International Journal of Biomedical and Advance Research
ISSN: 2229-3809 (Online); 2455-0558 (Print)
Journal DOI: https://dx.doi.org/10.7439/ijbar
CODEN: IJBABN **Original Research Article**

Effect of cyclophosphamide on liver in albino rats: A comparative dose dependent histomorphological study

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***Article History:**
Received: 14/02/2017
Revised: 09/03/2017
Accepted: 22/03/2017
DOI: https://dx.doi.org/10.7439/ijbar.v8i3.3953

Abstract

Cyclophosphamide is a commonly used anti cancer drug used in almost all regimens for treating a wide variety of carcinomas. It is a prodrug that gets activated in the liver subjecting the organ to a very high amount of the drug influx affecting its functions. The resulting hepatotoxic side effects of cyclophosphamide are dose-dependent as confirmed by studies based on enzymes levels. Recently, low dose cyclophosphamide regimen administered for prolonged periods is documented to be effective in controlling tumor growth and demonstrate a low overall toxicity profile with prolongation of survival. Therefore, the present study was conducted to compare and correlate histomorphological alterations in liver of adult Wistar albino rat induced by a bolus high dose and a divided low dose drug regime of cyclophosphamide administered intraperitoneally. The histomorphological analysis of liver from high dose experimental group demonstrated hepatocellular destruction associated with sinusoidal enlargement and inflammatory infiltration which was more extensive and widespread throughout the parenchyma. Whereas, these changes were observed to be absent or minimal in the low dose experimental group. In fact, the latter showed many binucleate cells and a number of basophilic nuclei containing mitotic figures marking a regenerative response. Hence, cyclophosphamide induces greater hepatodestructive changes at high doses but at low doses, it elicits a regenerative response. Since, prolonged anti cancer treatment with low dose cyclophoshamide has been proven to be efficacious, it is suggested that its dose regimen can be modified accordingly to provide maximum efficacy to the patients but with reduced concomitant toxicity.

Keywords: Cyclophosphamide, Hepatotoxicity, Inflammation.

1. Introduction

Cyclophosphamide is a synthetic anticancer drug that belongs to the nitrogen mustard group of alkylating agents that acts by adding an alkyl group (C_nH_{2n+1}) to DNA. It acts by attaching an alkyl group to the Guanine base of DNA, at number 7 nitrogen atom of the imidazole ring. Thus, causing irreversible cross-linkages in the DNA strands that leads to cell death in G2- and S-phases of the cell-cycle.[1] Due to this cytotoxic property it is used extensively in a variety of carcinomas singly or in combination with other drugs to treat a variety of leukemias, Hodgkin's lymphoma, Multiple myeloma, Mycosis fungigoides, Non-Hodgkin's lymphoma, breast cancer, ovarian cancer and Retinoblastoma. Cyclophosphamide is also used at lower doses for some autoimmune diseases such as systemic lupus erythematosus, severe rheumatoid arthritis, Wegener's granulomatosis, multiple sclerosis, some connective tissue disorders, minimal lesion glomerulonephritis, several forms of vasculitis, Nephrotic Syndrome and post organ transplantation for prevention of rejection of the organ.[2] Cyclophosphamide is a prodrug that gets activated to alkylating phosphoramide mustard in the liver and excreted primarily (70%) in urine in forms of metabolites.[3]

Adverse effects of cyclophosphamide include alopecia, nausea, vomiting, thrombocytopenia, mucosal ulcerations, brief spells of dizziness, transverse striations in the nails, increased skin pigmentation, pulmonary fibrosis, facial abrasions, leukopenia, hematuria, diarrhoea, hemorrhagic cystitis and petechial haemorrhage in lungs and small bowel.[1,4] General endocrinological imbalances in rats, ovarian failure, abnormal sperm production reduced fertility and outcome, decreased implantation, malformed and growth retarded fetuses have been observed in Sprague-Dawley rats.[1-2,5-8] Cyclophosphamide is also documented to cause hepatotoxicity in Fischer rats and cardiopulmonary toxicity in Mongrel dogs.[9-11]

In an attempt to reduce this high level of toxicity and check increase in the resistance to this drug, various studies have been conducted in the past to test the efficacy of low doses of cyclophosphamide in treatment of carcinomas with positive results. Chronic administration in a low dose through drinking water was safe and an effective form of low dose therapy in severe combined immunodeficient (SCID) mice, injected with human prostate, colon, melanoma and breast cancer cell lines subcutaneously.[12]

In a similar experiment, typical toxic effects like bone marrow suppression, decreased differential cell counts, and changes in gastrointestinal lining were observed to be minimal and transient with Low-dose cyclophosphamide dose regime in mice. Urologic side effects like hemorrhagic cystitis, was absent at low doses of cyclophosphamide. Weight of the animals was seen to decrease with the standard dose cyclophosphamide regimen whereas it was maintained in low doses.[13]

Intermittent bolus dose combined with daily low dose cyclophosphamide regimen was effective against both solid and hematologic tumour grown in male NIH Swiss athymic nude mice inoculated with human prostate cancer cells, female BALB/cJ inoculated with breast tumour cells and BALB/cJ induced by murine leukemia virus. Significant delay in tumour expansion and prolongation of median survival in mice were attributed to the antiangiogenic activity of low dose cyclophosphamide.[14] Combined therapy with metronomic chemotherapy and an angiogenesis inhibitor in mice models of advanced Non Squamous Cell Lung Carcinoma showed enhanced anti-tumor and anti-angiogenic effects as exhibited by decreased circulating endothelial cells, microvessel density and pericytes.[15]

In humans, low dose cyclophosphamide is proved to be as efficacious as the recommended standard dose in treating hormone-refractory metastatic prostate cancer (MHRPC) and docetaxel-resistant hormone refractory prostate cancer.[16-17] Prolongation of survival by sixty five months is reported even with stage IIIC platinum resistant ovarian cancer patient with poor performance status by administering low dose cyclophosphamide.[18]

Though liver is the major site of metabolism of cyclophosphamide yet, our search of literature suggested that only hepatic enzymes have been assayed to see the side effects of cyclophosphamide on the liver.

The present work was conducted to detect changes in liver morphology caused by single high dose and compare them with those by repeated low dose of cyclophosphamide in a laboratory animal, the albino rat.

2. Material and Methods

Inbred adult male Wistar albino rats (150-200 gm) were procured and arranged in three groups of six animals each housed with *ad libitum* access to food and water. The body weights were recorded before the onset of the experiment and prior to the sacrifice of animals. Experimental 1 Group rats were injected cyclophosphamide in a single dose of 150 mg/kg body weight **(High dose)** intraperitoneally. Experimental 2 group rats were injected cyclophosphamide in the dose of 50mg/kg body weight **(Low dose)** once a week for three weeks, intraperitoneally. Control animals were injected an equal volume of diluents (0.9% sterile saline solution) weekly for three weeks by the same route.

The animals were weighed and sacrificed on day twenty one, under anaesthesia. The weights of rats in control and both experimental groups were statistically analysed using paired sample t-test. The liver was dissected, processed for paraffin embedding. Sections (7 microns) were cut and stained with Haematoxylin and Eosin, and Periodic Acid Schiff. Histomorphological analysis of the tissue was done.

3. Result and observation

3.1 Observations with High dose

The mean body weight of animals was observed to be significantly decreased ($p < 0.05$) amounting to 7.58% of baseline **(Table 1).**

Table 1: Comparison of mean weight of rats before the experiment and prior to sacrifice in control and both experimental group

Group	Mean weight before the experiment (Grams)	Mean weight prior to sacrifice	P value (Paired t –test)	Significance	Percentage change in body weight
Control	185.00	189.17	0.224	Non Significant	2.2
Experimental-1 (High Dose)	175.83	162.50	0.005	Significant	7.58
Experimental-2 (Low Dose)	188.33	179.17	0.002	Significant	4.8

P value <0.05 is significant

Grossly, the liver appeared normal except for a few small dark areas that probably occurred due to hemorrhage. Microscopic examination 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 **(Fig 1a).** In the deeper parenchyma, density of hepatocytes appeared to be reduced. The distorted hepatocytes retained the lobular pattern of cords radiating from the central vein, but the cell plates were very thin and widely separated due to the dilated sinusoids in between them. The cytoplasm contained PAS negative vacuolations which coalesced to form a large vacuole in several hepatocytes compressing the nucleus to the other side **(Fig 1b).** Inflammatory cells mainly lymphocytes, and few neutrophils and basophils were mainly observed in the region of the portal triads suggesting interface hepatitis. A large number of lymphocytes were scattered throughout the hepatic parenchyma but formed distinct foci in some places between the hepatocytes known as focal hepatitis. Increased number of enlarged Kupffer cells was present in the endothelial lining of the dilated sinusoids congested with red blood cells **(Fig 1c).**

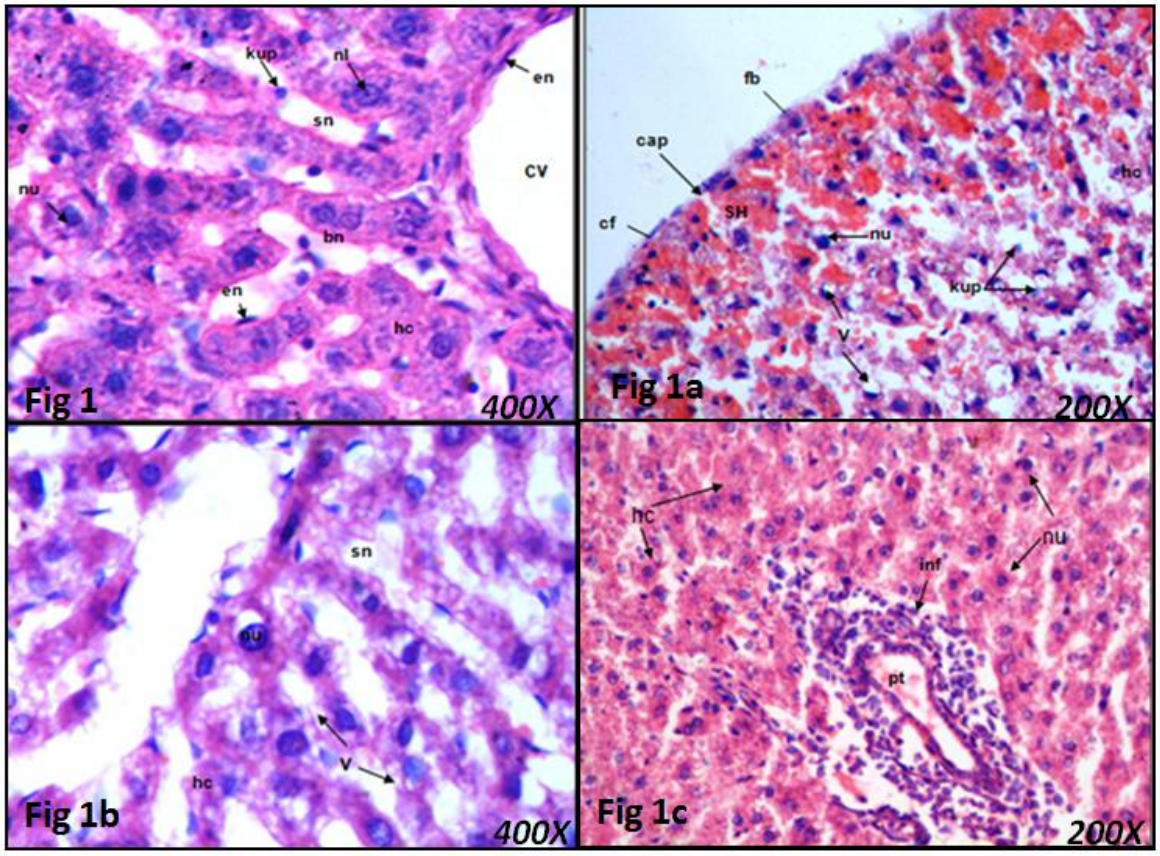

Fig 1. Control group. Normal histological appearance of hepatocytes (hc), nucleus (nu), nucleolus (nl), Central vein (cv), sinusoids (sn), . **Fig 1a.** H-E-stained sections in **Experimental group 1** showing thin capsule (cap) with subcapsular haemmorhage(SH), and small distorted nuclei with pyknotic nucleoli. **Fig 1b .** Vacuolations in hepatocytes in PAS stained sections pushing aside the nuclei .Sinusoids are enlarged. **Fig 1c.** Lymphocytic collection around portal triad (pt)

3.2 Observations with low dose

There was a significant ($p < 0.05$) decreased in the mean body weight among rats was but it was 4.86% of baseline. The thickness of capsule was maintained and similar to the capsule seen in the liver of the control animals. Minimal subcapsular haemorrhage was present. The cytoarchitecture deep to the capsule was maintained. Hepatocytes appeared normal in shape and size **(Fig 2a).** The cytoplasm stained slightly basophilic and very few vacuolations were appreciable. The sinusoids appeared mildly dilated. A few enlarged Kupffer cells were present among the endothelial lining of the sinusoids. Very few inflammatory cells were observed dispersed throughout the parenchyma of the liver **(Fig 2b).** The nuclei within the hepatocytes appeared rounded with 1-2 prominent darkly stained nucleoli. Mitotic figures were observed in some hepatocytes. A considerable number of cells were binucleate **(Fig 2c).**

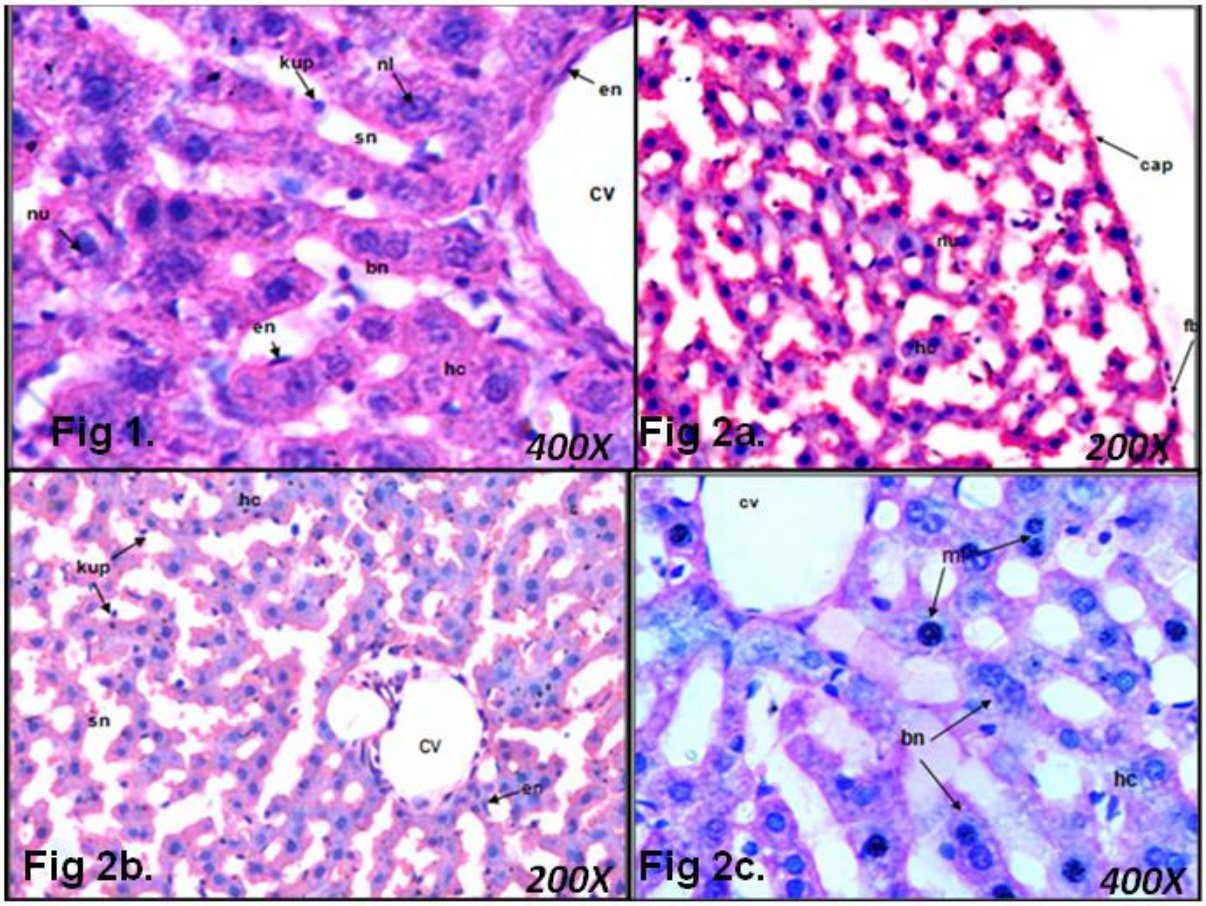

Fig 1. Control group. Normal histological appearance of hepatocytes (hc), nucleus (nu), nucleolus (nl), Central vein (cv), sinusoids (sn), . **Fig 2a.** H-E-stained sections in **Experimental group 2** showing no subcapsular haemmorhage, and maintained cytoarchitecture **Fig 2b .** No vacuolations and lymphocytic infiltration seen . **Fig 2c.** Binucleate hepatocytes (bn) seen with few nuclei containing mitotic figiures (mf).

4. Discussion

Cyclophosphamide caused significant decrease ($p < 0.05$) in mean weight of all experimental animals compared to the controls. This is in accordance with previous studies.[8,19] The reduction in weight of rats was 7.58% with high dose and 4.86% with low dose. Emmenegger *et al*. reported similar observation in mice treated with standard and low doses of cyclophosphamide.[13] The loss in weight is proportional to drug dose and may have resulted due to the degenerative changes seen in the liver parenchyma leading to loss of appetite and decrease in food intake.

Hepatotoxicity of cyclophosphamide is dose dependent and mainly caused by intermediary cytotoxic metabolites acrolien and phosphoramide mustard formed during its metabolism as depicted by elevation in serum enzymes levels.[20-21] Destruction of hepatocellular architecture associated with inflammatory infiltration were comparable to many previous studies.[21] The thinning of cell plates is explained by the compression of the hepatocytes in the cords adjacent to the dilated and congested sinusoids where the initial toxic effects probably occurred.[22] The hepatocellular destruction together with features of nuclear degeneration and apoptosis like

karyolysis, karyorrhexis and nuclear pyknosis and vacuolations occurred due to a direct effect of drugs.[23-24] These features were more profound and widespread in the high dose experimental group. Numerous hepatocytes with PAS negative cytoplasmic vacuolations, earlier reported to be fatty inclusions, were observed in high dose group.[21] No such fatty change was seen in the low dose experimental group.

Interface hepatitis and focal hepatitis as mentioned before are parallel to the ulcerations and lymphocytic infiltration observed in cardiac muscles in earlier animal experiments by O'Connell and Berenbaum[11] and Morais *et al*.[25] There 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. Characteristically, the liver of animals that received divided low doses demonstrated many nuclei that showed mitotic figures and a number of binucleate cells. 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.

5. Conclusion

Cyclophosphamide is one of the commonest chemotherapeutic agent but known to cause direct hepatic toxicity. The side effect profile of cyclophosphamide as determined by evaluating enzymes levels is proved to be dose-dependent. The present study also shows that the various histomorphological aberrations occurring in the liver, the primary organ of metabolism of cyclophosphamide are probably more extensive when the drug is given in bolus than when administered in divided doses. The extent of toxicity can be checked by administrating the drug in a divided regimen in place of a bolus dose as the efficacy of divided dose regimen is proven to be comparable and better than standard dose regimen with low toxicity profile. The dose regimen of cyclophosphamide can be modified accordingly to provide maximum efficacy to the patients but with reduced damage to liver at the same time.

Conflict of interest

The authors declared no conflicts of interest.

Funding

This study was conducted independent of any financial support.

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