Asian Journal of Pharmacy and Life Science ISSN 2231 – 4423
Vol.4 (3), July-Sept, 2014

Toxicological effects of cyclophosphamide and ameliorative role of ascorbic acid on the reproductive parameters of male *Rattus norvegicus*.

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ABSTRACT

The present study was aimed to investigate the toxicity of chemotherapeutic drug cyclophosphamide (CP) and possible chemo-protective effect of ascorbic acid (Vit-C) on cyclophosphamide induced toxicity on male albino rat in response to body weight, sperm motility, sperm count and testicular histological changes. Sixty adult male albino rats were divided into four groups of fifteen each. Group I served as the control received an intraperitoneal (i.p.) injection of physiological saline as vehicle. While, group II received 9 mg CP /kg body weight / alternative day for a 15, 30 and 60 days. However, group III animals were supplemented with Vit-C (100 mg/kg b.wt) via distilled water along with the CP dose for similar durations. Group IV received Vit-C only. After 15, 30 and 60 days testis and cauda epididymis were dissected out weighed and semen analysis was performed. Body weight, sperm motility and sperm count in male Rattus norvegicus were decreased significantly in all the experimental groups as compared to the control group; however we also found testicular histological changes in the same group. Whereas, ascorbic acid significantly ameliorates the effect of CP on the aforesaid parameters especially in 30 and 60 days treated groups. In conclusion ascorbic acid improved and protected against adverse effect of cyclophosphamide on body weight, sperm motility, sperm count and testicular histological changes. However, vitamin-C has protective effect against cyclophosphamide-induced reproductive toxicity. The mechanism is largely unknown but empirical supplementation of vitamin-C would be recommended before and during cyclophosphamide chemotherapy.

Key word: *Cyclophosphamide, Vitamin-C, Reproductive toxicity, Sperm motility, Sperm count, Rattus norvegicus.*

INTRODUCTION

Cyclophosphamide is an anticancer and immunosuppressive agent with a very narrow therapeutic index that undergoes a complicated process of metabolic activation and inactivation, [1, 2] which gets activated by cytochrome P450 enzymes and mixed by microsomal oxidase stem in liver. This process leads to gene rate active alkylating metabolites such as phosphamide mustard and acrolein [3]. These metabolites interfere with the growth of susceptible rapidly proliferating malignant cells by cross-linking with the DNA of tumor cells and prevent cell division. A range of adverse effects of cyclophosphamide have been reported, including nausea and vomiting, bone marrow suppression, hemorrhagic cystitis, urotoxicity, darkening of the skin, alopecia (hair loss) or thinning of hair, infertility etc [4]. It may also be associated with a risk of developing secondary malignancies [5] and also cause genotoxic effects by inducing chromosomal aberrations and development of micronuclei in the normal cells of the host [6]. It is proved that active metabolites of cyclophosphamide circulate through the blood and gains entry to tumor tissue to exert its therapeutic effects. However, it can also reach to other sensitive organs and normal cells to cause toxicities, thus, limiting its full therapeutic efficacy. Thus long-term side effects like gonadal toxicity have become an important issue in both men and women treated with cyclophosphamide [7, 8]. In a study adult male patients treated with cyclophosphamide have demonstrated diminished sperm counts and an absence of spermatogenic cycles in their

testicular tissue [9]. Long-term treatment with cyclophosphamide injures progeny, decreases the weight of the reproductive organs, and impairs fertility [10]. Some agents affect the fertility and other aspects of reproductive functions, for instance, treatments with anticancer drugs, such as cyclophosphamide, have been associated with oligospermia, azoospermia, seminiferous tubular atrophy [11, 12, 13] and increase in the levels of Follicle Stimulating Hormone (FSH) [14]. According to some authors [11, 12, 13] the cyclophosphamide induces alterations in human and rodent testicular functions, and promotes infertility. Previous studies on male rats have confirmed the potential of CP to cause oligospermia, azoospermia and histological alterations in the testis and epididymis [15, 16]. Decrease in weight of reproductive organ, impaired fertility, growth and development of next generation was also observed in cyclophosphamide treated male rats [10].

A number of studies has shown that Vit-C have anti-tumour, anti-inflammatory effect. It is an important intracellular reducing agent occurring in living tissue, its antioxidant properties protecting against the adverse effects of free radical reactions. Most animals synthesize their own vitamin C while, humans and few other animals, such as non-human primates, guinea pigs, and fruit bats do not. It is an active reducing agent involved in numerous biological effects and detoxification of many endogenous and exogenous compounds [17, 18]. It is necessary in the body to form collagen in bones, cartilage, muscle, and blood vessels, and aids in the absorption of iron[19]. Ascorbate inhibits CP-induced sister chromatid exchanges in mice reduces the incidence of CP-induced chromosomally aberrant cells and decreases mortality in pre implantation mouse embryos [20, 21, 22]. This vitamin has also been shown to decrease the frequency of chromosome damage induced by CP in human lymphocyte cultures [23]. However, there has been no convincing evidence of embryotoxicity induced by high doses of vitamin C *in vivo* [24]. Chemo-preventive and therapeutic role of vitamin C against cancers have been widely reported [25, 26]. The various reports on the protective function of dietary ascorbic acid against some anticancer agents-induced clastogenic effects [27, 28] and tissue toxicities [29, 30] have drawn increasing attention. Vitamin-C has a long history of adjunctive use in cancer therapy but the definite use of vitamin C for the treatment of cancer still remains inconclusive [31, 32].While many studies have reported the good therapeutic potential of vitamin C against cancer [31, 33].

Cyclophosphamide was chosen for this study because it is clinically utilized in treatments of oncologic patients, and also associated with variable periods of infertility in humans [34]. So, in this present study we have tried to evaluate the effects caused by cyclophosphamide administration, (9 mg/kg. b.wt.) and ameliorative role of ascorbic acid (100mg/kg. b. wt.) on body weight, sperm motility, sperm count, testicular histological changes of male albino rat, *Rattus norvegicus* were done after 15, 30 and 60 days.

MATERIAL AND METHODS

In this investigation, 60 disease free albino rats weighing 120 ± 10 gm were acclimatized and maintained at 23 ± 2^0C temperature with a 12 hours light-dark cycle in the Animal House, Laboratory of Endocrinology, Bioscience Department, Barkatullah University, Bhopal. The animals were fed with standard rat feed and water *ad libitum.*

Dose, preparation of drug, route and duration of administration:

Cyclophosphamide (CP) brand name-LEDOXAN known mutagenic and a pro-oxidant agent was purchased from local pharmacy market, Bhopal. 200mg CP was dissolved in 10 ml of distilled water to make stock solution and 9 mg/kg. b. wt. CP dose was introduced alternative day through intraperitoneal *(i.p.)* injection for a period of 15, 30 and 60 days.

Antidote:

Vitamin-C (ascorbic acid) analytical grade was used (obtained from VK Traders; MP Nagar Bhopal was dissolved in distilled water 100 mg/kg b.wt./animal/day) as antidote against cyclophosphamide in present investigation.

Experimental Design:

Total 60 albino rat were divided into four groups as under:

Ist Group: The animals of this group were received an intraperitoneal (*i.p.*) injection physiological saline as vehicle and served as control group.

IInd Group: The animals of this group were exposed with CP (9 mg/kg. b.wt.) through *i.p.* injection alternatively for 15, 30 and 60 day.

IIIrd Group: The animals of this group were exposed with CP (9 mg/kg. b.wt.) through i.p. injection and animals were also supplemented with ascorbic acid orally through distilled water for 15, 30 and 60 day.

IVth Group: This group of animals were treated with only ascorbic acid orally through distilled water and fed with normal diet. Five animals from each group were sacrificed on day of 16th, 31th and 61th by cervical dislocation, testis and cauda epididymis were quickly removed, cleaned, blotted, weighed and following parameters were done after the experimental investigation.

Parameters Estimated

Body Weight: The body weights of male albino rats were taken out initially by physical precision balance and expressed in gram after 15, 30 and 60 days of treatment which will denote the change in the their body weight.

Analysis of spermatozoa: Analysis of spermatozoa was done by opting method of Prasad *et. al.,* [35].

Histological study: The histopathological observation was done on testis, this organ was dissected out quickly and fixed in alcoholic Bouin's fluid for histopathological observation and was stained by Ehrlich's haematoxylin and eosin method [36].

Statistical analysis:

Standard error of mean (SEM) were calculated and the mean values of treated as well as the control groups were compared using Student's't' test ($p< 0.05$ to 0.001) [37].

RESULTS:

The body weight were lowered throughout the experiment in cyclophosphamide treated groups and the significant reduction values were observed in the later part of experiment *i.e.* up to 60 days as compared to the control group animals. While, there were insignificant changes in ascorbic acid supplemented along with cyclophosphamide groups and ascorbic acid alone treated groups in comparison to control group

The sperm motility and sperm count were lowered in cyclophosphamide treated group upto 15 days as compare to control group. However, in later part of the experiment *i.e.* after 30 and 60 days of treatment sperm motility and sperm count was significantly decreased (Table-2). While, the animal exposed with cyclophosphamide supplemented with ascorbic acid upto 15, 30 and 60 days showed significantly improvement in sperm motility and their count than that of cyclophosphamide treated groups. Whereas, ascorbic acid alone supplemented groups showed normal sperm motility and their count as similar to control group (Table-2).

Transverse section (T.S.) of control testis of male albino rat *Rattus norvegicus* showed normal testicular histo-architecture with well defined seminiferous tubule have spermatogenic cells *i.e.* different stages of spermatocytes, spermatids and large number of spermatozoan in the testicular lumen. Sertoli cells and interstitial cells were also well developed with prominent nuclei (Fig-1).While, the testis of animals exposed with Cyclophosphamide up to 15 days showed less compactly arranged, vacuolated seminiferous tubules with degenerative changes in Leydig cells and less number of spermatozoans in the lumen of the testis (Fig-3). Disruption in the seminiferous tubules characterized by highly atrophied and vacuolated spermatogenic cells along with severe degenerative and pycknotic changes in the

different stages of developing spermatozoans were seen in the testis of Cyclophosphamide exposed animals for 30 days (Fig-5). However, when the duration of Cyclophosphamide exposure was extended upto 60 days more severe degenerative and necrotic changes were noticed with reduced seminiferous tubules have more atrophied and degenerative spermatogenic cells and Leydig cells, Lumen were devoid of spermatozoans (Fig-7). While, when ascorbic acid was supplemented along with cyclophosphamide upto 15, 30 and 60 days the testis section showed ameliorated seminiferous tubule with visible different stages of spermatogenic cells with spermatozoans as compared to the groups exposed with cyclophosphamide (Figs-4, 6 & 8). Whereas the supplementation of Ascorbic acid alone showing normal testicular histo-architecture same as control groups (Fig-2).

DISSCUSSION:

Many drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organ systems including reproductive system. In a clinical context, testicular stem cell damage in patients exposed to chemotherapeutic drugs for a limited duration could result in long-term infertility or genetic alterations [38]. A strategy to diminish the side-effects of anticancer drugs with preservation of their chemotherapeutic efficacy is necessary. Effective anticancer and immunosuppressive therapy with CP is severely limited by reproductive toxicity as documented in a variety of species [39]. An oxidant mechanism may be involved in the reproduction toxicity, wherein CP and its metabolite acrolein cause inactivation of microsomal enzyme and result in increased reactive oxygen species generation and lipid peroxidation [40]. Our present study also revealed marked protective role of vitamin-C on cyclophosphamide induced male gonadal dysfunction. The CP represent here altered male body weight, disturbed sperm quality, disturbed testicular histology and increased incidence of apoptosis among germ cells. The findings of the present study pass in accordance with similar studies which previously reported that cyclophosphamide induced testicular androgenic and gametogenic dysfunction [41, 42, 43]. Other clinical trials found patients who had received cyclophosphamide showed a severe gonadal failure characterized by reduced testicular size, very low sperm count and some degree of Leydig cell impairment [44, 45]. Many authors have reported that CP exposure decreased body weight, relative organ weights and impair male fertility of various mammalian species, and alter growth and development of the next generation [10, 46, 47, 48]. Besides this, several workers have also reported that CP treatment significantly decreased relative organ weights of testis, epididymides and seminal vesicles, in male animals [16, 49, 50]. Similarly, in our study, it has been noticed that body weight decreased significantly after 30 and 60 days of CP exposed male albino rat (*Rattus norvegicus*). While, these values were recovered in AA along with CP treated rats. However, AA alone supplemented animals showed increased body weight in different intervals *i.e.* 15, 30 and 60 days. The reduced body weight may indicate less consumption of food material, protein catabolism and other essential contents of body [51]. Another reason in reduction of body weight may be due to the imbalance between orexigenic and anorexigenic circuits that regulate the homeostatic loop of body weight regulation, leading to cachexia [52]. The recovered body weight due to antioxidant potency of ascorbic acid against oxidative stress of cyclophosphamide may be inhibit the formation of ROS which prevents free radicals generation and may lead to prevention of DNA breakage, initiates protein biosynthesis and gluconeogenesis [53, 54, 55]. Recently Sallam *et. al.,* [56] also revealed that vitamin-C supplement stimulated weight gain in rabbits. However the recovery in body weight after AA supplementation along with CP treatment suggested the positive role of AA towards the body weight.

Beside this, the analysis of semen is one of the important clinical parameters of gonadal function. Normal semen is practically a guarantee of normal androgenicity. Semen is a composite solution consists basically of spermatozoa suspended in the seminal plasma.

It has been reported that the subchronic exposure to CP affected the normal development of sperm and changes the activity of some enzymes in testis and it directly damages Leydig cells and affects endocrine function of testis [44, 45, 57]. It is also reported that CP effects on DNA of testis and germ cell mutagenesis which directly effects sperm count and morphology in rats [58, 59, 60]. In connection to this, epididymal sperm count and motility decreased by CP treatment while the number of dead and abnormal sperms increased, confirming a previous report that CP induced an epididymis specific effect on sperm count and motility [61]. The decreased sperm count clearly shows the elimination of sperm cells at different stages of development and points to free radical attack through CP metabolism. In fact, oxidative damage to polyunsaturated fatty acids of cell membranes has long been considered to result in the impairment of membrane fluidity and permeability. This, results in damage of germ cells, spermatozoa and mature sperm [62]. It has also been reported that CP causes an increase in apoptosis at specific stages of germinal cycle [63], and also reflects death of spermatogenic cell. While, significant reduction in sperm motility may be due to the toxic effect of CP on the sperm flagellum through rapid loss of intracellular ATP [64]. It has been suggested that ATP may serve as an energy source for sperm motility and decrease in energy metabolism may be one of the limiting factors responsible for loss of sperm motility in CP-administered rats. A direct toxic effect of CP on the spermatogenesis in the seminiferous tubules may be considered as one of the mechanisms of action of CP in producing abnormal and dead sperms and we can also consider that spermatozoa are more susceptible to oxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity, these are susceptible to free radical attack, which results in disturbed sperm motility [39, 65, 66]. However, when AA was supplemented along with CP ameliorated values towards control groups were observed in sperm parameters throughout the experimental study *i.e.* 15, 30 and 60 days, the recovery was more prominent in 60 days treated groups as compared to 15 and 30 days groups. While, AA alone supplemented group, showed normal values of all sperm parameters *i.e.* sperm counts and sperm motility as similar to control group. There are several reports on the benefit of antioxidants in protecting male reproductive system from deleterious effects of reactive oxygen species and other free radicals generated during CP exposure [47, 67]. Two studies from the same researchers indicated that supplementation with lipoic acid as an antioxidant reduces CP-induced reproductive toxicity by the same mechanism [42, 43]. It also may be due to the decreased concentration of the steroid hormones *viz.* testosterone [68, 69, 50, 57].

Histological parameters such as tubule differentiation and spermiation indices can also give information about degree of testicular damage as a consequence of germ cell death. In general, massive germ cell loss caused by anticancer drugs is followed by a sharp decline in testicular histological parameters [70]. As shown in the present study, depletion of seminiferous epithelium and the consequent decrease of histological measurements caused by cytotoxic agents CP was confirmed in our report Structural development and maturation of germ cells and spermiation are important functions of Sertoli cells [71].Therefore, a potential explanation for failure of spermiogenesis in the CP-treated males is disruption of testosterone dependent junction of Sertoli cells with germ cells leading to their disorganization and separation. Additionally, FSH elevation can be an indication of spermiogenesis failure related to various causes including: testicular failure; genetic abnormalities and toxic exposure such as radiation, chemotherapy and heat [72]. Moreover, it indicates the abnormal Sertoli cell function resulted in reduced inhibin secretion [73]. A part from this, testicular tissue of cyclophosphamide treated group revealed disturbed spermatogenesis, severe diffuse damage of seminiferous tubules that reached to early atrophic changes with complete loss of the spermatogonial cells

together with degeneration and necrosis of Leydig cells. In addition, various degenerative and necrotic changes were observed among the epithelial cells lining prostatic acini. Testicular damage could be the cause of sperm abnormalities observed in our study. The former pathological alterations were more or less similar to a previous observation [74] who attributed those alterations to the oxidative stress induced by increasing lipid peroxidation production by cyclophosphamide as well as to the ability of the activated metabolites of cyclophosphamide (which are alkylating agents) to cause crosslinking of DNA strands, interfering with normal cell division in all rapidly proliferating tissues. On the other hand, Nandi *et al.* [60] related the increased germ cells apoptosis in testicular tissue to testosterone withdrawal, suggesting that testosterone may function as a cell survival factor, in some way protecting germ cells from apoptotic death. They also added that the molecular mechanism by which testosterone does so, however, has not yet been elucidated.

On other hands, we administrated vitamin-C during cyclophosphamide treatment to strengthen the antioxidant system, eliminate oxidative reaction and counteract or ameliorate the toxicity induced by cyclophosphamide. The present work showed that vitamin-C improved all studied parameter sand many reports are on the benefit of antioxidants in protecting male reproductive system from deleterious effects of reactive oxygen species and other free radicals generated during CP exposure [47, 67]. There is also evidence that Yukmijihwang-tang as a multi-herbal medicinal formula can improve reproductive toxicity of CP through reduction of oxidative stress [74]. Same researchers work also indicated that supplementation with lipoic acid as an antioxidant reduces CP-induced reproductive toxicity by the same mechanism [42, 43].

Therefore it is concluded that the co-administration of vitamin-C with cyclophosphamide therapy not only ameliorates reproductive toxicity through its antioxidant and androgenic activities but also it potentiates the preventive effects against the toxicity induced by cyclophosphamide.

CONCLUSION

Experimental data of our work suggested that cyclophosphamide treatment is associated with antigonadal activities as well as induction of oxidative stress in gonad that can be ameliorated significantly by vitamin-C.

Table-1: Body weight (gms) after different intervals *i.e*. 15, 30, and 60 days treatment of Cyclophosphamide, Cyclophosphamide (CP) + Ascorbic acid (AA) supplemented, Ascorbic acid (AA) alone and control male albino rat, *Rattus norvegicus*.

Groups →	Body weight (gms)			
Duration Days ↓	**Control**	**CP**	**CP+AA**	**AA**
0	100.2±2.247	101.8±1.803 ^{NS}	102.4±1.923^{NS}	102.8±2.515
15	106.6±3.054	95±2.019***	99.00±1.904 ^{NS}	107.3±3.12
30	114.24±2.109	87.8±0.962***	104.4±2.225**	116.3±1.237
60	123.00±2.716	84.00±0.935***	108.4±2.66***	124.3±2.288

± = Standard Error of Mean (SEM) of 5 animals.
NS = Insignificantly different from the control by Student's't' test.
** = Significantly different ($p< 0.01$) from the control by Student's't' test.
*** = Significantly different ($p< 0.00$) from the control by Student's't' test.

Table-2: Sperm motility and sperm count after different intervals *i.e*. 15, 30, and 60 days treatment of Cyclophosphamide, Cyclophosphamide (CP) + Ascorbic acid (AA) supplemented, Ascorbic acid (AA) alone and control male albino rat, *Rattus norvegicus*.

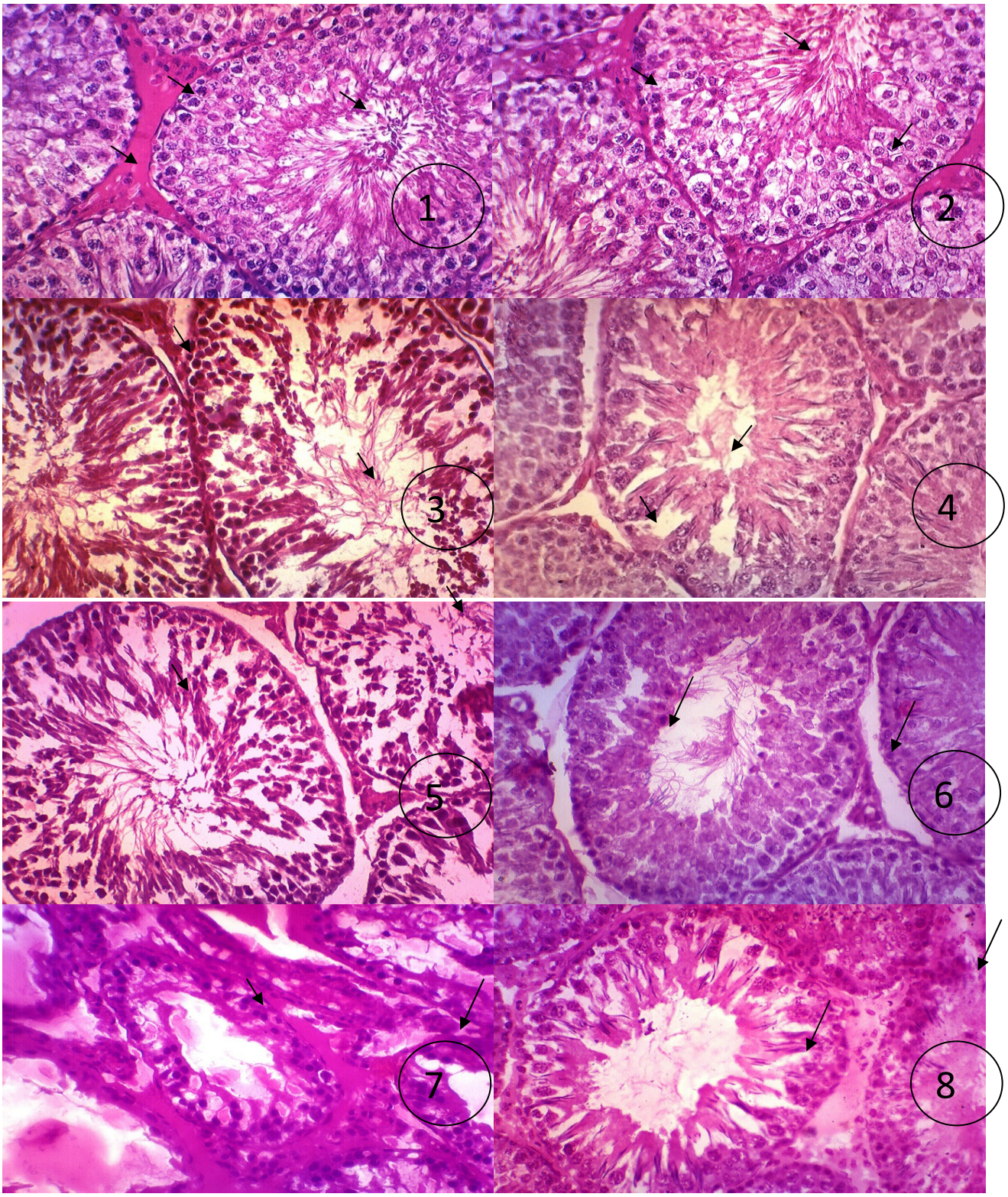
.N.	Parameters	Groups ↓ Duration →	15 Days	30 Days	60 Days
1.	**Sperm motility**	**Control**	59.585±2.617	61.859±2.727	64.258±2.052
	(%)	**CP**	2.25±0.39***	0.00±0.00***	0.00±0.00***
		CP+AA	36.979±2.153***	40.293±0.684***	43.04±1.844***
		AA	61.177±3.804	64.739±2.768	68.154±2.253
2.	**sperm count**	**Control**	61.26±3.276	69.3±3.156	77.83±3.101
	(millions/ml)	**CP**	28.3±1.546***	2.9±0.245***	0.00±0.00***
		CP+AA	43.7±0.796***	44.7±1.85***	45.05±1.088***
		AA	63.35±3.224	72.8±3.309	80.2±2.119

± = Standard Error of Mean (SEM) of 5 animals.
*** = Significantly different ($p < 0.00$) from the control by Student's 't' test.

ACKNOWLEDGEMENTS

Authors are very grateful to Senior research scholars of the Laboratory of Endocrinology, Department of Bioscience, Barkatullah University, Bhopal (M.P), especially Dr. Manohar Rao Gawande and Arunima Datta Banik.

 ISSN 2231 – 4423



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