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Assessment of 3, 4-Dichloroaniline Toxicity as Environmental Pollutant in Male Mice

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Abstract: The effect of 3,4-Dichloroaniline (3,4-DCA) on chromosome aberrations in both bone-marrow cells and spermatocytes, sperm quality and histopathological changes in male mice was investigated. Eighty male Swiss albino mice were divided into four groups (n = 20), one was considered as control and the other three groups were administered 3,4-DCA by gavage at the dose levels of 13.83, 27.67 and 55.33 mg/kg bw/day for 30 consecutive days. The doses were correspond, respectively, to approximately 1/32, 1/16 and 1/8 of LD_{50} value determined for male mice (442.6 mg a.i./kg bw). Results indicated that oral administration of 3,4-DCA significantly increased the frequency of structural and numerical types of chromosomal aberrations in both bone-marrow cells and spermatocytes. Similarly, there was a significant increase in count of abnormal spermatozoa in all 3,4-DCA treated groups of mice compared to respective control. On the other hand, 3,4-DCA caused a significant decrease in the sperm count and motility as compared to the control. Furthermore, histopathological changes in liver and testis were observed. All adverse effects were in a dose-dependent manner. In light of the aforementioned results, it can be concluded that 3,4-DCA has the potential to produce genotoxic effects, reproductive toxicity and histopathological alteration in male mice. Accordingly, strict limitations on the use of this compound must be put.

Key words: 3,4-DCA · Chromosome aberrations · Sperm quality · Histopathology · Mice

INTRODUCTION

The aromatic amine, 3,4-Dichloroaniline (3,4-DCA) is widely used as chemical intermediate in the synthesis of several herbicides (e.g. diuron, linuron and propanil), azo dyes for polyester fabrics, textile and pharmaceuticals [1-3]. It is also a degradation product of trichlorocarbanilide, a chemical used as active agent in the cosmetic industry [4]. Diuron, linuron, oxyfluorfen, 2-methyl-4-chlorophenoxyacetic acid, terbuconazol and difenoconazol can lead to the formation of chloroanilines including 4-chloroaniline and 3,4-dichloroaniline [5]. Thus, 3,4-DCA is a model environmental contaminant [6].

3,4-DCA often gets into the environment from agricultural activities, either subsequent to the application or to the industrial production processes of chemicals [7]. 3,4-DCA was detected in raw water at low levels but its concentration increased ~10 times after chloramination within a full scale drinking water treatment plant that used chlorinated agents as disinfectants in Spain [5]. It has been detected as an environmental contaminant in surface waters and in the effluents from dye-manufacturing plants [8]. DCA has been detected more frequently in environmental samples than its parent compounds [9]. It is not readily degraded by micro-organisms and, due to its persistence in the environment, is considered to be a reference xenobiotic [10]. DCA can persist in the environment as insoluble residues in soil and plants and can also photodimerise to form carcinogens [11]. DCA endangers growth, development and propagation of aquatic organisms and furthermore is potential harmful to humans [12, 13]. Studies *in vitro* and *in vivo* have shown that DCA has nephrotoxicity and reproductive toxicity to mammals [14, 15]. Consequently, DCA has been classified

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as a compound of environmental concern. Furthermore, due to its toxicity both to invertebrates and vertebrates and to its high production rate, it was included in the European Union priority List one of chemicals considered particularly troublesome for the environment [16]. Chromosomal aberrations may be used as an early warning signal for cancer development and it has been suggested that the detection of an increase in chromosomal aberrations, related to an exposure to genotoxic agents, may be used to estimate cancer risk [17]. Bone marrow, with its rapidly renewing cell populations, is one of the most sensitive tissues to cytotoxic agents [18]. In humans, the decline and well known regional differences in semen quality [19] and the increasing incidence of testicular cancers [20], during the last five decades, have been related to environmental contaminants [21]. Moreover, diuron, linuron, 3,4-DCA and 3,4-dichloroacetanilide (3,4-DCAc) have the capacity *in vitro* to connect to androgen receptors, permitting action as endocrine disruptors [22, 23]. On the other hand, very little information is available regarding sperm quality, cytogenetic and histopathological changes induced by this compound.

Therefore, this work has been designed to evaluate the potential effect of 3,4-dichloroaniline on chromosome aberrations in both bone-marrow cells and spermatocytes, sperm quality and histopathological changes in mice.

MATERIALS AND METHODS

Chemicals: 3,4-Dichloroaniline (3,4-DCA, 98%) was purchased from Tokyo Chemical industry Co, LTD, Japan. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Animals: Male Swiss albino mice weighing 28±5 g were purchased from the Theodor Bilharz Research Institute and were housed in polypropylene cages (43×30×15cm, five mice per cage). Mice were maintained under controlled temperature (23±1°C), 50–55% relative humidity, a photoperiod of 12 h light: 12 h dark and permitted to freely consume water and food *ad libitum*. The mice were acclimatized for 2 weeks before dosing.

Assessment of Oral LD_{50}: An acute oral toxicity study was performed in accordance with OECD [24]. Twenty animals were divided into four groups, each group contains five mice. 3,4-DCA was dissolved in corn oil and administered orally by gavage at four different doses i.e., 222.2, 333.3, 500 and 750 mg kg^{-1} bw. Following dosing the animals were allowed free access to food and water and observed for 14 days. The LD_{50} values were calculated according to the statistical method of Weill [25]. Results demonstrated that, The acute oral LD_{50} for male Swiss albino mice was determined as 442.6 mg a.i./kg bw, with 95% confidence limits of 342.58 to 571.78 mg/kg.

Experimental Design: Eighty male Swiss albino mice were divided into four groups (n = 20), according to approximately equal mean body weight. The first group served as vehicle control and was administered orally by corn oil, while the other 3 groups were administered 3,4-DCA by gavage at the dose levels of 13.83, 27.67 and 55.33 mg/kg bw. The doses were correspond, respectively, to approximately 1/32, 1/16 and 1/8 of LD_{50} value determined for male mice (442.6 mg a.i./kg bw). Dose concentrations were adjusted to a mean body weight (10 ml/kg bw). All groups orally administered 3,4-DCA or corn oil, once a day for 30 successive days and then 10 mice from each group were sacrificed for histopathological and chromosomal aberration analysis of both somatic (bone marrow) and germ (spermatocytes) cells. The remaining animals (10 mice) from each group were left without treating for 35 days from the last dose (duration of spermatogensis), then the animals were sacrificed for analysis of sperm count, motility and abnormalities.

Mitotic Index Determination: The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were also used for calculating the mitotic index. Randomly selected views on the slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. At least 1000 cells were examined in each preparation. The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells, multiplied by 100 [26].

Chromosomal Aberration Analysis: At the end of the treatment, animals of all treated groups were injected intraperitoneally with colchicine to arrest cell division at metaphase. Two hours after injection, animals were sacrificed by neck vertebra luxation for preparation of the chromosomes of bone marrow and spermatocyte cells. Chromosomes of bone-marrow cells were prepared by using the methodology of Yosida and Amano [27]. Both femurs were dissected out and cleaned of any adhering muscle tissue. Bone-marrow cells were collected from both the femurs by flushing in saline solution and then incubated at 37°C in hypotonic solution (KCl 0.56%) for 35min, fixed in methanol:glacial acetic acid (3:1).

The cells were resuspended in a small volume of fixative, dropped onto chilled slides, flame-dried and stained with 10% buffered Giemsa (pH 6.8).

Spermatocytes were prepared according to Brewen and Preston [28]. The tunica of the testis was removed and the tubules were transferred to a small Petri dish containing isotonic solution (2.2% sodium citrate) and teased out with curved forceps on a piece of mesh. The cell suspension was transferred into a conical tube and centrifuged. The supernatant was removed and the pellet was re-suspended in hypotonic solution (1.1% trisodium citrate) for 20 minutes and fixed in cold fixative. The cells were resuspended in a small volume of fixative, two or three drops of cell suspension were dropped on a clean slide stored in cold 70% ethanol and dried on hot plate at 60°C. Slides were stained with 10% buffered Giemsa (pH 6.8).

One hundred good metaphases for somatic and germ cells of each animal were examined microscopically for scoring different types of aberrations.

Sperm Characteristics

Sperm Collection: After 35 days of the last dose (duration of spermatogenesis), the animals were sacrificed by neck vertebra luxation. The epididymides from each mouse were removed and sperm was collected as quickly as possible when each mouse was dissected. The sperm count was assessed from right cauda epididymides while sperm motility and morphology were analyzed from the left one. Epididymis was excised and minced in 1 ml of phosphate buffered saline (pH 7.2) to obtain sperm suspension. The suspension was filtered through a nylon mesh [29].

Sperm Count: The cauda epididymal sperm count was performed according to Narayana *et al.* [29] using a Neubauer hemocytometric chamber. Layered slides with semen were viewed by bright-field microscope with magnification of 400×. The total sperm count in squares of 1 mm^2 each was determined to express the number of sperm/epididymis. Epididymal sperm counts were expressed as number of sperms per epididymis. To minimize the error, the count was repeated three times on each sample [30].

Sperm Motility: Approximately 10 µl of sperm suspension with a micropipette was layered onto a warmed microscope slide. Sperm motility was assessed by counting all progressive motile (effective), the non progressive motile (non-effective or sluggish) and the immotile (dormant) spermatozoa in the same microscopic field (400×). In each semen sample, 10 microscopic fields were examined with at least 100 sperm/field was counted. The number of motile sperm cells in each field was divided by the total number and the average of the fields was assayed. The percentage of motile spermatozoa was determined [31].

Sperm Abnormalities: To assess the spermatozoa morphological abnormalities, a drop of sperm suspension was smeared on a slide and air-dried and made permanent. The smeared slide was stained with 1% eosin Y and 5% nigrosin. Morphological sperm defects were evaluated and examined on optical microscope using 400× magnification [32]. At least 100 spermatozoa from different fields in each slide were examined and classified for criteria of morphological abnormalities (head, tail and tail-head) according to Filler [33]. Abnormal sperm cells were counted and the percentage was calculated.

Histological Study: At the end of experiment, liver and testis from each sacrificed mouse were dissected out; trimmed of excess fat. All tissues were fixed in 10% buffered formalin and were processed for paraffin sectioning by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks. Sections of about 5µm thickness were stained with Harris haematoxylin and eosin (H&E) for histological study [34].

Statistical Analysis: Statistical analyses were performed with SPSS software. Data were analyzed using one way analysis of variance (ANOVA) followed by Duncan's post hoc test for multiple comparisons between pairs. Results were reported as mean values ± S.D. and differences were considered as significant when ($P \leq 0.05$).

RESULTS AND DISCUSSION

There was no mortality, morbidity or distinctive clinical signs observed in any of the experimental animal groups during the study period.

Chromosomal Aberrations: The results of the present study showed that the treatment of male mice with 3,4-DCA induced significantly dose dependent reduction in the activity of mitotic index in both bone marrow cells and spermatocytes (Figure 1). The mitotic index factor is used to discover the toxicity for several physical and chemical agents which mostly affect the average of division or cleavage [35].

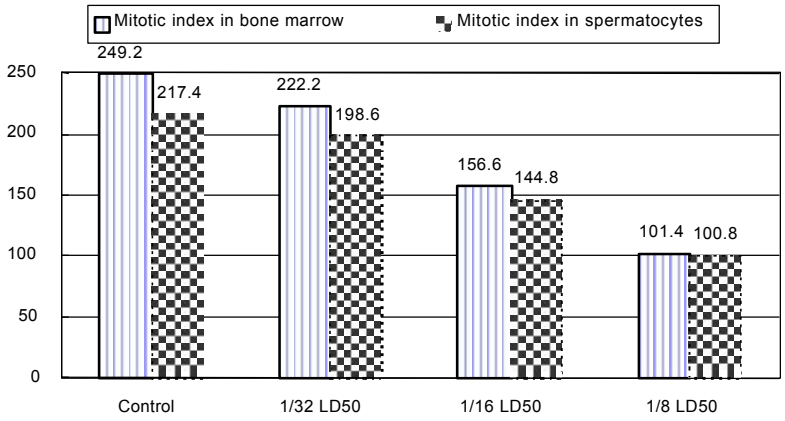

Fig. 1: Mean values of mitotic index in bone marrow and spermatocytes of control and 3,4-DCA treated male mice

Table 1: Mean values of different types of chromosomal aberrations in bone marrow cells of control and 3,4-DCA treated male mice

	Structural abnormalities									Numerical abnormalities				
Treatment	Gap	Break	Deletion	Centric fusion	End to end	Centro meric	Frag-ment	Ring form	Total structural aberrations	Endo-mitosis	Hypo-ploidy	Poly-ploidy	Total numerical aberrations	Total chromosomal aberrations
Control	$2.0^{a}\pm0.7$	$2.0^{a}\pm0.7$	$2.2^{a}\pm0.8$	$0.6^{a}\pm0.9$	$1.0^{a}\pm1.0$	$0.2^{a}\pm0.4$	$2.4^{a}\pm0.5$	$1.2^{a}\pm1.1$	$11.6^{a}\pm4.0$	$0.6^{a}\pm0.9$	$0.6^{a}\pm1.3$	$0.6^{a}\pm0.5$	$1.8^{a}\pm2.5$	$13.4^{a}\pm4.2$
1/32 LD_{50}	$4.2^{b}\pm0.4$	$4.2^{b}\pm0.8$	$3.2^{b}\pm0.4$	$1.2^{ab}\pm0.4$	$1.4^{a}\pm0.5$	$1.0^{ab}\pm1.0$	$4.4^{b}\pm1.1$	$2.8^{a}\pm0.8$	$22.4^{b}\pm3.3$	$0.6^{a}\pm0.9$	$1.0^{ab}\pm2.2$	$1.4^{ab}\pm0.5$	$3.0^{ab}\pm1.9$	$25.4^{b}\pm4.6$
1/16 LD_{50}	$5.4^{c}\pm0.5$	$6.4^{c}\pm0.5$	$3.8^{b}\pm1.0$	$2.2^{c}\pm0.4$	$2.6^{b}\pm0.5$	$1.4^{abc}\pm1.3$	$5.8^{bc}\pm0.4$	$5.0^{b}\pm1.2$	$32.6^{c}\pm1.6$	$1.0^{a}\pm1.0$	$1.8^{ab}\pm1.1$	$2.8^{cd}\pm0.8$	$5.6^{cd}\pm1.7$	$38.2^{c}\pm1.9$
1/8 LD_{50}	$6.6^{d}\pm0.5$	$6.8^{c}\pm1.6$	$6.8^{c}\pm0.4$	$3.6^{d}\pm0.5$	$5.2^{c}\pm0.8$	$2.4^{c}\pm0.9$	$7.0^{c}\pm1.5$	$6.4^{b}\pm1.9$	$44.8^{d}\pm5.6$	$1.4^{a}\pm1.3$	$3.0^{b}\pm1.7$	$3.6^{d}\pm1.1$	$8.0^{d}\pm2.0$	$52.8^{d}\pm5.9$

Values with different superscript letters within columns represent significant statistical differences (P < 0.05)

Table 2: Mean values of different chromosomal aberrations induced in spermatocytes of control and 3,4-DCA treated male mice

	Structural abnormalities				Numerical abnormalities			
Treatment	X-Y univalent	Autosomal	Chain	Total structral aberrations	Hypoploidy	Polyploidy	Total numerical aberrations	Total chromosomal aberrations
Control	$1.8^{a}\pm0.4$	$0.6^{a}\pm0.9$	$0.0^{a}\pm0.0$	$2.4^{a}\pm0.5$	$1.4^{a}\pm0.5$	$0.4^{a}\pm0.5$	$1.8^{a}\pm0.8$	$4.2^{A}\pm0.4$
1/32 LD_{50}	$3.2^{a}\pm0.8$	$2.2^{b}\pm0.4$	$0.0^{a}\pm0.0$	$5.4^{b}\pm0.5$	$2.0^{a}\pm0.7$	$1.0^{a}\pm1.0$	$3.0^{a}\pm1.2$	$8.4^{A}\pm1.5$
1/16 LD_{50}	$5.2^{b}\pm0.8$	$5.4^{c}\pm0.5$	$0.0^{a}\pm0.0$	$10.6^{c}\pm0.5$	$5.4^{b}\pm1.5$	$4.0^{b}\pm1.2$	$9.4^{b}\pm2.7$	$20.0^{B}\pm2.5$
1/8 LD_{50}	$9.6^{c}\pm1.8$	$6.8^{d}\pm1.1$	$0.6^{ab}\pm0.9$	$17.0^{d}\pm3.6$	$9.2^{c}\pm1.9$	$6.0^{c}\pm1.2$	$15.2^{c}\pm3.0$	$32.2^{C}\pm6.6$

Values with different superscript letters within columns represent significant statistical differences (P < 0.05)

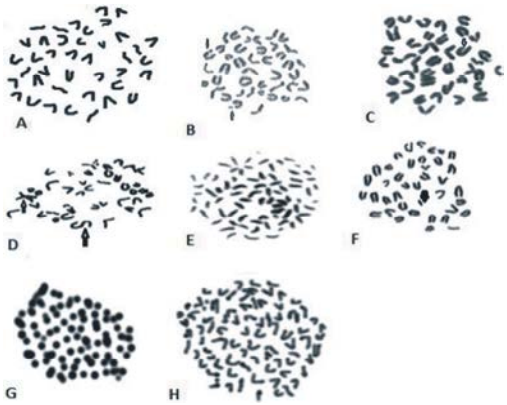

Fig. 2: Different types of chromosomal aberrations induced by 3,4-DCA in bone marrow cells: (A) control metaphase (B) gap and break (C) deletion (D) centric fusion, ring and end to end association (E) centromeric attenuation (F) fragment and hypoploidy (G) endomitosis (H) polyploidy

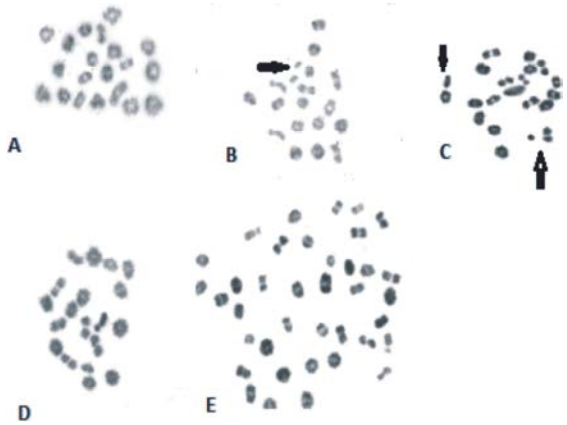

Fig. 3: Different types of chromosomal aberrations induced by 3,4-DCA in spermatocytes of male mice: A) control, B) autosomal univalent, C) x-y univalent, D) hypoploidy and E) polyploidy

Bauchinger *et al.* [8] tested the clastogenicity of 3,4-DCA in human lymphocytes *in vitro*. They indicated that 3,4-DCA might induce aneuploidy in mammalian cells by interaction with the mitotic apparatus. Meanwhile, there are very few published reports on the potential *in vivo* cytogenetic effects of 3,4-DCA. The results of the present study illustrated that oral administration of male mice by 3,4-DCA for 30 successive days induced dose dependent increases in the frequencies of structural and numerical and total chromosomal aberrations in both bone marrow cells and spermatocytes (Table 1&2). The observed structural chromosomal aberrations in bone marrow cells were gaps, breaks, deletions, centric fusion, end to end association, fragments, ring and centromeric attenuation (Figure 2A-E). The observed types of numerical aberrations were endomitosis, hypoploidy and polyploidy (Figure 2F-H). The most observed types of structural chromosomal aberrations in spermatocytes were autosomal univalent and X-Y univalent, while numerical aberrations were hypoploidy and polyploidy (Figure 3A-E).

The induction of structural chromosomal aberrations may result from: (i) direct DNA breakage, (ii) replication on a damaged DNA template, (iii) inhibition of DNA synthesis and other mechanisms such as topoisomerase II inhibition [36]. The chromosome malformations are either quantitative which include decrease or increase of the number of the chromosomes, or qualitative such as the exchange of the chromatid [37]. Our findings are in agreement with the result of Federico *et al.* [38] who showed that fenuron, chlorotoluron, diuron and difenoxuron at high concentrations induced an increasing number of chromosomal aberrations in non-metabolising Chinese hamster ovary cells. In addition, the four herbicides at the lowest dose-level induced chromosomal aberrations in the exposed epithelial liver cell line. Also, diuron has caused genetic damage in developing embryos and in bone marrow cells in mice [39].

Sperm Quality: Sperm count and motility were significantly reduced in mice following treatment with different doses of 3,4-DCA when compared to that of respective controls. There was also a significant difference in sperm count and motility among different 3,4-DCA treatment groups. Lowest sperm count and motility were found in high dose, followed by medium and low dose (Table 3). Sperm count is one of the most sensitive tests for assessment of spermatogenesis and fertility [40]. A decrease in sperm count in men over the last few decades is correlated with a steady increase in use of pesticides and other environmentally active chemicals [41]. It is possible that a steady decline in the sperm count over the years in the future might lead to oligospermia and infertility [42]. Suppression of gonadotrophins might have caused decrease in sperm density in testes [43].

Table 3: Mean values of sperm count and motility percentage in control and 3,4-DCA treated male mice groups

Treatment	Count 10^6/ml	Motility (%)
Control	$504.0^{a}\pm29.8$	$75^{a}\pm3.5$
1/32 LD_{50}	$359.8^{b}\pm51.4$	$67^{a}\pm6.7$
1/16 LD_{50}	$290.0^{c}\pm46.8$	$58^{b}\pm8.4$
1/8 LD_{50}	$220.8^{d}\pm24.5$	$36^{c}\pm5.5$

Values with different superscript letters within columns represent significant statistical differences ($P < 0.05$)

Sperm motility is one of the most important parameters used in the evaluation of sperm quality [44]. Sperm motility is also an important functional measurement to predict sperm fertilizing capacity. Any negative impact on motility would seriously affect fertilizing ability [45]. Marked inhibition of sperm motility in 3,4-DCA treated groups may be attributed to the low level of ATP content [46]. Sperm motility may be affected by altered enzymatic activities of oxidative phosphorolytic process. Oxidative phosphorolytic process is required for ATP production, a source of energy for the forward movement of spermatozoa. Full ATP pool is crucial for normal spermatozoa movement and a slight deprivation of ATP leads to reduction in motility, which may cause infertility [47].

Results showed also that there was dose dependent increase in the frequencies of sperm head and tail abnormalities in all 3,4-DCA treated groups of mice compared to respective controls. The frequency of abnormal spermatozoa also differed significantly among different 3,4-DCA treated groups, the highest number was in high dose followed by medium and low dose. Treated mice with 3,4-DCA at high and medium doses showed a significant increase in the frequencies of all types of abnormal spermatozoa, whereas the low dose treated mice showed significant increases in amorphous head, pin head and coiled tail type (Table 4 & Figure 4). These results are in agreement with those of Bo *et al.* [48] who reported that 3,4-DCA administered to male rats showed dose dependent decrease in sperm consistency, viability, motility and increase the sperm aberration rate. Sperm abnormality assay, a sensitive and reliable endpoint is widely used to identify germ cell mutagen [49]. The presence of abnormal sperm heads suggests induction of genetic damage in the male germ cells.

Table 4: Sperm abnormalities in control and 3,4-DCA treated male mice groups

	Type of sperm abnormalities						
Treatment	Hook less head	Amorphous head	Small head	Double head	Pin head	Coiled tail	Total sperm abnormalities
Control	$1.0^{a}\pm0.7$	$4.4^{a}\pm0.5$	$0.4^{a}\pm0.5$	$0.0^{a}\pm0.0$	$1.2^{a}\pm0.8$	$0.0^{a}\pm0.0$	$7.0^{A}\pm2.2$
1/32 LD_{50}	$1.4^{a}\pm0.5$	$8.4^{b}\pm0.5$	$0.6^{a}\pm0.5$	$0.0^{a}\pm0.0$	$3.2^{b}\pm0.4$	$2.0^{b}\pm0.0$	$15.6^{B}\pm1.5$
1/16 LD_{50}	$4.4^{b}\pm0.5$	$15.6^{c}\pm3.8$	$2.4^{b}\pm0.5$	$0.0^{a}\pm0.0$	$8.2^{c}\pm0.8$	$4.6^{c}\pm0.5$	$35.2^{C}\pm4.8$
1/8 LD_{50}	$7.8^{c}\pm0.8$	$23.4^{d}\pm1.1$	$4.2^{c}\pm0.8$	$0.6^{a}\pm0.9$	$10.4^{d}\pm1.7$	$10.4^{d}\pm1.1$	$58.8^{D}\pm2.6$

Values with different superscript letters within columns represent significant statistical differences ($P < 0.05$)

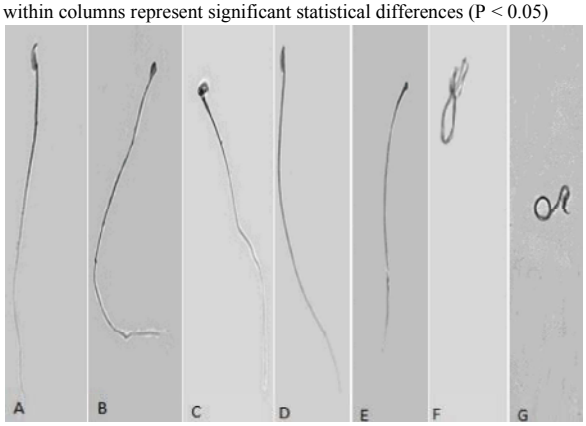

Fig. 4: Graph of sperm abnormalities of male mice treated with 3,4-DCA show A) normal sperm, B&C) amorphous head sperm, D) hock less head sperm, E) pin head sperm, F&G) coiled tail sperm

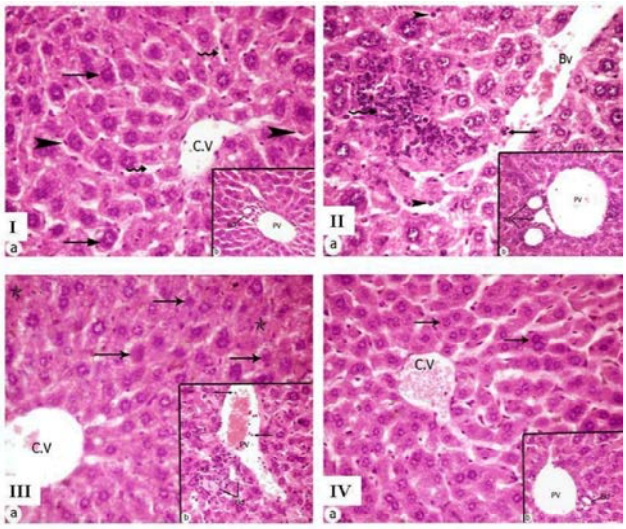

Fig. 5 I: Section in the liver of control mouse showing (a): normal architecture of hepatocyte radiating from central vein (C.V) with central vesicular nuclei (arrows). The hepatocytes are separated by narrow blood sinusoids (irregular arrows) and kupffer cells (head arrows). (b): Portal area between hepatic lobules with a branch of bile ductule (Bd) and branch of the portal vein (Pv). II) Section in the liver of mouse treated with 1/8 of 3,4-DCA LD_{50} value showing (a): aggregation of lymphocyte infiltration (irregular arrow), large size of kupffer cells (head arrows), some inflammatory cells (arrow) depressed in congested blood vessels (Bv). (b): dilatation of portal vein (Pv) and proliferation of bile ductules (Bd). III) Section in the liver of mouse treated with 1/16 of 3,4-DCA LD_{50} value showing (a): degeneration of hepatocyte (*), karyolysis of many nuclei (arrows). (b): proliferation of bile ductules (Bd), dilatation and congestion of portal vein (Pv) and depression of some inflammatory cells inside it (arrows). IV) Section in the liver of mouse treated with 1/32 of 3,4-DCA LD_{50} value showing (a): normal appearance of hepatocyte (arrows) and congested central vein (C.V). (b): normal portal area; bile ductule (Bd) and branch of the portal vein (PV). (H&E X 400)

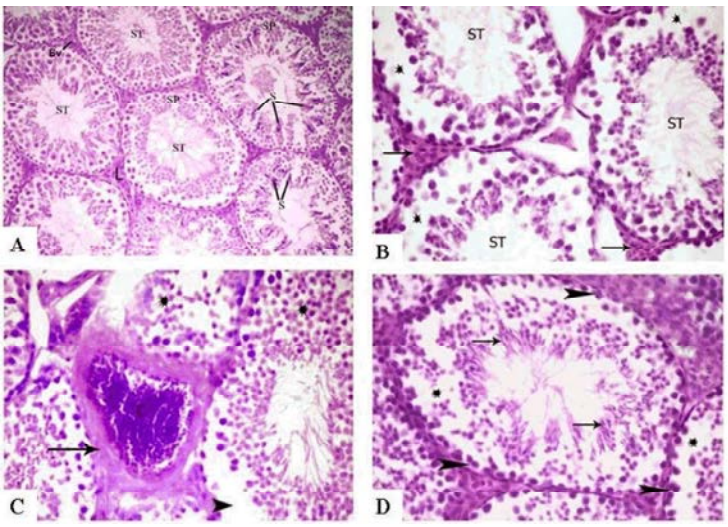

Fig. 6 A: Cross section in testis of a control mouse showing normal structure of the seminiferous tubules (st) with different types of spermatogenic cells (Sp) and spermatozoa (S). Also, interstitial tissue with Leydig cells (L) and blood vessels (Bv) are noticed. B) Cross section in testis of mouse treated with 1/8 of 3,4-DCA LD_{50} value showing irregular boundaries (short arrows) of seminiferous tubules (ST), degeneration of spermatogenic cells (*) accompanied with absence of spermatozoa and proliferation of Leydig cells (arrows). C) Cross section in testis of mouse treated with 1/16 of 3,4-DCA LD_{50} value showing disarrangement of spermatogenic cells (*) and reduction in some seminiferous tubules (head arrow). Thickening wall (arrow), dilatation and congestion of blood vessels (C) are also noticed. D) Cross section in testis of mouse treated with 1/32 of 3,4-DCA LD_{50} value showing normal appearance of primary spermatocytes (head arrows) and many of spermatozoa (arrows). Also, reduction of some spermatogenic cells (*) is noticed. (H&E, X 400)

Sperm head abnormalities may arise due to small deletions or point mutations, physiological, cytotoxic or genetic mechanisms [50], or alteration in testicular DNA which in turn disrupts the process of differentiation of spermatozoa [51].

Histopathological Examination: The evaluation of histopathological alterations in liver and testes of treated male mice basically confirmed the genetic results as well. The results of the histopathological examination revealed that control mice show normal architecture of the liver (Fig. 5 I). Liver of the mice administered with 1/8 of 3,4-DCA LD_{50} value exhibited aggregation of lymphocyte infiltration, large size of kupffer cells, some inflammatory cells, dilatation of portal vein and proliferation of bile ductules (Fig. 5 II). At medium dose treatment, the liver showed degenerated hepatocytes, karyolysis of many nuclei, proliferation of bile ductules, dilatation and congestion of portal vein (Fig. 5 III). At 3,4-DCA low dose, the liver revealed apparently normal portal tract and normal hepatocytes (Fig. 5 IV). The liver is an organ of detoxification which breakdown toxic substances and metabolites of the administered substances. This breakdown is carried out by the endoplasmic reticulum of the hepatocytes and as a result the hepatic cells get damaged severely [52]. 3, 4-DCA was found to induce free radical generation and antioxidant depletion and caused oxidative stress and lipid peroxidation in liver of crucian carp [1].

No histopathological changes were observed in the specimens collected from the control mice's testes which exhibited a normal testicular structure (Fig. 6 A). Testes of the high dose treatment exhibited degeneration of spermatogenic cells accompanied with absence of spermatozoa and proliferation of Leydig cells (Fig. 6 B). Similarly, testes of medium dose treatment showed disarrangement of spermatogenic cells and reduction in some seminiferous tubules, dilatation and congestion of blood vessels (Fig. 6 C). In contrast, the low dose treatment displayed normal appearance of primary spermatocytes and a reduction of some spermatogenic cells (Fig. 6 D). It is a well-known fact that the testosterone secreted by the Leydig cells is essential for the growth, division and differentiation of the germinal cells [52]. A testosterone deficit is expected to interfere with the completion of meiosis by a direct action on the

germ cells [53]. Thus, it seems possible that 3,4-DCA might have affected the Leydig cells which in turn reduced the production of the testosterone as a result the spermatogenesis gets inhibited. Also, toxicants have direct effect on sertoli cell function, which appears to be involved in the control of spermiation and when disturbed caused epithelial disorganization and subsequent tubular atrophy [54]. 3,4-DCA caused some histological damages in Esturine mysid *Mesopodopsis slabberi*, especially on eye, gonads and muscular tissue and the presence of 3,4-DCA accumulations, causing rather oval to spherical forms, which are visible in some essential structures. The destruction caused on the structure and organization of the muscular tissue and on gonads was also considerable [12].

CONCLUSION

Our data indicate that 3,4-dichloroaniline induces chromosome aberrations in both the bone marrow cells and spermatocytes of mice. The repression in mitotic index indicates the potential for 3, 4-dichloroaniline to induce growth arrest or to inhibit cell growth. These findings demonstrate that 3,4-dichloroaniline has a strong clastogenic/genotoxic potential. Also, our study indicated that 3, 4-dichloroaniline showed reproductive toxicity and histopathological alteration in male mice. Accordingly, strict limitations on the use of this compound must be put.

REFERENCES

1. Li, W., D. Yin, Y. Zhou, S. Hu and L. Wang, 2003. 3,4-Dichloroaniline-induced oxidative stress in liver of crucian carp (*Carassius auratus*). Ecotoxicology and Environmental Safety, 56: 251-255.
2. Kim, Y.M., K. Park, W.C. Kim, J.H. Shin, J.E. Kim, H.D. Park and I.K. Rhee, 2007. Cloning and characterization of a catechol-degrading gene cluster from 3,4-dichloroaniline degrading bacterium Pseudomonas sp. KB35B. J. Agric. Food Chem., 55: 4722-4727.
3. Wang, S., K. Poon and Z. Cai, 2012. Biodegradation and removal of 3,4-dichloroaniline by *Chlorella pyrenoidosa* based on liquid chromatography-electrospray ionization-mass spectrometry. Environmental Science and Pollution Research. Jun 6, DOI: 10.1007/s11356-012-0995-9.
4. Guilhermino, L., A.M.V.M. Soares, A.P. Carvalho and M. Celeste Lopes, 1998. Acute effects of 3,4-dichloroaniline to male wistar rats. Chemosphere, 37(4): 619-632.
5. Jurado-Sanchez, B., E. Ballesteros and M. Gallego, 2012. Occurrence of aromatic amines and N-nitrosamines in the different steps of a drinking water treatment plant. Water research, 46: 4543-4555.
6. Monteiro, M., C. Quintaneiro, M. Pastorinho, M.L. Pereira, F. Morgado, L. Guilhermino and A.M.V.M. Soares, 2006. Acute effects of 3,4-dichloroaniline on biomarkers and spleen histology of the common goby *Pomatoschistus microps*. Chemosphere, 62: 1333-1339.
7. Klütten, B., N. Kuntz and H. Ratte, 1996. Combined effects of 3,4-Dichlorianiline and food concentration on lifetable data of two related Cladocerans, *Daphnia magna* and *Ceriodaphnia quadrangular*. Chemosphere, 32: 2015-2028.
8. Bauchinger, M., U. Kulka and E. Schmid, 1989. Cytogenetic effects of 3,4-dichloroaniline in human lymphocytes and V79 Chinese hamster cells. Mutation Research Letters, 226(3): 197-202.
9. Giacomazzi, S. and N. Cochet, 2004. Environmental impact of diuron transformation: a review. Chemosphere, 56: 1021-1032.
10. Lao, S., 2003. Detoxification of 3,4-dichloroaniline in soybean by n-malonylation. Ph.D. thesis, University of Durham, UK.
11. Harvey, P.J., B.F. Campanella, P.M.L. Castro, H. Harms, E. Lichtfouse, A.R. Schaffner, S. Smrcek and D. Werck-Reichhart, 2002. Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. Environ. Sci. Pollut. Res., 9: 29-47.
12. Sardo, A.M., U.M. Azeiteiro, L. Pereira, F. Morgado and A.M.V.M. Soares, 2005. Histological evaluation of the exposure to 3,4-dichloroaniline in the estuarine mysid *Mesopodopsis slabberi*, under experimental conditions. Fresenius Environmental Bulletin, 14(7): 579-583.
13. Scheil, V., C. Kienle, R. Osterauer, A. Gerhardt and H.R. Köhler, 2009. Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology, 18(3): 355-363.
14. Hong, S.K., D.K. Anestis, J.G. Ball *et al*., 2002. *In vitro* nephrotoxicity induced by chloronitrobenzenes in renal cortical slices from Fischer 344 rats. Toxicology letters, 129(1-2): 133-141.
15. Zhang, B. and S. Lin, 2009. Effects of 3,4-Dichloroaniline on testicle enzymes as biological markers in rats. Biomedical and Environmental Sciences, 22: 40-43.

16. Commission of the European Communities, 1994. Commission Regulation (EC) No. 1179/94 of 25 May 1994 concerning the first list of priority substances as foreseen under Council Regulation (EEC) No. 793/93. Official J. Eur. Commun. 0003-0004.
17. Hagmar, L., A. Brogger, I.L. Hansteen, S. Heim, B. Hogstedt, L. Knudsen, B. Lambert, K. Linnainmaa, F. Mitelman, I.C. Reuterwall, C. Salomaa, S. Skerfving and M. Sorsa, 1994. Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res., 54: 2919-2922.
18. Bloom, J.C., 1993. Principles of hematotoxicology: Laboratory assessment and interpretation of data. Toxicol. Pathol., 21: 130-134.
19. Jorgensen, N., A.G. Andersen, F. Eustache, D.S. Irvine, J. Suominen, J.H. Petersen, A.N. Andersen, J. Auger, E.H. Cawood, A. Horte, T.K. Jensen, P. Jouannet, N. Keiding, M. Vierula, J. Toppari and N.E. Skakkebaek, 2001. Regional differences in semen quality in Europe. Hum. Reprod., 16: 1012-1019.
20. Jacobsen, R., E. Bostofte, G. Engholm, J. Hansen, J.H. Olsen, N.E. Skakkebaek and H. Moller, 2000. Risk of testicular cancer in men with abnormal semen characteristics: cohort study. BMJ, 321: 789-792.
21. Uguz, C., O. Varisli, C. Agca and Y. Agca, 2009. Effects of nonylphenol on motility and subcellular elements of epididymal rat sperm. Reprod. Toxicol., 28: 542-549.
22. Bauer, E.R., H.H. Meyer, P. Stahlschmidt-Allner and H. Sauerwein, 1998. Application of an androgen receptor assay for the characterization of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. Analyst, 123: 2485-2487.
23. Kojima, H., E. Katsura, S. Takeuchi, K. Niiyama and K. Kobayashi, 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by *in vitro* reporter gene assays using Chinese Hamster ovary cells. Environ. Health Perspect., 112: 524-531.
24. OECD, 2001. Guideline for Testing of Chemicals, No. 420, Acute Oral Toxicity-Fixed Dose Method. Organization for Economic Co-operation and Development. Paris, France.
25. Weill, C.S., 1952. Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. Biometrics, 8: 249-263.
26. Patlolla, P.B., K.A. Patlolla and P.B. Tchounwou, 2005. Cytogenetic effects of 1,1-Dichloroethane in mice bone marrow cells. Int. J. Environ. Res. Public Health, 2(1): 101-106.
27. Yosida, T.H. and K. Amano, 1965. Autosomal polymorphism in laboratory bred and wild Norway rats, *Rattus norvegicus*, found in Misima. Chromosoma, 16(6): 658-667.
28. Brewen, G.J. and V.R. Preston, 1987. Analysis of chromosome aberrations in mammalian cells. Chemical Mutagenicity, 5: 127-150.
29. Narayana, K., U.J.A. D'Souza and K.P.S. Rao, 2002. Ribavirin induced sperm shape abnormalities in Wistar rat. Mut. Res., 513: 193-196.
30. WHO, 1999. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th ed., Cambridge University Press, Cambridge, United Kingdom.
31. Kvist, U. and L. Bjorndahl, 2002. Manual on basic semen analysis, in: ESHRE Monographs 2, Oxford University Press, Oxford, UK.
32. Seed, J., R.E. Chapin, E.D. Clegg, L.A. Dostal, R.H. Foote, M.E. Hurtt, G.R. Klinefelter, S.L. Makris, S.D. Perreault, S. Schrader, D. Seyler, R. Sprando, K.A. Treinen, D.N. Veeramachaneni and L.D. Wise, 1996. Methods for assessing sperm motility, morphology and counts in the rat, rabbit and dog: a consensus report. ILSI Risk Science Institute Expert Working Group on Sperm Evaluation. Reprod Toxicol., 10(3): 237-44.
33. Filler, R., 1993. Methods for evaluation of rats epididymal sperm morphology, In: R.E. Chapin and J.H. Heindel (Eds.), Male Reproductive Toxicology, Academic Press Inc., San Diego, CA, pp: 334-343.
34. Delafield, F., 1984. Haematoxylin and eosin for general staining. Staining of the animal tissues practical and theoretical. London, Oxford University Press.
35. De-Bonis, S., D.A. Skoufias, L. Lobea, R. Lopez, G. Robin, R.L. Margolis, R.H. Wad and F. Kozielsk, 2004. *In vitro* screening for in heritors of the human mitotic kinetin Egg 5 with antimitotic and antitumor activities. Mol. Cancer, 3: 1079-1090.
36. Jha, A.M., M. Kumar and Abha, 2009. *In vivo* cytogenetic effects of 2-trans hexenal on somatic and germ cells of laboratory mice. Egyptian Journal of Biology, 11: 7-12.

37. Anderecsu, N., D. Stoicanscu, A. Belengen, S. Farcas, C. Popa, M. Stolan and V. Belengeanu, 2010. Unbalanced karyotype in human fetus due to a recurrent familial translocation. Muta. Res., 34: 233-237.
38. Federico, C., S. Motta, C. Palmieri, M. Pappalardo, Librando and S. Vand Saccone, 2011. Phenylurea herbicides induce cytogenetic effects in Chinese hamster cell lines. Mutation Research, Genetic Toxicology and Environmental Mutagenesis, 721(1): 89-94.
39. Cox, C., 2003. Diuron. Journal of Pesticide Reform, 23(1): 12-20.
40. Sanchez-Pena, L.C., B.E. Reyes, L. Lopez-Carrillo, R. Recio, J. Moran-Martinez, M.E. Cebrian and B. Quintanilla-Vega, 2004. Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol. Appl. Pharm., 196: 108-113.
41. Carlsen, E., A. Giwerchman, N. Keiding and N.E. Skakkebaek, 1995. Declining semen quality and increasing incidence of testicular cancer: is there a common cause? Environ. Health Perspect., 103(7): 137-139.
42. Al Hamdani, N.M.H. and H.N. Yajurvedi, 2010. Cypermethrin reversibly alters sperm count without altering fertility in mice. Ecotoxicology and Environmental Safety, 73: 1092-1097.
43. Joshi, S.C., R. Mathur and N. Gulati, 2007. Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat. Toxicol. Indust. Health, 23: 439-444.
44. Barratt, C.L., M.J. Tomlinson and I.D. Cooke, 1993. Prognostic significance of computerized motility analysis for *in vivo* fertility. Fertil Steril, 60(3): 520-525.
45. ElMazoudy, R.H., A.A. Attia and N.S. El-Shenawy, 2011. Protective role of propolis against reproductive toxicity of chlorpyrifos in male rats. Pesticide Biochemistry and Physiology, 101: 175-181.
46. Bai, J.P. and Y.L. Shi, 2002. Inhibition of $Ca^{(2+)}$ channels in mouse spermatogenic cells by male antifertility compounds from Tripterygium wilfordii Hook. f. Contraception, 65(6): 441-5.
47. Bett, R.A., D.A. Bradley, R.B. Christenses, C.A. Paulsen, W.J. Bremner and A.M. Matsumoto, 1996. Combined administration of Levonorgestrel, testosterone induces more rapid, effective suppression of spermatogenesis than testosterone alone A promising male contraceptive approach. J. Clin. Endocrinol. Metab., 81(2): 757-762.
48. Bo, Z., L. Ran and Z. Xin, 2009. Reproductive toxicity of 3,4-dichloroaniline on sperms of rats. Journal of Environment and Health, 26(2): 107-109.
49. Rai, S.P. and K.K. Vijayalaxmi, 2001. Tamoxifen citrate induced sperm shape abnormality in the *in vivo* mouse. Mutat. Res., 492: 1-6.
50. Odeigah, P.G.C., 1997. Sperm head abnormalities and dominant lethal effects of formaldehyde in albino rats. Mutation Research, 189: 141-148.
51. Bruce, W. and J. Heddle, 1979. The mutagenicity of 61 agents as determined by the micronucleus, *Salmonella* and sperm abnormality assays. Canadian Journal of Cytology and Genetics, 21: 319-334.
52. Sharma, S., R.P. Goyal, G. Chakravarty and A. Sharma, 2008. Toxicity of tomato red, a popular food dye blend on male albino mice. Experimental and Toxicologic Pathology, 60: 51-57.
53. Steinberger, E., 1971. Hormonal control of mammalian spermatogenesis. Physiol. Rev., 51: 1-22.
54. Bedwal, R.S., M.S. Edwards, M. Katoch, A. Bahuguna and R. Dewan, 1994. Histological and biochemical changes in testes of zinc deficient Bal B/C strain mice. Indian J. Experim. Biol., 32(4): 243-47.