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(RESEARCH ARTICLE)

A histopathological study on the effects of cyclophosphamide on the hepatic tissue of female golden hamsters

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Abstract

The aim of this study was to study the acute toxic effect of cyclophosphamide on the histological structure of the liver tissue in Syrian golden hamsters. Twenty-seven adult female hamsters were injected intraperitoneally with 0, 100 and 200 mg/kg body weight cyclophosphamide. The dosing was administrated on alternating days for one week as an acute dosing schedule and humane sacrifice was done on day 7. Hepatic tissue was collected and processed with paraffin technique for histological examination then stained with hematoxylin and eosin stains (H&E) and Periodic acid–Schiff stain (PAS). The control tissues showed normal histological structure of the liver tissue. With H&E histological changes appeared in the tissue from the hamsters dosed with 100 mg/kg body weight in the form of; slight fatty changes with vacuolated cytoplasm in some areas. The higher dose showed diffuse fatty infiltration, central vein congestion and loss of hepatocyte architecture in addition to sinusoidal congestion. With PAS stain for both low and high doses showed a decrease in glycogen amount due to presence of degenerative hepatocytes and necrosis. We recommend for the patients who undergo long term therapy with cyclophosphamide to perform clinical and biochemical tests at regular intervals to indicate early on any hepatic dysfunction.

Keywords: Cyclophosphamide; Liver; Hepatotoxicity; Acute Toxicity; Hamsters; Histopathology

1. Introduction

Cyclophosphamide, also known as Cytoxan, is an alkylating anti-neoplastic drug and is an affiliate of the oxazophorine group [1]. Its importance is in its antitumor activities against many types of cancers as; adeno-carcinoma, retino-blastoma, malignant lymphomas, myeloma, breast-carcinoma, neuro-blastoma, and leukemia [2,3,4]. Cyclophosphamide also has significant immunosuppressive action and is used clinically to treat autoimmune diseases. However, despite its wide clinical use, cyclophosphamide also retains many adverse effects, including hepatotoxicity in humans and experimental animals [1]. Metabolic activation of cyclophosphamide is a cytotoxic alkylating agent which produces highly reactive carbonium ions that react with the electron-rich areas of susceptible molecules, such as nucleic acids and proteins and it is converted to its active form in the liver [1]. The first step in this activation involves the ring hydroxylation of cyclophosphamide to yield 4-OH-CPA, an unstable metabolite which readily equilibrates with its ring-opened tautomer, aldophosphamide [5]. These equilibrium products are then either detoxified to form 4-keto-CPA and carboxyphosphamide or spontaneously converted to phosphoramide mustard, a bifunctional alkylating metabolite, and acrolein. Cyclophosphamide is inactivated by N-dechloroethylation, resulting in N-de-chloro-ethylated metabolites and the byproduct chloroacetaldehyde [1]. Metabolic conversion of cyclophosphamide leads to the formation of two cytotoxic metabolites, phosphoramide mustard and acrolein. Phosphoramide mustard is believed to have antineoplastic activity. Nevertheless, acrolein, is a highly reactive metabolite with a short biological half-life, which means it may be responsible for cyclophosphamide -induced liver damage [1]. The resultant cytotoxic metabolites and toxic byproducts are detoxified by various aldehyde dehydrogenases and by conjugation with glutathione (GSH) via GSH S-transferases

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[6]. Nevertheless, cyclophosphamide requires metabolic activation by the hepatic microsomal cytochrome P450 oxidase system for both its therapeutic and toxic action [1]. In experimental studies, cyclophosphamide produced chromosome damage and micronuclei in Chinese hamsters, rats, and mice, in addition to gene mutations in the mouse spot test and in the transgenic lacZ construct of Muta Mouse. Displaying it acts as both a mutagen and a carcinogen [7,8,9]. The primary target of cyclophosphamide in its mutagenic, teratogenic, and anti-neoplastic effects is DNA. Cytotoxicity of cyclophosphamide is applied via the cross-linking of cellular DNA, and there is occurrence of inter strand and DNA-protein cross- links following drug exposure, but no single strand breaks [10]. The cytotoxic action of cyclophosphamide results mainly due to phosphoramide mustard-induced DNA cross-linking [11], which that binds the N-7 position of guanine to the phosphate backbone of DNA [12, 13]. The effectiveness and toxicity of cyclophosphamide on humans differs greatly from one patient to another and is mainly, related to pharmacokinetic and pharmacogenetic mechanisms [14,15,16,17]. A relationship has been found between high doses of cyclophosphamide and its toxic metabolites (phosphoramide mustard and acrolein) and hepatotoxicity [15, 18]. Moreover, this hepatotoxicity has been linked to high levels of other metabolites such as O-carboxyethyl-phosphoramide mustard and 4- hydroxycyclophosphamide [15, 16]. Furthermore, it has been shown that cyclophosphamide -induced adverse reactions could be a result of cholinesterase inhibition [18]. Hepatotoxicity induced by cyclophosphamide is characterized by cytolysis and cholestasis. It occurs with oral or intravenous administration and it has dose dependent changes. Mainly, there are three histological patterns that have been demonstrated: massive hepatic necrosis, necrosis of perivenous hepatocytes and diffuse hepatocellular damage with mild steatosis [13]. Therefore, a growing interest is directed towards the present study to emphasis the cyclophosphamide impact on the liver of female golden hamsters (*Mesocricetus auratus*).

Aim

To evaluate the acute toxic effects of cyclophosphamide metabolites on the histological structure of the liver of female Golden Hamsters (*Mesocricetus auratus*).

2. Material and methods

Twenty-seven adult female Hamsters were obtained from a farm in El-Bayda city. The weight range when dosed was from 150 - 250 grams. The animals were bred at the animal house of the Histology department, Faculty of Medicine, University of Benghazi- Libya. The animal ethical committee of the University of Benghazi approval had been taken and all humane procedures had been taken. They were injected intra-peritoneally with cyclophosphamide purchased as a powder from a local pharmacy, and diluted with normal saline. The dry substance of cyclophosphamide was dissolved in 50ml of physiological normal saline and vigorously shaken after the addition of the solvent. The chemical was administrated to the animals as an acute dosing schedule, ensuring that the least suffering and stress will be applied to the animals at all times. Two doses were given, 100 mg/kg body weight and 200 mg/kg body weight based on the literature of similar compounds [19], alongside the control 0mg/kg body weight. The dosing was administrated on alternating days for one week. Meaning they were dosed on day 1, day 3 and day 5. Hepatic tissue, from the left lobe were collected from all the sacrificed animals and sliced transversely and put in 10% neutral buffered formalin and used for histological examination. Tissue processing was done by using paraffin technique [20], the slides were stained with (H&E and PAS) then examined under a light microscope.

3. Results

Microscopically, the liver sections of the control group stained with H&E, showed a normal lobular architecture. The hepatocytes radiated from the central vein forming anastomosing plates of liver cells, separated from each other by vascular spaces, hepatic sinusoids. The hepatocytes appeared polyhedral with acidophilic cytoplasm, round central nucleus as shown in Figure 1.A. Portal tracts or triad contained normal structures including branches of the portal vein, branches of the hepatic artery, and branches of the bile duct (Figure 1.B). In the hamsters treated with the low dose 100mg/kg body weight (therapeutic dose of cyclophosphamide) histological changes in the slides stained with the hematoxylin and Eosin shown an area of mild degenerated hepatocytes, beginning of a dilated congested central vein, mild lymphocytic infiltration and loss of hepatic architecture Figure 2.A. The bile duct had a normal histological structure as clearly demonstrated in Figure 2.B. In Figure 2.C. shown fat cells infiltration between the plates of hepatocytes, with vacuolated appearance of cytoplasm in the hepatocytes, and also clear normal hepatic nucleus structure showing normal distribution of euochromatin and heterochromatin and dark nucleolus. In the hamsters treated with the therapeutic dose of cyclophosphamide the histological changes in the PAS stained slides were established showing, a formation of a dilated vein with a mild collection of blood cells, also illuminated is an area of minor degenerated hepatocytes, and mild lymphocytic infiltration. The glycogen granules in the hepatocytes are also presented (Figure 3.A). In a section of the liver triad it presented very thin compressed portal tract with a normal portal duct and collapsed artery, furthermore, it displayed mild inflammatory infiltrate and hepatocytes with normal

structural cords and pink glycogen granules in the cytoplasm (Figure 3.B). Fat cells infiltration, with vacuolated appearance of cytoplasm in hepatocytes, and normal hepatic nucleus with glycogen is shown in Figure 3.C. The toxicity (at high dose cyclophosphamide) is associated with acrolein which induce; cell death, apoptosis, and necrosis. Histopathological examination of liver tissue of this group (treated with 200mg/kg body weight) was characterized by the alteration of liver architecture and hepatocyte necrosis and degeneration around the central veins of the liver with pyknotic nucleus and eosinophilic cytoplasm with marked inflammatory cell infiltration (Figure 4). Figure 5.A, displays, an area of congested hepatic cords, a large dilated central vein very congested filled with red blood cells (RBCs) called red venous congestion, and Hemosiderin-laden macrophages which are associated with bleeding and the breakdown of red blood cells. However, in Figure 5.B presents thickening wall of a portal vein with inflammatory cells and surrounded by fibrotic area. Normal hepatic cords are furthermore presented. In Figure 5.C shows a liver section from the toxic group, stained with PAS, displaying a dilated congested vein filled with red blood cells and inflammatory cells, and a thickening wall of portal vein surrounded by a fibrotic area. Also, shown are pyknotic nuclei in some hepatic cells, degeneration and disorganization of hepatocytes.

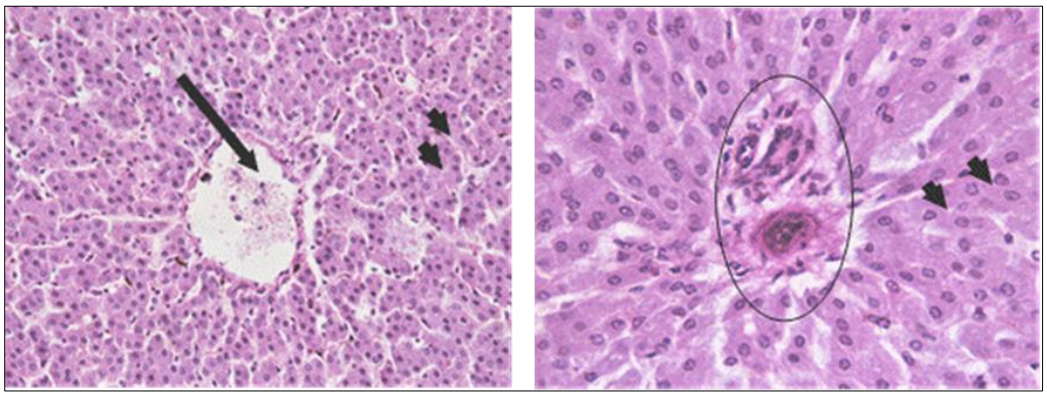

Figure 1 Photomicrograph of the liver section of control hamster showing, lobular architecture: A. normal central vein (long arrow), and hepatocytes with normal structures (arrow heads). H and E stain, (X200). B. normal portal triad (circle) and hepatocytes with normal structures (arrow heads). H and E stain, (X400)

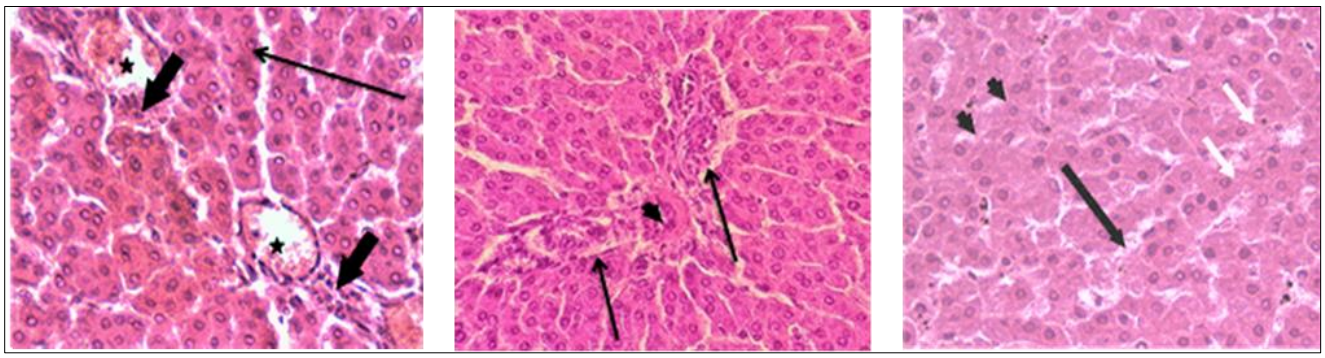

Figure 2 Photomicrograph of the liver section of hamster treated with (therapeutic dose) of cyclophosphamide showing, A. an area of mild degenerated hepatocyte (long arrow), beginning of a dilated congested central vein (star), and mild lymphocytic infiltration (thick arrows). B. mild lymphocytic infiltration at the portal tract, (long arrows) and a normal structure of the bile duct is clear in the section (arrow head). C. showing fat cells infiltration (long arrow), with vacuolated appearance of cytoplasm in hepatocytes (arrow heads), and normal hepatic nucleus structure showing normal distribution of euochromatin and heterochromatin and dark nucleolus (white arrows). H and E stain, (X400)

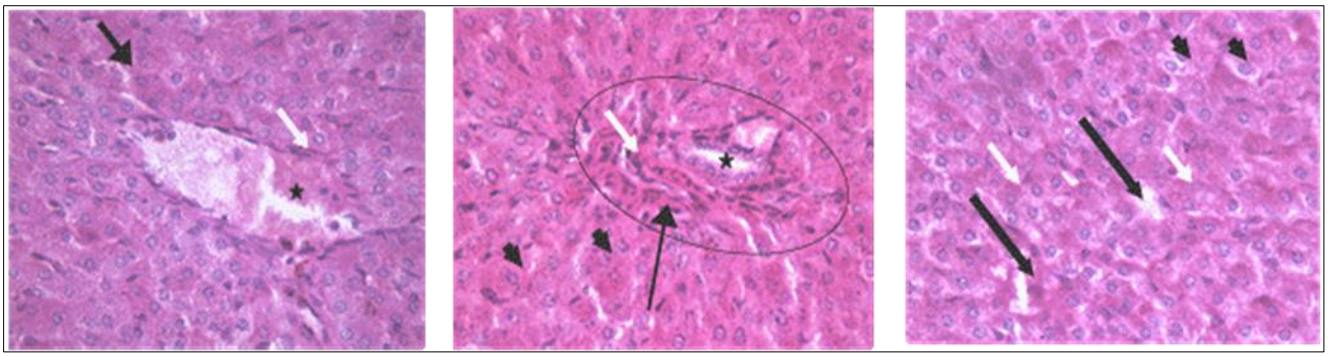

Figure 3 Photomicrograph of the liver section of hamster treated with (therapeutic dose) of cyclophosphamide showing, A. creation of a dilated vein with a mild collection of blood cells (black star), an area of minor degenerated hepatocytes (short arrow), and mild lymphocytic infiltration (white arrow). B. very thin compressed portal tract (circle) with a portal duct (star) and collapsed artery (white arrow) and mild inflammatory infiltrate (long arrow). Hepatocytes with normal structural cords and pink glycogen granules in the cytoplasm (arrow heads). C. fat cells infiltration (long arrow), with vacuolated appearance of cytoplasm in hepatocytes (arrow heads), and normal hepatic nucleus with glycogen (white arrows). PAS stain, (X400)

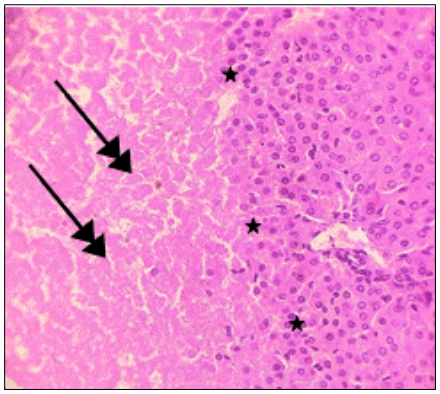

Figure 4 Photomicrograph of the liver section of hamster treated with a (toxic dose) of cyclophosphamide showing, necrosis areas (double arrows), inflammatory cell infiltration (black stars), surrounded by an area of degenerated hepatocytes with double nucleus (thick arrow) and beginning of a dilated congested central vein (white star). PAS stain, (X200)

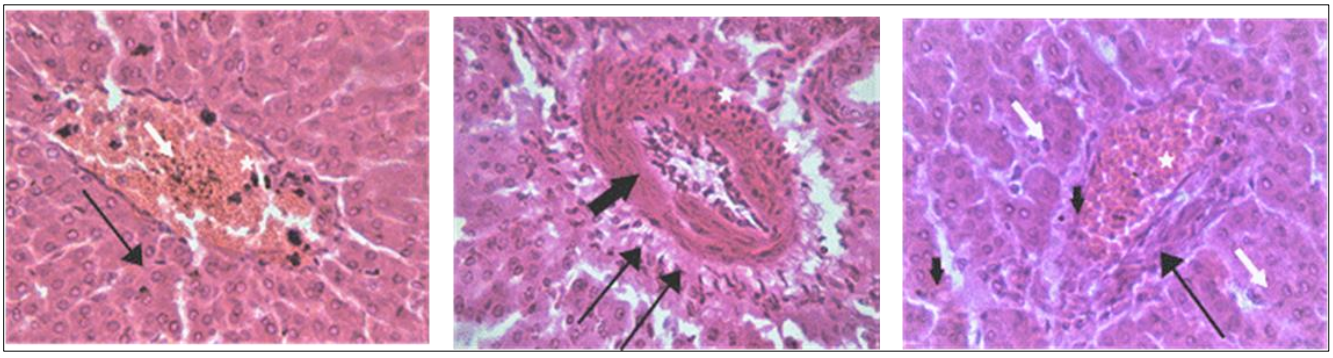

Figure 5 Photomicrograph of the liver section of hamster treated with a (toxic dose) of cyclophosphamide showing, A. an area of congested hepatic cords (long arrow), a large dilated central vein very congested filled with RBCs called red venous congestion (white star), and Hemosiderin-laden macrophages which are associated with bleeding and the breakdown of red blood cells. (White arrows). H and E stain, (X400). B. thickening wall of portal vein (thick arrow) with inflammatory cells (white stars), surrounded by fibrotic area (long arrows), normal hepatic cords also shown. (H & E stain). C. dilated congested vein with red blood cells and inflammatory cells (star), and thickening wall of portal vein

surrounded by fibrotic area (long arrows), pyknotic nuclei in some hepatic cells (head arrows), degeneration and disorganization of hepatocytes (white arrows) (PAS stain, X400)

4. Discussion

Our results showed the acute toxic effect of CP was clear in the liver tissue in both the therapeutic and toxic doses, the effects were evident on the liver tissue, even if the time of dosing was just one week. The hepatotoxicity of cyclophosphamide that was found in this study, is related to the production of 4-hydroxycyclophsphamide and acrolein [21, 22]. Likewise, researchers observed that exposition to toxic metabolites of CP leads to escalation liver toxicity and mortality [21]. This would affect the weight of the animals if treated for longer as it indicates toxicity, and was also found by other studies [21, 23]. Fatty changes of liver tissue with low dose cyclophosphamide (100mg/kg body weight) found in this study and furthermore induced liver necrosis with the high dose of cyclophosphamide is associated with the cyclophosphamide metabolites specifically acrolein [22] which induces; cell death [24], apoptosis [25], and necrosis [26], was correspondingly found in our research. Moreover, found in our study that cyclophosphamide prompts liver congestion (hemorrhage), and there was sinusoidal enlargement and inflammatory cell infiltration. Thick blood vessels, fibrosis and compressed blood vessels was moreover established. Additionally, in this study the histopathological study of the liver with PAS stain for both low and high dose decreased glycogen amounts found in the hepatocytes, due to presence of degenerative hepatocyte and necrosis [24]. When comparing our results with other studies that demonstrated in the histopathological study of liver that the low dose group displayed histological changes that appeared after 6 weeks in the form of; slight fatty changes in some areas, but the portal triads and central vein were normal [26], which was similar to the present study. Also the study showed comparable results to the present study as, marked fatty changes, with central vein congestion and loss of hepatocytes' architecture [26]. Concerning the high dose group, similarly to our study, the changes were more evident in liver tissues, by means of there was diffuse fatty infiltration, central vein congestion and loss of hepatocyte architecture [24] in both studies. With high dose of cyclophosphamide there was also destruction to endothelium of sinusoids in addition to sinusoidal congestion. Therefore, the changes that occurred in the liver of Albino rats after administration of cyclophosphamide were in the form of fatty changes, hemorrhages and central vein congestion. These changes were also noticed in our study. These changes differ according to the dose of drug and the period between administration of the drug and histological findings [26]. Microscopic examination of other researchers revealed thinning of the capsule, destruction and subcapsular haemorrhage at a few sites. The cytoarchitecture beneath the capsule was completely disrupted. The hepatocytes appeared abnormal in shape with amorphous eosinophilic cytoplasm and pyknotic nuclei [27]. Our results showed histological changes that were similar to previous literatures, similar to scientists who studied the effect of cyclophosphamide in different doses on the liver of Wistar albino rats the results of the study showed that the liver histopathology of the high dose there was hepatocellular destruction associated with sinusoidal enlargement and inflammatory infiltration, this experimental study demonstrated that histopathological changes resulting from cyclophosphamide differs according to doses [27]. Destruction of hepatocellular architecture associated with inflammatory infiltration were comparable to many previous studies [28]. The hepatocellular destruction together with features of nuclear degeneration and apoptosis like karyolysis, karyorrhexis and nuclear pyknosis and vacuolations occurred in the histopathological slides of the toxic dosed due to a direct effect of drugs [29, 30]. The histopathological changes with high dose of cyclophosphamide in some studies, revealed that marked destructive changes are observed in liver tissue as the cyto-architecture beneath liver capsule was completely disrupted with presence of sub-capsular hemorrhage at few sites [27]. While cyclophosphamide if administered at low doses, stimulates a regenerative response. Therefore, for effective use of cyclophosphamide as anti-cancer treatment, it should be administered in low doses for prolonged period to avoid liver toxicity. As it was evident from this study and others that hepatotoxicity of cyclophosphamide is dose related [15, 27]. When compared with other researchs revealed that liver injury and liver dysfunction occurs with extensive exposure to metabolites of cyclophosphamide. Additionally, as a result of hepatotoxicity, sinusoidal obstruction syndrome arises and leads to elevated levels of aminotransferase and bilirubin [31]. Infrequently, administration of regular doses of cyclophosphamide could cause clinically obvious liver and kidney injuries. These types of injuries are initiated within the first few weeks after cyclophosphamide therapy, and are manifested primarily as rise in serum enzymes levels. The destruction in most cases is self-limited, and mainly resolves on drug cessation. Nevertheless, reappearance of these manifestations may follow re-exposure to this drug [20]. In addition, recent studies assessed the effect of cimetidine compared to cyclophosphamide -induced liver toxicity. Rats were sacrificed after fasting for a night. Biochemical parameters related to liver function and histopathology of liver tissue were assessed. Concerning rats which received cyclophosphamide, there was a rise in liver parameters as; alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase, gamma, glatamyl transferase, total bilirubin, conjugated bilirubin and malondialdehyde [32]. Alternatively, there was a drop in levels of liver superoxide dismutase, catalase, glutathione and glutathione peroxidase in the same group. Histopathological examination of liver tissue of this group revealed that there was hepatocyte necrosis around the central veins of the liver. However, cyclophosphamide -induced liver damage was significantly ($P<0.05$; 0.01) improved in a dose-

dependent way in rats administered with cimetidine preceding to the administration of cyclophosphamide [33]. Commonly, on treating cancer cases with cyclophosphamide, minor and temporary elevations in serum aminotransferase levels were perceived. The rise in the level of enzymes is dose related effect, it is more seen in cases treated with heavy doses of cyclophosphamide via the intravenous route [29]. Histologically, it has been reported that cyclophosphamide -treated rats have hepatic tissue periportal inflammation, hemorrhage, and congestion [23]. Interface hepatitis and focal hepatitis as mentioned before are parallel to the ulcerations and lymphocytic infiltration observed in cardiac muscles in earlier animal experiments in 1974 [34] and 1999 [35]. Similar to our results, here were no distinct collections of leukocytes observed in the low dose group, only a few lymphocytes were seen distributed throughout the parenchyma of the liver [27]. Typically, the liver of animals that received divided low doses demonstrated many nuclei that showed mitotic figures and a number of binucleated cells (this was also found in our experiment). These features are known to be associated with the activity and regenerative response of the liver against any injury. This may probably explain an ongoing regeneration and repair in the liver in low dose experimental group that maintained its cytoarchitecture and the number of hepatocytes [27]. Cyclophosphamide therapeutic use as an anti-cancerous drug should be restricted due to its side effects [36]. Cyclophosphamide is known to produce highly reactive carbonium ions that react with the electron-rich areas of susceptible molecules, such as nucleic acids and proteins causing damage to the DNA and RNA of the cells. But it is a prodrug i.e, converted to its active form in the liver [36]. In somatic cells, cyclophosphamide has been shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells. It has also produced chromosome damage and micronuclei in rats, mice and Chinese hamsters [37]. DNA is the primary target in terms of the teratogenic, mutagenic, and antineoplastic effects of cyclophosphamide. Effects of cyclophosphamide on DNA have been reported widely in mammalian cells, both of somatic and germ cell origin [37]. Cyclophosphamide is thought to exert its cytotoxicity via the cross-linking of cellular DNA, and studies demonstrated that following drug exposure there is occurrence of interstrand and DNA-protein cross-links, but no single strand breaks [9]. The cytotoxic action of cyclophosphamide results mainly due to phosphoramide mustard induced DNA cross-linking [10]. Exposure of cells to phosphoramide mustard results in the induction of a mixture of interstrand, DNA protein cross links and enlargement of cells. Phosphoramide mustard considerably increases the quantity of cross linked DNA after incubation with intact LM4 cells or nuclei isolated from these cells [38]. Although, acrolein is described to bind to proteins, to form DNA adducts, to create basic locations, and to induce DNA single strand breaks [39]. In somatic cells cyclophosphamide has been shown to produce gene mutation, chromosomal aberration, micronuclei and sister chromatid exchanges in a variety of cultured cells. Chromosome analysis of mice was performed in unfertilized metaphase II-oocytes the investigation demonstrated the dose-dependent frequency of the induced types of chromosomal abnormalities [40]. Studies also showed that cyclophosphamide interrupts meiotic events before pachynema stage. The various abnormalities seen were disomy, nullisomy and diploidy spermatozoa with double size than normal cells [40]. This was observed in the present study in the binucleated hepatocytes in the tissue dosed with the toxic administration, although no study was inducted to compare between the controls and treated, but it could be recommended.

5. Conclusion

Our study corroborates that the cyclophosphamide induces Hepatotoxicity. The histopathological changes of animals under therapy in the form of fatty changes of liver with low dose cyclophosphamide, and induced liver necrosis with the higher dose (toxic dose) of cyclophosphamide is associated with acrolein which induces; cell death, apoptosis, and necrosis. Furthermore, we concluded that cyclophosphamide induces liver congestion (hemorrhage), and there was sinusoidal enlargement and inflammatory cell infiltration. Thick blood vessels and fibrosis were also established in addition to compressed blood vessels. Besides results were found with PAS stained slides for both low and high doses of cyclophosphamide decreased glycogen amounts, owing to the presence of degenerative hepatocytes and necrosis. Liver damage lowers the antioxidant defense system and increases the reactive oxygen species ROS, that induces lipid peroxidation and liver peroxidative damage in patients undergoing chemotherapy which will be reflected as histopathological changes in the liver and other organs when examined under the microscope. The results of this study supports the cyclophosphamide induced hepatotoxicity which can be measured by histopathological changes. The dose regimen of cyclophosphamide can be modified accordingly to provide maximum efficacy to the patients but with reduce damage to the liver at the same time.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest between the authors.

Statement of ethical approval

Animal experiments were designed and conducted in accordance with the guidelines of institutional animal ethical committee.

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