International Journal of Research and Review
Vol. 9; Issue: 1; January 2022
Website: www.ijrrjournal.com
Research Paper *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Cyclophosphamide Induced Histological Changes in Testis of Swiss Albino Mice

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DOI: https://doi.org/10.52403/ijrr.20220133

ABSTRACT

Background: Cancer is one of the leading causes for morbidity and mortality. The most often used anticancer drug is Cyclophosphamide, the continuous usage of which may affect the fertility outcome in young boys. Henceforth an attempt is made in this experimental animal study design on Swiss albino mice to look into the histological changes in the testis tissue post exposure to the Cyclophosphamide.

Subjects & Methods: Healthy male Swiss albino adult mice (n=43), which were 6 to 8 weeks old, with average weight of 32±5 g were obtained from Central Animal Research Facility, Manipal were used for the study. They were maintained in well ventilated polypropylene cages containing paddy husk.

The adult male mice were divided into two groups, control (n=7) and test (n=36). In the test group, the mice were injected with Cyclophosphamide at variable doses as per body weight. From each group certain no. of mice was sacrificed on different days. A parallel control group was kept for each of the treatment intervals. Similarly, the 12 prepubertal male mice, control (n=3) and test (n=9) which were of 2 weeks old with average weight of 8±2 g were also considered. The testicular tissue was then extracted and used in histological study.

Results: A significant damage was noticed in the spermatogenic cells of the testicular epithelium. However, Leydig cells were least affected.

Conclusion: The experimental design studied here throws light on dose and time dependent toxic effect of one of the most commonly used anticancer drug- cyclophosphamide.

Keywords: Cyclophosphamide, Testis histology, Swiss Albino mice.

INTRODUCTION

Cancer is one of the leading causes for morbidity and mortality. The most often used anticancer drug is Cyclophosphamide, the continuous usage of which may affect the fertility outcome in young boys. The major drawback of chemotherapeutic agents is their lack of specificity on tumor cells. The rapidly proliferating normal cells of the body are also affected by these agents. Therefore, post-chemotherapy, the normal body tissues are particularly affected in a dose-dependent manner by majority of these drugs.

Henceforth an attempt is made in this experimental study design on mice to look into the histological changes in the testis tissue post exposure to the Cyclophosphamide (CP).

Cyclophosphamide (CP) is odorless, fine white crystalline powder that is soluble in ethanol and water. The drug is a strong immunosuppressive, acting on cells with high mitotic activity, inhibiting the immune response. The cytotoxic effect of

cyclophosphamide is more marked on B cells and humoral immunity than on T cells and cell mediated immunity. It is used as first line drug in cancer diseases such as acute leukemia, chronic lymphocytic leukemia, multiple myeloma, ovarian carcinoma, Ewing's sarcoma and small cell carcinoma of lung. It is used as second line drug for prostate, breast and cervical carcinoma and also in chronic myeloid leukemia. Cyclophosphamide is also used in autoimmune diseases like Rheumatoid arthritis, Wegener's Granulomatosis, and Nephritic syndrome in children. It's also used as a second line drug in maintenance regimen for immunosuppression in organ transplantation whenever first line drugs are intolerable.

It is well known to cause extensive damage to the germinal epithelium (1). A number of studies have reported permanent azoospermia in 99–100% of patients with Hodgkin's lymphoma who were treated with 6–8 courses of combination chemotherapy regimens such as COPP - cyclophosphamide, vincristine, procarbazine, and prednisolone (2). Leydig cells are much less vulnerable to damage post chemotherapy than germ cells, likely due to their slow rate of turnover. When Leydig cell dysfunction occurs prior to or during puberty, affected individuals will experience delayed and/or arrested pubertal maturation and lack of secondary sexual characteristics (3).

Cyclophosphamide treatment causes significant systemic toxicity due to overproduction of Reactive Oxygen Species (ROS) that cause oxidative stress (4). Majority of time the vital organs like brain, heart, lungs, spleen, liver and kidney are also affected other than gonadotoxicity. The seminiferous epithelium is highly susceptible to damage caused by toxins, as a result the young patients with cancer suffer prolonged and sometimes permanent reduction in sperm count or azoospermia and are most often infertile. The most sensitive testicular cells to chemotherapeutic drugs are differentiating spermatogonia. The death of these cells results in depletion of later stages of germ cells and reductions in sperm counts. The eventual recovery of sperm production depends on the survival of the spermatogonial stem cells and their ability to differentiate to spermatozoa. If stem cells survive and their microenvironment is not affected, recovery of spermatogenesis will occur rapidly.

In mice it is shown that the interval needed for the recovery of fertility is directly proportional to the death of the spermatogonial stem cells. There is also evidence that these agents can produce damage to somatic tissue. In rat testes, even though numerous stem spermatogonia survived the cytotoxic treatment, complete recovery of seminiferous epithelium was not observed. Recovery from moderate doses is possible sometimes after a prolonged period of azoospermia; however high doses cause permanent azoospermia. Thus the toxic effect of this drug was dose and time dependent. The effect of drug on sterility also depends on the state of gonadal function at the time of exposure (5). To better elucidate the action of cyclophosphamide on spermatogonial stem cell survival, studies using experimental animal models are needed.

MATERIALS & METHODS

Animal model:

Healthy male Swiss Albino (*Mus musculus*) mice from inbred colony maintained at Central Animal Research Facility, Manipal (Reg No 94/1999/CPCSEA) were used for the study. Among them, 43 were adult mice (6 to 8 weeks), with average weight of 32±5 g and 12 were pre-pubertal age group (2 weeks, with average weight of 8±2 g). The mice were maintained in well ventilated polypropylene cages containing paddy husk. Animals were given food and fresh drinking water *ad libitum*. The protocol for the animal use was approved from the Institutional Animal Ethics Committee, Manipal University, Manipal (IAEC Ref No -79/2013).

Experimental design:

The adult male mice (n=43) were divided into two groups; control (n=7) and test (n=36). In the test group, the mice were injected with cyclophosphamide (CP) at a dose of 50, 100, 200 and 250 mg/kg body weight. From each group, 3-4 mice were sacrificed on day 7, 28 and 42. A parallel control group was kept for each of the treatment intervals. Similarly, the prepubertal mice (n=12) were divided into two groups; control (n=3) and test (n=9). The prepubertal test groups were injected with 50, 100 and 200 mg/kg body weight of CP. After a gap of one week, 3 mice from each of group were sacrificed to study the testicular and epididymal histology.

The control animals were injected Phosphate Buffered Saline (PBS) solution. The mice of test groups were injected with freshly prepared cyclophosphamide solution which was purchased from SIGMA ALDRICH (C0768, St. Louis, MO 63103, USA) by dissolving it in PBS Solution. The conscious mouse was restrained manually and the drug was injected intra-peritoneally as per their body weight.

The treated mice were kept under observation. The food and water was supplied to all cages and weekly twice the cages were cleaned. During the observation period, the activity and signs of toxicity like hair loss, fatigue or any other changes in their activity was recorded. Weekly weight of mice was recorded right from the day of drug/saline injection.

Collection of testes:

On the specific periods, animals were weighed and sacrificed by cervical dislocation. The mice were dissected as per guidelines given in "The Laboratory Mouse-Handbook of experimental animals" by Hans Hedrich (2004). Briefly, the mouse was restrained in supine position, by pinning it on the wax board. A midline skin incision was given on the abdomen with the help of scalpel. The skin incision was further extended laterally towards the knee in the lower 1/3rd of abdomen, with the help of toothed forceps and scissors. The skin incision finally appears like inverted Y. After retracting the skin to the sides, the peritoneum was cut in similar manner and was pinned to the wax board during which the intra-abdominal and pelvic organs are well exposed. Once testis was identified, the gonads are separated with the help of blunt forceps and Vannas Scissors. The separated organs were cleared off from the adhering connective tissue, excised and were placed in PBS Solution. The dry testicular weight was taken and the tissues and the epididymis were later fixed in modified Bouin's solution (0.2% picric acid/2% (v/v) formaldehyde in PBS) and then transferred to 70% alcohol for histological examination where the stained sections were studied under Olympus Research Microscope.

Statistical Analysis: The data was represented as mean ± standard error and was calculated using Microsoft Excel and Origin 6.0 software (Microcal™ Origin®, Version: 6.0, Northampton, MA 01060, USA). The statistical significance of the data was analyzed using one way ANOVA test using GraphPad InStat Software (Version: 3.06, 32-bit for Windows). 'p' value < 0.05 was considered as statistically significant.

RESULT

In the present study histological changes induced by CP in testicular tissues of pre-pubertal and adult mice was studied. Further, both time-dependent and dose-dependent changes were assessed in the tissue sections.

Pre-pubertal testis:

Effect on germ cell count and histology: The control testicular sections had 53.78 ± 1.58 germ cells which was significantly lower in the CP treated mice. At 7 day post treatment interval, in 50 mg CP treated mice the number of germ cells present was 29.43 ± 0.99 and was significantly lower than the control mice ($p < 0.01$). Increasing the CP dose further did not

result in any further depletion of germ cells. However, they had significantly lower number of germ cells ($p< 0.01$) compared to control (Figure 1).

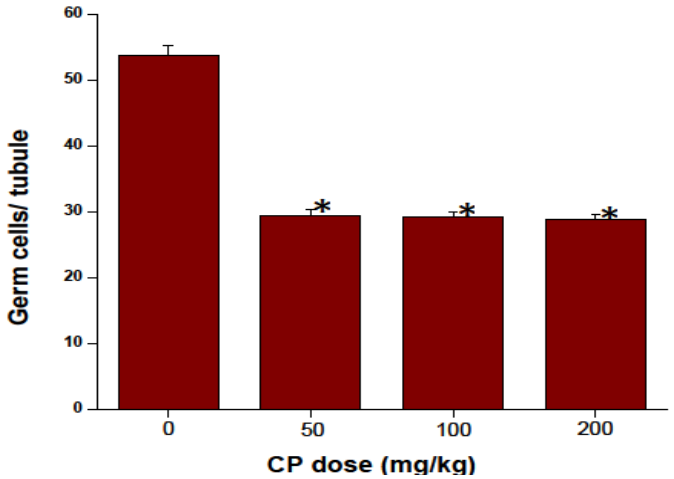

Figure 1: Germ cell count in the seminiferous tubules of pre-pubertal mice treated with various doses of cyclophosphamide at 7 day post-treatment interval

**p< 0.01 compared to control*

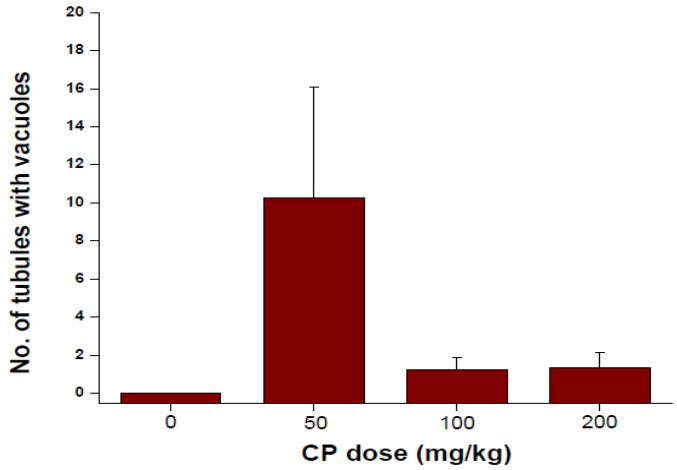

Figure 2: Effect of various doses of cyclophosphamide on vacuolization in seminiferous tubules of pre-pubertal mice at 7 day post-treatment interval

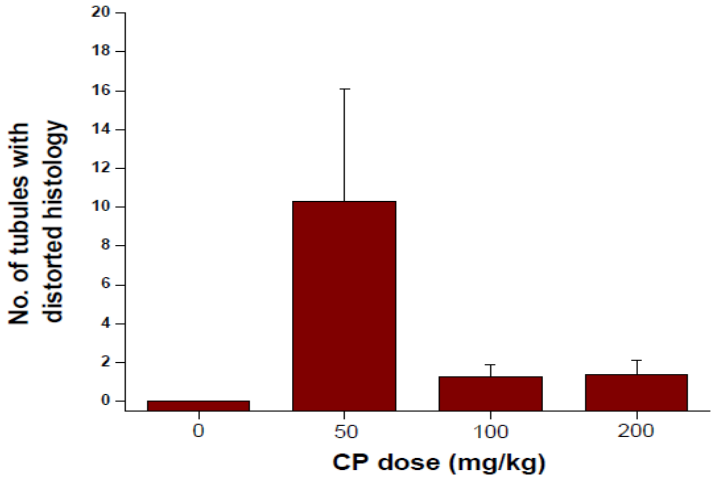

Figure 3: Effect of various doses of cyclophosphamide on the testicular tissue organization at 7 day post-treatment interval

In addition to this, the testicular tissues had higher degree of vacuolations in mice treated with CP especially at 50 mg/kg dose. However, the difference was

statistically not significant. At 100 and 200 mg/kg dose, the vacuolization was lower than at 50 mg/kg body weight (Figure 2). Similarly, the tubules with distorted cellular organization were higher in 50 mg/kg dose (Figure 3).

Adult testis:

Effect on germ cells: The adult testis of control mice had 48.29 ± 0.99 germ cells (Figure 4). CP administration resulted in a dose-dependent depletion of germ cells in the adult testes. Even at the lowest dose tested (50 mg/kg), a significant depletion of the germ cells (24.01 ± 0.94) was observed in adult testes ($p < 0.001$). At the highest dose administered (250 mg/kg), the testis had only 10.51 ± 0.39 germ cells in it which was significantly lower than all other groups.

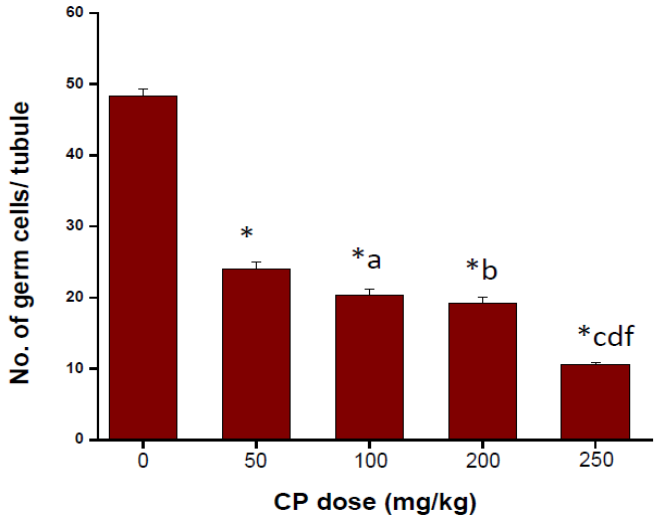

Figure 4: Effect of various doses of cyclophosphamide on the germ cell count in adult testicular tissue of mice at 7 day post-treatment interval

**p< 0.001 V/s control; [a]p< 0.05, [b]p< 0.01, [c]p< 0.001 V/s 50 mg;[d]p< 0.001 V/s 100mg; [f]p< 0.001 V/s 200mg*

When the germ cell count was assessed in testicular sections of mice at 28 days post CP administration, it was observed that in mice treated with 50 mg/kg of CP, the germ cells repopulated in the testis and was similar to that of control testis (Figure 5). However, in mice treated with CP above 100 mg/kg body weight, the germ cells were still significantly lower than the control and 50 mg/kg group ($p < 0.001$).

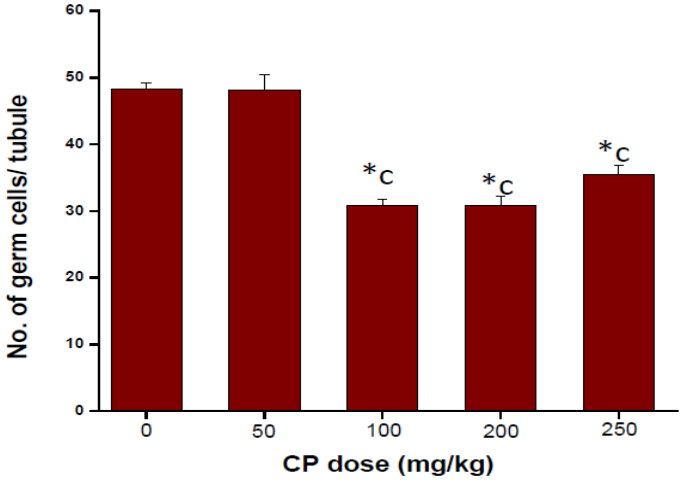

Figure 5: Effect of various doses of cyclophosphamide on the germ cell count in adult testicular tissue of mice at 28 day post-treatment interval

**p< 0.001 V/s control; [c]p< 0.001 V/s 50 mg/ kg*

Similarly, at 42 days after the CP administration, recovery in the CP-induced depletion of germ cells was even evident in the mice treated with CP up to 200 mg/kg (Figure 6). But in mice treated with 250 mg/kg of CP, the germ cells were still significantly lower than the control mice ($p< 0.001$) and the remaining CP treated mice ($p< 0.001$, $p< 0.01$, $p< 0.01$ compared to 50, 100 and 200 mg/kg dose respectively).

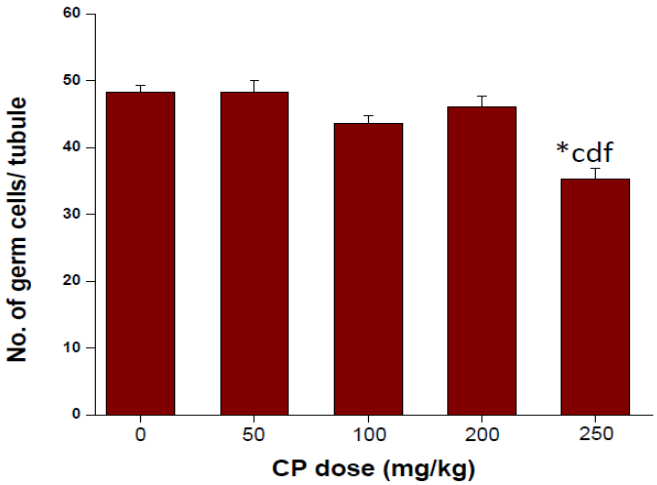

Figure 6: Effect of various doses of cyclophosphamide on the germ cell count in adult testicular tissue of mice at 42 day post-treatment interval

**p< 0.001 V/s control; [c]p< 0.001 V/s 50 mg;[d]p< 0.01 V/s 100mg; [f]p< 0.01 V/s 200mg*

Effect on spermatogenesis: Cyclophosphamide mainly targets the proliferating cells in the testis. In adult testis spermatogonial cells and spermatocytes are the proliferating cells. Hence, in this study to understand the effect of CP on spermatogenesis seminiferous tubules were assessed and classified as tubules having all the representative cells of the spermatogenic cycle and tubules lacking all the cell types of spermatogenic cycle.

At 7 day post treatment interval, in control testis all the tubules had complete spermatogenic cells (Figure 7). A dose-dependent increase in the tubules with incomplete spermatogenesis was observed in CP treated group indicating its adverse effect on spermatogenesis as early as 7 days after the treatment. In mice treated with 50 mg/kg of CP 14.05 ± 4.25 tubules were having partial spermatogenesis which further increased to 17.28 ± 3.51, 19.11 ± 0.53 and 20.41 ± 3.47 in 100, 200 and 250 mg/kg group respectively. All these groups had significantly higher number of tubules with incomplete spermatogenesis.

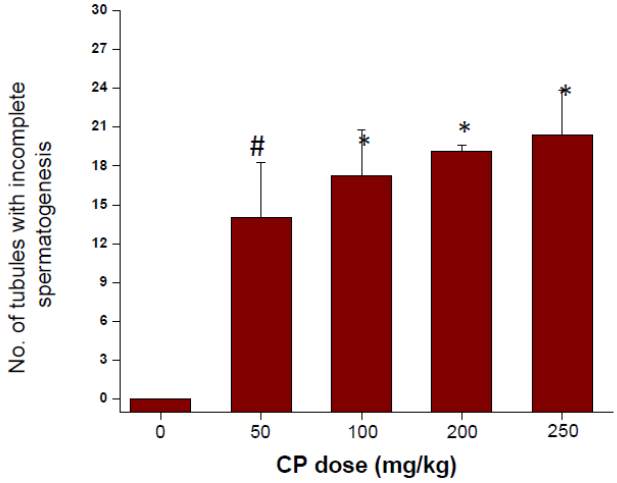

Figure 7: Incomplete spermatogenesis in seminiferous tubules of adult mice treated with various doses of cyclophosphamide at 7 day post-treatment interval

*[#]p< 0.05; *p< 0.001 V/s control*

At 28 day post-treatment interval a similar effect of CP was observed on the spermatogenesis. However, at lower doses (50 and 100 mg/kg dose), though the number of tubules with incomplete spermatogenesis was higher than in the control group, the difference was not statistically significant (Figure 8). In comparison to 7 day data, a decrease in the tubules with incomplete spermatogenesis was observed in these groups at this interval. This indicates that by this period of time the testicular tissue can partially overcome the adverse effects of lower doses of CP. However, at 200 and 250 mg/kg dose, even at this period a significantly higher percentage of tubules still had incomplete spermatogenesis ($p < 0.001$) compared to control as well as the lower doses ($p < 0.05$ and 0.01).

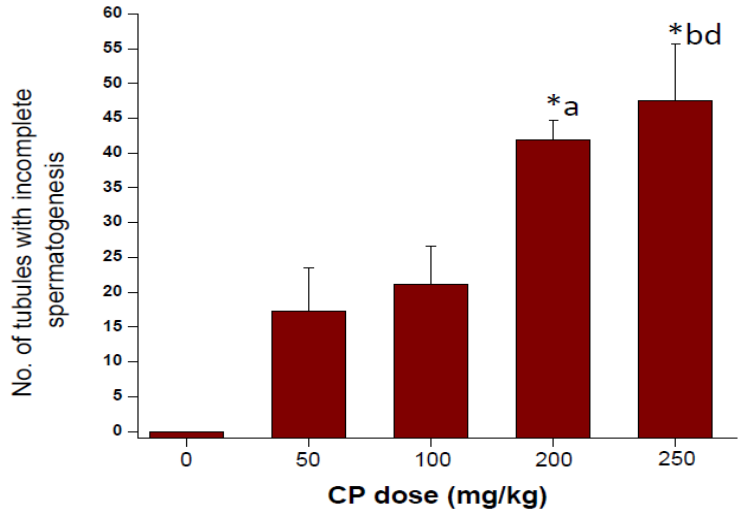

Figure 8: Incomplete spermatogenesis in seminiferous tubules of adult mice treated with various doses of cyclophosphamide at 28 day post-treatment interval

**p< 0.001 V/s control; [a]p< 0.05, [b]p< 0.01 V/s 50 mg;[d]p< 0.05 V/s 100*

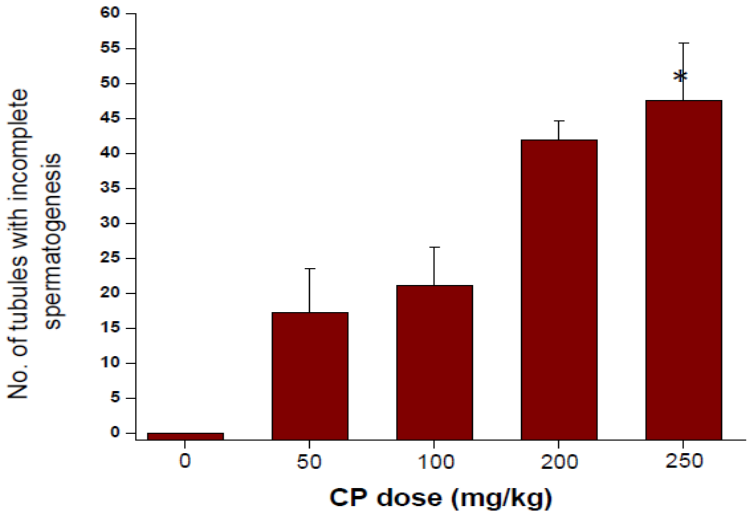

Figure 9: Incomplete spermatogenesis in seminiferous tubules of adult mice treated with various doses of cyclophosphamide at 42 day post-treatment interval

**p< 0.01 V/s control*

Similarly, at 42 day post-treatment interval, at lower doses (50 and 100 mg/kg dose), though the number of tubules with incomplete spermatogenesis was higher than in the control group, the difference was not statistically significant (Figure 9). In comparison to 7 day data, a decrease in the tubules with incomplete spermatogenesis was observed in these groups at this interval. This indicates that at lower doses by this period of time the testicular tissue can partially overcome the adverse effects

of CP. However, at 200 and 250 mg/kg dose, even at this period a significantly higher percentage of tubules still had incomplete spermatogenesis ($p < 0.001$) compared to control as well as the lower doses ($p < 0.05$ and 0.01).

In addition to this, the vacuolization and distorted histology was observed in testicular tissues of mice exposed to various doses of CP. Distorted histology was commonly observed in mice exposed to lower doses specifically at early intervals (Figure 11) while the vacuolated histology was common feature of mice exposed to higher doses of CP (Figure 10).

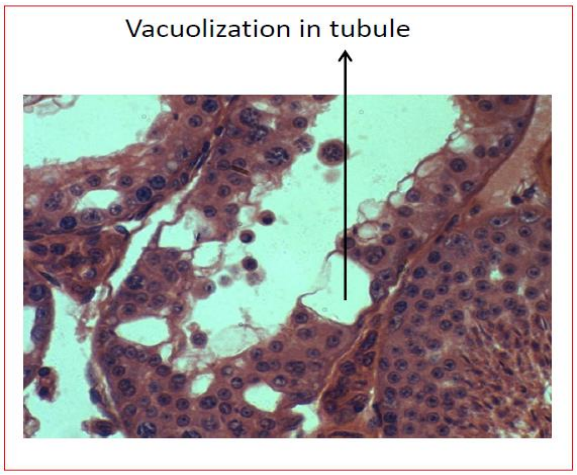

Image 3: Representative image to show vacuolization and incomplete spermatogenesis in testicular tissue of mice exposed to CP

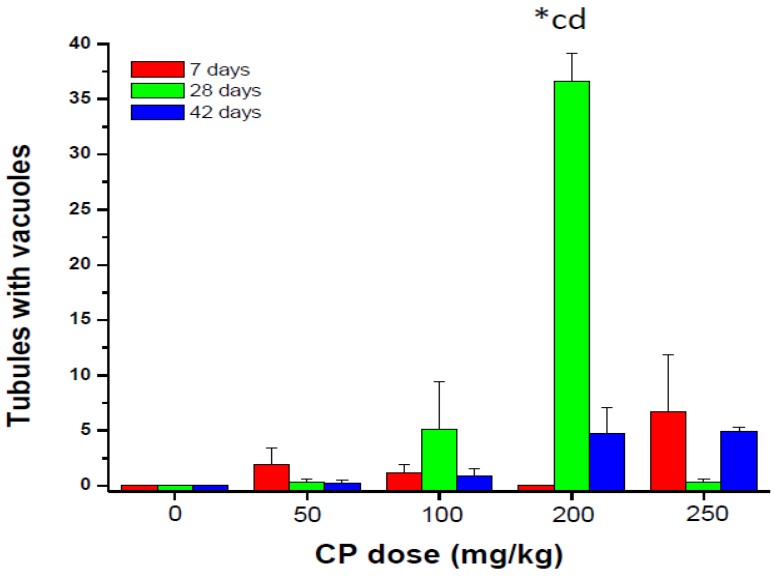

Figure 10: Vacuolization in testicular tissue of mice exposed to various doses of CP at different times intervals after exposure
** p< 0.001 V/s control, [c]p< 0.001 V/s 50mg, [d]p< 0.001 V/s 100mg, [f]p< 0.001 V/s 250mg*

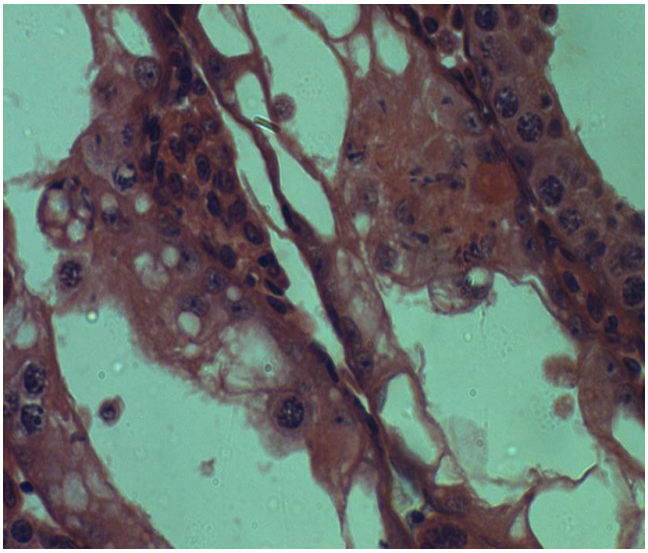

Image 4: Representative image to show distorted spermatogenesis in testicular tissue of mice exposed to CP

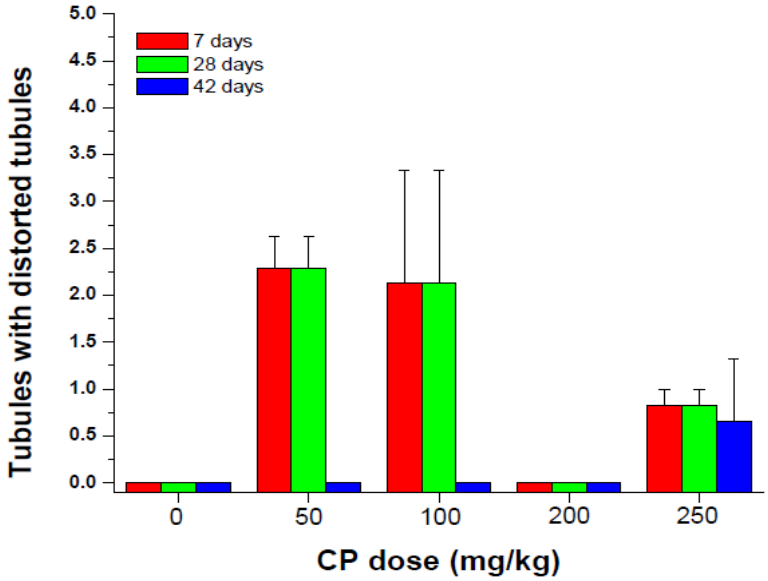

Figure 11: Distorted spermatogenesis in testicular tissue of mice exposed to various doses of CP at different times intervals after exposure

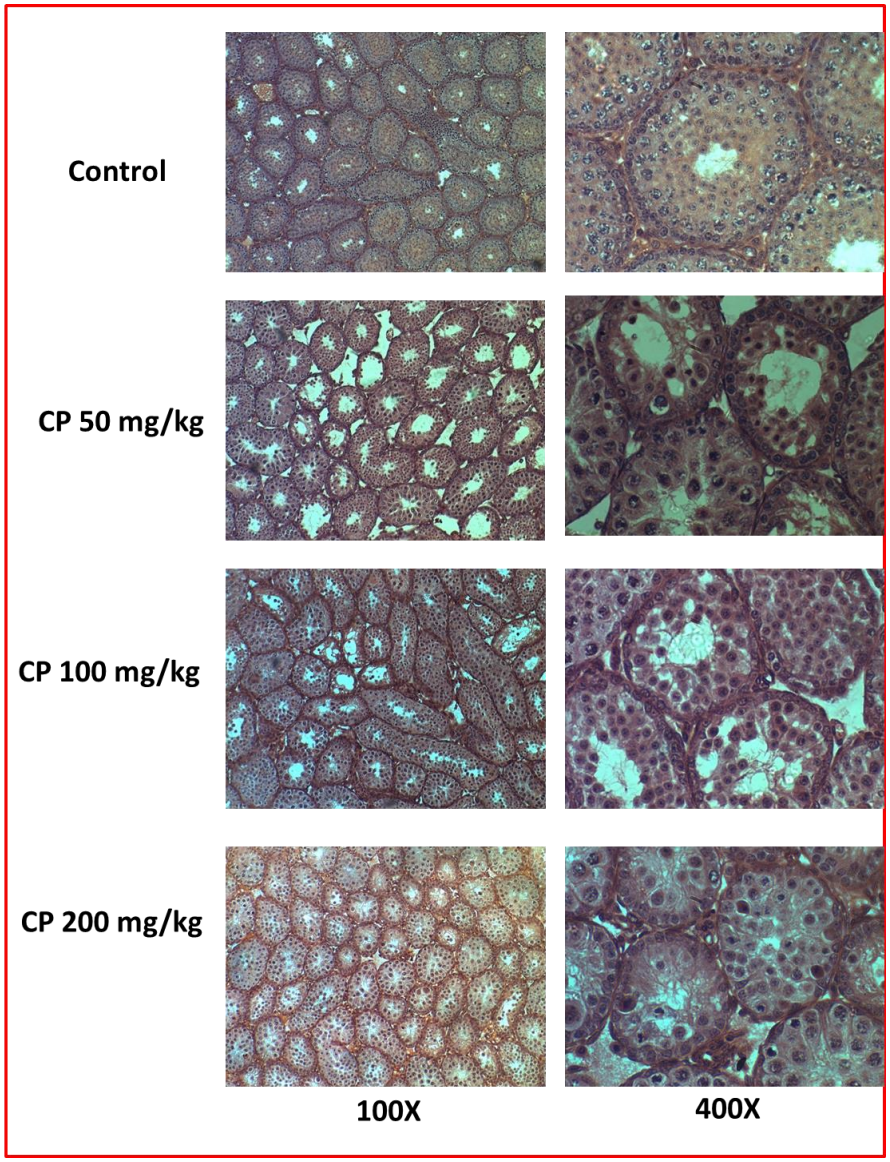

Image 5: Representative images showing histological changes in the pre-pubertal testis from mice treated with various doses of CP at 7 day post-treatment interval

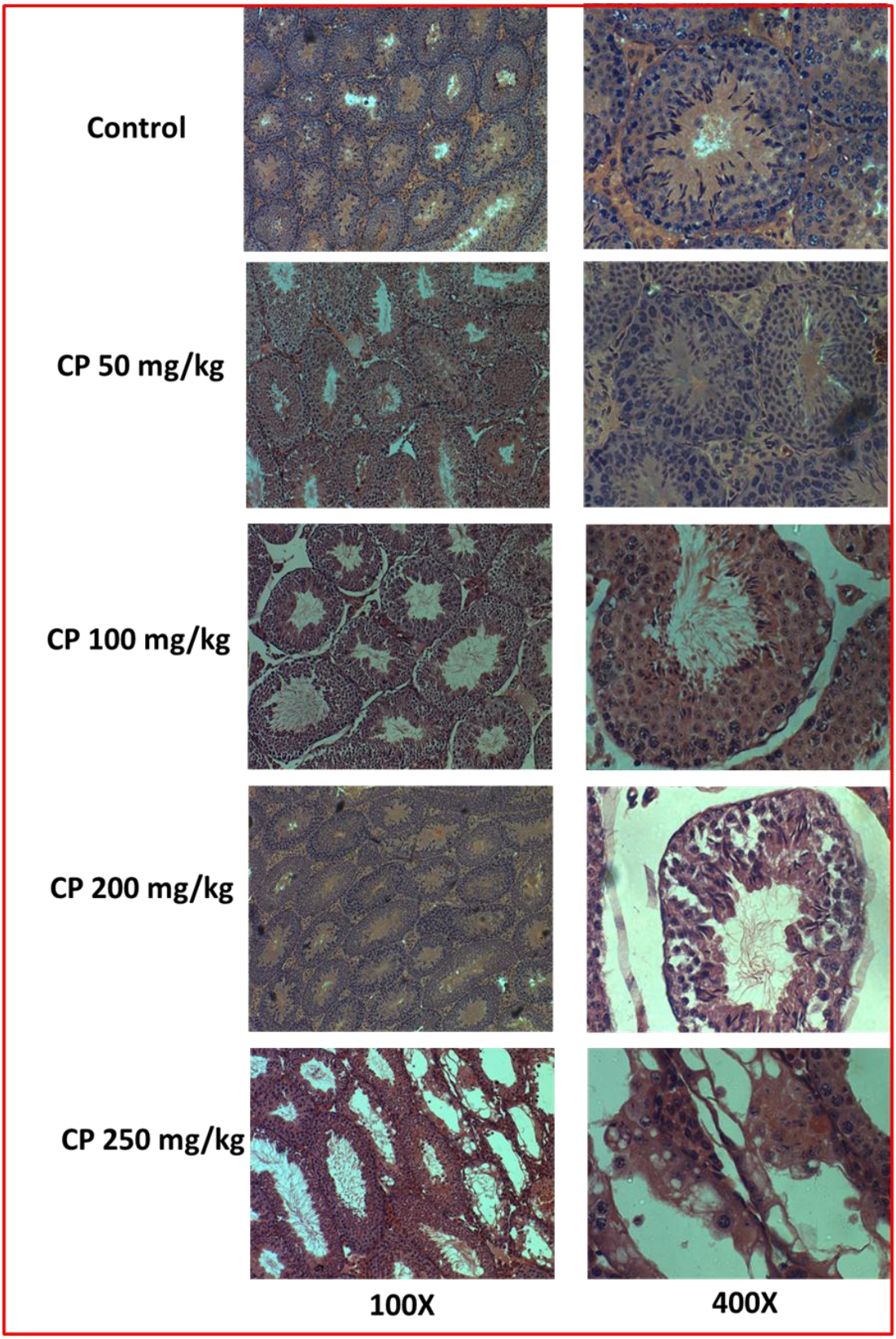

Image 6: Representative images showing histological changes in the adult testis from mice treated with various doses of CP at 7 day post-treatment interval

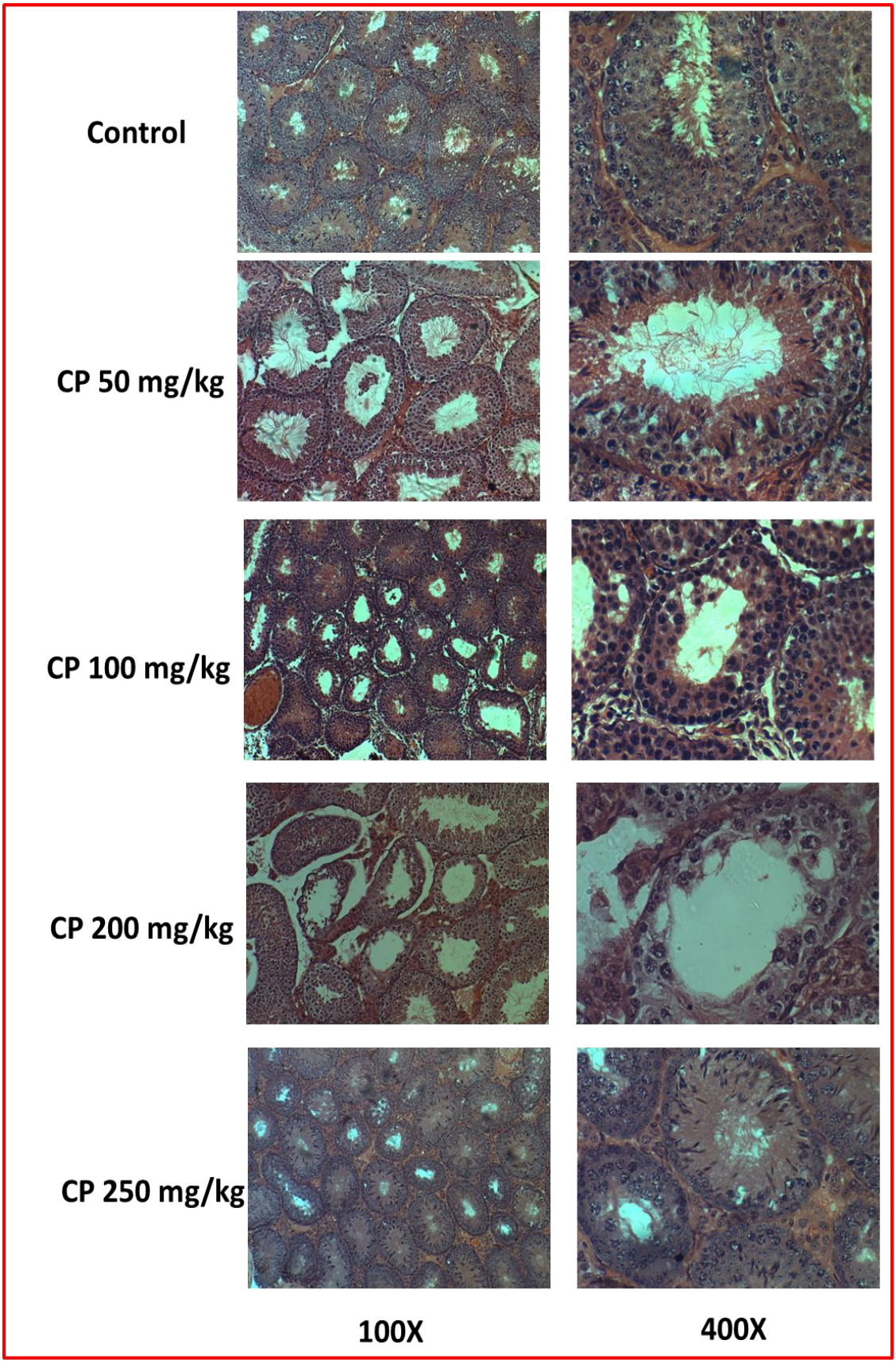

Image 7: Representative images showing histological changes in the adult testis from mice treated with various doses of CP at 28 day post-treatment interval

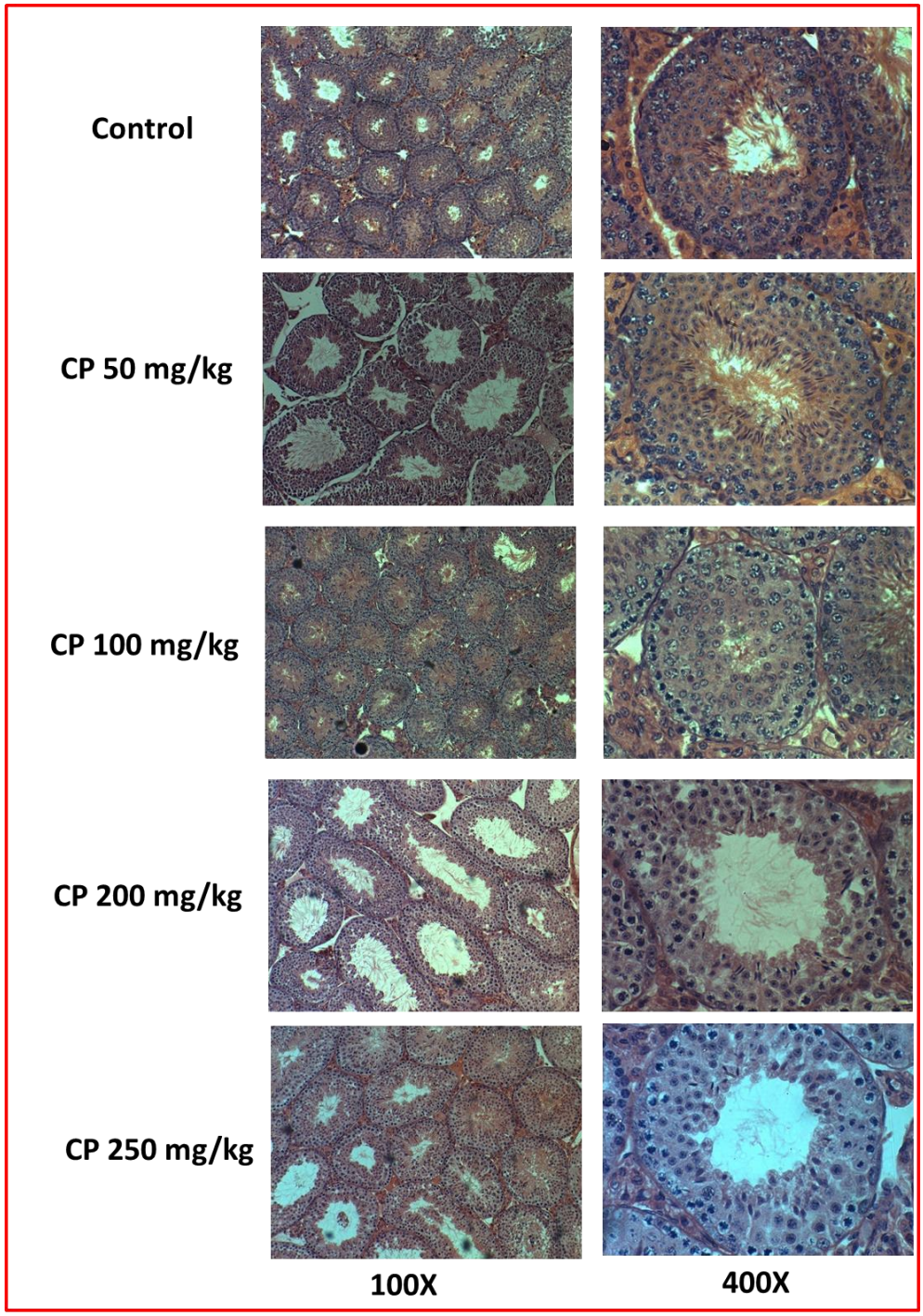

Image 8: Representative images showing histological changes in the adult testis from mice treated with various doses of CP at 42 day post-treatment interval

DISCUSSION

In the present study the histological changes induced by cyclophosphamide in testicular of pre-pubertal and adult mice was assessed. The testis was found to be more sensitive to cyclophosphamide-induced adverse effects. This phenomenon was true in both pre-pubertal and adult mice.

Earlier studies based on histological assessment of testicular tissue and measurement of basal follicle-stimulating hormone (FSH) levels from a small number of patient samples have suggested that the immature testis was relatively resistant to chemotherapy (2.6,7,8). On the contrary, more recent studies have demonstrated that both prepubertal and pubertal testes are

highly vulnerable to cytotoxic agents used in cancer therapy (9.10). The result of the present study agrees with these reports. Even at lowest dose (50 mg/kg), CP administration significantly depleted the germ cells at 7 day post-treatment interval. Similar observation has been made by earlier studies (11, 12).

In the adult mice, the exposure to 50 mg/kg of CP resulted in depletion of the germ cells from the testis. The depleting effect of CP increased with increase in CP dose. At 250 mg/kg, which is close to the LD_{50} value, the number of germ cells was as low as ~10 per tubule. Similar results were also obtained in previous studies on BALB/c mice by Cunha et al (1987), where the study showed decrease in differentiating spermatogonia followed by an orderly maturation depletion of later germ cell stages and repopulation from surviving cells post CP treatment. However, with increase in the time gap after CP administration, there was a gradual increase in the number of germ cells in CP treated mice compared to that of 7 day post-treatment interval.

The mechanism of action of CP is well understood. Under the influence of cytochrome P450 isoenzyme 2B, highly reactive secondary metabolites phosphoramide mustard and acrolein are generated in liver. They are known cause nucleotide base mispairs and DNA/DNA or DNA/protein cross-linking that lead to major disruptions in nucleic acid function and the inhibition of DNA synthesis (13). In addition, cyclophosphamide-induced nucleic acid damage can result in cell cycle arrest or cytotoxicity (14,15). CP also disturbs the redox balance in the cell by inducing free radical generation and depleting the cellular antioxidants (5,16). Therefore, it is obvious that the highly proliferating germ cells are depleted following CP treatment as observed in this study. Reduction in the testicular weight is generally attributed to the decreased germ cell population and sperm production (17). Study by Elangovan et al., (2006) showed that mice treated with higher dose (200mg/kg) of CP showed increased damaged and decreased number of spermatogonial cells in testicular sections. His results also showed that the spermatozoa in testis respond differentially to the damaging effects of acute and chronic cyclophosphamide exposure. Moreover, his study also showed that the size of the seminiferous tubule was decreased with increasing concentration of CP. Similar findings were also seen in few other studies (16,18).

At 42 day post treatment interval, which marks one complete spermatogenic cycle in mice, except at 250 mg/kg dose group, the germ cell count in CP treated group did not differ compared to control tubules. This suggests that the testicular tissue was able to repopulate the germ cells depleted by CP. The spermatogonial cells can mitotically divide to keep adequate number of germ cells in the testicular compartment. Such response in testis after CP administration is well documented in the literature. Previous studies have shown that the sperm count recovers in mice following CP administration even though the quality remains poor (19,20). The repopulation of the germ cells in testis and the spermatogenesis is directly related to the dose of CP administered, treatment schedule followed and the recovery period given after the CP insult (21). In this study, single dose of CP was administered, which was sufficient to cause histological changes in the testis of pre-pubertal and adult mice.

The adult testicular architecture is properly defined with spermatogenic cells at different stages of spermatogenesis with fixed kinetics. Therefore, it is easy to understand and identify the different stages of spermatogenic cells in the sections of seminiferous tubules. The seminiferous tubule with incomplete spermatogenic cells is a most common feature of testicular histology following gonadotoxic treatment. Even though these cells have differential sensitivity to the cytotoxic insult, depending upon the dose and the treatment schedule, apart from the germ cells, even the

spermatocytes are depleted. In the present study the testicular sections revealed significant depletion of spermatogonia and spermatocytes which was evident even after 42 days of gap. The focal spermatogenesis was observed in majority of the tubules from testicular tissues of mice exposed to higher doses of CP. Similar observations were made from earlier studies. The results obtained in our study correlates with previous study Cunha et al., (1987), where the cytotoxic effects of CP recovered spermatogenesis and was observed in testicular sections taken 57 to 71 days post CY treatment. At this time, recovery of all germ cell types in most, but not all, tubules indicates that numerous stem spermatogonia survive the cytotoxic effects of CP, which was similar to our results taken 42 days post CP treatment.

Studies done by Hafez et al., (2006) states that the interstitial spaces of high dose treated groups were widened and filled with thickened and congested blood vessels, with moderately edematous interstitial tissue, which was also noticed in testicular sections in present study. Vacuolation and distortion of tubules was additional feature noticed in testicular sections of higher doses of CP, these findings are similar to the findings obtained in a previous study (18). Moreover, the oedematous vacuoles together with the abnormal aggregation of some late spermatids were explained by El-Hafez, (1980) to be due to the disturbance in the spermiogenesis and the tubule itself has lost its power of contraction to push the mature spermatids into the lumen. Some authors have found the formation of multinucleated giant cells in the testicular lumen of treated groups. The rise in these cells was said to be indicative of retarded cell maturation and differentiation at some stages of spermatogenesis (22).

CONCLUSION

The experimental design on male Swiss albino mice was to throw light on dose and time dependent toxic effect of one of the most commonly used anticancer drug-cyclophosphamide, on testicular histology of pre-pubertal and adult age group. From the results of the present study it is clearly evident that cyclophosphamide affects the male reproductive system. Specifically, the testicular histology is altered considerably in a dose-dependent manner. Pre-pubertal and adult testis tissue shows similar response to the toxic effects of cyclophosphamide indicating its possible consequences on the testicular function. The histological changes in the adult testis are reversible at the lower doses. However, the quality of the spermatozoa produced and its influence on the fertility potential are still under experiment.

ACKNOWLEDGEMENT

I wish to acknowledge the Department of Anatomy and Department of Clinical Embryology, Kasturba Medical College and Hospital, Manipal,for their support and training me during the study period.

Conflict of Interest: None

Source of Funding: None

Ethical Approval: Approved

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How to cite this article: Supriya, Kalthur SG, Kalthur G et.al. Cyclophosphamide induced histological changes in testis of Swiss Albino Mice. *International Journal of Research and Review*. 2022; 9(1): 276-290. DOI: *https://doi.org/10.52403/ijrr.20220133*

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