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CHLORAMBUCIL -TREATMENT INDUCED OXIDATIVE STRESS IN TESTICULAR TISSUE OF SWISS ALBINO MICE

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ABSTRACT
Chlorambucil (CLB) is an alkylating agent used against chronic leukemia. This study was carried out to investigate the adverse effect of chlorambucil treatment on testicular tissue of mice. Twenty Swiss albino mice were randomized divided into four groups (n=5). Group 1 served as control received vehicle while 2, 3 and 4 groups received 1mg/kg, 3mg/kg and 5mg/kg body weight of chlorambucil (CLB) respectively for 28 days via intraperitoneal injection. Testosterone and Luteinizing hormone were estimated. Oxidative and anti-oxidative biomarker enzymes such as lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) levels were evaluated. Effect on sperm parameters was carried out. The results showed that treatment with chlorambucil at 3mg/kg and 5mg/kg caused a significant increase at $p>0.05$ in the luteinizing hormone and testosterone levels. CLB at 3mg/kg and 5mg/kg body weight caused significant increase in LPO with simultaneous decrease in CAT, SOD and GSH levels. The findings revealed that chlorambucil treatment resulted in generation of oxidative stress in the testicular tissue of treated mice in dose dependent manner.

KEYWORDS: Chlorambucil, Oxidative stress Testis, Mice, Reproductive toxicity.

INTRODUCTION
Chlorambucil, an anticancer drug used in the treatment of Non-Hodgkin lymphoma, is also used as an immunosuppressive drug for various autoimmune and inflammatory conditions such as nephritic syndrome. Chlorambucil acts by interfering with DNA replication, damaging the DNA leading to cell arrest and cellular apoptosis.[1] Chlorambucil is also classified as an alkylating agent with severe toxic effects. Exposure to this cytotoxic chemotherapy compound has been reported to lead to deleterious effects in most vital organs due to its inability to differentiate between normal cells from cancerous cells. As most chemotherapy drugs are have been reported to lead to dysfunction of vital organs and have lasting effects on reproductive health, it was thought necessary to evaluate the possible adverse effect of chlorambucil on reproductive system of male mice; an excellent model to monitor the potential damage induced by chemical agents.[2]

MATERIALS AND METHODS
Dose Grouping
Male swiss albino mice weighing about 20 g maintained under standard conditions of humidity (50 ± 5%), temperature (25 ± 2^{o}C) and dark and light cycles (12 h each) with free access to food and water were randomlyselected into 4 groups (n=5). Group 1 served as control while 2, 3 and 4 groups received 1mg/kg, 3mg/kg and 5mg/kg of Chlorambucil compound prepared in Dimethysulfoxide (DMSO) via intraperitoneal injection respectively.

Tissue samples for biochemical studies
At autopsy, reproductive organ mainly, testes was removed; blotted free of blood and adhering tissues and stored at -20^{0}C for further analyses. Second part of testicular tissue was fixed in 10% formal saline for microscopy analyses.

Tissue Biochemistry of Testicular Homogenates
10% testicular homogenates (w/v) was prepared in chilled 100 mM Tris-HCL buffer (pH 7.4) using Cole Parmer tissue homogenizer and the homogenate was used to measure Reduced Glutathione (GSH), lipid peroxidation, superoxide dismutase (SOD) and catalase (CAT) levels were also measured according to the standard protocols.[3,4,5,6] Protein contents in the samples were estimated by the Lowry's method using bovine serum albumin as standard.[7] All the parameters were expressed as per mg protein.

Lipid Peroxidation (LPO) Level
The lipid peroxidation was estimated by a spectrophotometric method in terms of thiobarbituric

acid reactive substances. Briefly, one volume of homogenate was mixed with two volumes of stock solution (15% w/v trichloroacetic acid in 0.25 N HCL and 0.375% w/v thiobarbituric acid in 0.25 N HCL) in a centrifuge tube, vortexed and heated for 15 min at 95^{o}C in water bath. The mixture was cooled and centrifuged at 5000 rpm for 5 min and the absorbance of the supernatant was read at 532 nm.[8]

Superoxide dismutase (SOD) level

Superoxide dismutase (SOD) was measured using modified method of Kakkar *et al.*, (1984).[9] Assay mixture containing sodium pyrophosphate buffer (pH-8.3, 0.052 M), nitroblue tetrazolium (300uM), NADH (780 uM) and appropriately diluted enzyme in total volume of 3 ml was incubated at 37^{o}C for 90 sec. The reaction was stopped by the addition of glacial acetic acid. The reaction mixture was missed vigorously by adding in n-butanol and was allowed to stand for 10 min before the collection of butanol layer. The absorbance was measured at 560 nm. The SOD activity was calculated in units/ml/mg protein.

Catalase (CAT) level

Catalase was measured by the method described by Aebi (1983).[4] Assay mixture consisting of 0.01 M phosphate buffer (pH-7.0), 0.2 M hydrogen peroxide (H_2O_2) and tissue homogenate was incubated at 37^{o}C for 1 min. the reaction was stopped by adding of potassium dichromate (5% w/v) and acetic acid. The remaining hydrogen peroxide was determined by measuring chromium acetate after heating the assay mixture in a boiling water bath for 15 min. The absorbance was read at 570 nm against .The enzymatic activity was measured in µMol/min/mg protein.

Glutathione (GSH) level

Glutathione (GSH) activity was estimated by centrifuging an aliquot of 10% homogenates of the tissues in 100 mM Tris-HCl buffer (pH-7.4) containing 0.16 M KCl at 1000 x g for 5min. the supernatant was used to measure the rate of reduction of 5'5'- dithiobis – (2 nitrobenzoate) to 2-nitor-5 thiobenzoate. The absorbance was taken at 412 nm. Glutathione content was expressed in µM/mg protein.[10]

Hormone Assay

The testicular testosterone and luteinizing hormone levels from each group were measured.[11] Briefly, testicular proteins were extracted with phosphate buffer (50 mM, pH -7.4) and centrifuged at 10,000 g for 20 mins, the supernatant was used to estimate Testosterone and LH levels using ELISA and were expressed in ng/ml.

Sperm Motility, Count and Morphology

Cauda epididymidis was removed from each mouse and cleaned off from the epididymal fat pad, and minced in a pre-warmed Petri dish containing 500 µl phosphate buffer saline solutions (PBS, pH – 7.4) at 37^{o}C. Sperm motility was estimated by putting a drop of sperm suspension on a clean slide and covered with a cover slip and analyzed by the computer assisted sperm analyser (CASA) by Hamilton Throne. The motility was expressed as percentage incidence.[12] For Sperm count, an aliquot of this suspension was charged into the Neubauer's counting chamber and the spermatozoa were counted under light microscope. Total sperm count was calculated as the average of the spermatozoa count (N) in each chamber X multiplication factor (10^6) X dilution factor and was expressed in millions/ml.[13] The sperm morphology was also evaluated (2)(Wyrobek & Bruce, 1975). Briefly, smear of sperm was made on a clean slide and stained with haematoxylin and eosin and were examined under a light microscope with an oil immersion lens. The morphology of spermatozoa was scored.[14]

Statistical analysis

All statistical comparisons between the groups were made using analysis of variance (ANOVA) by prism statistic software. Results were presented asmean ± SEM (Standard Error Mean). Values of $p < 0.05$ were considered as statistically significant.

3.0 RESULTS

Effect of Chlorambucil treatment on luteinizing hormone level

Luteinizing level of mice exposed to chlorambucil showed an increase in all groups in dose dependent manner. The effect was significantly high ($p<0.05$) at 5mg/kg when compared with the control.

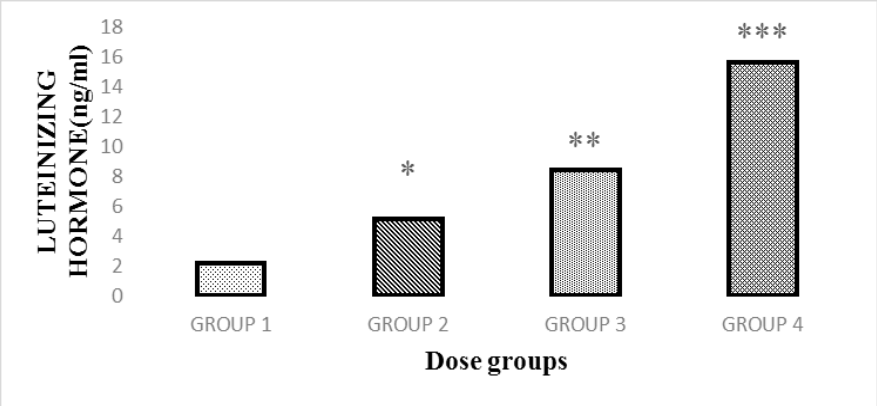

Figure 1: **Bar chart showing effect of chlorambucil treatment on the level of luteinizing hormone in the testis of mice. Data represents the mean ± and significant changes when compared with mice in control group 1. * (p<0.05), ** (p< 0.01), *** (p<0.001).**

Effect of Chlorambucil treatment on Testosterone level

Testosterone level of mice exposed to chlorambucil treatment shows a decrease in all groups in dose dependent manner. The effect was significantly low (p<0.05) 5mg/kg when compared with the control.

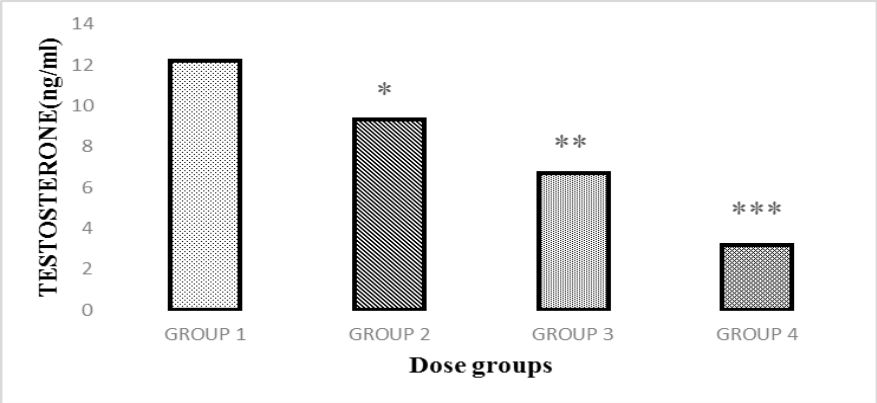

Figure 2: **Bar chart showing effect of chlorambucil treatment on the level of testosterone in the testis of mice. Data represents the mean ± and significant changes when compared with mice in control group 1. * (p<0.05), ** (p< 0.01), *** (p<0.001).**

Sperm motility, count and morphology

Chlorambucil treatment caused significant decrease (p <0.001) in epididymal sperm count and motility (Figures 3 & 4) across the chlorambucil treatment groups. A marked increase (p <0.01) in abnormal morphology was observed in epididymal spermatozoa (Figure 5) in all chlorambucil treated mice.

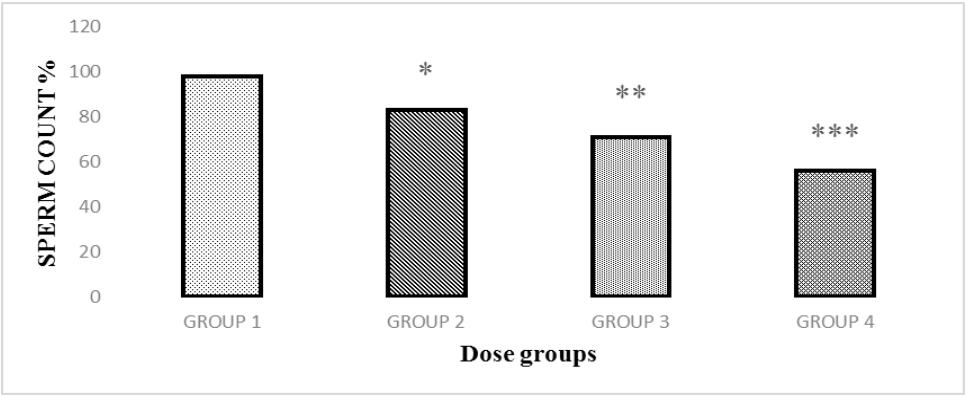

Figure 3: **Bar chart showing effect of chlorambucil treatment on the Sperm count of mice. Data represents the mean ± and significant changes when compared with mice in control group 1. * (p<0.05), ** (p< 0.01), *** (p<0.001).**

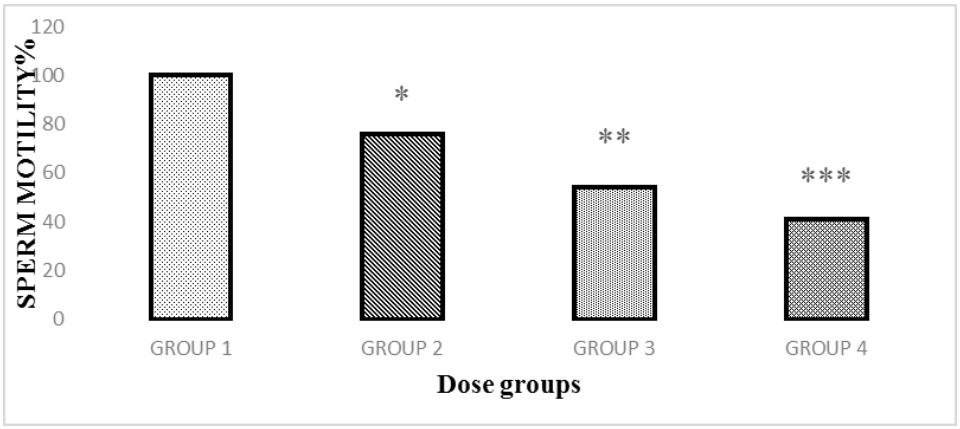

Figure 4: **Bar chart showing effect of chlorambucil treatment on the Sperm motility of mice. Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), *** (p<0.001).**

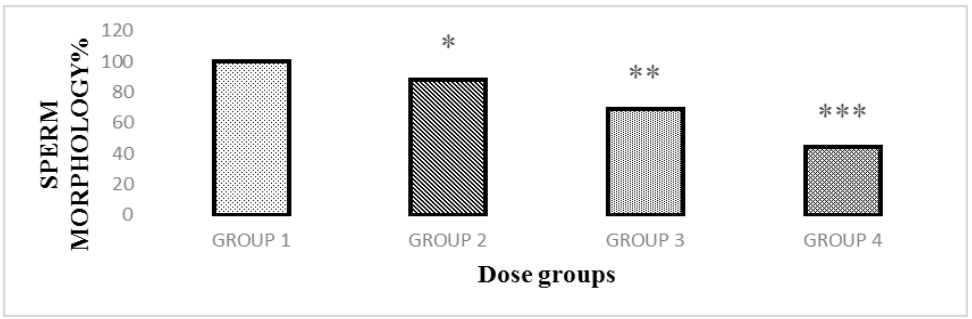

Figure 5: Bar chart showing effect of chlorambucil treatment on the Sperm morphology of mice. Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), * (p<0.001).**

Testicular Enzymes activities

Chlorambucil treatment resulted in significant increase (p <0.01) in the concentration of thiobarbituric acid reactive substances in testis tissue homogenate of mice. Chlorambucil induced depletion in the protein level in testis in a dose dependent manner. Oxidative stress was induced by Chlorambucil treatment as confirmed by the significant decrease (p <0.01) in GSH, CAT, and SOD levels in the testicular tissues (Figure 6, 7, 8 & 9). However, significant increase (p <0.01) in reduced glutathione (GSH) was observed in the epididymis of mice following withdrawal of Chlorambucil treatment.

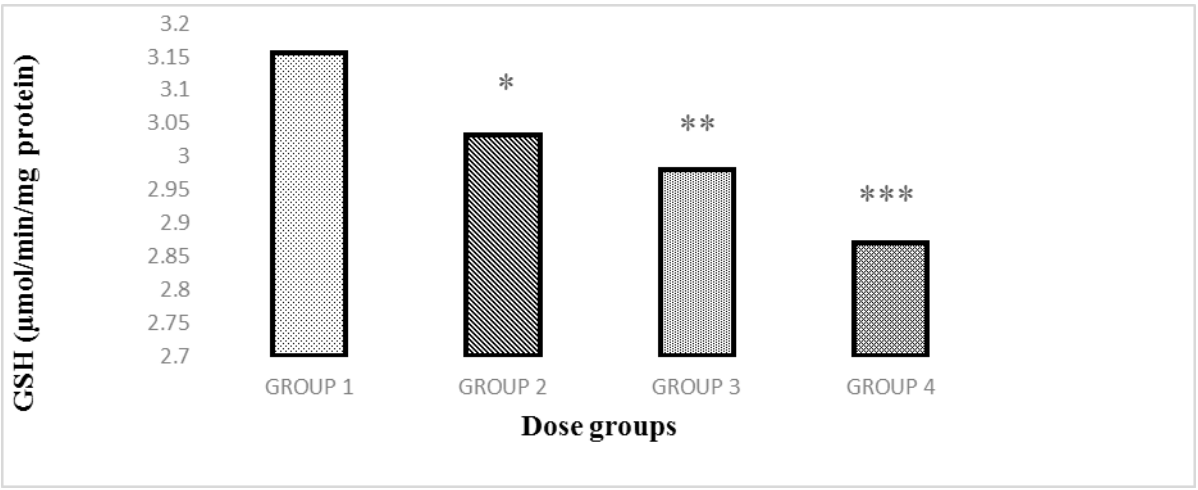

Figure 6: Bar chart showing effect of chlorambucil treatment on reduced glutathione level (GSH). Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), * (p<0.001).**

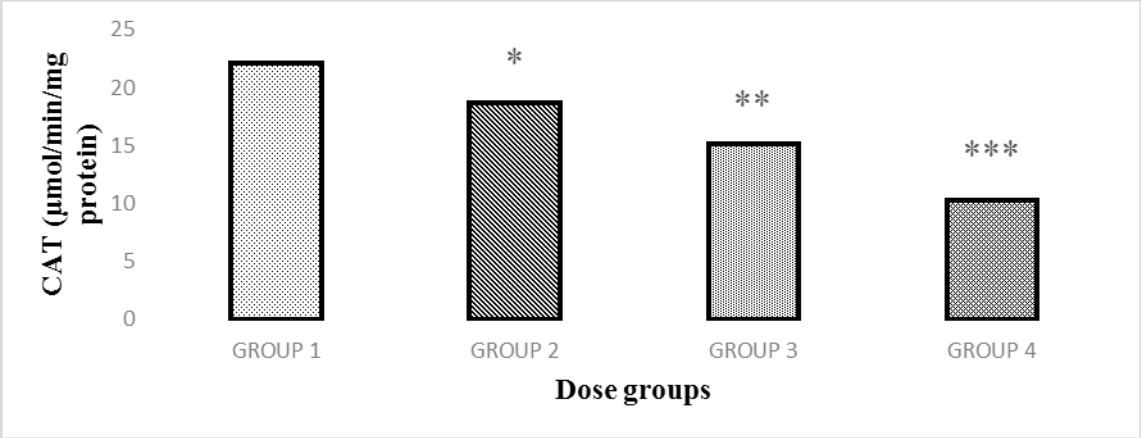

Figure 7: Bar chart showing effect of chlorambucil treatment on Catalase (CAT) level. Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), * (p<0.001).**

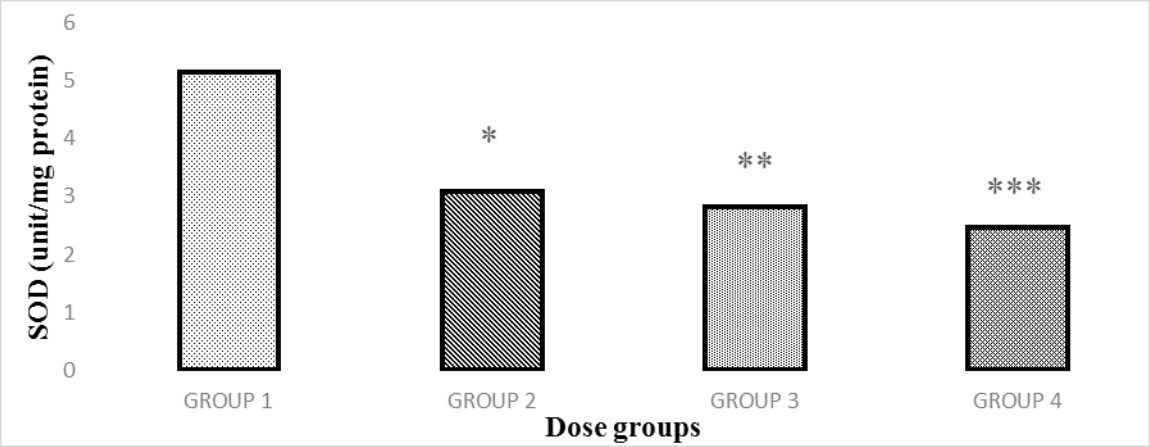

Figure 8: Bar chart showing effect of chlorambucil treatment on Superoxide dismutase (SOD) level. Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), * (p<0.001).**

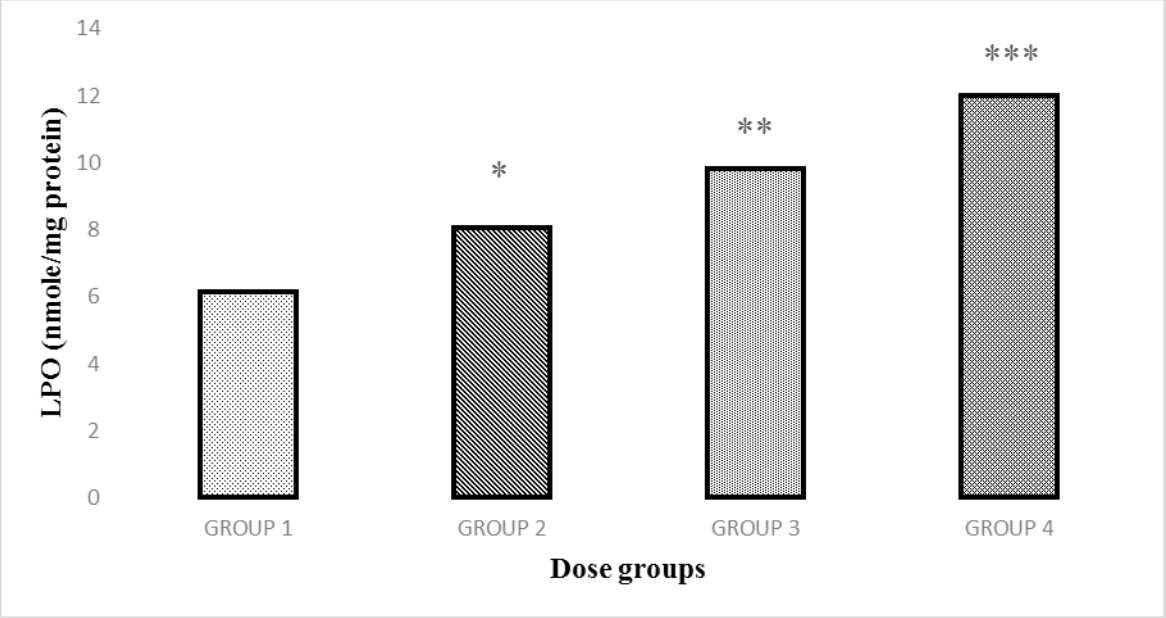

Figure 9: Bar chart showing effect of chlorambucil treatment on Lipid peroxidation. Data represents the mean ± and significant changes when compared control group 1. * (p<0.05), ** (p< 0.01), * (p<0.001).**

DISCUSSION AND CONCLUSION

Number of factors have been said to greatly increase the quality and quantity of sperm production and infertility risks which include chemotherapy drugs for cancer, antibiotics and toxic substances etc. These factors resulted in generation of reactive oxygen species and oxidation of germ cells in the testis leading to reduce sperm concentration.[15,16] Testis is the major organ for male sexual development and fertility. It secretes hormones to promote male-specific traits and produces sperm for reproduction. Several studies indicate that the human male reproductive capacity has deteriorated considerably during the past few decades .This decreasing trend in male fertility has led to speculation that recent environmental, dietary and/or lifestyle changes are interfering with a man's ability to produce spermatozoa. In view of this, present study was designed to investigate effect of chlorambucil, an anticancer drug on testis of mice *in-vivo*. This study revealed that the treatment of mice with chlorambucil increased the level of luteinizing hormone however, decrease level of testosterone was seen indicating that Chlorambucil interference with androgen supply, a similar finding was also reported in mice after treatment with salinomycin compound.[17]

Superoxide-dimutase and catalase are the two major radical scavenging enzymes. SOD is the main enzymatic defense against the superoxide anion. This enzyme detoxifies the superoxide anion, thus converting it into H_2O_2 and water. A decreased in SOD activities was observed in the testes treated with chlorambucil. The significant decrease in the level of SOD in mice treated with chlorambucil revealed that the testicular tissue is undergoing oxidative stress. Catalase is a heme protein that catalyzes the decomposition of hydrogen peroxides and protects tissues from hydroxyl radicals. A decreased in catalase level was observed in all the chlorambucil treated groups in dose dependant pattern. Cellular systems are sheltered from cell damages caused by reactive oxygen species (ROS) by various defenses consisting of antioxidants with diverse functionalities. When the ROS present in the cellular system subdue these defense system, oxidative stress is induced, and this could result in cellular injuries and ultimately development of diseases.

A decline in Glutathione level was observed in testis of chlorambucil induced rats. The decrease in GSH level may probably be due to increased formation of lipid peroxides on chlorambucil exposure testis. GSH is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by glutathione.

Decreased mobility of spermatozoa is mostly due to some deformity of the sperm. It can be caused by a disorder in the production of sperm. The sperm motility of mice in the treated groups in this study was significantly ($P<0.05$) reduced thus showed that the chlorambucil affect the motility ability of spermatozoa by causing deformation of the cells and rendered them less motile. The present study demonstrated that chlorambucil disrupted epididymal sperm quality. chlorambucil treatment groups resulted in decrease of epididymal sperm motility parameters and increases the abnormal sperm morphology. Sperm motility depends on mitochondrial functions and thus spermatozoa having adequate mitochondrial integrity should have robust motility. Mitochondrial damage during chemical exposure could be one of the major reasons for reduced sperm motility. Mitochondrial dysfunction can result in the formation of ROS.[1] Alteration in the mitochondria membrane potential is known to be a major cause of precipitation of apoptosis in many cell types.

Sperm count is one of the most sensitive tests for spermatogenesis, since it gives the cumulative result of all stages in sperm production, and it is highly correlated with fertility.[18] Result showed that chlorambucil is cytotoxic to the spermatozoa since it decreases the sperm count significantly in a linear manner. The decrease in the sperm count in the present study may be due to the decreased levels of intra testicular testosterone because testosterone level is directly linked to spermatogenesis.[19] It is also possible that the Sertoli cells might have been affected and the other possibility might be due its effect on the epididymal function. According to Russel and Russel, (1996)[20] male germ cells are very ideal and easy for the study of the genotoxicity of drugs since they exist in different phases of cell development and differentiation. Chlorambucil treatments resulted in more than double the percentage of abnormal sperms, hence it could be considered as a mutagen. Currently it is widely accepted that the induction of sperm abnormality mainly takes place through point mutation. Therefore, it is possible that change in the sperm structure might have been due to point mutation. Conclusively, based on the available data, it can be said that exposure to chlorambucil lead to oxidative stress in the male reproductive organs mainly testis and causes disruption of the normal spermatogenesis. chlorambucil impaired spermatogenic process by inducing oxidative stress testis thereby disrupt spermatogenesis.

COMPETING INTERESTS- The author has declared that no competing interests exist.

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