Research Paper

Volume : 5 | Issue : 4 | April 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Cyclophosphamide Induced Testicular Toxicity – A Comparison Between Acute and Subchronic Doses

Medical Science

KEYWORDS : Sperm abnormality, Sperm count, Motility, Gonado-somatic index

Sujayraj R.S	Dept. of Post-Graduate Studies and Research in Biosciences, Mangalore University, Mangalagangothri – 574 199, Karnataka, India.
*** Prashantha Naik**	Dept. of Post-Graduate Studies and Research in Biosciences, Mangalore University, Mangalagangothri – 574 199, Karnataka, India. * Corresponding author
Vishma B.L	Dept. of Post-Graduate Studies and Research in Biosciences, Mangalore University, Mangalagangothri – 574 199, Karnataka, India.

ABSTRACT

Cyclophosphamide (CP), a broad spectrum anticancer drug has been evaluated for its toxic potency on male reproductive system comparing between acute and sub-chronic therapeutic doses. Two groups of Swiss albino mice, one representing the acute and the other one sub-chronic group, were treated i.p. with CP at 50mg/kg and 15mg/kg/week for consecutive 5 weeks, respectively. 35-days post treatment sampling was done after the first administration of CP, by collecting the semen from both the cauda epididymides. The semen samples were analyzed for sperm abnormality, count and motility, by following the standard protocols. Gonado-somatic index, i.e., ratio of the body weight to testes weight was also determined. Both the acute and subchronic doses of CP induced the adverse effects on reproductive system in terms of high frequency of abnormal sperm, reduction in sperm count and motility at the significant levels when compared with the solvent control ($p<0.001$). Comparison of the results between acute and subchronic treated groups indicated that the magnitude of the CP-induced effects on sperm morphology, count and GSI ($p<0.05$) was statistically found to be higher in the latter group. Further, the effect of subchronic dosing was statistically found to be more on the sperm motility ($p<0.01$). Thus, subchronic dosing of CP even at a lower dose imparts more toxic effects on the testes and spermatogenesis than that of the acute higher dose treatment.

1. Introduction:

Toxicological studies prior to release of commercial agents, particularly for pharmaceutical products are important for their safer utility and FDA approval. In preclinical analysis of pharmaceuticals, study of various factors affecting the toxicity and magnitude of the effects is essential for clear cut understanding of the pharmacokinetic features of the agents. Among various factors, dose and treatment regimen are the most important ones as they determine the therapeutic as well as side effects of the drugs.[1] Cyclophosphamide with varied trade names such as Endoxan, Cytoxan, Neosar, Procytox, Revimmune, Cycloblastin is commonly used in chemotherapy for many cancers, and also for autoimmune disorders, light-chain (AL) amyloidosis and certain renal diseases.[2] Depending upon type of the cancers/disorders, patient conditions, therapeutic efficiency required, CP is administered either acute or subchronic at different doses. On the other side, in spite of potential therapeutic property, CP is also known for numerous side effects, including reproductive toxicity. Considering its potential toxicity on fertility of patients suffering from cancer, cryopreservation of semen samples prior to chemotherapy is routinely carried out as a part of Assisted Reproductive Toxicology (ART). CP-induced adverse effects on male reproductive system is well known based on many clinical studies. [3,2,4] and laboratory experiments [5,6,7] There are some reports focusing on CP for its testicular toxicities. Aguilar-Mahecha[5] evaluated the effects of acute and chronic CP treatment on meiotic progression and the induction of DNA double-strand breaks in rat spermatocytes. CP has been evaluated for its adverse effects on male reproductive system considering both dose and time factors in rats [8], and dose-response relationship in mice. [7,9] Juma et al.[10] made a comparative analysis of intravenous and oral administration of CP for its pharmacokinetics and alkylating activity in man. CP has been evaluated for its side effects in different dosage schedules in breast cancer patients.[11] Though reports on CP-induced testicular toxicities and protective effects against CP-induced adverse effects on reproductive system[12,13,14] are plenty, studies for comparison between acute and sub-chronic doses are very limited. Therefore, the present study was taken up to evaluate the adverse effects of CP on spermatogenesis at its acute and subchronic doses, using Swiss albino mice as the test system.

2. Material and Methods:

2.1 Chemicals:

Cyclophosphamide (Cycloxan, CAS No. 50-18-0;Batch No. KB 9124003, manufactured by Biochem Pharmaceutical Industries LTD. Mumbai, India) was used as the test chemical. Eosin-Y stain, Phosphate buffered saline (PBS), were procured from SRL, India.

2.2. Experimental Animals:

Swiss albino mice, *Mus musculus* species (8–10 weeks old; 30±2 g body weight), bred and maintained in the animal house of Biosciences department, Mangalore University were used as the experimental model. A total of five healthy male mice were used for each treatment and control group. Experimental animals were maintained in a good hygienic condition in an air-conditioned room at a temperature of 24^0C ($\pm1^0$C) with 12 h light/dark cycle and 50±5% humidity. They were fed with standard mice pellets (Lipton, India) and water *ad libitum*. Animals were taken care and handling of the animals were in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.[15] Experimental animals were sacrificed by cervical dislocation and dissected to collect the testes and cauda epididymides; the latter was processed for certain semen parameters. The study was undertaken with the prior approval from the Institutional Animal Ethics Committee (Ref. No. MU/AZ/99/2013-14/IAEC, dt.2.4.2013).

2.3. Dose and Treatment Schedule:

Three groups of animals were taken, one for acute (50 mg/kg b.wt.), another for sub-chronic (15 mg/kg/week for 5 consecutive weeks), and the other one served as the negative control (2.0 ml distilled water). The selection of CP dose was based on the therapeutic dosage (http://www.rxlist.com/cytoxan-drug/indications-dosage.htm). The selected doses of CP were dissolved in distilled water taken as the vehicle control and administered intraperitoneally (i.p.) in 0.2 ml quantity, and 35-days post treatment sampling was done. In case of subchronic dosing, first treatment of CP was considered as the day zero and dissection was done on 35^{th} day.

2.4. Sperm Abnormality Assay:
Animals were sacrificed by cervical dislocation; the testes were dissected out and weighed. Both the cauda epididymides were removed and placed in a watch glass containing 1.0 ml PBS (pH-7.2). The cauda epididymides were minced thoroughly, and the suspension obtained was filtered through a cheese cloth to remove the tissue debris. The filtered suspension was added with 1% aqueous Eosin Y at 10:1 ratio and kept for 20 minutes for staining. A drop of the sperm suspension was taken on a clean microscope slide, smeared and air dried. The air dried slides were observed under 40X objective of a compound microscope with a green filter. A total of 2000 sperm per animal (500 sperms/slide) was analyzed from each treatment and control groups, to determine the frequency of abnormal sperm in a population of normal ones. Spermatozoa with abnormal shape were scored according to the criteria of Wyrobek and Bruce [16]. Individual type and total teratospermia were expressed in terms of percentage.

2.5. Sperm Count:
An aliquot (0.05ml) from the sperm suspension (1ml) was removed before staining and was diluted 40 times with PBS (1:40) and mixed thoroughly. A sample of the diluted sperm suspension was introduced to the Neubaur counting chamber and the total sperm count in 8 squares of 1 mm^2 was determined and multiplied by $5X10^4$ to calculate the number of sperm per epididymides.[17]

2.6. Sperm Motility:
Sperm motility was determined by assessing at least five microscopic fields to classify 100 spermatozoa (400 magnifications). The motility was determined by using the basic procedure established for routine human semen analyses and graded fast progressive, slow progressive and no progressive or immotile. [18] The latter three types of sperm were taken as the impaired motility. The percentage frequency of motile sperm was determined by subtracting the total number of sperm with impaired motility from the total number of sperm scored for motility.

2.7. Gonado-somatic Index (GSI):
Body weight of the animals on 35^{th} day of the treatment and testes weight collected after dissection were taken. GSI was determined by using the formula: GSI = Testes weight/ Total body weight) x 100. [19]

2.8. Statistical Analysis:
All the values of the results are expressed mean with standard deviation. The results were subjected to statistical analysis for the significance comparing with the solvent control by employing one-way ANOVA with Dunnett's post hoc test.

3. Results:
3.1 Sperm abnormality:
The results obtained are presented in the table No.3.1. and figure 3.2. CP at its both acute and sub-chronic doses induced the statistically high frequency of abnormal sperm when compared with the solvent control ($p<0.001$). Comparison between the acute and subchronic dosage, the frequency of abnormal sperm was more in the latter dose ($p<0.05$).

3.2 Sperm Count, Motility and GSI:
Table No. 3.2 and figure 3.2 and 3.3 show the results obtained for sperm count, motility and GSI. The sperm count and percentage frequency of motile sperm and reduction in GSI both in acute and subchronic doses were drastically decreased as indicated by the p value ($p<0.001$) in comparison with the solvent control. In the subchronic treatment, the count and GSI were decreased at a significance of $p<0.05$; while the frequency of motile sperm was decreased with the $p>0.01$, when compared with the acute dose treatment.

4. Discussion:
In perspective of clinical significance of CP and its prescription at various dose and dosage schedule, the present work has been carried out to comparatively evaluate the spermatotoxicity in response to acute and subchronic treatment. CP is already well known for its various adverse effects, including on male reproductive system. In the present study, both single and sub-chronic dosing of CP induced adverse effect on spermatozoa as indicated by the increased frequency of abnormal sperm accompanied with the reduction in the count and motility at a statistical significant level ($p<0.001$). The results are in parallel with the previous reports, where acute[20, 21] and multiple doses of CP[9,8] have been demonstrated to induce the aforesaid effects in rodents. CP being a cytotoxic and not a target specific drug, its adverse effect on testes / spermatogenesis is expected. Production of abnormal sperm occurs due to many reasons in response to chemical exposure. Chemical agents may damage the genetic material and/ or affect the differentiation during spermatogenesis leading to affect the sperm morphology and other parameters.[22,23] As far as CP is concerned, it is known to impart the toxic effects by both the mechanisms, i.e. through mutation (genetic) and by interfering with the differentiation of spermatozoa (physiological). CP-induced DNA damage /chromosomal aberration in somatic and germ cells, has been well studied and reported elsewhere. In brief, during spermatogenesis, CP through circulation reaches testes and epididymides where it induces DNA and chromatin damage leading to affect the morphology.[24,25] Point mutation of genes involved in spermatogenesis and loss of a fragment of the genome leads to teratospermia.[26] In addition, CP is a potent inducer of oxidative stress [27], which in turn affects the quality of the sperm (morphology, motility and count) by peroxidation of polyunsaturated fatty acids in plasma membranes of spermatozoa. [28]

In general, whether acute or subchronic treatment, as the dose increases, magnitude of the toxicities increase, which has been proven for CP also with reference to its effect on male reproductive parameters.[7, 9] However, it is interesting to study the magnitude of the effect at higher acute dose and lower subchronic dose in comparison. It is important to assess the impact of the drug due to single and multiple exposure not only for the sake of curiosity, but also in view of practical applicability. In the present study, potency of CP-induced adverse effects in semen parameters such as head deformity, count, motility and GSI were found to be statistically high in subchronic exposure when compared with the single dose exposure. The current observation is in consistent with the previous report [8], where the extent of CP-induced toxic effects on spermatozoa has been shown to be more in lower multiple doses than that of the acute higher dose in rats. Species difference is also an important factor for the pharmacokinetic and toxicological results. [29, 1] However, having obtained similar kind of results in rats as reported by Wtwt[8] and in mice in the current study, it may be inferred that in case of man also the magnitude of toxic effects are more in chronic or subchronic treatment at lower doses than that of the higher acute dosage. In this regard, studies may be taken up for the confirmation using the clinical samples derived from patients under chemotherapy.

The possible reason for the higher toxic effect in subchronic treatment is that at single acute dose, CP induced DNA damage through its main metabolic product phosphoramide mustard, which forms DNA crosslinks both between and within DNA strands at guanine N-7 positions leading to abnormality in sperm parameters, and also by oxidative stress during the initial stage of spermatogenesis; whereas in subchronic treatment, there must be continual insult by CP and its metabolites during spermatogenesis leading to higher rate of toxic effects. This assumption

Volume : 5 | Issue : 4 | April 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

is based on the rate of body clearance of CP or its metabolites. LC-MS/MS analysis of the sample collected by various bleeding techniques indicated that the terminal elimination half-life values ($t_{1/2}$) after i.p. treatment of CP were within the range 0.49 - 0.52 hr in mice. [30] Gas chromatographic analysis of plasma derived from patients under CP chemotherapy indicated that the mean total body clearance was 66.6 ml kg^{-1} h^{-1} after intravenous dosing and 93.1 ml kg^{-1} h^{-1} following oral dosing.[10] Jameson et al. [31] proposed that the total duration of spermatogenesis requires approximately 4.5 cycles in mammals, and for the mouse this is 35 days. Further, it has been advocated that germ cells that are exposed to chemical agents at late spermatogonial stage take seven weeks to reach the cauda epididymis after undergoing a series of changes to produce the matured sperms.[32] Observation for sperm parameters at 35-days post treatment sampling represents the initial stage of damage, i.e., particularly at the DNA/chromosomal level. In case of subchronic exposure, the treatment (15 mg/kg/ week) affects the spermatogenesis continually at different stages starting from the initiation to the ultimate differentiation, which overall affect the abnormality in sperm parameters as observed in the present study. In other words, CP imparts toxicity during the different stages of spermatogenesis, and longer the tissue is exposed to the drug, greater is the number of cells affected. However, damage during the initial stage of spermatogenesis is a crucial step due to high mitotic activity of spermatogonial cells in testes[33], which was the plausible reason for getting the statistically high significant effect both in acute and subchronic dosage when compared with the solvent control ($p<0.001$). In the acute treatment, both primary and secondary spermatocytes are affected with the mutagenic agent and not the spermatid as CP is cleared from the body before maturation of the spermatozoa which requires more time. [34]

Thus, from the present study and based on the similar kind of previous reports, it can be inferred that special care should be taken in case of CP chemotherapy with subchronic dosing since it leads to adverse effects in higher magnitude than those of acute dosing.

Table 3.1: Percentage Frequency[A] of Different Types of Abnormal Sperm Induced by CP at single and multiple doses after 35 Days of Treatment:

Dose Mg/kg b.wt	Amorphous	Banana shaped	Hookless	Folded	Double headed	Double tailed	Abnormal sperms ±(SD)
D.W.	0.88	0.07	0.31	0.06	0.02	0.03	1.37±0.51
CP-50	2.2	0.45	1.38	0.69	0.22	0.8	5.74±1.36*
CP-15x5	3.9	0.59	1.42	0.52	0.29	0.6	7.32±1.53*[a]

[A] =2000 sperm/animal; 5 animals/group
*p<0.001 – Control *Vs* CP-50 and Control *Vs* CP-15x5
[a] p<0.05 - CP-50 Vs CP-15x5 (Dunnett's post hoc test).

Table 3.2: [A]Effect of acute and Sub-chronic Doses of CP on GSI, Sperm Count and Motility After 35 Days of Treatment:

Dose (mg/kg)	Gonadosomatic Index (GSI)			Sperm count/ (x106) ±SD	%Motile spermC
	Body weight (g) Mean± SD	Testes weight (g) Mean±SD	[B]GSI Mean± SD		
D.W.	31.36 ± 2.29	0.226±0.008	0.721±0.06	12.26 ± 2.9	82.31±9.82
CP-50	30.72 ± 2.58	0.198 ±0.005	0.644±0.05[b]	08.19 ± 1.6[c]	63.26±8.87[c]
CP-15x5	31.23 ± 2.42	0.195 ± 0.006	0.624±0.06[c*]	06.25 ± 1.4[c*]	47.41±6.29[c**]

[B] GSI = (Testes weight/ Total body weight) x 100
D.W. – Distilled water; CP-Cyclophosphamide; GSI – Gonadosomatic Index
[C] From a total of 100 sperm scored
[A] =5 animals/group
[b] p<0.001; [c] p<0.001 – Control *Vs* CP-50 and Control *Vs* CP-15x5
*p<0.05; **p<0.01 – CP-50 *Vs* CP-15x5 (Dunnett's post hoc test).

Figures:

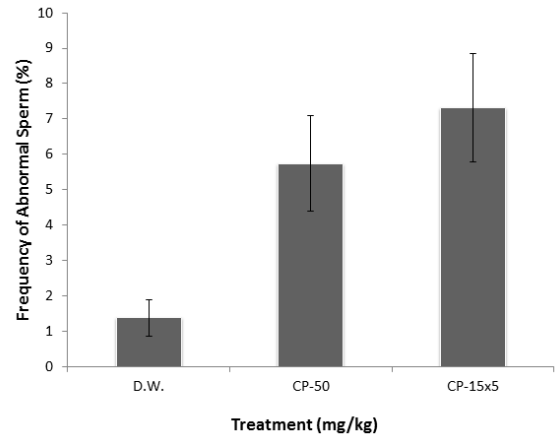

Fig. 3.1: Frequency of Abnormal Spermatozoa Induced by Cyclophosphamide at Single and Sub-chronic Doses

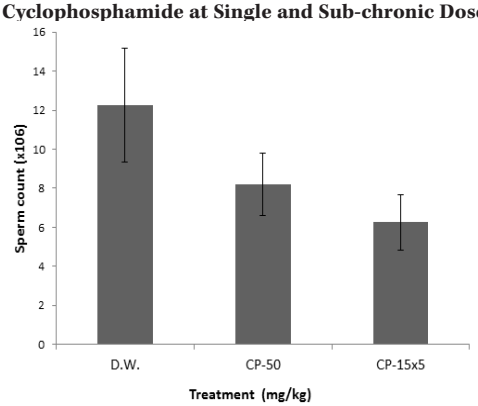

Fig. 3.2: Effect of Cyclophosphamide and Controls on Epididymal Sperm Count

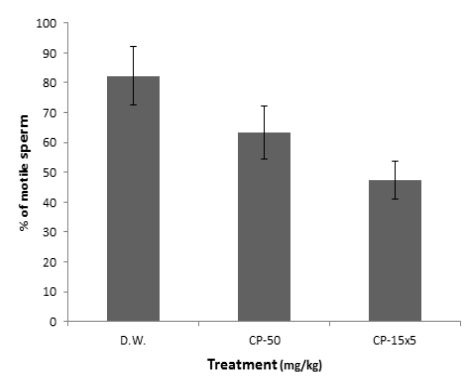

Fig 3.3: Frequency of Motile Spermatozoa in the Presence of Cyclophosphamide

Acknowledgment: We thank Mangalore University's DST-PURSE (Department of Science & Technology - Promotion of University Research and Scientific Excellence) for providing the short term fellowship to one of the authors (Sujayraj R.S.) to carry out this work.

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