = -0.016$). Interestingly, significant positive correlation ($r = 0.699$) was observed between leukocytes and testicular cells in group III (Table 3). Cause and

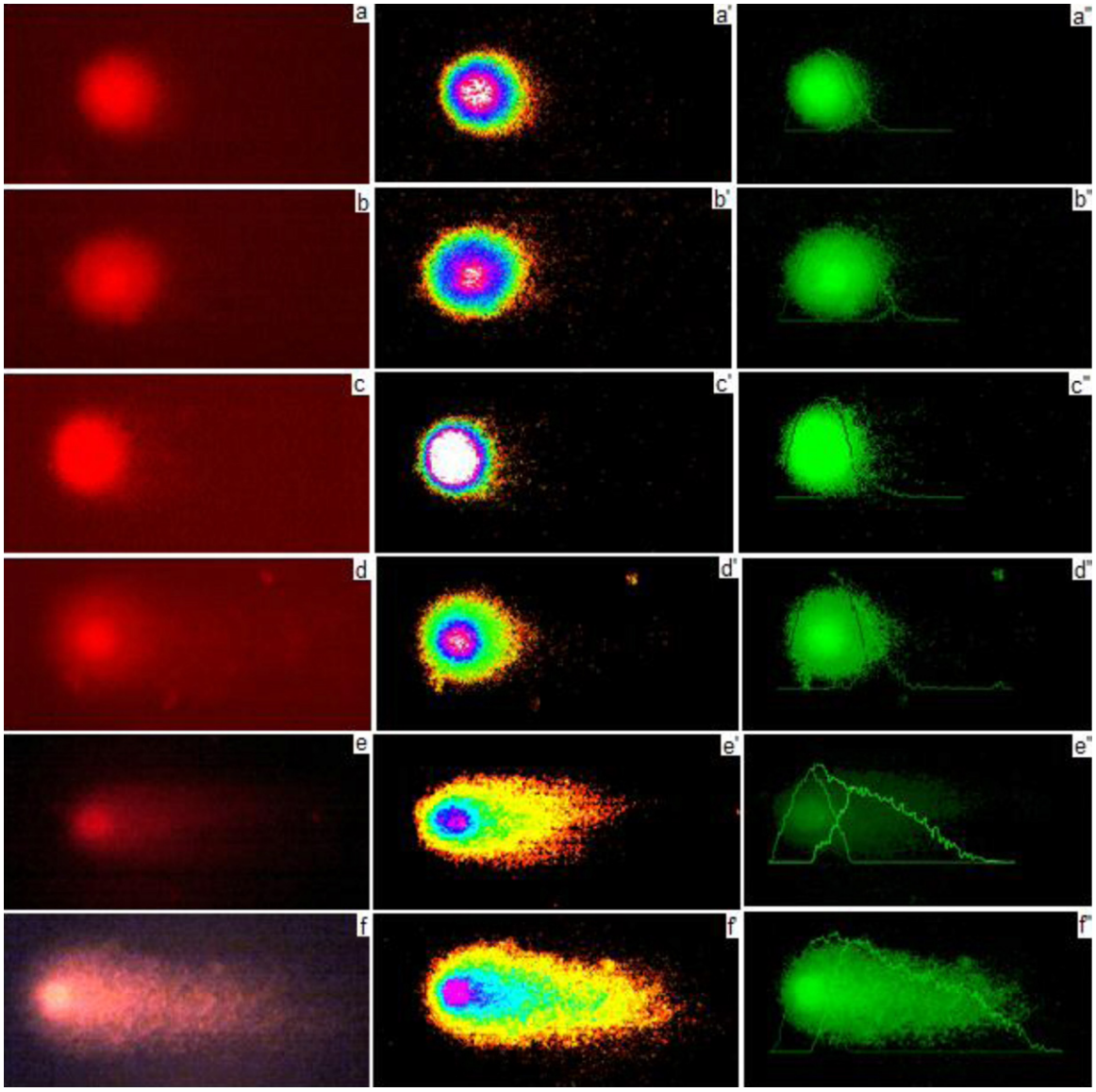

Fig. 1. SCGE analysis of leukocytes produced typical comets of varying tail length under alkaline condition. (a) Sham operated control (group I), (b) vas occlusion (group II), (c) vas occlusion reversal by DMSO (group III), (d) vas occlusion reversal by $NaHCO_3$ (group IV), (e) 20 μM H_2O_2 induced damage and (f) 50 μM H_2O_2 induced damage. Single (′) and double primes (″) are pixel distribution and intensity graph. Analyzed and viewed in various spectrum by CometScore freeware version 1.5 (TriTek, Sumerduck, VA, USA).

Table 2
Olive moment, tail length and %DNA (mean ± SD) observed in testicular cells were examined in comets of group I ($n = 5$, 360 and $n = 5$, 450 days), group II ($n = 10$), group III ($n = 10$), group IV ($n = 10$), hydrogen peroxide induced damage H_2O_2 20 μM ($n = 5$, 360 and $n = 5$, 450 days), and H_2O_2 50 μM ($n = 5$, 360 and $n = 5$, 450 days).

	Vas occlusion			Reversal		
	Olive moment (px)	Tail length (px)	%DNA	Olive moment (px)	Tail length (px)	%DNA
Group I	2.15 ± 0.42	5.56 ± 1.22	4.05 ± 1.59	1.98 ± 0.65	5.06 ± 1.04	4.16 ± 1.29
Group II	2.20 ± 0.69	5.67 ± 2.05	4.61 ± 1.01	–	–	–
Group III	–	–	–	1.08 ± 0.63	4.80 ± 3.70	3.66 ± 1.61
Group IV	–	–	–	2.12 ± 0.43	4.88 ± 1.27	3.98 ± 1.11
H_2O_2 20 μM	24.49 ± 1.51*	60.86 ± 6.26*	44.68 ± 2.94*	24.59 ± 1.19*	56.67 ± 9.61*	45.14 ± 4.30*
H_2O_2 50 μM	38.80 ± 3.53*	177.07 ± 7.71*	60.44 ± 3.18*	37.41 ± 4.06*	182.49 ± 10.23*	60.65 ± 2.68*

±SD, standard deviation; px, pixel.
* Significantly different ($p < 0.001$) when compared to test samples.

effect dependent DNA damages in leukocytes and testis were correlated for strength of association between group I and group II, group I and groups III–IV, group II and groups III–IV, group III and H_2O_2 induced positive controls and group IV and H_2O_2 induced positive controls (Tables 4 and 5). Strikingly, the correlation of DNA damages in leukocytes between group III and 20 μM H_2O_2 induced positive control showed positive correlation ($r = 0.333$), similarly, group IV and 20 μM H_2O_2 induced positive control showed positive correlation ($r = 0.356$) in testicular cells (Tables 4 and 5).

Although the percentage of DNA damages were insignificant regardless of RISUG injection and reversal by DMSO/$NaHCO_3$, DNA damage in leukocytes was greater than the rate of damage calculated for testicular cells (Fig. 3). It was also evident that the DMSO-reversal animals showed higher standard deviation for percent DNA damage in leukocytes; nevertheless, the mean value

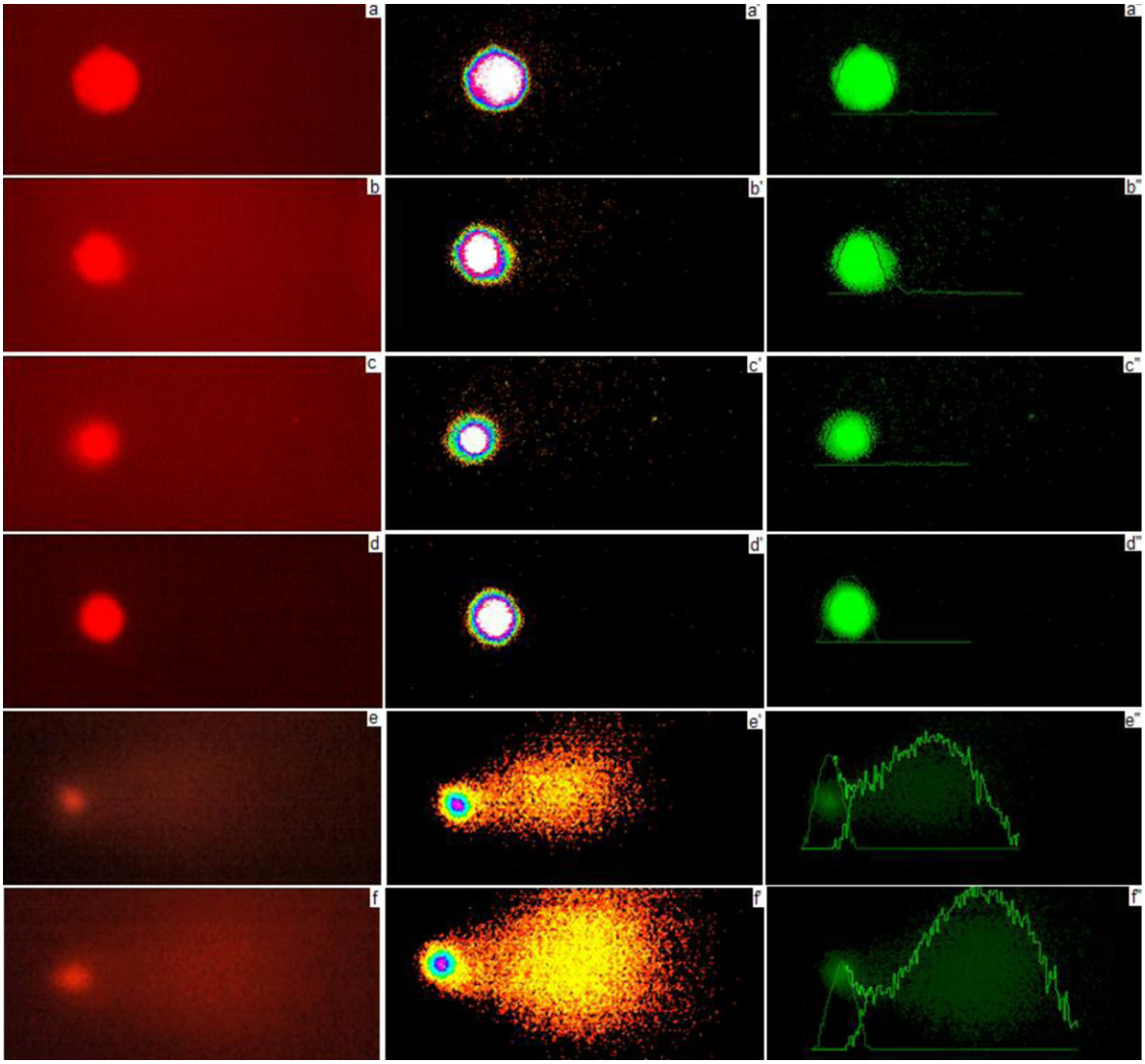

Fig. 2. Comet appearance of testicular cells under alkaline condition. (a) sham operated control (group I), (b) vas occlusion (group II), (c) vas occlusion reversal by DMSO (group III), (d) vas occlusion reversal by $NaHCO_3$ (group IV), (e) 20 μM H_2O_2 induced damage and (f) 50 μM H_2O_2 induced damage. Single (′) and double primes (″) are pixel distribution and intensity graph. Analyzed and viewed in various spectrum by CometScore freeware version 1.5 (TriTek, Sumerduck, VA, USA).

Table 3
Correlation analysis of %DNA damages in groups between leukocytes and testis.

Testis	Leukocytes						
	Group I (360 days)	Group I (450 days)	Group II	Group III	Group IV	20 μM H_2O_2	50 μM H_2O_2
Group I (360 days)	$r = -0.417$	–	–	–	–	–	–
Group I (450 days)	–	$r = -0.701$	–	–	–	–	–
Group II	–	–	$r = -0.016$	–	–	–	–
Group III	–	–	–	$r = 0.699$	–	–	–
Group IV	–	–	–	–	$r = -0.395$	–	–
20 μM H_2O_2	–	–	–	–	–	$r = -0.092$	–
50 μM H_2O_2	–	–	–	–	–	–	$r = -0.795$

Group I, group II, group III, group IV – experimental groups; 20 μM/l and 50 μM/l H_2O_2 induced damage.

Table 4
Correlation analysis of %DNA damages in leukocytes between groups.

Leukocytes	Leukocytes					
	Group I (450 days)	Group II	Group III	Group IV	20 μM H_2O_2	50 μM H_2O_2
Group I (360 days)	$r = 0.525$	$r = 0.645$	–	–	–	–
Group I (450 days)	–	–	$r = 0.058$	$r = 0.706$	–	–
Group II	–	–	$r = 0.122$	$r = 0.352$	–	–
Group III	–	–	–	–	$r = 0.333$	$r = -0.059$
Group IV	–	–	–	–	$r = -0.296$	$r = -0.191$

Group I, group II, group III, group IV – experimental groups; 20 μM/l and 50 μM/l H_2O_2 induced damage.

Table 5
Correlation analysis of %DNA damages in testicular cells between groups.

Testis	Testis					
	Group I (450 days)	Group II	Group III	Group IV	20 μM H_2O_2	50 μM H_2O_2
Group I (360 days)	$r = 0.660$	$r = -0.420$	–	–	–	–
Group I (450 days)	–	–	$r = 0.048$	$r = 0.686$	–	–
Group II	–	–	$r = -0.396$	$r = -0.056$	–	–
Group III	–	–	–	–	$r = -0.485$	$r = 0.191$
Group IV	–	–	–	–	$r = 0.356$	$r = -0.683$

Group I, group II, group III, group IV – experimental groups; 20 μM/l and 50 μM/l H_2O_2 induced damage.

stayed within control range. Comparative analysis of all parameters in groups III and IV, revealed that reversal by DMSO leads to more overall damage in leukocytes then testis (Fig. 4).

4. Discussion

Previous studies revealed necrospermia following vas occlusion with RISUG as evidenced by damage in acrosomal membrane, loss of segmented columns and numeric aberration in centriole of the neck, degeneration of mitochondrial sheath and axoneme in the mid-piece and absence of plasma membrane in the mid-piece and tail [22]. This status gradually leads to azoospermia and loss of fertility [22]. Studies carried out in Langur monkeys indicated that complete reversal of RISUG is achievable by non-invasive methods [4,23–27]. However, the same technique may not be feasible in humans because the ductus deferens is difficult to palpate reliably proximal to the scrotum. Therefore, attempts have been made toward reversal of vas occlusion with SMA/RISUG using DMSO or $NaHCO_3$ [5,6]. Long-term effects of RISUG and its reversal by DMSO and $NaHCO_3$ have not been investigated in detail.

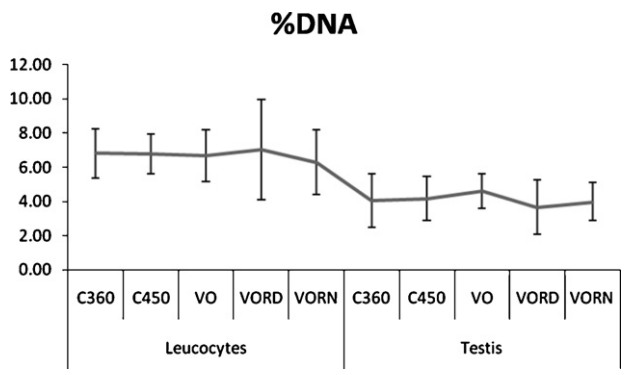

Fig. 3. Percentage DNA damage in leukocytes and testis following treatment with RISUG and reversal by DMSO and $NaHCO_3$, with reference to sham-operated control (360 (C360) and 450 days (C450)). VO, vas occlusion; VORD, vas occlusion reversal DMSO; VORN, vas occlusion reversal $NaHCO_3$, values are in percentage (%) scored by CometScore freeware version 1.5 (TriTek, Sumerduck, VA, USA).

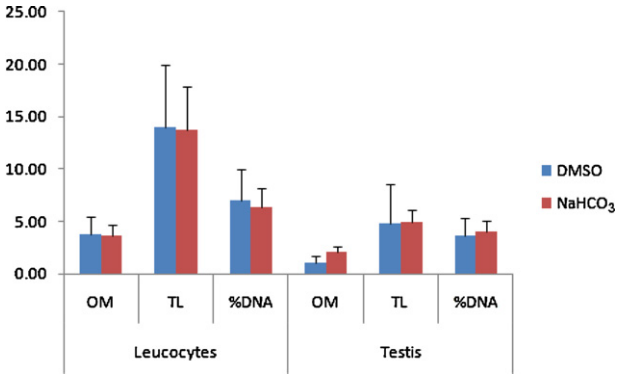

Fig. 4. Comparative analysis of mean values of olive moment (OM), tail length (TL) and %DNA damage following DMSO and $NaHCO_3$ reversal in both leukocytes and testis. Olive moments and tail lengths are measured in pixels (px), values of %DNA damage are in percentage (%), scored by CometScore freeware version 1.5 (TriTek, Sumerduck, VA, USA).

The present study did not observed signs of DNA damage by the COMET assay for either RISUG or reversal by DMSO/$NaHCO_3$. One drawback of this assay is that it does not distinguish DNA damage caused by potentially mutagenic strand breaks *versus* strand breakage secondary to physiological cell death (apoptosis) [28]. Both tissue samples examined here, leukocytes on one hand and testicular cells on the other, showed background DNA damage perhaps due to normal physiology and metabolism [29]. Single-stranded breaks can arise from various factors including UV rays, X-rays, ionizing radiation, and oxidative stress which may result due to by-products of normal metabolite processes [29–34]. DNA damage in cells could also arise from several other factors such as its life cycle, age, air pollution and diet [35–37]. In the present study, blood samples drawn for SCGE analysis contained cell types such as neutrophils, eosinophils and monocytes that may be in some cases apoptotic. Similarly, testicular samples contained a mix of cell types such as Sertoli cells, Leydig cells, spermatogonia, spermatocytes, spermatids and premature sperm with basal apoptosis [38]. These differences in cellular composition can explain different amounts of DNA damage encountered in leukocytes *versus* testicular sample preparations.

The SCGE technique has confirmed to be a highly sensitive, especially for low level DNA damage in many single cell analyses [13,14,39]. In this study the low concentration of H_2O_2 exposure provides evidence of SCGE to detect DNA breakage for as low as 20 μM [40]. Different patterns of comet tails indicate DNA migration varied between triangular, circular and oval in shape. The nature of cells and the degree of DNA damage reflect the appearance of comets, it is also found that the induced damage shows higher degradation of DNA in testicular cells comparing to lymphocytes [14]. We have noticed higher percentage of DNA in tails of comets exposed to H_2O_2 for both the leukocytes and testicular cells, the damage in leukocytes were, however, lesser (<20%) than the damage in testicular cells. The test samples were also noted with slight amount of DNA in tail, though it showed less damage in testicular cells compared to leukocytes. Our results indicate that the testicular cells are more sensitive to induced DNA damage or resistant to basal metabolic damage. Overall percentage of DNA damage in sham operated control, vas occlusion and vas occlusion reversal by either DMSO or $NaHCO_3$ showed less then 7% for both leukocytes and testicular cells, which was insignificant to imply any response related to RISUG or its reversal.

It is known that reactive oxygen species induce several types of damages in DNA, including single- or double-stranded breaks, alkali-labile sites, and a variety of species of oxidized purines and pyrimidines, which are easily detected by SCGE or comet assay [41]. The present study indicates insignificant amount of DNA damage in all four groups which, establish limited to no treatment specific oxidative stress. The grade of DNA degradation in leukocytes, however, varies within groups, it was speculated that leukocytes have greater exposure to free radicals and other DNA damaging factors than that of the testicular cells. We found a positive correlation within leukocytes between sham operated control and vas occlusion, however, slightly negative correlation was observed within testis for the same. Since RISUG occludes the vas deferens it possibly leads to a normal degeneration of spermatozoa in testis to achieve a precise homeostasis during germ cell renewal, proliferation and exportation [39,42].

We demonstrate here a successful reversal and 100% resumption of fertility by both DMSO and $NaHCO_3$ [6]. During comparative analysis of reversal by DMSO and $NaHCO_3$ we observed a higher variation in tail length and percent DNA damage in leukocytes for DMSO reversal animals than $NaHCO_3$ reversal. This may be due to the capacity of DMSO to penetrate cell membranes and possibly reaching to the blood vessels and consequently leading to immunogenic expression and apoptosis of leukocytes. Substances dissolved in DMSO are quickly absorbed [43]. Correlation analysis also revealed a positive correlation for percent DNA damage between leukocytes and testis for DMSO reversal. However, to some extent percent DNA damage in testis for $NaHCO_3$ reversal also indicated positive correlation with 20 μM H_2O_2 induced damage. Thus, considering the amount of DNA damage observed in both reversal methods and its correlation with DNA damage in leukocytes, suggest $NaHCO_3$ as a better alternative for reversal of vas occlusion by RISUG over DMSO.

5. Conclusion

We conclude from the evidence presented by modified SCGE or comet assay that vas occlusion with RISUG and its reversal by DMSO/$NaHCO_3$ are highly unlikely to produce any genotoxic activity in leukocytes and/or testis, when injected under prescribed dose regimen.

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