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Research Article

Toxicopathological evaluation of sub-therapeutic exposures of cyclophosphamide in Wistar rats

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Abstract

Toxicopathological effects for sub-therapeutic doses of cyclophosphamide (CP) were studied in rats after 28 days repeated dosing. A total of 24 male Wistar rats were procured and randomly allotted to four groups of six rats each. The control group rats received normal saline and treatment group rats received CP orally at 1.5 (low dose), 3.0 (mid dose) and 6.0 (high dose) mg/kg for 28 days. No treatment related clinical signs, mortality, body weight and feed intake were noticed. A significant reduction of TEC, PCV, Hb and monocytes in high dose group, and TLC in mid dose group were noticed. Highly significant reduction of PLT and TLC were noticed in high dose group. Highly significant increase in total cholesterol, ALT and CK in high dose group and significant increase in mid dose group were noticed. A significant increase in BUN, and decrease in total protein and albumin were noticed in high dose group. Highly significant reduction of B-cell and T-cell markers in high dose group and significant reduction in mid dose group were noticed. Bone marrow cytology smear revealed increased myeloid to erythroid ratio and micronucleated polychromatic erythrocytes. At necropsy, sacrificed rats from all dose groups did not reveal any changes except reduced spleen and thymus weights, and increased liver weight in high dose group. Histopathological examination of lymphoid and visceral organs from high dose group revealed architectural alterations, whereas other dose groups did not reveal any alterations.

Keywords: Bone marrow cytology, Cyclophosphamide, Haemato-biochemistry, Immunotoxicity, Wistar rats

Highlights

- A 28 days repeated dose toxicity study was conducted for CP at sub-therapeutic doses in Wistar rats
- Haematological values of CP treated rats revealed anaemia, thrombocytopenia, leucopenia and monocytopenia
- CP caused hypercholesterolemia, hypoproteinemia, hypoalbuminemia, and increased ALT, CK and BUN values
- CP induced immunosuppression, myelosuppression and more micronucleated polychromatic erythrocytes
- Gross and histopathological examination of lymphoid and visceral organs revealed architectural alterations in CP treatments

INTRODUCTION

Cyclophosphamide (CP) is a common cytostatic agent and has been widely used in the treatment of various types of cancers and non-malignant diseases (Ayza *et al*., 2022). It interferes with nucleic acid and generates secondary pathological events, which are closely associated with genomic instability and tumor generation (Swift and Golsteyn, 2014). Genome instability refers to an elevated rate of DNA damage and associated mutations, strand breaks in DNA, DNA replication, cell cycle control or chromosome segregation (Kovalchuk, 2021). The genome instability exhibits during aging, carcinogenesis, immune deficiencies, infertility, cardiovascular diseases, metabolic syndromes and neuromuscular diseases (Georgoulis *et al*., 2017). The cancer patients treated with CP are at a greater risk of genomic damage (Zhang *et al*., 2021).

CP is a strong immunosuppressive agent that inhibits cell proliferation, kills antigen-sensitive lymphocytes, prevents macrophage function and causes atrophy of immune organs (Moghe *et al*., 2015; Raj and Gothandam, 2015; Jordan and Waxman, 2016; Du *et al*., 2024; Ye *et al*., 2024). CP either alone or in combination with other medications used in the treatment of Hodgkin's and non-Hodgkin's lymphomas, autoimmune diseases like systemic vasculitis, lupus erythematosus and sclerosis (Ayza *et al*., 2022). It can cause damage to antioxidant capacity of tissue, liver (Mahmoud *et al*., 2017), kidney (Jiang *et al*., 2020) and impede the growth and development of body (Hou *et al*., 2007). CP therapy

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mostly associated with immune suppression, oxidative stress, reduced body weight and visceral organ weights and multi-organ toxicity (Hou *et al*., 2007; Moghe *et al*., 2015; Khan, 2017; Du *et al*., 2024; Ye *et al*., 2024). Though the CP is used widely against malignant and non-malignant diseases, the toxicological evaluation of sub-therapeutic doses need to be elucidated fully. Hence, the present study was undertaken to unveil the underlining toxicity of CP at sub-therapeutic doses in Wistar rats.

MATERIALS AND METHODS

Experimental design: A 28 days repeated dose oral toxicity study was conducted in male Wistar rats according to OPPTS (1996) and OECD407 (2008). A total of 24 male Wistar rats of six to eight weeks old, weighing 180-200 g was procured from M/s In Vivo Bio Sciences, Bengaluru, India. The rats were randomly distributed into four groups of six rats each. Rats from group I served as control and received vehicle as normal saline orally for 28 days. Rats from groups II, III and IV received CP orally at 1.5, 3.0 and 6.0 mg/kg for low, mid and high dose groups respectively for 28 days. These sub-therapeutic doses of CP were selected based on oral LD_{50} value (160 mg/kg) in rats (Hou *et al*., 2007). Five gram of CP (C-2236) was purchased from M/s TCI Chemicals, Chennai and formulations were prepared on weekly basis and then stored at 2-8°C until used. Rat feed and purified drinking water were provided *ad libitum*. Temperature and relative humidity were maintained between 18°C and 26°C and 30% and 70% respectively. Light and dark cycles were controlled to give approximately 12 hours light and 12 hours dark.

Clinical observations: Rats were observed twice daily for any unusual clinical signs, morbidity and mortality during entire treatment period.

Body weight and feed intake: Body weights and feed intake were recorded once in every 3 to 4 days during treatment period and fasting body weight was recorded on 29th day for all animals.

Haematology: Blood samples were collected from overnight fasted rats on 29th day in K_3EDTA coated vacutainer. Blood parameters were analyzed using haematology analyser Vet Scan HM2, USA. Differential leucocyte counts were determined from the blood smears prepared manually.

Serum biochemistry: Serum samples were extracted from overnight fasted rats on 29th day and analyzed for biochemical parameters using M/s Biosystems A50, India. Serum electrolytes were analyzed in UV-VIS double beam spectrophotometer (M/s Systronics, Model 2202, India) by using kits.

Immune function test: The T-cell and B-cell markers were analyzed on 29th day for all rats. Blood samples were collected in heparinized vacutainer and analyzed for T-cell markers (CD4% and CD8%) using Flow Cytometer (M/s BD FACS Celesta, Nodel). Serum samples were tested for IgM antibodies using rat IgM enzyme linked immunosorbent assay kit (M/s IDEXX Laboratories, USA).

Bone marrow cytology: Bone marrow cytology (BMC) smears were prepared from sacrificed rats on 29th day. The smears were air-dried, fixed in absolute methanol and then stained with Wright's-Giemsa stain (Bolliger, 2004). The slides were examined for myeloid to erythroid (M: E) ratio and induction of micronucleus in polychromatic erythrocytes (MNPCE).

Gross pathology: All rats were sacrificed on 29th day using over dose of chloroform and detailed necropsy was conducted. The lymphoid organs and major visceral organ weights were recorded.

Histopathology: All organs from each rat was collected and preserved in 10% neutral buffered formalin. Tissues were trimmed, processed, embedded in paraffin blocks, sectioned and stained with haematoxylin and eosin (H&E) stain (Bancroft and Gamble, 2008).

Statistical analysis: The data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (Snedecor and Cochran, 1989) using Graph Pad Prism software version 5.02.

RESULTS

Clinical observations: The rats from control and CP treated groups did not show any obvious clinical signs, morbidity and mortality.

Body weight and feed intake: No significant changes were noticed in the body weight and feed intake after repeated dosing of CP in rats. However, a mild reduction of body weight was noticed at high dose group.

Haematology: Mean±SE haematological values of

different dose groups of rats are presented in Table 1. A significant (P<0.05) reduction of TEC, Hb, PCV and monocytes in high dose group, and TLC in mid dose group were noticed. Highly significant (P<0.01) reduction of PLT and TLC were noticed in high dose group.

Table 1. Mean±SE haematological parameters of Wistar rats treated with different doses of CP

S. No.	Haematological parameters	Vehicle Control (n=6)	Low dose (n=6)	Mid Dose (n=6)	High Dose (n=6)
1.	TEC (x10^6/µL)	8.65^a±0.1	8.46^a±0.2	8.39^a±0.11	7.63^b±0.8
2.	Hb (g/dL)	15.8^a±0.5	15.5^a±0.3	14.8^a±0.12	14.2^b±1.5
3.	PCV (%)	47.1^a±0.2	47.0^a±0.7	43.1^a±0.46	40.6^b±4.6
4.	MCV (fL)	54.4±0.5	55.6±0.6	51.4±0.46	57.1±6.0
5.	MCH (pg)	18.3±36.8	18.3±0.2	16.9±0.15	18.6±2.0
6.	MCHC (g/dL)	33.6±0.7	32.9±0.1	33.0±0.21	32.7±3.4
7.	RDW (%)	16.8±0.2	16.7±0.2	15.9±0.11	17.3±1.8
8.	PLT (x10^3/µL)	919.4^a±0.1	956.3^a±42.6	924.9^a±12.33	759.8^b±78.6
9.	TLC (x10^3/µL)	16.9^a±2.0	16.1^a±0.94	11.9^b±0.6	9.7^c±0.9
10.	Neutrophils (%)	17.4±2.9	17.8±2.11	16.7±1.2	16.1±1.9
11.	Lymphocytes (%)	72.6±7.6	74.8±2.28	75.7±1.3	77.5±1.7
12.	Monocytes (%)	5.6^a±1.1	4.0^{ab}±0.46	4.3^{ab}±1.0	3.2^c±1.1
13.	Eosinophils (%)	4.3±0.6	3.1±0.3	3.3±0.7	3.4±0.5
14.	Basophils (%)	0	0	0	0

Means with same superscripts within a row do not differ (P>0.05) from each other.

Serum biochemistry: Mean±SE serum biochemical values of different dose groups of rats are presented in Table 2. A highly significant (P<0.01) increase in total cholesterol, ALT and CK in high dose group, and significant (P<0.05) increase in mid dose group were noticed. A significant (P<0.05) increase in BUN, and decrease in total protein and albumin were noticed in high dose group.

Table 2. Mean±SE serum biochemical parameters of Wistar rats treated with different doses of CP

S. No.	Biochemical parameters	Vehicle Control (n=6)	Low dose (n=6)	Mid Dose (n=6)	High Dose (n=6)
1.	Glucose (mg/dL)	104.60±1.78	94.89±1.71	98.63±2.31	91.23±2.42
2.	Total cholesterol (mg/dL)	94.20^c±5.10	96.84^c±3.43	129.67^b±12.71	133.41^a±4.67
3.	Triglycerides (mg/dL)	69.70±2.71	74.44±3.03	71.93±4.41	75.71±4.18
4.	Total protein (g/dL)	7.72^a±0.12	7.22^a±0.10	7.25^a±0.15	6.46^b±0.22
5.	Albumin (g/dL)	3.34^a±0.05	3.34^a±0.11	3.14^a±0.10	2.40^b±0.07
6.	BUN (mg/dL)	18.45^b±0.49	18.65^b±1.37	21.40^b±0.56	22.33^a±0.97
7.	Creatinine (mg/dL)	0.83±0.03	0.73±0.02	0.87±0.03	0.84±0.04
8.	ALT (U/L)	80.75^c±4.09	78.53^c±3.77	96.43^b±8.80	109.02^a±11.38
9.	AST(U/L)	170.73±14.53	163.07±11.86	155.60±11.46	164.84±13.67
10.	ALP (U/L)	230.30±7.61	231.78±9.36	200.95±15.85	226.48±8.22
11.	GGT (U/L)	10.50±1.05	11.11±0.95	14.22±0.79	11.13±0.44
12.	Creatinine kinase (U/L)	720.70^c±34.92	741.00^c±57.09	839.90^b±92.15	945.33^a±28.56
13.	Total bilirubin (mg/dL)	3.01±0.31	3.38±0.25	3.33±0.13	2.61±0.31
14.	Direct bilirubin(mg/dL)	2.03±0.15	2.18±0.11	2.22±0.09	2.00±0.16
15.	Calcium (mg/dL)	8.76±0.15	8.32±0.23	9.15±0.39	9.41±0.57
16.	Phosphorus (mg/dL)	4.90±0.26	4.44±0.24	5.44±0.45	5.34±0.35
17.	Magnesium (mg/dL)	1.67±0.09	1.65±0.11	1.35±0.05	1.49±0.05
18.	Sodium (mmol/L)	106.96±1.21	107.03±1.24	105.12±0.87	104.05±1.04
19.	Potassium (mmol/L)	6.06±0.18	5.91±0.36	6.01±0.22	6.44±0.13
20.	Chloride (mmol/L)	88.31±0.75	86.87±0.90	85.97±0.96	86.69±1.06

Means with same superscripts within a row do not differ (P>0.05) from each other.

Immune function test: Mean±SE immune suppression values of different dose groups of rats are presented in Table 3. A highly significant (P<0.01) reduction of

Table 3. Mean±SE immune suppressive parameters of Wistar rats treated with different doses of CP

Sl. No.	Immune suppressive parameters		Vehicle Control (n=6)	Low dose (n=6)	Mid Dose (n=6)	High Dose (n=6)
1.	B-cells	IgM (µg/mL)	425.13^{a}±14.52	419.85^{a} ±16.38	386.59^{b}±12.21	350.77^{c}±18.88
2.	T-Cells	CD4 (%)	7.82^{a}±2.06	7.56^{a}±2.97	5.31^{b}±1.21	3.31^{c}±1.01
3.		CD8 (%)	4.45^{a}±3.73	4.42^{a}±3.11	3.48^{b}±1.03	2.29^{c}±0.82
4.		CD4/CD8	2.32^{a}±1.01	1.98^{a}±0.62	1.67^{b}±0.51	1.47^{c}±0.13

Means with same superscripts within a row do not differ (P>0.05) from each other.

B-cell (IgM) and T-cell (CD4%, CD8%, CD4/CD8 ratio) markers in high dose group, and significant (P<0.05) reduction in mid dose were noticed.

Bone marrow cytology: BMC smears revealed mixed population of myeloid cells, erythroid cells, few megakaryocytes and plasma cells (Fig. 1). The myeloid to erythroid ratio for vehicle control, low, mid and high dose groups recorded as 1.02±0.15, 1.38±0.11, 1.66±0.15 and 1.88±0.10 respectively. Mean±SE percent MNPCE for control, low, mid and high dose groups observed as 0.75±0.08, 1.29±0.17, 1.67±0.18, 1.81±0.21 respectively. A significant (P<0.05) increase in micronuclei induction was observed in CP treated groups (Fig. 2).

Table 4. Mean±SE absolute organ weights (g) of Wistar rats treated with different doses of CP

Sl. No.	Organs	Vehicle Control (n=6)	Low dose (n=6)	Mid Dose (n=6)	High Dose (n=6)
1.	Liver	8.868^{a}±0.60	9.038^{a}±1.44	9.043^{a}±1.38	9.243^{b}±1.28
2.	Kidneys	2.047±0.33	2.073±0.20	2.177±0.21	2.211±0.19
3.	Heart	0.995±0.08	0.943±0.08	0.967±0.09	0.993±0.06
4.	Brain	1.999±0.09	1.952±0.05	1.954±0.13	1.938±0.08
5.	Adrenals	0.077±0.01	0.069±0.02	0.065±0.01	0.071±0.01
6.	Spleen	0.888^{a}±0.54	0.692^{ab}±0.31	0.665^{ab}±0.12	0.534^{c}±0.11
7.	Thymus	0.503^{a}±0.16	0.495^{a}±0.18	0.448^{ab}±0.08	0.387^{c}±0.19
8.	Testes	3.301±0.16	3.156±0.20	3.106±0.34	3.131±0.27
9.	Epididymis	0.984±0.09	0.949±0.11	0.884±0.17	0.941±0.06

Means with same superscripts within a row do not differ (P>0.05) from each other.

Gross pathology: Gross pathological examination of sacrificed rats from all dose groups did not show any morbid changes. Mean±SE absolute organ weights (g) of different dose groups of rats are presented in Table 4. A significantly (P<0.05) decreased spleen and thymus weights, and increased liver weight were observed in high dose group. However, other organ weights did not differ between control and CP treated groups.

Histopathology: The lymphoid organs (spleen, thymus and lymph nodes) from high dose group revealed lymphoid cell depletion, lymphocytolysis and haemorrhages. Additionally, spleen showed extra medullary haematopoiesis (Fig. 3), thymus showed lack of cortico-medullary differentiation (Fig. 4) and mesenteric lymph node showed lymphocytolysis in sinuses (Fig. 5). Liver showed vacuolar degeneration of hepatocytes, sinusoidal congestion, centriacinar necrosis and biliary hyperplasia (Fig. 6). Lungs revealed interstitial pneumonia, alveolar macrophages and haemorrhages (Fig. 7). Heart revealed myocardial necrosisand mononuclear cell infiltration (Fig. 8), and kidneys showed tubular degeneration and interstitial congestion.

DISCUSSION

Clinical observations: The rats from CP treated groups did not show any obvious clinical signs in this study are similar to the reports of Hou *et al*. (2007) who also did not observe any unusual signs even at a higher dose of 10 mg/kg of CP for 30 days dosing in rats.

Body weight and feed intake: The present study showed unaltered body weight gain and feed intake up to 6 mg/kg. However, a reduction in body weight was noticed at 5 to 10 mg/kg of CP for 30 day oral dosing in rats (Hou *et al*., 2007; Jalali *et al*., 2012) and 80 mg/kg of CP for 3 days intraperitoneal injection in mice (Yang *et al*., 2019; Du *et al*., 2024; Ye *et al*., 2024). It indicates that either low-dose long-term exposure or high-dose short-term exposure of CP causes weight loss with less feed intake in rats and mice.

Haematology: Blood pictures in CP treated rats revealed anaemia, thrombocytopenia, leucopenia and monocytopenia. These features are in accordance with

Toxicopathological effects of cyclophosphamide in Wistar rats

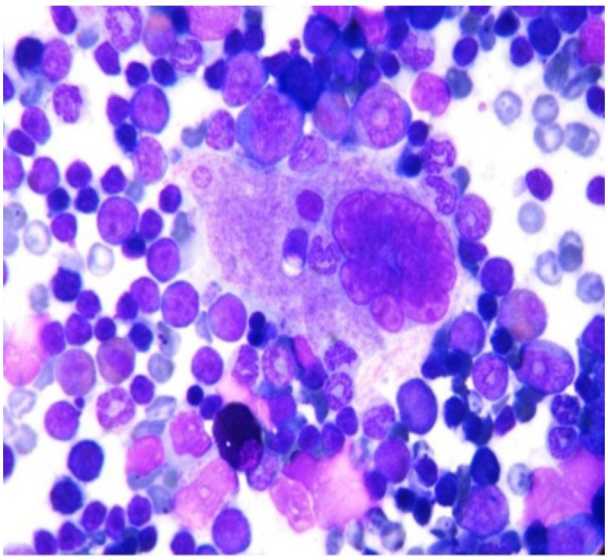

Fig. 1. BMC smear from high dose group rat revealing erythroid cells, few MNPCEs and megakaryocyte. Wright Giemsa 1000x

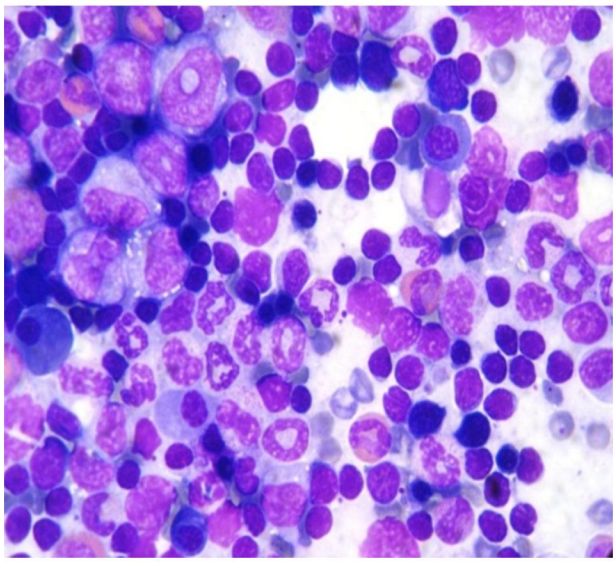

Fig. 2. BMC smear from high dose group rat showing mixed population of myeloid cells, plasma cells and few MNPCEs. Wright Giemsa 1000x

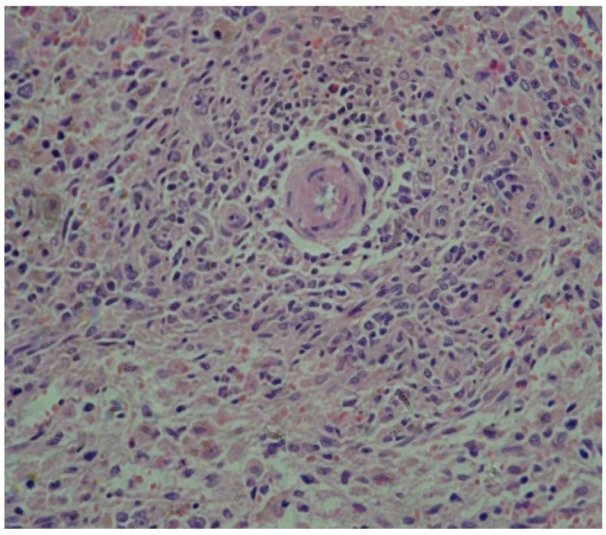

Fig. 3. Spleen from high dose group rat showing lymphoid cell depletion and extra medullary haematopoiesis. H&E 400x

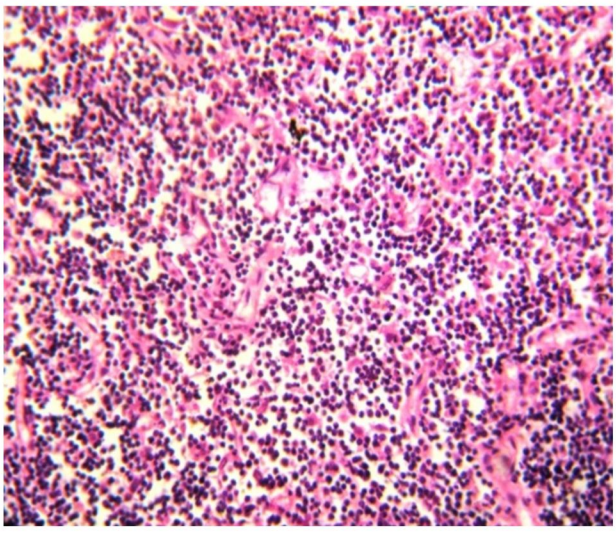

Fig. 4. Thymus from high dose group rat showing lack of cortico-medullary differentiation, lymphoid cell depletion and lympho cytolysis. H&E 200x

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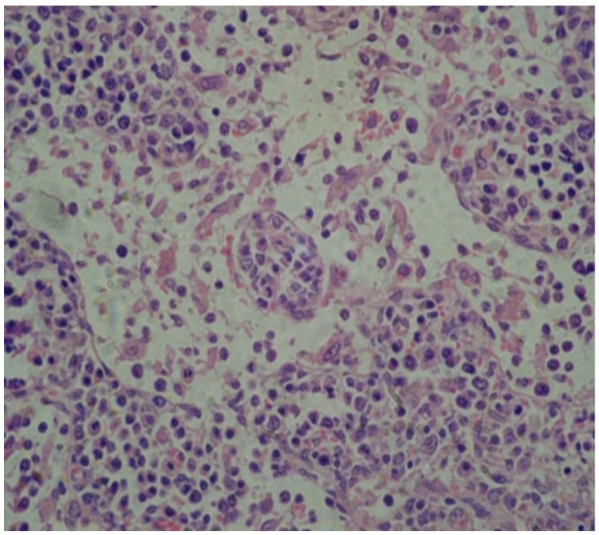

Fig. 5. Mesenteric lymph node from high dose group rat showing lymphoid cell depletion and lymphocytolysis in sinuses. H&E 400x

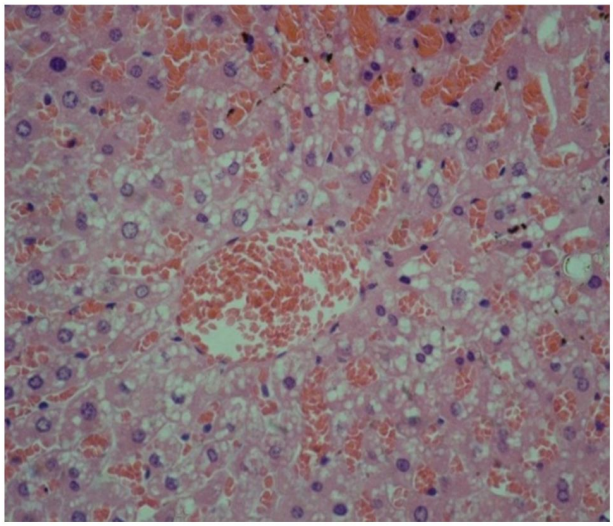

Fig. 6. Liver from high dose group rat showing vacuolar degeneration of hepatocytes, sinusoidal congestion and centriacinar necrosis. H&E 400x

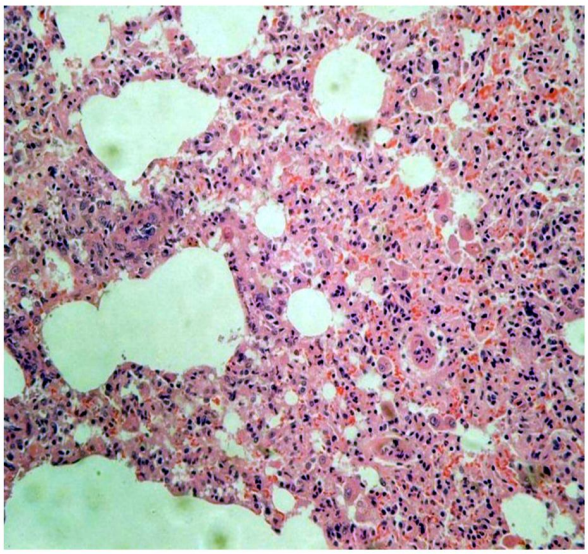

Fig. 7. Lung from high dose group rat showing interstitial pneumonia and alveolar macrophages. H&E 200x

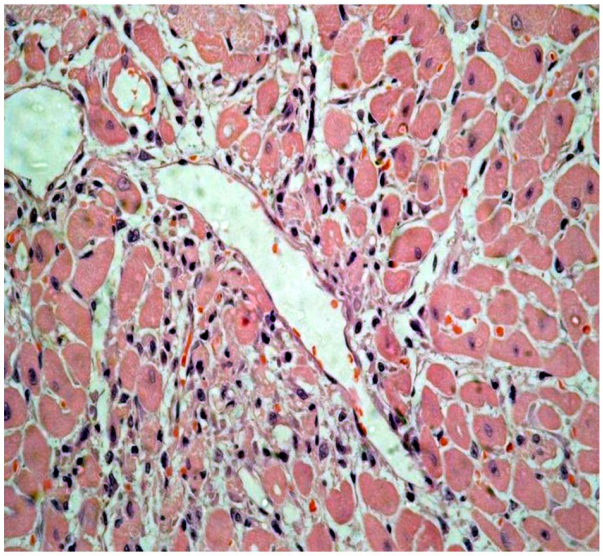

Fig. 8. Heart from high dose group rat showing necrosis and mononuclear cell infiltration. H&E 400x

previous workers (Hou *et al*., 2007; Zhang *et al*., 2021) who observed similar findings in rats treated with CP at a higher dose rate of 5 to 40 mg/kg. Reduced TLC in CP treated rats showed that TLC is more sensitive to CP when compared to other blood components (Khan, 2017; Cengiz, 2018; Alrumaihi *et al*., 2019). CP is a strong immunosuppressive agent that inhibits cell proliferation and kills antigen-sensitive lymphocytes (Du *et al*., 2024; Ye *et al*., 2024).

Serum biochemistry: Hypercholesterolemia, hypoproteinemia, hypoalbuminemia and increased ALT, CK and BUN values observed in this study is attributed to deleterious effects of CP on liver and kidney functions (Zhang *et al*., 2021). CP might destroy the balance of oxidation and antioxidation ability in liver, impaired kidney function and disturbed amino acid metabolism (Zhang *et al*., 2021). These alterations reflected histologically in liver and kidney of this study attributed to toxic effects of CP at higher doses.

Immune function test: The immunosuppressive effects of CP on B-cells and T-cells observed in this study is agreed with earlier reports at 10 mg/kg of CP in rats (Hou *et al*., 2007) and 40 to 80 mg/kg of CP in mice (Zhang *et al*., 2021; Du *et al*., 2024; Ye *et al*., 2024). CP is metabolized by cytochrome P450 mixed function oxidase in liver and produced metabolites like phosphoramide mustard and acrolein. Acrolein is an active metabolite responsible for causing immunosuppression by interferes with antioxidant defense system and produce highly reactive oxygen-free radicals (Moghe *et al*., 2015; Oyagbemi *et al*., 2016; Sherif, 2018; Ayza *et al*., 2022).

Bone marrow cytology: The M:E ratio recorded in this study is similar to findings of Bolliger (2004) who reported M:E ratio in normal rats varies from 1.07 to 1.93. A trend in increased myeloid series and decreased erythroid series of cells in CP treated groups of this study might be due to myelosuppressive activity of CP in bone marrow (OECD474, 2016). An increased incidence of micronuclei induction noticed in CP treated groups of this study concurred with previous reports. According to Krishna *et al*. (1995), a 17-fold increase in MNPCEs has been recorded in mice dosed 40 mg/kg of CP. This clearly indicates the clastogenic potential of CP in inducing MNPCE in rats and mice. Hence, CP can be suggested as positive control for mammalian micronucleus test (OECD474, 2016).

Gross pathology: Reduced weight of spleen observed in this study is similar to previous reports in rats at 10 mg/kg (Hou *et al*., 2007) and mice at 40 to 80 mg/kg of CP treatments (Zhang *et al*., 2021; Du *et al*., 2024; Ye *et al*., 2024). CP is highly toxic to spleen which causes atrophy and degenerative changes (Du *et al*., 2024; Ye *et al*., 2024). The reduced splenic weight reflected the immunotoxicity and immunosuppressive effects of CP in rats (Hou *et al*., 2007; Raj and Gothandam, 2015; Meng *et al*., 2017; Zhao *et al*., 2020).

Histopathology: The alterations seen in lymphoid organs of this study are also observed by earlier workers in rats (Hou *et al*., 2007) and mice (Zhang *et al*., 2021; Du *et al*., 2024; Ye *et al*., 2024). The multi-organ toxicity in CP treatment could be due to production of highly reactive oxygen-free radicals, which causes hepatic and renal damage (Oyagbemi *et al*., 2016; Sherif, 2018; Ayza *et al*., 2022). The liver damage caused by CP lead to digestive abnormalities that making reduced body weight and immunosuppression.

The altered haemato-biochemical values, immunosuppressive effects, micronuclei induction and multi-organ toxicities due to CP treatments indicated dose limitations in clinical applications. Hence, CP can be used with lower doses or in combination with other medications to avoid side effects.

Conflicts of interest: The authors declare that there is no conflict of interest.

Data availability statement: The data supporting to research findings are preserved by the corresponding author and will be made available upon request.

Author's contribution: RM: Overall responsible for planning and execution of the research work and writing the manuscript; SS: Technical support.

Ethical approval: The study protocol was approved by Institutional Animal Ethical Committee (IAEC/12/VC&RI-NKL/2018) and experiment was carried out as per the guidelines of Committee for Control and Supervision of Experiments on Animals (CCSEA), India.

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