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Reproductive Toxicology

Disperse Red 1 (textile dye) induces cytotoxic and genotoxic effects in mouse germ cells

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ABSTRACT

Disperse Red 1 (DR1), which is widely used in the textile industry, is an azo dye that contributes to the toxicity and pollution of wastewater. To assess the toxic effects of DR1 on reproduction, sexually mature male mice (*Mus musculus*, strain CF-1) were orally (gavage) treated with single doses of the compound at 20, 100 and 500 mg/kg body weight. Testicular features and sperm parameters were evaluated 8.3, 16.6 and 24.9 days after treatments. In addition to testicular toxicity caused by the dye, the data clearly showed an increased frequency of sperm with abnormal morphology and decreased fertility. An increased amount of DNA damage was also detected in testis cells 16.6 and 24.9 days after treatments with 100 and 500 mg/kg. This study demonstrated the toxic and genotoxic effects of DR1, indicating the harmful activity of this dye on reproductive health.

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1. Introduction

The widespread use of dye compounds in several industries has increased due to consumer demands for color diversity and resistance to light exposure, washing and perspiration [1]. After the manufacturing process, dye compounds are released into rivers, and even after routine treatment, these compounds are not completely eliminated [2]. Moreover, data show that chlorinated products generated during conventional water treatment can be more mutagenic than pure dyes [3]. Studies on the mutagenic and carcinogenic potentials of some dyes found in Brazilian rivers have shown that these compounds are able to induce mutations in *Salmonella typhimurium* [4], chromosome aberrations in *Allium cepa* [5] and preneoplastic lesions in the colons of Wistar rats [6].

Azo dyes belong to a large family of synthetic dyes that are highly resistant to natural degradation. These compounds are able to induce several toxic effects, such as genotoxicity, mutagenicity, cytotoxicity and carcinogenicity [7]. Nevertheless, the mutagenicity of azo dyes cannot be generalized to the chemical group, and each dye must be studied individually [8]. In particular, the azo dye Disperse Red 1 (DR1) (n-ethyl-n-(2-hydroxyethyl)-4-(4-nitrophenylazo)aniline), which is widely used in the textile industry, contributes to the toxicity and pollution of wastewater, and its chlorination products after water treatment are even more dangerous [3]. The literature shows that exposure of human lymphocytes and a human hepatoma (HepG2) cell line to DR1 *in vitro* at concentrations of 1.0 and 2.0 μg/mL increases the frequency of micronuclei and also causes mutations in the Salmonella assay (13 revertants/μg) [8]. High toxicity was also detected in the water flea *Daphnia similis* (EC$_{50}$: 127 μg/L) [9] and in *Girardia tigrina* (DR1 1 mg/L) [10].

With regard to reports on the reproductive toxicity of dyes, the azo textile dye Congo Red (1 g/kg/day) was toxic to both male and female mice [11]. Prenatal exposure to this dye (0.5 g/kg/day) also induced damage in the gonads, reducing fertility in female mice [12]. Recently, Suryavathi et al. [13] showed that textile dyes in untreated wastewater caused sterility in rats and mice.

Prior studies linking textile dyes and reproductive toxicity led us to ask whether the widely used Disperse Red 1 azo dye could also induce germ cell damage and to determine the possible mechanisms of inducing DNA damage. Therefore, this study aimed to evaluate the toxicological and toxicogenetic activities of Disperse Red 1 in male mice.

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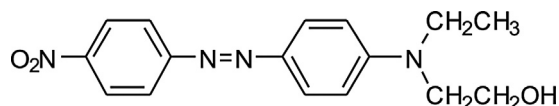*E-mail address:* fernandesfh@gmail.com (F.H. Fernandes).

Fig. 1. Chemical structure of Disperse Red 1.

2. Materials and methods

2.1. Chemical

Disperse Red 1 (CAS No. 2872-52-8) was purchased from Sigma (St. Louis, MO, purity >95%) (Fig. 1).

2.2. Animals and treatments

The study protocol (Protocol 0647) was approved by the Bioethics Committee of the Faculty of Medicine, University of Chile.

Three-month-old adult male CF-1 mice were obtained from the animal facilities of the University of Chile Medical School. The animals were maintained in a room with controlled temperature ($22 \pm 2\,°C$) and light (12 h light 12 h dark cycle) and had *ad libitum* access to commercial food and tap water.

The animals were distributed into five groups with five animals in each: Group 1, the negative control, received only tap water and basal diet during the entire experimental period; Group 2, the positive control, was treated with an intraperitoneal (ip) injection of n-methyl-n-nitrosourea (MNU – 50 mg/kg/body weight [b.w.]); Groups 3, 4 and 5 were orally (gavage) treated with single doses of DR1 at 20, 100 or 500 mg/kg body weight (b.w.), respectively. Before euthanasia, animals were anesthetized with an ip injection of ketamine (44 mg/kg b.w.). Euthanasia was performed by cervical dislocation 8.3 (199.2 h) 16.6 (398.4 h) and 24.9 (597.6 h) days after DR1 treatment, times that are approximately 1, 2 and 3 cycles of the seminiferous epithelium, respectively [14]. As observed by Oakberg [15], most cells sampled 16.6 and 24.9 days after dye

exposure corresponded to differentiating type A spermatogonium, but only a few cells had differentiated at the initial phase after 8.3 days (Fig. 2).

2.3. Testicular endpoints

2.3.1. Histology

After euthanasia, both testes were removed and separated from the epididymis and related tissues. The right testis was weighed and used for the comet assay, and the left testis was fixed in Bouin solution, washed in 70% ethanol and stored until it was embedded in Paraplast (Tyco Healthcare Group, Mansfield, Massachusetts, USA) and cut into 5 μm cross-sections.

A serial section was stained with hematoxylin and eosin; images from transverse sections of testicular tissue were obtained under a light microscope at 200× magnification, and the epithelial height, tubular diameter and tubular lumen diameter were measured (50 tubules/mouse for each parameter) using the Axion Vision 4.9.1 (Carl Zeiss, Jena, Thuringia, Germany) image analysis software. Seminiferous tubule histopathology was performed under a light microscope at 1000× magnification. The endpoints considered were: epithelial vacuolization, cell depletion and tubular atrophy.

2.4. Sperm endpoints

2.4.1. Cell sampling, epididymal cauda weight and sperm count

Sperm were sampled from cauda epididymis 16.6 and 24.9 days after DR1 treatment to show the effect of dye exposure on intermediate spermatogonium, pachynema phase cells and spermatids, and mature spermatozoa, respectively [15]. The cauda epididymis was removed and weighed, and a sperm suspension was then obtained using the protocol described by Fornés and Bustos-Obregón [16]. Sperm were counted (in duplicate slides) under a light microscope at 200× magnification using a Makler® chamber. A total of 100 squares were considered, and results are expressed as 10^6 sperm/g cauda epididymis.
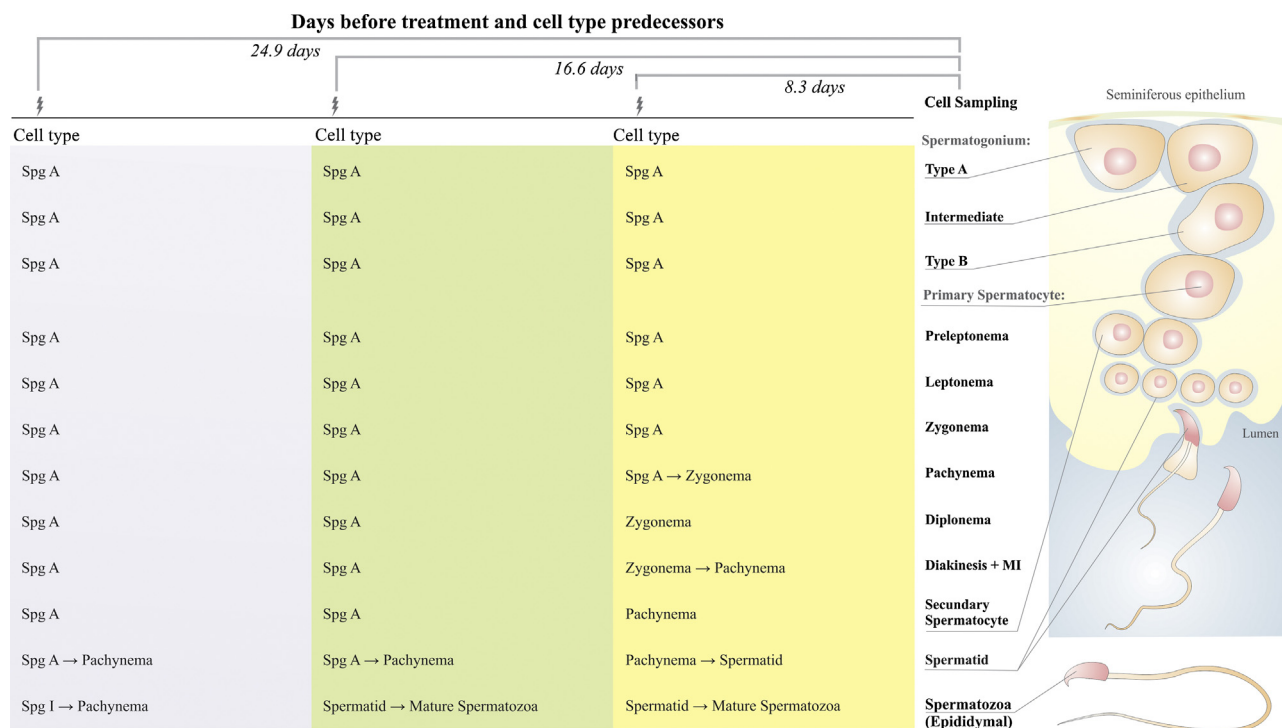

Fig. 2. Schematic diagram showing cell types predecessors from each sampling times (8.3, 16.6 and 24.9 days). A Spg – spermatogonium type A; I Spg – intermediate spermatogonium; MI – meiosis I (according to Oakberg 1957).

2.4.2. Sperm morphology

A 200 μL volume of sperm suspension was mixed with 200 μL of PBS and centrifuged at 2000 rpm for 10 min. Pellets were then dropped on slides and stained with hematoxylin–eosin. Analysis was performed in a light microscope at 1000× magnification. Three hundred spermatozoa per animal were evaluated, and their morphologies were classified as normal or abnormal according to Vigil and Bustos-Obregon [17]. The results are expressed as % abnormal sperm.

2.4.3. Sperm DNA integrity

The integrity of the sperm DNA was evaluated using the acridine orange fluorescence test following the methodology described by Tejada et al. [18]. Slides were analyzed in an epifluorescence microscope at 524 nm and 400× magnification. Three hundred spermatozoa per animal were counted, and the results are expressed as the percentage of sperm with normal DNA integrity (green head) and the percentage of sperm with a loss of DNA integrity (a spectrum of yellow-orange to red head).

2.5. Comet assay

The comet assay was used for detecting primary DNA damage (single- and double-strand breaks and alkali-labile sites) in testicular cells isolated as previously described by Anderson et al. [19]. The alkaline version of the assay was performed according to the technique described by Tice et al. [20]. Briefly, 10 μL of the cell suspension was mixed with 75 μL of low-melting-point agarose and was placed onto slides that had previously been covered with a thin layer of normal-melting-point agarose. Thereafter, the slides were immersed into cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCI, Triton X-100 and DMSO) at 4 °C overnight and were subsequently incubated in an alkaline buffer (pH 13) for 20 min to allow the DNA to unwind and for alkali-labile site expression. Electrophoresis was conducted in the same alkaline buffer at 4 °C for 20 min at 25 V (0.86 V cm^{-1}) and 300 mA. Then, the slides were neutralized in 0.4 M Tris–HCI (pH 7.5) for 15 min, fixed in absolute ethanol and stored at room temperature until analysis. The slides were stained with acridine orange (3 μg/mL) and then examined in an epifluorescence microscope at 524 nm and 400× magnification. A total of 100 randomly selected nucleoids per treatment was analyzed and classified based on tail length: 0 – no tail, no damage (<5%); 1 – low level of damage (5–20%); 2 – intermediate level (20–40%); 3 – high level (40–95%); and 4 – totally damaged (>95%) (Fig. 3I). The DNA damage index (DDI) was obtained using the following formula [20]:

$$DDI = \frac{(\text{nucleoid class } 0 \times 0) \pm (\text{nucleoid class } 1 \times 1) \pm (\text{nucleoid class } 2 \times 2) \pm (\text{nucleoid class } 3 \times 3) \pm (\text{nucleoid class } 4 \times 4)}{\text{Number of nucleoids analyzed}}$$

Haploid, diploid and tetraploid cells were classified based on the nuclear diameter [21] (Fig. 3II). To verify the distribution of cell types among treatments, 200 cells from each animal were classified as haploid, diploid and tetraploid. Total (haploid, diploid and tetraploid cells) and haploid (individually analyzed) cells were scored at different intervals of seminiferous epithelium cycles: 1, 2 and 3 cycles or 8.3, 16.6 and 24.9 days after treatment with DR1, respectively. In addition, the time that germ cells of the germinal epithelium (type A, intermediate and B spermatogonial cells, and pre-leptonema, leptonema, zygonema, pachynema, diplonema and diakinesis cells; primary spermatocytes) take to convert into haploid cells was estimated. These times were calculated on the basis of the approximate duration of spermatogenesis [15].

2.6. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA; *post hoc* Dunnet) and Kruskal–Wallis tests (*post hoc* Dunn). All results are expressed as the means ± standard deviations. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Histology

Fig. 4 shows photomicrographs of testis transverse sections from mice treated with DR1 and controls. Histological abnormalities (*i.e.*, vacuolation in the seminiferous epithelium and loosening of the tubular basement membrane) were clearly observed 16.6 and 24.9 days after dye exposure in those animals treated with the highest concentrations (100 and 500 mg/kg) (Fig. 4H and D). At the lower concentration (20 mg/kg), DR1 induced high spermiation (large numbers of spermatozoa can be observed in the lumen) when animals were analyzed 24.9 days after treatment (Fig. 4F).

3.2. Sperm endpoints

Spermatozoa sampled 16.6 days after DR1 treatment (500 mg/kg) presented a higher (*P* < 0.01) percentage of abnormal morphology (flattening of the dorsal acrosomal region, alterations of the apical region of the acrosome, folding of the tail over the head and anomalies of the flagellum) than those of the control (Table 1, Fig. 3 III). A significant increase in the cauda epididymis weight, spermatozoa number and luminal diameter at 20 mg/kg, an increase in sperm abnormalities and decreases in testicular weight and DNA integrity were detected 24.9 days after treatment with 500 mg/kg DR1.

3.3. Comet assay

No differences in the number of haploid and total (haploid + diploid + tetraploid) cells were observed among the groups at the three sampling times (8.3, 16.6 and 24.9 days after exposure to DR1). Significant increases in DNA damage were detected in all types of cells (haploid, diploid and tetraploid) 16.6 and 24.9 days after treatments with 100 and 500 mg/kg DR1 (Fig. 5B, C, E and F). Increased damage induced by 20 mg/kg DR1 was only observed for the sum of all cells (total cells) 24.9 days after exposure (Fig. 5F).

4. Discussion

The potential consequences of DNA damage in male germ cells may be severe for offspring because mutations in the paternal genome may be passed on to them [22,23]. However, little is known about the effects of azo textile dyes on reproductive cells. Therefore, this study was designed to evaluate the toxic effects of DR1 on mouse spermatogenesis and the reproductive tract. Although DR1 did not induce any changes in the numbers of diploid and tetraploid cells, reduced testicular weight, vacuolation in the seminiferous epithelium and loosening of the tubular basement
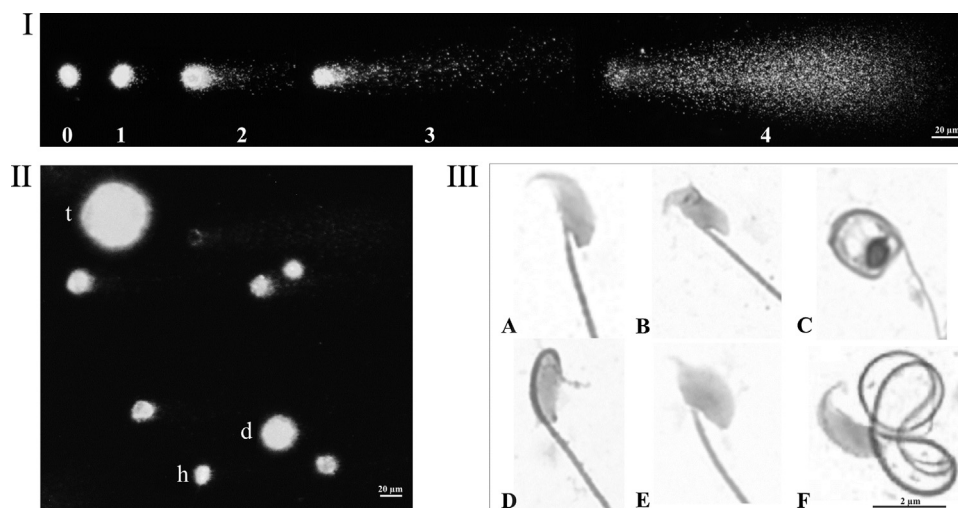
Fig. 3. Photomicrographs showing germ cells and the damage induced by Disperse Red 1. I – Nucleoids classified according to their tail length (comet assay): 0 – no tail, no DNA damage; 1 – low level of damage; 2 – intermediate level; 3 – high level; 4 – totally damaged. II – Nucleoids classified according to the nuclear diameters (DNA content): t – tetraploid; d – diploid; h – haploid cells. III – Sperm morphology: A – normal; B = normal; B – flattening of the dorsal region; C and D – tail folded over the head; E – alterations of the apical region; F – anomalies of the flagellum.
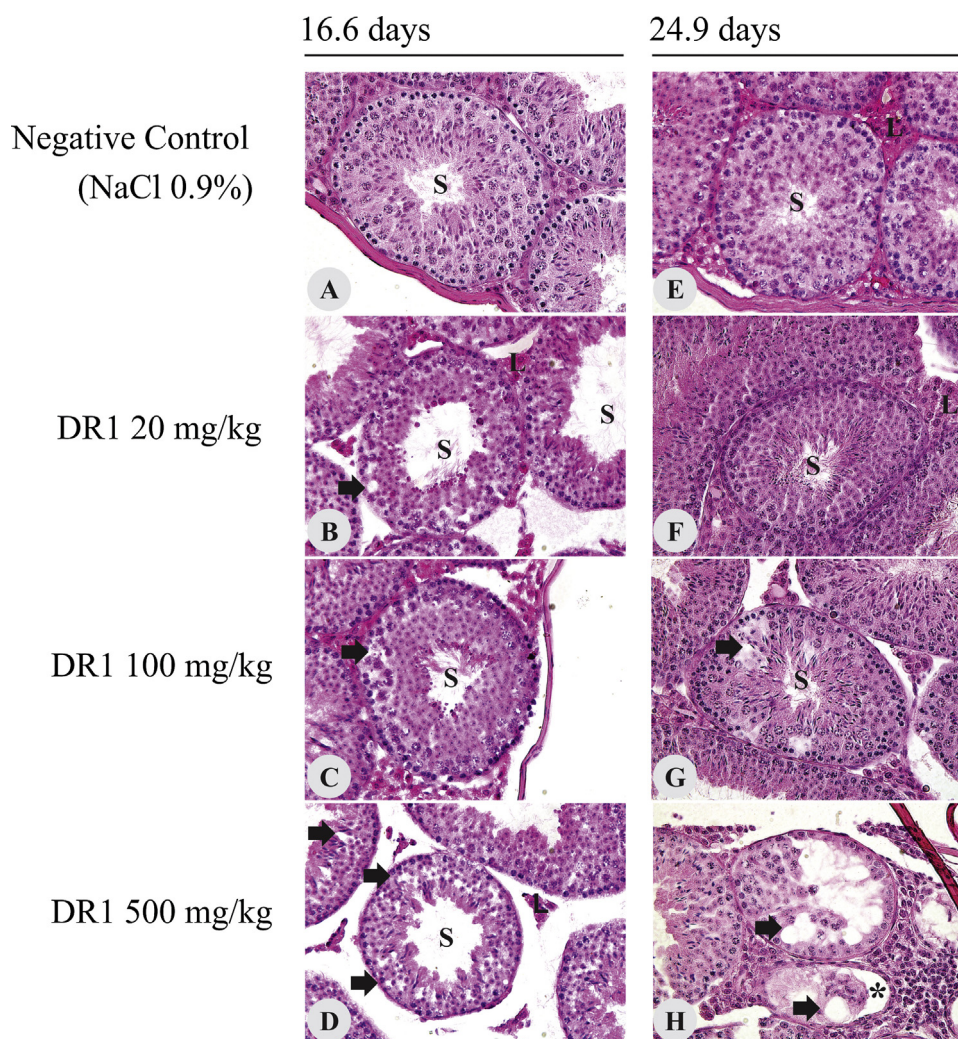

Fig. 4. Photomicrographs of mouse testis transverse sections at 16.6 (A–D) and 24.9 (E and F) days after treatment with Disperse Red 1 (DR1). S – spermatozoa; L – Leydig cell; arrow – vacuolization; * – loosening of the tubular basement membrane. Hematoxylin–eosin staining, 400×.

Table 1
Frequencies (means ± SD) of morphological and spermatic alterations 16.6 and 24.9 days after treatment with Disperse Red 1 (DR1).

Endpoints/treatments	MNU	Control (0.9% NaCl)	DR1 (mg/kg)		
			20	100	500
16.6 days (*2 cycles of seminiferous epithelium*)					
Anatomical features					
Testicular weight (mg)	138.4 ± 10.3	120.3 ± 10.4	130.6 ± 19.8	145.2 ± 9.7	139.8 ± 16.7
Cauda epididymis weight (mg)	58.6 ± 6.7	60.8 ± 10.0	51.9 ± 9.0	60.8 ± 8.8	52.4 ± 4.4
Sperm					
Sperm count (10^6 sperm/g)	152.7 ± 15.3	81.3 ± 4.0	71.8 ± 20.4	73.6 ± 23.3	79.4 ± 26.0
Normal sperm DNA integrity (%)	94.0 ± 2.0	99.5 ± 0.5	94.3 ± 7.56	90.9 ± 7.21	95.6 ± 3.15
Abnormal sperm morphology (%)	16.6 ± 4.8	8.1 ± 2.3	13.71 ± 4.6	15.7 ± 4.9	30.0 ± 11.7[**]
Histometric parameters					
Tubular diameter (μm)	168.6 ± 8.5	164.5 ± 13.9	177.7 ± 8.6	174.2 ± 10.8	173.2 ± 9.4
Epithelial height (μm)	52.4 ± 3.3	51.9 ± 6.8	59.7 ± 2.4	54.5 ± 3.4	54.0 ± 5.1
Luminal diameter (μm)	69.9 ± 7.4	67.0 ± 7.8	61.0 ± 5.6	69.7 ± 5.2	70.6 ± 5.8
24.9 days (*3 cycles of seminiferous epithelium*)					
Anatomical features					
Testicular weight (mg)	60.5 ± 15.1	108.0 ± 7.2	101.9 ± 9.7	97.1 ± 4.8	84.4 ± 9.1[**]
Cauda epididymis weight (mg)	41.2 ± 6.8	45.7 ± 8.2	71.1 ± 16.6[*]	39.6 ± 3.7	45.4 ± 3.2
Sperm					
Sperm count (10^6 sperm/g)	103.0 ± 33.5	67.7 ± 15.1	114.0 ± 27.7[*]	63.4 ± 20.6	69.0 ± 7.0
Normal sperm DNA integrity (%)	93.9 ± 2.9	99.7 ± 0.2	97.3 ± 2.2	96.9 ± 1.1	92.5 ± 2.2[**]
Abnormal sperm morphology (%)	25.2 ± 7.5	10.3 ± 3.8	19.8 ± 5.9	18.1 ± 4.6	60.5 ± 8.8[**]
Histometric parameters					
Tubular diameter (μm)	107.5 ± 7.9	127.9 ± 6.2	117.6 ± 9.8	120.6 ± 5.8	120.1 ± 4.1
Epithelial height (μm)	36.9 ± 6.4	43.5 ± 1.6	40.5 ± 2.9	42.0 ± 2.2	41.4 ± 1.1
Luminal diameter (μm)	38.0 ± 4.2	46.4 ± 5.4	33.6 ± 3.5[*]	40.4 ± 3.6	42.4 ± 6.3

MNU – n-methyl-n-nitrosourea (50 mg/kg b.w.; positive control); *$P < 0.05$, **$P < 0.01$ compared to the negative control.
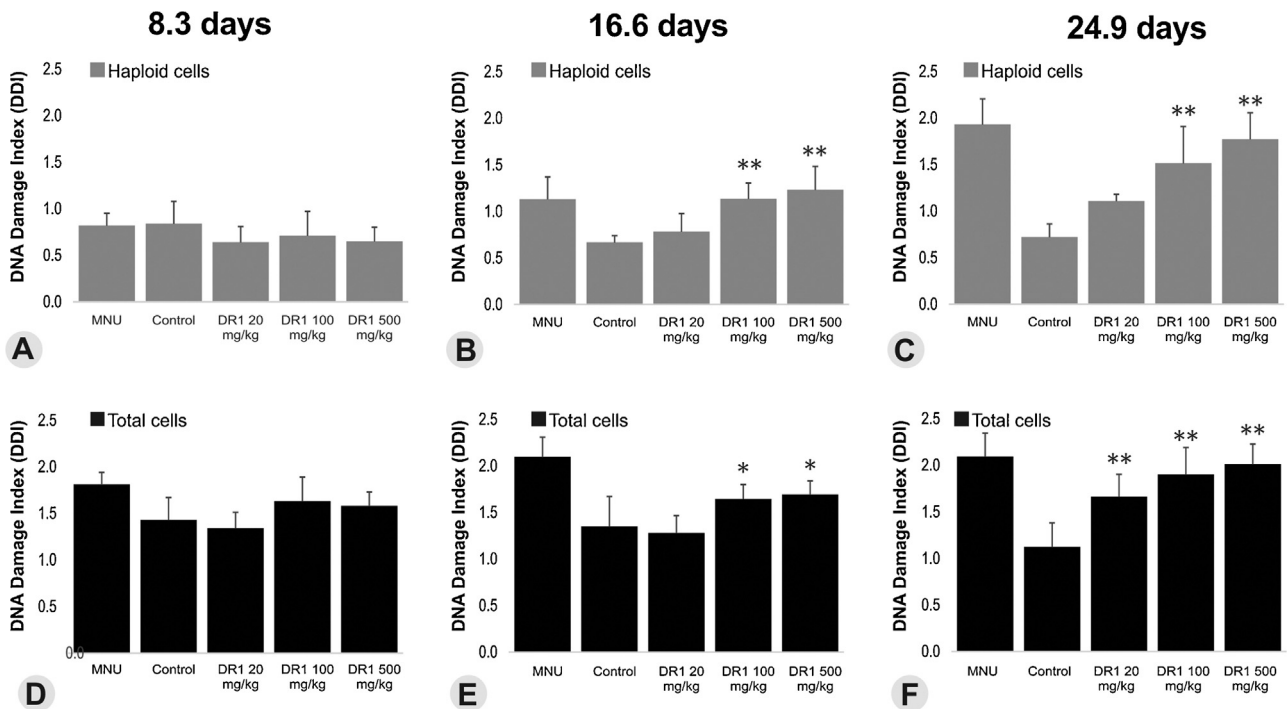

Fig. 5. DNA Damage Index (DDI) in germ cells of male mice treated with Disperse Red 1 (DR1). A, B and C show the mean damage in the haploid cells (only the haploid cells were included in this score); D, E and F show the mean damage in all cells (including haploid, diploid and tetraploid cells; classified according to their nuclear diameter) of the seminiferous tubule population. Control – 0.9% NaCl; MNU – n-methyl-n-nitrosourea (50 mg/kg b.w.; positive control).*$P < 0.05$, **$P < 0.01$ compared to the negative control.

membrane were detected. Vacuolization is the most common morphological response of Sertoli cells to various injuries [24], and vacuolization seemed to be associated with the reduction in testes weights detected mainly after treatment with the highest DR1 concentration (500 mg/kg). Indeed, the decrease in testicular weight induced by DR1 may be ascribed to widespread testicular damage.

Reductions in protein and cholesterol testicular content, for example, were already linked to decreased reproductive organ weights and complete sterility in albino rats exposed to textile discharge water [13]. Testis weight reduction was also described after rat and mouse exposure to dyes derived from benzidine, dimethylbenzidine and dimethoxybenzidine [11].

For the sperm endpoints (morphology and DNA integrity), the clearest reproductive toxic effects were observed 24.9 days after treatment with DR1 at 500 mg/kg. As described by Oakberg [15], sperm collected from the cauda epididymis at days 16.6 and 24.9 were spermatids and primary spermatocytes in the pachynema stage, respectively, at the moment of treatment (Fig. 2). These results indicate that primary spermatocytes were more sensitive to DR1 toxic effects than spermatids. In pachynema, homologous chromosomes are completely paired, and at this stage breakage and exchange of DNA occur (crossing over), but cell differentiation and DNA compaction occurs in spermatids before they become spermatozoa.

Although a checkpoint in pachynema may prevent failures in meiosis [25], cells at this stage may accumulate more damage because DNA repair fails after crossing over. Recent published data demonstrated clear evidence of changes in the DNA structure caused predominantly by DR1 intercalation [26]. Corroborating this finding, the present study showed an increased amount of primary DNA damage (single- and double-strand breaks and alkali-labile sites) in male germ cells (haploid, diploid and tetraploid) sampled 16.6 and 24.9 days after DR1 treatment. According to Oakberg [15], the haploid cells analyzed at these time points (secondary spermatocytes, spermatids or spermatozoa) would be undifferentiating spermatogonia types A, intermediate or B or primary spermatocytes (at prophase I or pre-leptonema – pachynema phases) at the time of DR1 treatment, whereas the diploid and tetraploid cells (spermatogonia types A, intermediate or B, or primary spermatocytes at prophase I) may only be spermatogonia A at the time of treatment (Fig. 2). Therefore, the sampling times of 16.6 and 24.9 days after DR1 treatment mean that the scored cells went through two and three cycles of seminiferous epithelium, respectively [14].

Another interesting result was the increased sperm concentrations and cauda epididymis weight observed 24.9 days after exposure to DR1 at the lowest dose (20 mg/kg). One possible explanation for this finding could be an endocrine-disrupting effect of the dye. A recent study showed that some textile dyes (mainly blue and red dyes) are potential endocrine-disrupting agents able to induce estrogenic or anti-estrogenic activities [27], by binding to a specific hormone receptor, mimicking the hormone or blocking normal biological response [28]. Other suggestion could be the mild aggression caused by DR1 20 mg/kg in contrast to the severe damage (cell death) induced by the two highest doses.

According to Ribas-Maynou et al. [29], the alkaline comet assay is one of the best techniques for predicting human male infertility. As previously described, this version of the comet assay can detect several DNA lesions, including alkali-labile sites and single- and double-strand breaks. Although single breaks are rapidly repaired, double-strand breaks are unlikely to persist for many rounds of mitosis and meiosis (e.g., spermatogenesis) [30,31]. Therefore, it is possible that the alkali-labile sites (converted to breaks when in contact with alkaline electrophoresis solution; pH > 13) induced by DR1 in spermatogonia A persisted and could be detected 24.9 days after treatment. Similarly, Olsen et al. [32] demonstrated that benzo[a]pyrene-induced DNA adducts in male mouse germ cells can persist in spermatozoa and give rise to de novo mutations. DNA damage is often associated with abnormal sperm morphology [33,34]. However, sperm with a normal appearance can also carry DNA lesions that can increase the risk of genetic disease in the offspring [35,36]. It is important to emphasize that the proportion of cells that were microscopically classified as haploid, diploid and tetraploid in the control group was similar to that previously reported by Wellejus et al. [37] using cytometry.

To the best of our knowledge, there are no data in the literature describing DR1 genotoxicity in mammalian germ cells in vivo. Nevertheless, some studies have reported the mutagenic activities of several dyes, including DR1, in somatic cells. Increased frequencies of micronuclei have been observed in human lymphocytes and human hepatoma cells (HepG2) after in vitro exposure to DR1 [8], suggesting that this dye is a potential clastogen to somatic cells. It was also demonstrated that Disperse Orange 1, Sudan I and 17 other dyes are genotoxic in various somatic cells [38–40]. In conclusion, our data demonstrated the toxic effects of DR1 on mouse spermatogenesis, revealing the potentially harmful effects of this dye on reproductive health. Nevertheless, the actual caution level for DR1 needs to be clearly stated.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

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