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The Pathological Features of Cyclophosphamide Induced Multi-Organs Toxicity in Male Wister Rats

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ABSTRACT
Cyclophosphamide has the ability to induce multi-organ toxicity. The current study was conducted to investigate the toxicity effects of one dose and two doses of intraperitoneal administration of cyclophosphamide at dose of 50 mg/kg of body weight in male Wister rats. Fifteen male rats were assigned into three group five rats each group namely, control, 1-dose, and 2-doses. 1-dose group rats received one dose of cyclophosphamide at dose of 50 mg/kg of body weight, and 2-doses group rats received two doses of cyclophosphamide at dose of 50 mg/kg of body weight. Liver, lungs, kidneys, spleen and testes weight were recorded and collected for histopathological examination. The results showed a decrement in testes and spleen weight of 2-doses treated rats. The histopathology results showed pathological changes in all examined organs of cyclophosphamide treated rat. The fibrosis lesion was observed in 2-doses group's affected organs. Testicular injury in the 2-doses group involved 10% of the affected testis. In conclusion, cyclophosphamide treatment at dose of 50 mg/kg of body weight to male Wister rats induced multi-organs toxicity. The rats that received two doses of cyclophosphamide as dose of 50 mg/kg of body weight induced fibrosis in affected organs and elevated the testicular injury.

Keywords: Cyclophosphamide toxicity, liver toxicity, testes toxicity and kidneys toxicity

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INTRODUCTION

Chemotherapy has been used for cancer treatment for almost 70 years by targeting the proliferation potential and metastasising ability of tumour cells. Despite the progress made in the development of potent chemotherapy drugs, their toxicity to normal tissues and adverse side effects in multiple organ systems as well as drug resistance have remained the major obstacles for the successful clinical use (Sagar *et al*., 2007; Maor, Malnick, 2013).

Cyclophosphamide is an anti-cancer chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic as well as some autoimmune diseases. Cyclophosphamide is an alkylating anticancer agent adds an alkyl group to the guanine base of DNA, and this leads to the synthesis of aberrant couples of cytosine-thymine forcing the DNA reparation system of the cells to remove the modified guanine, triggering cell apoptosis (Matalon, Ornoy, Lishner, 2004; Patti, Lo Fermo, 2011; Altayli *et al*., 2012).

With increased success rate of cancer treatment, due in part to the aggressive use of high combination drug therapies, there has been growing concern about the long term side effects (carcinogenic) of these alkylating agents and other neoplastic drugs. There are several reports indicating the carcinogenic effects of cyclophosphamide in humans and animals.

Cyclophosphamide have the ability to induce liver, kidneys and lung injury in human and animal. (McDonald *et al*., 2003; Chen *et al*., 2011; Rehman *et al*., 2012; Ozkok *et al*., 2012; Shokrzadeh *et al*., 2015). Testicular toxicity and damage are significant toxic effects of cyclophosphamide in human and animals (Elangovan *et al*., 2006; Rezvanfar *et al*., 2008; Kim *et al*., 2013).

The current study was conducted to investigate the toxicity effects of one dose and two doses intraperitoneal administration of cyclophosphamide at dose of 50 mg/kg of body weight in male Wister rats

MATERIALS AND METHODS

Animal

Sixteen-week olds male Wister rats of body weight range between 240- 270 g were obtained from veterinary medicine faculty, university of Kufa. The rats were placed in polypropylene plastic cages (five rats per cage) with wood chips for bedding and housed in an animal room with controlled conditions in animal house at Veterinary Medicine faculty, University of Kufa.

The rats were provided with tap water and fed with commercial chow daily *ad libitum* and allowed to acclimatise for one week period.

Cyclophosphamide

Cyclophosphamide injection IP 1G (Endoxan-N 1G, Baxter) was obtained from local pharmacy and was used to induce toxicity.

Experimental design

Fifteen male Wister rats were assigned into three groups five rats each group, namely control, 1-dose group and 2-doses. Control group rats (control group) received two intraperitoneal injections of normal saline in day one day eight of experimental period. 1-dose group rats received a one intraperitoneal injection of cyclophosphamide at dose of 50 mg/kg of body weight in day one of experimental period. 2-doses group rats received two intraperitoneal injections of cyclophosphamide at dose of 50 mg/kg of body weight in day one and day eight of experimental period. The rats were euthanized three weeks post-intraperitoneal injections of cyclophosphamide or normal saline by inducing a respiratory failure using chloroform (TABLE I).

TABLE I: Experimental design

Group	Treatment	Rout	Time of dose
Control	Two doses of normal saline	I.P injection	Day one 1st dose and day eight 2nd dose
1-dose	One dose of cyclophosphamide at dose of 50 mg/kg	I.P injection	Day one
2-doses	Two doses of cyclophosphamide at dose of 50 mg/kg	I.P injection	Day one 1st dose and day eight 2nd dose

Gross pathology

Complete gross examination was conducted to detect any gross. The spleen, liver, lungs, kidneys, heart and testes were blotted dry and weighed immediately after necropsy.

Histopathology methods

Liver, lungs, kidneys and testes samples were collected and fixed in 10% formalin for 48 hours. After fixation, samples were sliced to 0.5 cm thick and placed in plastic cassettes for dehydration, before embedded in. The tissue samples were sectioned at 4 μm thickness and stained with Haematoxylin and eosin stain following the standard procedure. Tissue section were observed using a light microscope at 40x, 100x, 200x, 400x and 1000x magnifications.

RESULT

Gross pathology

The weights of heart, lungs, liver, testes, spleen and kidneys showed no increasing or decreasing in the organs weight of 1-dose group rats compared with control group rats. The heart, lungs, liver and kidneys weight of group 2-doses rats also did not and increasing or decreasing compared with control group rats. However, the testes and spleen weights of 2-doses group rats were decreased compared with control group rats (TABLE II). The statistical analysis was conducted using one way ANOVA test. The statistical analysis results showed no significant ($p>0.05$) differences in organs weight of 1-dose and 2-doses groups compared with control group. The heart, lungs, liver and kidneys weight of 2-doses group did not show a significant ($p>0.05$) differences compared with control group. Testes and spleen weight of 2-doses group showed a significant ($p<0.05$) decrement compared with control group (TABLE II).

TABLE II: Heart, lungs, liver, testes, spleen, and kidneys weights (unit: gram)

Groups	Heart		Lungs		Liver		Testes		Spleens		Kidneys	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0.94	±0.02	2.49	±0.05	12.68	±0.33	3.30	±0.10	1.22	±0.03	2.68	±0.05
1-dose	1.14	±0.10	2.66	±0.09	12.57	±0.45	2.74	±0.08	1.12	±0.06	2.53	±0.24
2-doses	1.03	±0.17	2.40	±0.44	9.97	±2.75	2.09[a]	±0.94	0.83[a]	±0.01	2.36	±0.57

* The superscript latter a indicate the significant ($p<0.05$) differences in 2-doses group compared with control group.

**n*= 5 rats

Histopathology results

The histopathological examination of current study showed a pathological changes with different degrees severity in lungs, liver, kidneys and testes of 1-dose and 2-doses groups.

Testes

The testes lesion was observed in both 1-dose and 2-doses groups. This lesion was characterized by spermatocytes and spermatogonia apoptosis or necrosis, where the seminiferous tubules appeared totally or partially empty. The involvement of this lesion was observed in about 10% of 2-doses group rat's testes and few numbers in 1-dose group rat's testes (FIGURE 1).

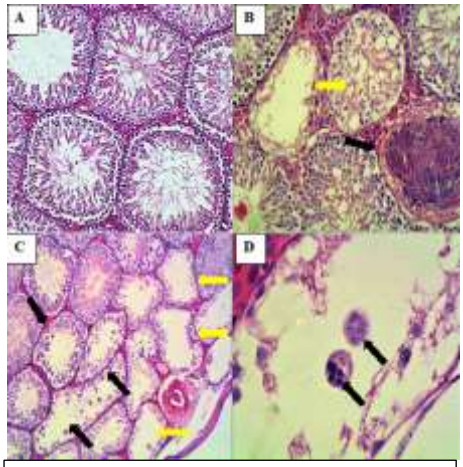

Figure 1: photomicrograph of testes of control and cyclophosphamide treated rats

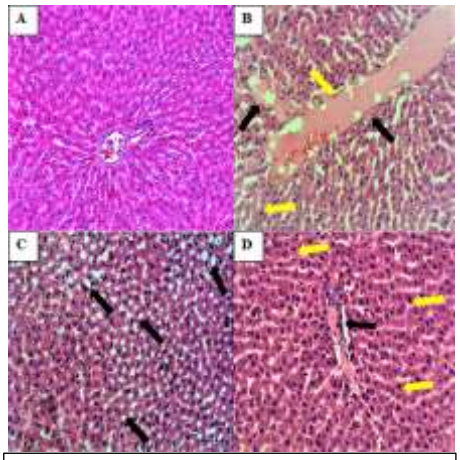

Figure 2: Photomicrograph of liver of control and cyclophosphamide treated rats.

Liver

The liver lesion was observed in 1-dose and 2-doses groups rats. The liver lesion was manifested by presence of fatty liver change in liver hepatocytes with presence of inflammatory cells in hepatic sinusoid. Also, mild liver amyloidosis was observed in both treated groups (FIGURE 2). However, this lesion involved in small focal of liver parenchyma of 1-dose group and was more extend in liver of 2-doses group. Hepatic blood vessels congested also observed. Early liver fibrosis was observed in liver of 2-doses group, where certain numbers of fibroblasts and collagen fibre was noted in liver parenchyma (FIGURE 2).

Lungs

The lungs lesion was observed in 1-dose and 2-doses groups rats. The lungs lesion was characterized by thickening of alveolar walls with presence of inflammatory cells in alveolar capillaries. Hyaline membrane features also observed in lung parenchyma. These lesion was observed in 1-dose and 2-doses groups rats (FIGURE 3).

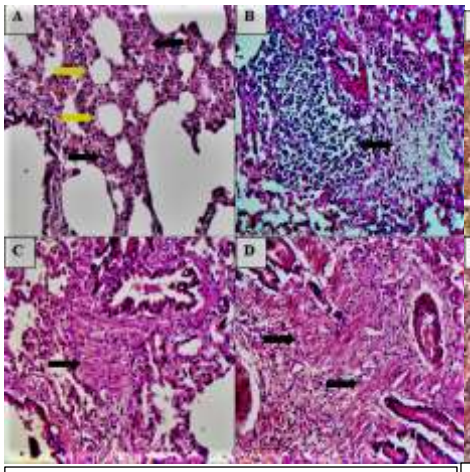

Figure 3: Photomicrograph of lung of cyclophosphamide treated rats

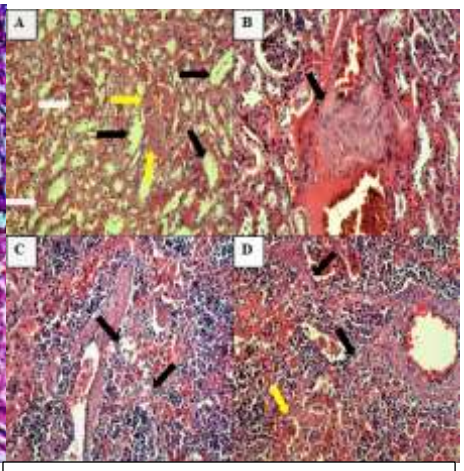

Figure 4 :- Photomicrograph of kidney and spleen of cyclophosphamide treated rats.

Enlargement of bronchus-associated lymphoid tissue (BALT) with presence of necrotic lymphocytes. Also, fibrosis of BALT areas was observed in 2-doses group rats, where fibroblast with presence of collagen fibre was observed within lymphoid tissue (FIGURE 3). Lung interstitial fibrosis was noted in 2-doses group rats and did not observed in 1-dose group rats, where the fibrous tissue was observed within acini structures in lung parenchyma (FIGURE 3).

Spleen

The spleen lesion was observed in 2-doses group rats. These lesion was characterized by presence of lymphocytes necrosis in lymphatic follicles and periarterial lymphoid sheaths (PALS) of white pulp areas. Spleen fibrosis was observed in red pulp areas represented by thickening of splenic cords with presence of fibrous tissue in spleen parenchyma (FIGURE 4).

Kidney

The kidney lesion was observed in both groups of 2-doses and 1-dose. The kidney lesion was characterized by tubular injury extended in cortex and medulla of kidney. Kidney fibrosis was observed in 2-dose group (FIGURE 4).

DISCUSSION

Cyclophosphamide is an anti-cancer chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic as well as some autoimmune diseases (Matalon, Ornoy, Lishner, 2004; Patti, Lo Fermo, 2011; Altayli *et al*., 2012). Several studies revealed that cyclophosphamide able to induce multi organs injuries including liver, lung, spleen, kidneys and testes (Chen *et al*., 2011; Rehman *et al*., 2012; Ozkok *et al*., 2012; Shokrzadeh *et al*., 2015). Where, the mechanism of cyclophosphamide to induce toxicity was due to an oxidative stress and the generation of toxic reactive oxygen species (ROS) (Manda, Bhatia, 2003; Motawi, Sadik, Refaat, 2010). It has been reported that oxidative DNA damage is caused by a hydro peroxide derivative of cyclophosphamide through generation of H_2O_2 (Murata *et al*., 2004).

The current study was conducted to reveal the effects of dose frequency on cyclophosphamide toxicity. Testes injury including spermatocytes necrosis or apoptosis induced by cyclophosphamide have been reported. Kothari *et al*. (2010) reported that ROS play a critical role in the pathogenesis of reproductive disorders, particularly in the pathological mechanism of male infertility. The present study showed that testes weight was decreased significantly ($p<0.05$) in 2 doses treated rats compared with 1 dose treated rats or control group. Also, the histopathological results showed that 2 doses of cyclophosphamide induced a moderate to severe testes lesion in 2 doses treated rats compared with 1 dose treated rats. Where, the total empty seminiferous tubules of testes was observed in more than 10% of total seminiferous tubules compared with 1 dose treated rats, which the empty seminiferous tubules was observed in few numbers indicating that more frequent of doses able to induce more toxicity in testes.

The histopathological results of liver showed that the liver injury was observed in both 1 dose and 2 doses treated rats. Where, several studies indicated that cyclophosphamide able to induce hepatocytes necrosis as well as liver fibrosis (Chen *et al*., 2011; Shokrzadeh *et al*., 2015). In current study, liver hepatocytes necrosis was observed in both groups that treated with cyclophosphamide. However, early liver fibrosis was observed in rats that treated with 2 doses of cyclophosphamide compared with rats that treated with 1 dose of cyclophosphamide revealing risk of multi-doses administration to this drug. Where, the liver fibrosis severity depended on the size and frequency of cyclophosphamide dose (Chen *et al*., 2011).

The cyclophosphamide treatment can induce a lung lesion including interstitial fibrosis was documented (Ozkok *et al*., 2012). The lung histopathology results of this study showed the ability of cyclophosphamide to induce lung lesion in both treated groups, where this lesion characterized by thickening of alveolar wall with infiltration of inflammatory cells. Lymphocytes necrosis of bronchus associated lymphoid tissue (BALT) with presence of fibrous tissue in BALT of 2 doses treated rats' lung. Also the interstitial fibrosis of lung was observed in 2 doses treated rats compared with one dose treated rats. The 2 doses treatment showed more efficacy to induce lung fibrosis compared with 1 dose treatment, which showed an alveolar lesion only.

The spleen as a secondary lymphoid tissue the white pulp pf spleen consist of mainly lymphocytes. The cyclophosphamide as anti-cancer and immune suppressor drug targeting the lymphocytes causing a necrosis of these cells (Araghi *et al*., 2018). In the current study the spleen weight of 2 doses treated rats was decreased significantly compared with other groups of this study. The histopathology results spleen of 2 doses treated rats showed lymphocytes necrosis of white pulp with presence of splenic fibrosis and white and red pulp. However, the significant spleen lesion was not observed in 1 dose treated rats. The kidney injury was observed in cyclophosphamide treated rats. However, the kidney fibrosis was also observed in 2 doses treated rats compared with 1 dose treated rats.

CONCLUSION

Cyclophosphamide treatment at dose of 50 mg/kg of body weight to male Wister rats induced multi-organs toxicity including testes, liver, lungs, spleen and kidneys compared with control group. However, the rats that received two doses of cyclophosphamide as dose of 50 mg/kg of body weight induced fibrosis in affected organs and elevated the testicular injury compared with one dose treated rats indicting the risk of multi-dose administration of cyclophosphamide to induce fibrosis in these organs.

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LIST OF FIGURES

1. FIGURE 1: photomicrograph of testes of control and cyclophosphamide treated rats.

A/ Testis of control rat. Normal testis histology. B/ Testis of 1-dose treated rat. Note the total empty seminiferous tubules (yellow arrow) due to necrosis of spermatocytes and spermatogonia. Also, seminiferous tubules lumen was filled with debris of necrotic cells and fibrotic tissue (black arrow) was observed. C/ Testis of 2-doses treated rat. Several total empty seminiferous tubules (yellow arrows) was observed with the presence of partial empty seminiferous tubules (black arrows), where this tubules under necrosis process. D/ Testis of 2-doses treated rat. Spermatocytes under apoptosis process, note the apoptotic bodies (black arrows) inside the cytoplasm of affected cells. H&E. A&B: x100, C: x40 and D: x400.

2. FIGURE 2: Photomicrograph of liver of control and cyclophosphamide treated rats.

A/ Liver of control rat. Normal liver histology. B/ Liver of 1-dose treated rat. Note the mild amyloidosis in liver parenchyma (black arrows), where the deposit amyloid protein replacing the necrotic hepatocytes. Signs of nuclear pyknosis (yellow arrows) were also observed. C/ Liver of 2 dose-treated rat. Note the fatty liver changes in liver parenchyma represented in several vacuoles (black arrows) inside cells cytoplasm of between the hepatocytes aggregation. D/ Liver of 2 dose-treated rats. Early liver fibrosis (black arrow) was observed, where the fibrous tissue noted in the liver parenchyma. Signs of nuclear pyknosis (yellow arrows) were also observed. H&E. A, B, C, and D: x100.

3. FIGURE 3: Photomicrograph of lung of cyclophosphamide treated rats.

A/ Lung of 1-dose treated rat. Thickening of the alveolar wall (black arrows) in the lung parenchyma, where the alveolar capillaries were congested with the presence of inflammatory cells within the alveoli leads to narrowing of air spaces of alveoli (yellow arrows) due to presence of inflammation. B/ Lung of 2-doses treated rat. Necrosis of lymphocytes of BALT forming a large space (black arrows) in inside BALT. C and D/ Lung of 2-doses treated rat. Interstitial lung fibrosis (black arrows) was observed in affect lungs, which observed within the alveolar structures. H&E. A: x100, B:x40 and C&D: x100.4.

4. FIGURE 4: Photomicrograph of kidney and spleen of cyclophosphamide treated rats.

A/ Kidney of 1-dose treated rat. Necrosis of epithelial cells of renal tubules forming spaces in renal parenchyma (black arrows). Deposition of amyloid protein (yellow arrows) in replacing the tubular structure of the kidney with the observation of haemorrhage (white arrows) within the renal tubules. B/ Kidney of 2-doses treated rat. Kidney fibrosis (black arrow) was observed in the cortex area of the kidney, which manifested by the presence of fibrous tissue replacing the proximal and distal convoluted tubules. Also, haemorrhage was observed close to affected areas. C/ Spleen of 1-dose treated rat. Lymphocyte necrosis was observed in the white pulp area forming small spaces (black arrows), which led to loss of the normal architecture of white pulp. D/ Spleen of 2-doses treated rat. Spleen fibrosis was observed in white pulp (black arrow) and red pulp (yellow arrows) of the spleen. H&E. A, B, C and D: x100.