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Genotoxic effect of the tricyclic antidepressant drug clomipramine hydrochloride in somatic and germ cells of male mice

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ABSTRACT

Objective: To assess the genotoxic potential of the antidepressant drug clomipramine hydrochloride (CH) through different mutagenic end points.
Methods: The study included chromosomal aberration analysis of bone marrow cells, primary spermatocytes, morphological sperm abnormalities and histopathological changes of liver cells using both light and electron microscopy. Three doses (0.195, 0.26 and 0.65 mg/20 g body weight) were tested. Each dose was given orally to mice for different periods of time (the doses are equivalent to the recommended daily intake doses in man).
Results: The tested doses of CH applied for 5 and 30 days increased the frequencies of chromosomal aberrations with dose and time dependant manner. The two high doses, 0.26 and 0.65 mg/20 g body weight revealed significant effect in comparison to the control. Dose -dependent increase in morphological sperm abnormalities and decrease in sperm count were recorded after CH treatment for 5 consecutive days. Pathological changes in liver tissue reached to sever damage were recorded after treatment with the medium and the high doses for 30 days. Ultrastructural examination showed that the low dose had little differences in liver histological architecture as compared to the control group, while prominent pathological changes in nuclei as well as dilated rough endoplasmic reticulum were observed in mice treated with the medium or the high dose of the drug.
Conclusions: It is concluded that CH has genotoxic effect in somatic and germ cells of mice as well as damaging effect on liver tissue after treatment with the medium and the high doses. However, the usage of low dose (especially for short time, 5 days) can be utilized as a safe therapeutic dose.

1. Introduction

Depression is one of the most common diseases around the world. It represents a significant burden for both individuals and the society. The prevalence rate of the disease in a population may reach 14%. Depression episode may occur at any age from childhood to elderly. At best to date, only 50% of the depressed patients undergo treatment and among them less than 50% fully recovered with the existing drugs[1,2].

Tricyclic antidepressants (TCAs) are some of the most commonly prescribed drugs worldwide[3]. Although, in some cases the efficacy of TCAs in the acute-phase of depression was lower than initially thought[4], the continuation of therapy in patients reduces the risk of relapse[5].

However, some research studies pointed on the side effects that accompanied the continued use of TCAs, *e.g.* cardiac diseases, heart failure[6] and the risk of type 2 diabetes[7]. Seizures and inhibition of monoamine oxidase (in brain) have been reported to occur in patients with TCA medications. Also, the drugs are known to produce a number of toxic effects on organs containing self renewing cell population such as bone marrow, skin and gastrointestinal tract[8].

Clomipramine hydrochloride (CH) is one of the most used TCA drugs. It contains two benzene rings in its chemical structure.

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Benzene is an important toxic material, as it is metabolized in the liver by cytochrome P450 2E1 to various phenolic metabolites which accumulate in the bone marrow[9]. These metabolites can produce reactive free radical species. Redox cycling of these free radicals produces active oxygen that may damage cellular DNA and cause DNA adducts. Consequently this can lead to inducing of chromosome aberrations[10]. Several studies have been demonstrated that benzene and its metabolites might significantly contribute in inducing chromosome aberrations in somatic and germ cells as well as sperm abnormalities[11,12]. Therefore, the present study was designed to evaluate the potential genotoxic effect of CH in male albino mice. This evaluation included cytogenetic assays, sperm parameters and histopathological examination of liver cells.

2. Materials and methods

2.1. Experimental animals

Male Swiss albino mice (*Mus musculus*) aging 3 months old and weighing about 20 g were obtained from Schistosome Biological Supply Centre in Theodor Bilharz Research Institute, Cairo, Egypt. The animals were housed in stainless steel wire mesh cages on a bedding of wood chips. They were kept in an ambient temperature of (25 ± 3) ℃ on a light/dark cycle of 12/12 h and supplied with food and water *ad libitum*.

2.2. Ethics

Handling of animals and the anesthetic procedures were performed according to the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt and being sure that animals did not suffer at any stage of the experiment.

2.3. Chemical drug

CH (Supranil) was a pure powder, purchased from Alpha Chem Advanced Pharmaceutical Industries S.A.E Company.

The drug was dissolved in sterile distilled water and orally administrated at three dose levels of 0.195, 0.26, and 0.65 mg/20 g body weight of mouse. These doses are equivalent to the doses of acceptable daily intake of CH for human (75 mg, 100 mg and 250 mg/70 kg body weight), after modification on the basis of relative surface area between species according to Paget and Barnes formula[13].

2.4. Experimental design

For chromosomal aberration analysis in somatic and germ cells and histopathological examination, mice were divided into six groups (5 mice/group). Three groups were orally administrated the tested doses daily and samples were taken after 5 days of treatment. The other three groups were treated for 30 days.

For sperm shape abnormalities, the method of Fahmy *et al.*[14] was followed and mice were orally administrated CH for 5 successive days. Samples were collected 35 days after the 1st dose of treatment.

In all experiments, a concurrent control group was taken for each treatment. Both treated and control animals were sacrificed by cervical dislocation.

2.5. Cytological preparation

2.5.1. Chromosome aberration analysis

Mice were *i.p.* injected with 0.5 mL of colchicine (0.05%)/kg body weight 2 h before sacrificed. Femurs were removed and the bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared using the method of Fahmy *et al.*[14]. Fifty metaphase spreads per animal were analyzed for scoring different types of chromosomal aberrations. Also, metaphase spreads from spermatocyte cells were prepared according to Al-Ashaal *et al.*[15] and Russo[16] for meiotic chromosomal analysis.

The mitotic and meiotic indices of bone marrow and spermatocyte cells respectively were investigated by recording the number of dividing cells/1 000 cells/ animal.

2.5.2. Sperm shape abnormalities

Sperm were prepared according to the recommended method of Fahmy *et al.*[14] and smears were stained with 1% Eosin Y. At least 600 sperm per animal were assessed for morphological abnormalities. The sperm abnormalities were evaluated according to the standard method of Fahmy *et al.*[14]. Epididymal sperm count was also determined by hemocytometer as described by Pant and Srivastava[17].

2.5.3. Histopathological examination

2.5.3.1. Light microscopy

Specimens of liver from all animals were dissected immediately after death and fixed in 10% neutral buffered formal saline for at least 72 h. Specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol (70%, 80%, 90%, 95% and finally absolute alcohol), cleared in xylene, impregnated in soft paraffin wax at 55 ℃ and embedded in hard paraffin. Serial sections of 6 μm thick were cut and stained with haematoxylin and eosin[18] for histopathological investigation. Images were captured and processed using Adobe Photoshop Version 8.

2.5.3.2. Electron microscopy

Specimens of liver were removed immediately after death and fixed by immersion in 4% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3 for 6 h. The ultrathin sections of 70–100 nm were prepared and examined under JEOL 100 S transmission electron microscope at 80 kV accelerating voltage according to reported methods of Peng *et al.*[19].

2.6. Statistical analysis

The obtained data were subjected to analysis of variance (Two-way ANOVA). Least significant differences (LSD) were used to compare between means at probability 0.05.

3. Results

The frequency of chromosomal aberrations induced in bone marrow cells after treatment with different doses of CH was summarized in Table 1. The results showed that the mean value of aberrations was not statistically significant at all doses treated for 5 days. In addition, dose-related relationship and significant chromosomal aberrations after repeated treatment for 30 days were recorded. The mean value of chromosomal aberrations reached 8.0 ± 0.6, 17.4 ± 0.8 and 22.0 ± 0.6 after 30 days of treatment with the low (L), medium (M) and the high (H) doses respectively compared with 1.8 ± 1.2 for the control group. Breaks/fragments, deletion and aneuploidy were the most prominent aberrations induced after treatment with CH. The results also revealed that the treatment with CH caused significant depression in the rate of cell division in all examined doses (Table 1). Mice treated with the high dose had the lowest rate of mitotic index in comparison with other doses and the control.

The results in Table 2 show that the treatment with low dose

Table 1

Percentage of metaphases with different types of chromosomal aberrations induced in mice bone marrow after CH treatment.

Treatment and doses	Structural aberrations (%)				Numerical aberrations (%)			Chromosome aberration	Mitotic index
	Gaps	Breaks and/or fragment	Deletion	Ring	Endomitosis	Aneuploidy	Polyploidy	(mean ± SE)	(mean ± SE)
Control (untreated)	0.4	0.8	0.4	-	0.8	0.4	0.8	1.8 ± 1.2^{a}	294.40 ± 1.83^{a}
CH (5 days)									
5 × L	0.4	0.8	0.8	-	0.8	0.4	0.4	1.8 ± 0.8^{a}	289.00 ± 2.39^{a}
5 × M	0.4	0.8	0.4	-	0.8	0.4	0.8	1.8 ± 0.9^{a}	276.00 ± 3.81^{b}
5 × H	0.8	0.8	1.2	-	0.8	0.8	0.4	2.4 ± 0.3^{a}	261.80 ± 1.23^{c}
CH (30 days)									
30 × L	0.4	8.8	0.4	-	2.4	3.6	0.4	8.0 ± 0.6^{c}	222.80 ± 2.50^{d}
30 × M	2.8	12.8	6.0	1.2	4.8	4.8	2.4	17.4 ± 0.8^{d}	131.80 ± 3.02^{e}
30 × H	2.0	13.2	11.2	2.0	6.8	6.4	2.4	22.0 ± 0.6^{d}	95.60 ± 1.64^{f}

The number of examined metaphases was 250 (50/mouse). L: Low dose; M: Medium dose; H: High dose. Values with different superscript letters within a column represent significant statistical differences ($P < 0.05$).

Table 2

Percentage of metaphases with different types of chromosomal aberrations induced in mouse spermatocytes after CH treatment.

Treatment and doses	Structural aberrations (%)			Numerical aberrations (%)		Chromosome aberration	Mitotic index
	X-Y univalent	Autosomal univalent	Chain	Polyploidy	Aneuploidy	(mean ± SE)	(mean ± SE)
Control (untreated)	2.8	0.4	0.8	1.2	1.2	3.20 ± 0.71^{a}	85.40 ± 0.92^{a}
CH (5 days)							
5 × L	2.8	1.2	0.8	2.0	3.2	5.00 ± 0.55^{a}	82.40 ± 1.51^{ab}
5 × M	4.0	2.4	2.0	2.4	4.4	7.60 ± 2.39^{ab}	79.60 ± 0.65^{ab}
5 × H	5.6	12.8	7.6	1.2	4.0	15.60 ± 1.18^{c}	76.80 ± 0.81^{b}
CH (30 days)							
30 × L	10.8	6.4	4.4	2.4	6.0	15.00 ± 1.56^{c}	66.40 ± 0.30^{c}
30 × M	12.0	9.2	5.6	4.0	7.2	19.00 ± 1.20^{c}	62.40 ± 0.55^{cd}
30 × H	14.8	14.0	8.0	7.2	9.2	26.60 ± 0.80^{d}	57.00 ± 0.70^{d}

The number of examined metaphases was 250 (50/mouse). L: Low dose; M: Medium dose; H: High dose. Values with different superscript letters within a column represent significant statistical differences ($P < 0.05$).

Table 3

Sperm abnormalities induced in male mice after oral treatment with CH.

Treatment and doses	Head abnormalities (%)				Tail abnormalities (%)		Sperm abnormalities	Sperm count (×10^{6})
	Amorphous	Without hook	Triangle	Banana	Forked	Coiled	(mean ± SE)	
Control (untreated)	1.40	2.37	0.67	0.40	0.30	0.50	34.0 ± 1.9^{a}	3 658.4 ± 21.0^{a}
CH doses								
L	2.40	2.70	1.00	1.20	1.07	1.30	58.6 ± 2.9^{b}	3 161.6 ± 19.0^{b}
M	4.27	3.90	18.30	1.77	2.10	3.10	101.8 ± 1.9^{c}	2 713.6 ± 13.3^{c}
H	7.77	4.97	3.50	3.00	3.50	6.07	172.6 ± 1.8^{d}	2 522.4 ± 40.8^{d}

The number of examined sperm was 3 000 (600/mouse). L: Low dose; M: Medium dose; H: High dose. Values with different superscript letters within a column represent significant statistical differences ($P < 0.05$).

of CH for 5 days led to insignificant increase in chromosomal aberrations in mouse spermatocytes compared to control. Treatments with higher doses (0.26 and 0.65 mg/20 g body weight) showed significant increase in chromosomal aberrations which was dose- and time-dependant. X-Y univalent was the most prominent type of aberrations. Its percentage reached 14.8% in mice treated with the high dose of CH compared with 2.8% for control. Autosomal univalent, chain, polyploidy and aneuploidy also showed significant elevation in dose- and time-dependant manner. Significant meiotic delay was observed at all doses as compared to control and the high dose of CH recorded the lowest rate of meiotic index.

Table 3 shows that the treatment with CH at different dose levels induced significant increase in sperm shape abnormalities. The mean value of abnormalities/mouse (600 sperm) reached 58.6 ± 2.9, 101.8 ± 1.9 and 172.6 ± 1.8 after treatment with the three tested doses respectively compared to 34.0 ± 1.9 for the control which indicated a dose-related relationship. Significant reduction in sperm count was recorded after treatment with different doses of CH compared to control with dose-related relationship.

For histopathological examination, the normal structure of liver tissue (control group) was identified in Figure 1. The low dose of CH caused the same effect after 5 and 30 days of treatment as light pathological changes in liver tissue compared to control. The effect of the medium and the high doses of the drug after 30 days of treatment were more pronounced as compared with their effect after 5 days. High dose treatment for 30 days gave the most dramatic effects among all groups, which represented as degeneration, vacuoles, dilatation of sinusoids, and there were also lymphocytic infiltration, distinguishing of kupffer cells and karyolysis of some parenchymal cells (Figures 2 and 3).

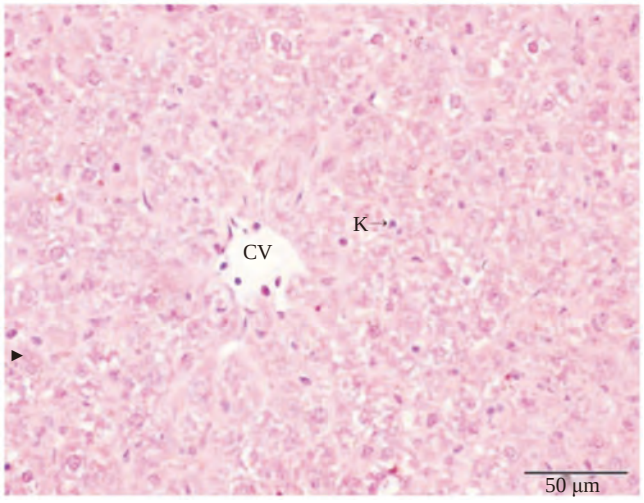

Figure 1. Section of the liver of the control mouse showing radially arranged hepatic cords around the central vein (CV).
Normal hepatocytes with centrally located nuclei, some cells are binucleate (►). Normal sinusoids with (Kupffer cells) (H & E).

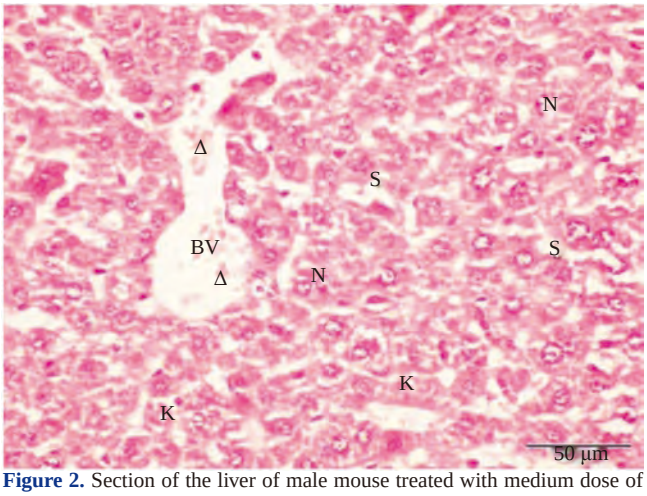

Figure 2. Section of the liver of male mouse treated with medium dose of clomipramine for 30 days.
N: Hepatocytes' nucleus appeared normally; S: The sinusoids between hepatocytes were dilated; BV: Blood vessel manifest dilatation with slight congestion (Δ); K: Kupffer cells (H & E).

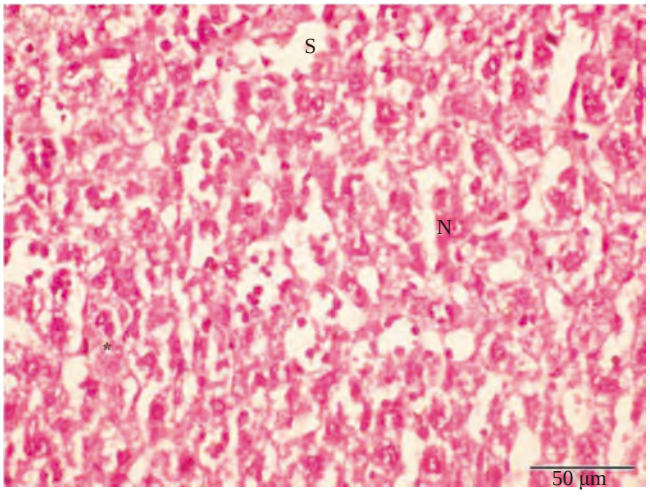

Figure 3. Section of the liver of male mouse treated with high dose of Clomipramine for 30 days.
N: Nucleus; S: The sinusoids between hepatocytes were dilated with increased number of kupffer cells. Inflammatory cells were visible everywhere. Early sign of karyolysis (*) appeared. (H & E).

Histopathological examination of liver cells by electron microscope is represented in Figures 4–7. The normal structure of liver tissue (control group) was identified in Figure 4. Similar to control, the treatment with the low dose for 30 days indicated normal nucleus and nucleoplasm. The cytoplasmic matrix showed rarefaction. Peroxisomes, lysosomes, and neutrophils were distinguished (Figure 5), whereas, the treatment with the medium dose showed dilatation of rough endoplasmic reticulum (rER) and appearance of collagen fibers. Blood vessels were highly congested with erythrocytes. The nuclear envelope showed invaginations in addition to chromatin migration and condensation (Figure 6). In high dose, irregular nuclear envelope, dilatation of rER and hepatocytes congestion with erythrocytes were recorded (Figure 7).

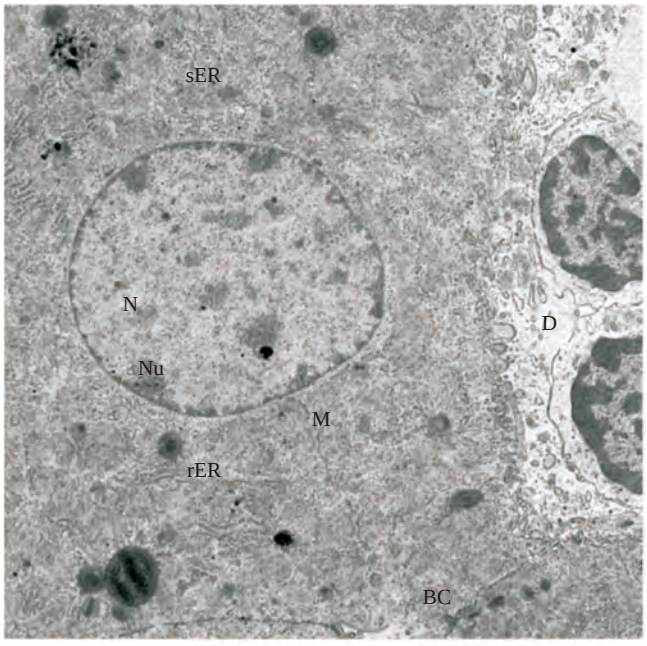

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Figure 4. An electron micrograph from a section in the liver of control group. Hepatocytic nucleus (N) having normal euchromatin and heterochromatin that is typically located close to regular shaped nuclear envelope. The cytoplasm contains parallel cisternae of rER, which studded with ribosomes. Smooth endoplasmic reticulum (sER) can be detected. Numerous rounded and oval mitochondria (M) were distributed throughout the cytoplasm. Two functional surfaces of the hepatocyte are appeared, facing bile canaliculus (BC) at the bottom of the figure and facing the perisinusoidal space of disse (D) and sinusoid (S) from the left. Nu: Nuclei.

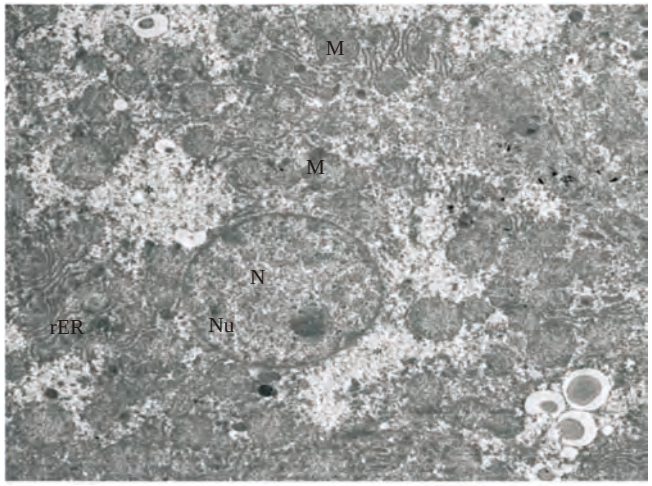

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Figure 5. An electron micrograph from a section in mice treated with low dose of CH for 30 days.
Hepatocyte nucleus (N) with normal chromatin and nuclear envelope. Normal mitochondria (M) appear in close association with the rER. Peroxysom appeared disscutered within the cytoplasm. Primary and secondary lysosomes are distinguished. Nu: Nuclei. Rarefaction (*) of cytoplasmic matrix was well distinguished.

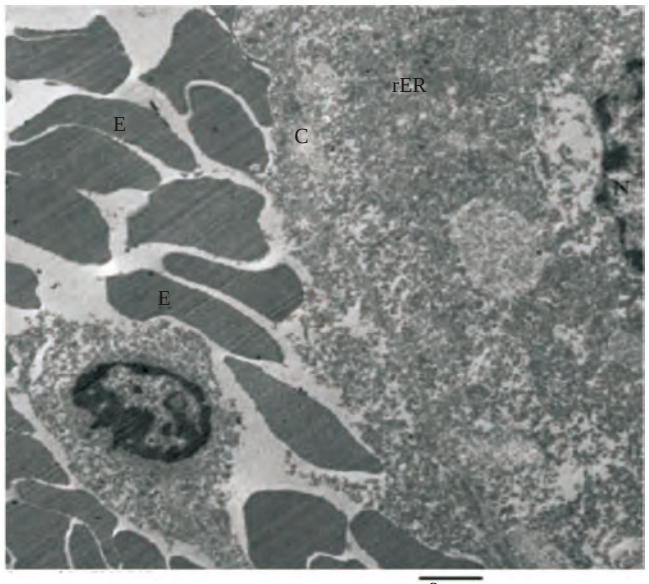

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Figure 6. An electron micrograph from a section in the liver of animals treated with medium dose of CH for 30 days. Showing highly congested blood vessel erythrocytes (E). Portions of collagen fibres (C) were also appeared.

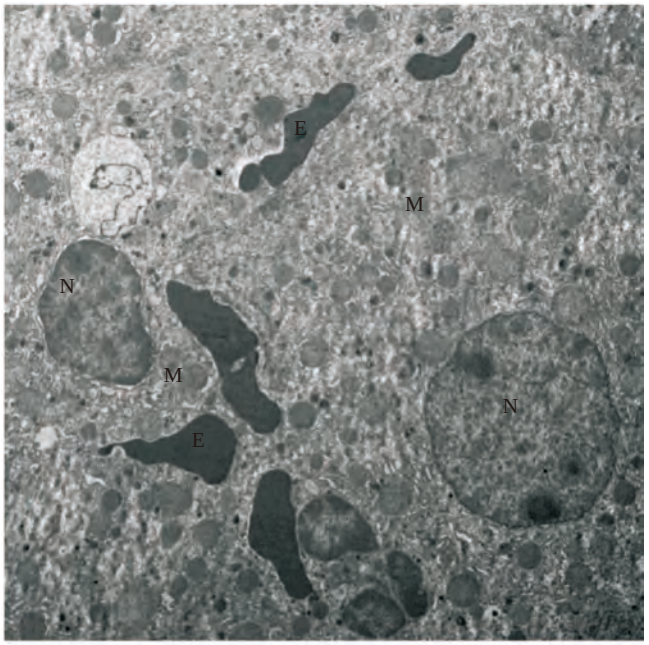

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Figure 7. An electron micrograph from a section in the liver of animals treated with the high dose of CH for 30 days.
Shrinkage of nucleus (N), binucleate hepatocyte bearing irregular nuclear envelopes. Erythrocytes (E) and peroxisomes are the distinguishable of this hepatocyte segment. Dispersed endoplasmic reticulum were recognized.

4. Discussion

An *in vivo* study in somatic and germ cells of mice was performed to evaluate the genotoxic side effects of the antidepressant drug CH. The results of the present work showed that the treatment with CH caused an increase in chromosomal aberrations in bone marrow cells and spermatocytes, and decreased in mitotic and meiotic activities represented by mitotic and meiotic indices with dose and time-dependant manner. To our knowledge, the genotoxic effect of CH in mammalian tissues has not been previously discussed. However, based on chemical structure of CH, the drug contains two benzene rings. Benzene is considered to be toxic, clastogenic and carcinogenic component[12,20]. This component is metabolized in liver by cytochrom P450 2E1 to various phenolic metabolites that can generate reactive oxygen species (ROS) which interact with cellular DNA causing DNA adducts that lead to chromosomal aberrations[10,21]. Moreover, phenols may undergo transformation process after penetration of the cell which can lead to increase of the toxicity of compounds by forming electrophilic metabolites and lipid peroxidation (LPO). This results in DNA damage causing the genotoxicity[21]. Therefore, the genetic deleterious effects of CH observed in the present study might be due to the effect of benzene and its metabolites which are the major constituents of the chemical structure of the CH drug.

Our findings were supported by the study performed by Ji *et al*.[11], who reported that occupational exposure to benzene has been associated with chromosomal aneuploidy in somatic and germ cells. Moreover significant increase in both structural and numerical aberrations was recorded in bone marrow cells of mice exposed to phenols[22]. In germ cells, the abnormal number of chromosomes (sperm aneuploidy) were estimated by two or three times in men exposed to benzene than those found in men who had not been exposed[23,24]. Higher frequencies of micronuclei were recorded in benzene metabolites-treated human lymphocytes[25] and in Chinese hamster V79-derived cells[26]. Micronucleus assay as chromosomal aberration is a cytogenetic form of measuring chromosomal damage[27]. Other TCAs *e.g.* imipramine and desipramine have genotoxic effect as evidenced by increase in sister chromatid exchanges in mouse bone marrow cells[28]. The drug amitriptyline induced chromosomal aberrations in a dose-dependent manner in bone marrow and mouse spermatocytes and a decrease in both mitotic and meiotic activity[29].

The effect on sperm morphology was significantly related to the ability of any drug or agent to induce genotoxic and mutagenic changes. In the present study, CH induced significant increase in the mean value of sperm abnormalities and decrease in sperm count. Such effect increased with increasing the dose. The same results were obtained in mice after treatment with the TCA drug amitriptyline[29]. In the present study, both head and tail abnormalities were recorded. The head-shaped abnormalities most probably reflect a change in DNA content[30]. Also Daris *et al*.[31] reported that head abnormalities especially amorphous heads are related to elevated degree of DNA fragmentation. The tail is responsible for sperm movement, so the distortion of tail or coiling may limit its movement and decrease fertility[15].

Sperm abnormalities detected in the present study could be due to oxidative stress pathway which generates ROS and LPO. The sperm have high contents of polyunsaturated fatty acids in plasma membrane and thus they are highly sensitive to oxidative stress. The production of ROS, LPO and altered membrane can affect sperm DNA leading to sperm abnormalities[32].

Histopathological examination of liver cells using both light and electron microscope revealed the same effect. The results showed that mice treated with clomipramine at low dose showed little differences as compared to control. These differences were confined to cytoplasmic inclusions. Whereas, prominent pathological changes in nuclei as well as dilated rER were observed in mice treated with the medium or the high dose of the drug. The treatment with high dose for 30 days gave the worst results of changes in hepatic cells. The generation of ROS and LPO play an important role in causing liver damage[33,34].

In general, the results of chromosome aberrations, sperm abnormalities and count as well as the deleterious effect on hepatic tissue showed that the effect of clomipramine was dose- and time-dependent. In conclusion, clomipramine is a genotoxic agent for both somatic and germ cells and should be taken under special precautions and medical supervision. Low dose and short period of treatment are preferred to avoid genotoxic side effects.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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