

Original article

# Carbon Tetrachloride-Induced Testicular Toxicity and Histopathological Alteration in Male Swiss Albino Mice

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## ABSTRACT

Recently there has been an increased association between toxic substances present in the environment and male infertility. Carbon tetrachloride (CCL<sub>4</sub>) is widely used as a chemical intermediate and as a feedstock in the production of chlorofluorocarbons. CCL<sub>4</sub> is highly toxic to the liver, kidney, testicle, brain and other tissues. Therefore, the present study was designed to identify its effect on the reproductive system in adult male mice. Thirty adult male albino mice were divided into three equal groups (n=10): the first group control, the second group received 0.1ml/100g body weight olive oil and the third group received 0.1ml/100g/body weight CCL<sub>4</sub> intraperitoneally every alternate day for three weeks. The results showed that CCL<sub>4</sub> causes a significant decrease in body weight, sperm motility, sperm count and testosterone level, while it leads to a significant increase in the number of abnormal sperm morphology. Additionally, CCL<sub>4</sub> caused apparent alterations in the histological structure of the testes. In conclusion, CCL<sub>4</sub>-induced reproductive toxicity in male mice.

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## INTRODUCTION

In recent years, there has been a decrease in human fertility due to toxic substances present in the environment and drugs. These toxicants, including environmental pollutants and clinically useful drugs, can cause severe cellular damage to different organs of our body by producing highly reactive substances such as free radicals [1]. CCl<sub>4</sub> is a colorless, volatile and very stable toxic organic compound that is rapidly absorbed by humans and animals after being released into the environment through toxic emission into air, water and soil. CCl<sub>4</sub> is particularly toxic to the kidney, testicle, brain, heart, lung, and other tissues, especially the liver. It has been demonstrated that treatment with CCl<sub>4</sub> can damage the structural and functional integrity of the male reproductive system in experimental animals due to oxidative toxicity [2]. CCl<sub>4</sub> has frequently been utilized as a model for studying reactive oxygen species (ROS) induced damage in several organs such as the lung, testes, and liver [3], kidney, and liver [4]. According to numerous findings, CCl<sub>4</sub> injection led to morphological and functional reproductive problems in male rodents due to oxidative toxicity [2]. The toxicity of CCl<sub>4</sub> is based on the biotransformation of the cytochrome P450 (CYP) system into the trichloromethyl radical (CCl<sub>3</sub>•), which is then further converted into the trichloromethyl peroxy radical (CCl<sub>3</sub>O<sub>2</sub>•). The strong toxicity of CCl<sub>4</sub> in liver tissues is explained by the high expression of CYP genes in hepatic tissues [5]. However, there is mounting evidence suggests that CYP expression, including both steroidogenic and non-steroidogenic CYP enzymes, is significant in reproductive organs [5]. Once CCl<sub>4</sub> is absorbed, it is widely distributed among tissues, especially those with high lipid content, reaching peak concentrations within <1–6 h, depending on the exposure concentration or dose [6]. High levels of polyunsaturated fatty acids are necessary for spermatozoa to

produce the fluid plasma membrane that is necessary for fertilization. However, this also makes them more vulnerable to destruction by ROS [7]. Furthermore, as CYP isozymes are found in the testis, prostate [8], epididymis [9] and germ cells [10], it is plausible that CCL<sub>4</sub> damages the lipids in these tissues and cells through oxidative stress [11]. The study aimed to determine the toxic effects of CCL<sub>4</sub> on the testis and sperm parameters of male mice.

## METHODS

### *Animals and diet*

Thirty Swiss albino male mice 8 to 10 weeks of age weighing 25-30 g were used for this study. These mice were inbred in the animal house of the Zoology Department/ Faculty of Science / University of Tripoli. The mice were housed in plastic cages containing wooden flakes and had free access to water and a standard diet (ad libitum).

### *Experiments and treatment*

The experiments were performed on a total of 30 adult Swiss albino mice. The animals were divided into three groups (ten mice/ group), the first group, which served as the control group did not receive any treatment. The second group was injected with the solvent (0.1ml olive oil/100gm/body weight). The third group, which served as the experimental group was injected intraperitoneally with CCL<sub>4</sub> at a dose of 0.1 ml/100 gm/body weight (10 ml of CCL<sub>4</sub> + 90 ml of olive oil) every alternate day for three weeks using the method of Alkreathy et al. [12] with some modifications. At the end of the treatment period, all mice were sacrificed by cervical dislocation and sperm analysis was evaluated.

### *Seminal Fluid collection*

Groups of treated and untreated mice were killed by cervical dislocation. The sperm of each mouse was obtained by squeezing the vasa deferentia gently into 1 ml of normal saline in a small dish. The specimen was mixed gently with a special dropper to distribute the seminal fluid. Sperm suspension was incubated for 15 minutes at 32 °C to allow sperm separation [13].

### *Assessment of sperm count*

Sperm count was made using the method by [14]. Sperm count in the vasa deferentia of control and treated mice was determined using an improved Neubauer hemocytometer. The number of spermatozoa in the squares was counted under the microscope (ZEISS) at 400X magnification. Sperm count was expressed as million per milliliters.

### *Examination of sperm morphology*

For the sperm morphology test, two smears were made from each mouse and allowed to dry in air. Smears were stained with 1% eosin Y in water for 10 minutes. From each mouse, 500 sperms were examined at 400 magnifications for morphological abnormalities. Results were expressed as a percentage of abnormal sperm [15]. Also mutation factor and mutation indices were calculated by the following equations [16]:

$$\text{Mutation factor (MT)} = \frac{\text{frequency of abnormal sperm heads(treated)}}{\text{frequency of abnormal sperm heads(control)}}$$

$$\text{Mutation Index (MI)} = \frac{\text{frequency of abnormal sperm heads(treated - control)}}{\text{frequency of abnormal sperm heads(control)}}$$

### *Determination of sperm motility*

Sperms from both treated and untreated mice were examined according to Ficsor and Ginsberg [17] by using the improved Neubauer hemocytometer (American Optical Co., Buffalo, N.Y). Numbers of motile and non-motile sperms of treated and untreated mice were counted under 400X magnification. The number of motile and non-motile spermatozoa was expressed as a percentage of the total number of counted spermatozoa.

### *Determination of serum testosterone levels*

Testosterone levels were determined by an enzyme-linked immunosorbent assay commercial kit, following the procedures outlined by the manufacturer (BioChek). Clotted blood samples were centrifuged for 15 minutes at 3,000 rpm to separate the serum and were stored at -20°C until measurement of testosterone hormone.

### Histological study

For histological examination, specimens from testes tissue were taken immediately after sacrificing mice and fixed in 10% formalin solution. The fixed specimens were then trimmed, washed, dehydrated, and embedded in paraffin. Sections of 7  $\mu\text{m}$  thickness were cut with a manual microtome, and stained with Hematoxylin and Eosin (H&E). The stained sections were examined under the microscope and the different cell types were carefully studied and photographed. Testis sections from each study group were evaluated for structural changes [18].

### Ethical approval

All experiments in this study complied with the bioethical research established by the Libyan National Committee for Biosafety and Bioethics and its methodology conforms to the published guide Principles of Laboratory Animal Care [19].

### Statistical analysis

The results were expressed as mean  $\pm$  standard error (SE). The data was analyzed statistically using one-way analysis of variance (ANOVA) to test for any differences between the mean values of all groups. ANOVA was followed by post hoc Turkey test used for multiple comparisons. A value of  $P \leq 0.05$  was considered statistically significant. These statistics were done using SPSS package version 20.

## RESULTS

### Body weights

The results of this study showed that administration of  $\text{CCl}_4$  for three weeks caused a slight change in the body weight of albino male mice when compared with the control group (Table 1). Body weights of the control group, olive oil group (solvent) and treated group were  $28.5 \pm 0.8\text{g}$ ,  $27.9 \pm 0.8\text{g}$  and  $25.5 \pm 1.5\text{g}$  before injection respectively, while at the end of the experiment, the body weights were  $29.5 \pm 1.1\text{g}$ ,  $26.3 \pm 0.9\text{g}$  and  $24.5 \pm 2.0\text{g}$  correspondingly. The decrease in body weight was observed in the solvent group and  $\text{CCl}_4$  group.

**Table 1. Effect of carbon tetrachloride treatment on mean value of mice body weights (g)**

Groups	Weight before treatment (g)	Weight after treatment (g)
Control	$28.5 \pm 0.8^a$	$29.5 \pm 1.1^a$
Olive Oil	$27.9 \pm 0.8^a$	$26.3 \pm 0.9^b$
$\text{CCl}_4$	$25.5 \pm 1.5^b$	$24.5 \pm 2.0^b$

### Sperm parameters

The effect of  $\text{CCl}_4$  on sperm motility, count and abnormal sperm shape is shown in (Table 2). The results indicated that the administration of  $\text{CCl}_4$  dose (0.1ml/100g/body weight) caused a significant ( $p \leq 0.05$ ) decrease in both sperm count and sperm motility with associated significant ( $p \leq 0.05$ ) increase in the percentage of abnormal sperms as compared with the control group (Table 2). The sperm morphology test of treated mice with  $\text{CCl}_4$  showed different sperm phenotype abnormalities such as ring tail, tight tail, bent mid piece, normal sperm, fused head, and amorphous head (Figure 1).

**Table 2. Effects of carbon tetrachloride on sperm parameter of male mice**

Groups	Count ( $10^6/\text{ml}$ )	Normal sperm shape	Motile (%)
Control	$25.8 \pm 0.9^a$	$432.6 \pm 11.1^a$	$80.8 \pm 0.7^a$
Olive Oil	$26.3 \pm 1.0^a$	$429.0 \pm 7.0^a$	$65.0 \pm 1.5^b$
$\text{Ccl}_4$	$21.5 \pm 1.3^b$	$377.9 \pm 4.2^b$	$36.9 \pm 1.6^c$

Values are presented as means  $\pm$ SE ( $n=10$ ). The mean difference is significant at the  $P \leq 0.05$  level. a, b and c show a significant difference at ( $P \leq 0.05$ ).

### Sperm morphology assay

In addition to sperm morphology test, a sperm morphology assay showed a significant increase in mutation factor and mutation index in the treated group of  $\text{CCl}_4$  (0.1ml/100g/b.w) when compared with the control group (Table 3).

**Table 3. Effect of carbon tetrachloride on the frequency, mutagenicity of abnormal sperm of male mice**

Groups	Frequency of abnormal sperm (%)	Mutation factor	Mutation index
Control	13.5±2.2 <sup>b</sup>	0.00±0.00 <sup>c</sup>	00.0±00 <sup>b</sup>
Olive Oil	14.2±1.4 <sup>b</sup>	1.08±0.20 <sup>b</sup>	0.09±0.19 <sup>b</sup>
CCl <sub>4</sub>	24.4±0.8 <sup>a</sup>	1.87±0.42 <sup>a</sup>	0.88±0.41 <sup>a</sup>

**Testosterone level in the blood**

The mean value of testosterone levels in the control, olive oil, and treated group were 2.76±0.21, 2.77±0.11 and 1.84±0.08 ng/ml, respectively (Table 4). Testosterone level was significantly reduced in CCl<sub>4</sub> (P≤0.05) in comparison with the control group.

**Table 4. Effect of carbon tetrachloride on the level of testosterone (ng/ml) of male mice.**

Groups	Control	Olive Oil	CCl <sub>4</sub>
Testosterone levels (ng/ml)	2.76±0.21 <sup>a</sup>	2.77±0.11 <sup>a</sup>	1.84±0.08 <sup>b</sup>

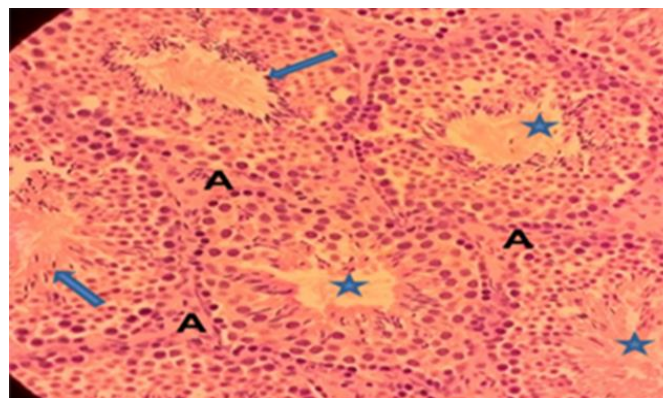
The mean difference is significant at the P ≤ 0.05 level. a and b Significant difference (P ≤ 0.05) as compared with control.



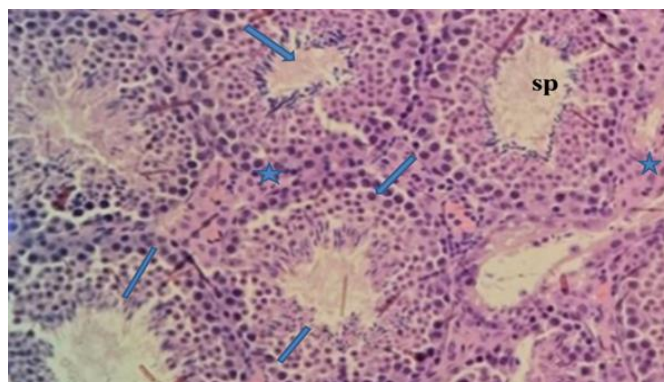
**Figure 1. Different abnormal sperm morphology of treated mice with CCl<sub>4</sub>. a- Ring tail, b-Tight tail c-Bent mid piece, d-Normal sperm, e- Point head, f-Amorphous head.**

**Histological observation**

Sections of tissue testes of the control (figure 2) and olive oil (figure 3) groups respectively showed the normal histological features of seminiferous tubules; closely packed seminiferous tubules, separated from each other by narrow interstitial spaces containing interstitial cells of Leydig. These seminiferous tubules contain spermatogenic cells and Sertoli cells. The spermatogenic cells were formed of spermatogonia, primary spermatocytes and spermatids.

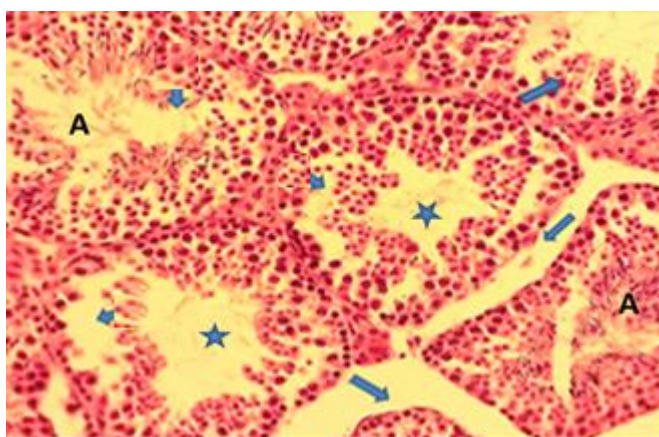


**Figure 2: Photomicrograph of the testicle section of a mouse from the control group showed Seminiferous tubules (Arrows) lined by normal spermatogenic cells, Interstitial Space (A) is clearly visualized and contains leydig cells, (H&E, 40x).**



**Figure 3.** Photomicrograph of the testicle of a mouse injected with olive oil shows the interstitial cells: ledig cells (asterisk), normal structure of the seminiferous tubules (blue line), normal sperms in the lumen(sp) (H&E, 40x).

The administration of CCl<sub>4</sub> for 4 weeks resulted in marked degeneration of Spermatogenic layers, mild disturbance in seminiferous tubules structure with occasional loss of germ cells and dilation of interspaces between seminiferous tubules when compared with the control, disappearance of Leydig cells (figure 4).



**Figure 4.** Photomicrograph of the testicle of a mouse from the treated group with carbon tetrachloride showed disappeared Interstitial cells (long arrows) and disturbance of seminiferous tubule structure (short arrows). Disturbance and Reduction in the number of spermatogonia, primary spermatocytes and Sertoli cells, few sperms (A) in the lumen of seminiferous tubules (H&E, 40x).

## DISCUSSION

Exposure to environmental chemicals can affect male fertility in several ways. A variety of hazardous chemical substances produced during industrial operations such as CCl<sub>4</sub> which accumulate inside the body can cause reproductive dysfunction [20]. Evaluation of sperm parameters is used as a marker for recognizing male reproductive toxicants. In the present study, reproductive and histopathological studies were performed on the testes of adult male mice after intraperitoneal administration of CCl<sub>4</sub>. The present study showed a significant decline in the body weight of the CCl<sub>4</sub>-treated group as compared to the control group. The significant reduction in body weight may be due to a decrease in food intake, which leads to a decrease in the body weight of the CCl<sub>4</sub>-treated group. The results of the current study were compatible with other studies [12, 21-23] which elucidated that CCl<sub>4</sub> compounds caused a decline in the body weight of treated animals compared to the control.

There is a direct association between sperm count, the extent of spermatogenesis, and fertility in animals [24,25]. Sperm parameters (sperm count, motility, and viability) are crucial for male fertility as they are key markers for spermatogenesis, epididymal maturation, and sperm fertilization capacity [26]. In the present study, the administration of CCl<sub>4</sub> caused a significant decrease in sperm count and percentage of motility compared with the control group. However, a significant increase in abnormal sperm morphology was noticed in the CCl<sub>4</sub>-treated group than in the control group. These results are consistent with previous studies [22,23,27] which found similar effects following CCl<sub>4</sub> administration with different routes

Sperm cells are vulnerable to damage caused by free radicals produced during the metabolism of CCl<sub>4</sub>. It is now widely accepted that the toxic effects of CCl<sub>4</sub> are a result of the overproduction of ROS. When Excessive ROS

production occurs, it can lead to the peroxidation of unsaturated fatty acids in the sperm membrane, causing a loss of motility [28], damage to the acrosomal membranes, and oxidation of DNA. These effects ultimately lead to a loss of fertilization capacity [29]. Since sperm cells are rich in polyunsaturated fatty acids, they are highly susceptible to oxidative stress [30]. While a small amount of ROS is necessary for sperm's physiological functions, such as capacitation, hyperactivation, and acrosomal reaction, high levels of ROS can lead to infertility. This is due to not only lipid peroxidation or DNA damage but also the inactivation of enzymes and oxidation of proteins in spermatozoa. Oxidative stress is primarily caused by lifestyle factors, but it can also be due to immature spermatozoa, inflammatory factors, genetic mutations, and altering levels of sex hormones. Since oxidative stress occurs due to the lack of antioxidants and has adverse effects on semen, lifestyle modification, and antioxidant regimens can be useful therapeutic approaches to overcome this problem [31].

In addition, CCl<sub>4</sub> injection showed an increase in mutational effects on sperm morphology, which was in agreement with a study by [16]. Additionally, the results of this study showed that there was a significant decrease in the serum level of testosterone in the CCl<sub>4</sub>-treated group compared to the control group indicating harmful changes in the Leydig interstitial cells of testes, which are responsible for testosterone biosynthesis and secretion. It has been reported that CCl<sub>4</sub> can affect Leydig cells directly or indirectly by altering the response of the gonads to FSH and LH [32]. Leydig cells produce testosterone in response to LH, which then binds to Sertoli cells to stimulate spermatogenesis through FSH [33]. In rats treated with CCl<sub>4</sub>, testicular dysfunction was observed due to the pituitary gland's failure to secrete FSH and LH [34]. Moreover, CCl<sub>4</sub> may exert its toxic effects via multiple pathways, such as oxidative stress, lipid peroxidation and necrosis of testicular cells [35].

In the control group Seminiferous tubules lined by normal spermatogenic cells, interstitial space is visualized and contains Leydig cells. However, in the group exposed to CCl<sub>4</sub>, the interstitial cells disappeared and there was a disturbance in the structure of seminiferous tubules. This led to a reduction in the number of spermatogonia, primary spermatocytes, and Sertoli cells, ultimately resulting in a decrease in the formation of sperms in the lumen of seminiferous tubules. The histological findings of this study are in line with several other studies that have previously reported similar histopathological changes in testes treated with CCl<sub>4</sub> [23,36]. Keshtmand et al. [22] and Rahmoni et al. [27] also obtained similar results, observing marked testicular damage in male rats treated with CCl<sub>4</sub>, in addition to decreased sperm motility, viability, and testosterone levels.

## CONCLUSION

Based on the analysis of sperm parameters and testicular tissue, these results indicate that exposure of male mice to CCl<sub>4</sub> resulted in severe testicular tissue and had a negative impact on sperm parameters within a short period of time. It is evident that CCl<sub>4</sub> exhibits considerable toxic effects on vital organs. Therefore, it is essential to develop preventive measures to counteract the toxicity of CCl<sub>4</sub>.

## Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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## سمية الخصية الناجمة عن رابع كلوريد الكربون والتغيرات النسيجية المرضية في ذكور الفئران البيضاء السويسرية

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قسم الأحياء النمائية، قسم علم الحيوان، كلية العلوم، جامعة طرابلس، طرابلس، ليبيا.

### المستخلص

في الأونة الأخيرة، كان هناك ارتباط متزايد بين المواد السامة الموجودة في البيئة والعقم عند الرجال. يستخدم رابع كلوريد الكربون (CCL4) على نطاق واسع كمادة كيميائية وسيطة ومادة وسيطة في إنتاج مركبات الكربون الكلورية فلورية. CCL4 شديد السمية للكبد والكلية والخصية والدماغ والأنسجة الأخرى. ولذلك صممت الدراسة الحالية للتعرف على تأثيره على الجهاز التناسلي لدى ذكور الفئران البالغة. تم تقسيم ثلاثين فأراً أبيضاً بالغاً من الذكور إلى ثلاث مجموعات متساوية (العدد = 10): المجموعة الأولى السيطرة، والمجموعة الثانية تلقت 0.1 م / 100 جم من وزن الجسم زيت الزيتون والمجموعة الثالثة تلقت 0.1 مل / 100 جم / وزن الجسم من CCL4 داخل الصفاق كل بديل. يوم لمدة ثلاثة أسابيع. أظهرت النتائج أن CCl4 يسبب انخفاضاً ملحوظاً في وزن الجسم وحركة الحيوانات المنوية وعدد الحيوانات المنوية ومستوى هرمون التستوستيرون، في حين يؤدي إلى زيادة كبيرة في عدد الأشكال غير الطبيعية للحيوانات المنوية. بالإضافة إلى ذلك، تسبب CCL4 في حدوث تغييرات واضحة في البنية النسيجية للخصيتين. في الختام، السمية الإنجابية الناجمة عن CCl4 في ذكور الفئران.

**الكلمات الدالة.** ذكور الفئران، رابع كلوريد الكربون، التستوستيرون، الخصية، معلمات الحيوانات المنوية.