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QUANTITATIVE MORPHOMETRIC STUDY ON CYCLOPHOSPHAMIDE TESTICULAR TOXICITY IN ADULT ALBINO RATS

(With 12 Tables and 13 Figures)

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دراســة كمية قياسية على التأثير الضار لعقار السيكلوفوسفاميد بخصية الجرذ الأبيض البالغ

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يستخدم عقار السيكلوفوسفاميد بصورة واسعة في علاج الأورام وحالات اضطراب المناعة، ومن الآثار الجانبية لاستخداماته هي التغيرات التي يحدثها في الوظيفة الإنجابية للذكر، فعقار السيكلوفوسامايد له أثار ضارة على النسيج الجرثومي وتكوين النطف بالخصية، كما أن الجرعات العالية منه قد تسبب عدم وجود النطف الناضجة، مما ينتج عن ذلك حدوث العقم في الإنسان، وقد اتجهت الدراسة الحالية إلى تقييم الآثار الضارة لهذا العقار على خصية الفأر وإمكانية ارتداد هذه الآثار السلبية. وأجريت هذه الدراسة على ثلاثين جرذاً ذكراً ناضجاً حيث تم تقسيمهم إلى ثلاث مجموعات؛ مجموعة ضابطة تم حقنها بمحلول الملح الفسيولوجي، ومجموعة معالجة بعقار السيكلوفوسفاميد (100مجم/ كجم من وزن الفأر) مرة أسبوعياً ولمدة خمس أسابيع متتالية، حيث تم قتلها وفحصها بعد انتهاء الجرعات مباشرةً، أما المجموعة الثالثة فهي المجموعة التي أعيدت للتأهيل حيث تم قتلها وفحصها بعد 6 أسابيع من التوقف عن إعطاء العقار. وقد تم إعداد قطاعات شبه رقيقة من المجموعات الثلاثة وأجريت عليها دراسات مورفولوجية وكمية قياسية مع تحاليل إحصائية للحصول على تقييم دقيق لدراسة الخلل الضار الذي يسببه عقار السيكلوفوسفاميد على تكوين خلايا النطف بخصية الفأر وإمكانية الشفاء من هذا الضرر بعد التوقف عن تناول العقار. وقد أظهرت النتائج أن عقار السيكلوفوسفاميد يحدث تغيرات شكلية عديدة بالخصية ومنها ظهور إنبيوبات خصوية صغيرة الحجم مشوهة أو منكمشة مع وجود انتكاسات انحلالية أو فجوات بخلايا أمهات النطف، وخلايا النطف الأولية، وأرومات النطف مع تمدد جليّ بالنسيج البيني. وأظهرت الدراسات القياسية العددية تناقصاً معنوياً في الحجم النسبي للأنبيوبات الخصوية وقطرها، وأيضاً في ارتفاع النسيج الجرثومي المبطن لها، كما لوحظ تناقص معنوي في أعداد الخلايا الجرثومية بأنواعها المختلفة بالحيوانات المعالجة بالعقار مقارنةً بحيوانات المجموعة الضابطة. وقد وجد أن خلايا أمهات النطف (أ) كانت أكثر تأثراً بالتغيرات الضارة التي يحدثها العقار حيث أظهرت تغيَّرات انحلالية واضحة في التركيب مع وجود تناقص معنوي في العدد مقارنةً

بالمجموعة الضابطة. وبالنسبة لخلايا النطف الأولية وكذا أرومات النطف فقد أبديا أيضاً تناقصاً معنوياً في أعدادهما، أما خلايا سرتوللي وخلايا ليدج فكانتا أقل تأثراً بالعقار حيث أبدت تغيرات شكلية أقل مع نقص غير معنوي في أعدادها، أما النسيج البيني المنتشر بين الأنبيوبات الخصوية فقد أظهر زيادةً معنوية في الحجم النسبي له عند مقارنته بالمجموعة الضابطة. وبالنسبة للحيوانات التي تم تأهيلها بعد التوقف عن تناول العقار (مجموعة إعادة التأهيل) فقد أظهرت زيادة معنوية في جميع القياسات الإحصائية السابق ذكرها عندما قورنت بالمجموعة المعالجة وكان ذلك مصحوباً بالتحسن في التغيَّرات الشكلية الناجمة عن تناول هذا العقار، وبهذا أصبح الارتداد عن التغيرات الشكلية والقياسية إلى المستوى الطبيعي واضحاً في هذه المجموعة. وقد استنتج من هذه الدراسة أن الآثار الضارة لعقار السيكلوفوسفاميد على الخصية قد يكون بسبب تأثير هذا العقار المباشر والضار على نوعية أو أعداد الخلايا الجرثومية المنشئة للنطف مما يؤدي إلى إنتاج خلايا نطفية ناضجة مشوهة أو ميتة، وبالتالي حدوث نقص أو فشل في الخصوبة للذكر، وأن ارتداد الآثار السلبية الناجمة عن العقار بالخصية بعد فترة التأهيل والتوقف عن تناول العقار يلقي الضوء على دور هذه الفترة في الاستشفاء من آثار العقار الضارة والعودة بالخصية إلى الوضع الطبيعي، من حيث إنتاج خلايا نطفية ناضجة طبيعية كماً وشكلاً.

SUMMARY

A side effect of cyclophosphamide (CP), an alkylating agent widely used to treat tumors and autoimmune disorders is the alteration of male reproductive function. CP is extremely dangerous to the germinal epithelium and damage to spermatogenesis. High doses can cause azoospermia which can result infertility in humans. This study is focused on the evaluation of toxicological effect of cyclophosphamide on male rat testis and the possible reversibility of these toxic effects. Thirty adult male rats were divided into three groups: vehicle-treated (control), CP-treated and rehabilitated groups. CP was administrated intraperitoneally (100mg/kg/week) for five successive weeks, and semithin sections from testicular tissue were prepared and examined after last injection (treated group) and six weeks later (rehabilitated group). A quantitative morphometric study and statistical analysis were applied for accurate and efficient assessment of spermatogenic impairment induced by CP. The CP treated group showed various morphological alterations in the testis such as reduction in the size and distortion in the shape of the seminiferous tubules with degeneration and vacuolation in spermatogonia, spermatocytes and spermatids associated with marked interstitial oedema. In addition, the CP induced significant decreases in volume proportion, diameter and epithelial height of the seminiferous tubules together with a significant reduction in the number of different germ cells in the treated animals. Interestingly, spermatogonia A

appeared to be target cells for the damaging effect of CP in the testis that presented severe degenerative changes in structure along with significant reduction in number in the treated group animals. Furthermore, the number of primary spermatocytes and round spermatids decreased significantly in CP- treated group. The Sertoli and Leydig cells, however, appeared to be less affected to CP toxicity; exhibiting insignificant decrease in number as well as less morphological alterations. The Interstitial spaces of treated group animals showed a highly significant increase in its volume proportion, when compared to the control animals. Meanwhile, the rehabilitated group showed significant increases for aforementioned variables in comparison to the CP- treated group, associated with reversal of morphological changes towards normalcy. Based on the results from the present study it is concluded that the morphological alterations induced by CP toxicity were further substantiated by morphometric findings in the testicular tissue of rat. A direct toxicity of CP to the number and quality of spermatogenic compartment may be considered as one of the mechanisms of action of CP in producing the abnormal and dead sperms that alter fertility. By the reversal of morphological and stereological changes towards normalcy, the role of rehabilitation is illuminated in CP induced testicular damage**.**

Key words: *Testicular toxicity, Cyclophosphamide, Morphometry, Spermatogenic cells*

INTRODUCTION

Cyclophosphamide (CP) is in the nitrogen mustard group of alkylating chemotherapeutic agents. It is one of the most frequently used antitumor agents for the treatment of a broad spectrum of human cancers, including leukemia, lymphoma, germ cell tumors and carcinomas of breast, lung, and cervix (Friedman *et al.*, 1999). It is also used as an immunosuppressive agent in the treatment of arthritis, nephrotic syndrome, multiple sclerosis and other nonmalignant diseases, as well as for organ transplantation (Perini *et al.*, 2007; Uber *et al.*, 2007). The therapeutic activity of CP is the result of chemically reactive metabolites, phosphoramide mustard, that alkylate DNA and protein, producing cross-links, and inability to synthesize DNA, and ultimately kill cancer cells (Dollary, 1999; wang, *et al.*, 2007). The cytotoxic effect of cyclophosphamide targets rapidly dividing cells, as well as the cancerous cells, makes the highly proliferative testis, especially, a good

target for damaging effects in humans and experimental animals (Fraiser *et al.,* 1991; Ahmad *et al.,* 2008).

The CP is extremely dangerous to the germinal ephitelium and causes damage and impairment to spermatogenesis in the testis (Clopi *et al.,* 2004; Selvakumar *et al.,* 2006a; Elangovan *et al.,* 2006). It causes a certain degree of oligozoospermia to azoospermia associated with increased levels of follicular stimulating hormone (FSH) (Ridola *et al.,* 2009)which can result in human infertility (Tripathi and Jena, 2008). Further, it has been reported that chronic low dose administration of CP can decrease the weight of reproductive organs (Das *et al.,* 2002), impair male fertility (Trasler *et al.,* 1986), increase the post implantation loss and fetal malformation and can also alter growth and development of the next generation in rats (Higuchi *et al.,* 1995).

Animal studies have revealed that treatment of rats/mice with CP led to decreased DNA synthesis in spermatogonia and protein synthesis in spermatids as well as biochemical and histological alterations in the testis and epididymis (Meistrich *et al.,* 1995; Kaur *et al.,* 1997). CP induces morphological alterations such as reduction in size of the seminiferous tubules, number of the seminiferous tubules, degeneration and vacuolation spermatogonia, spermatocytes and spermatids in dose dependent manner (Tripathi, Jena, 2008). It has already been reported that CP treatment causes impairment of sperm in mice, lowered the sperm parameters in rats and induces abnormality in sperm head morphology (Elangovan *et al.,* 2006; Codrington *et al.,* 2007).

It was found that CP treatment resulted in a decreased number of early germ cells and reduced spermatogenesis (Schrader *et al.,* 2001&Tripathi and Jena, 2008). The morphometric study of different stages in spermatogenic cycle indicates that testicular toxicity can be detected from day seven, even after a single oral administration of CP at the dose 100mg/kg in rat (Matsui *et al.,* 1995). Spermatogonia are the target cell type affected at time of CP injection (Russell and Russell 1991 & Elangovan *et al.,* 2006) and a decrease in the number of spermatogonia can be detected at day 3 after CP exposure (Matsumoto et al., 2000). The death of stem spermatogonia is most likely attributed to the mechanism of azoospermia (Meistrich, 1986). A complete loss of germinal cells and spermatocytes were suggested at the highest dose of 200mg/kg of CP (Tripathi and Jena, 2008).

The precise mechanism by which CP causes testicular toxicity is unknown; however, It has already been reported that CP induced apoptosis in a stage specific manner in spermatogonia and spermatocyte

of rat testes (Cai *et al.,* 1997). CP has been shown to inhibit proliferation of germ cells due to its DNA and chromosome damaging effect during spermatogenesis (Andreson *et al.,* 1995; Schimenti *et al.,* 1997). Further, CP has been shown to alter the expression of stress response genes, most dramatically in round spermatids (Aguilar-Mahecha *et al.,* 2002; Chamorro- Cevallos *et al.,* 2008) and to have varying effects on spermatogenesis-related genes in the rat testes (Fukushima *et al.,* 2005). It has been postulated that the cyclophosphamide-induced changes in gene expression during spermatogenesis were a reflection of damaged chromatin (Hales *et al.,* 2005). Recently, CP alters the sperm chromatin structure as well as the composition of sperm head basic proteins in male rats (Codrington *et al.,* 2007).

There was tendency of recovery in the spermatogenesis observed over time, at lower doses of CP- treated groups as compared to higher doses (Meistrich *et al.,* 1992; Elangovan *et al.,* 2006). Recovery of spermatogenesis has to begin at the beginning after drug induced azoospermia and continues until the germ cells repopulate the testes adequately for the spermatogenic process of differentiation and maturation (Ahmad *et al.,* 2008). The presence of spermatogonia stem cells is a prerequisite for being able to stimulate recovery. Spermatogenesis fails to recover not because stem spermatogonia are killed, but rather because surviving stem spermatogonia fail to differentiate (Kangasniemi *et al.,* 1996). Hormonal manipulation using GnRH (gonadotrophin releasing hormones) agonists or systemic testosterone has been shown to have a cytoprotective effect on spermatogenic cells during treatment with chemotherapy in rats (Meistrich *et al.,* 2000). Astaxanthin (AST) treatment in mice (Tripathi and Jena, 2008), Yukmijihwang-tang (YJT) in rat (Oh *et al.,* 2007) or Spirulina (SP) treatment (Chamorro-Cevallos *et al.,* 2008) can also protect against adverse reproductive effects of CP thereby, enhancing spermatogenesis.

MATERIALS and METHODS

I- Experimental animals

Thirty adult male albino rats, three months old, were used in this study. They were obtained from the animal house of Assiut University and maintained in the animal house under normal conditions, with free access to food and water in the normal daily light and darkness cycle.

II-Drug preparation:

Vials containing 200 mg of the pure powder form of cyclophosphamide (CP) dissolved in 10 ml dextrose 5%were used, so each one ml of the resulting solution contained 20 mg of the drug.

III-Experimental protocol:

The experimental animals were divided into 2 groups:

Group A: Control group (10 animals); rats were injected intraperitoneally (I.P.) with dextrose 5%.

Group B: Experimental group (20 animals); rats were treated by I.P. injection of CP in a dose equal to 100mg/kg body weight once weekly for 5 successive weeks (Velez et al., 1989). At the end of the treatment period the treated animals were subdivided into 2 subgroups; (a) and (b), 10 animals each.

Animals of subgroup (*A)* were sacrificed after last injection to investigate the toxic effect of CP administration on the testis, while animals of subgroup (*B*) Left for another 6 weeks without any treatment and then sacrificed to investigate the rehabilitation effect on the testis.

1. Tissue preparation for histological processing:

The animals were first anaesthetized by ether and intracardially perfused with 10 ml of heparnized isotonic solution in a concentration of 130 IU/10 gm body weight (Ye et al., 1993), to facilitate testicular perfusion via the cardiac route. This was followed by intracardiac fixation with 2.5% cacodylate buffered gluteraldehyde. The wall of the scrotum was dissected and the testis was extracted.

2. Weight and volume of the testis:

The weights of the testes were measured using a satorial balance. The volumes of the testes were determined by means of water displacement technique (El-Shennawy 1991).

3. Histological technique for semithin sections:

The excised testes were homogenized in 5% gluteraldehyde in 0.1 M sodium cacodylate buffer at 4oC for 2 – 48 hours and were set for histological processing. The testes were sliced and the testicular samples were routinely trimmed, post-fixed in 1% osmium tetraoxide, dehydrated and then embedded in epon for preparation of semithin sectioning. The semithin sections (1.0 micron) were stained by2% aqueous toluidine blue and examined under a light microscope.

4. Stereological methods:

Histological quantification was performed by image analysis system (Leica Q500). The following parameters were determined for the tested groups of animals:

1. Volume proportion of the seminiferous tubules and interstitial spaces.

2. The diameter of the seminiferous tubules.
3. The epithelial height of the seminiferous tubules.
4. The cell number per cross section of a seminiferous tubule for Sertoli cells, spermatogonia, primary spermatocytes and round spermatids.
5. Number of Leydig cells per high power field.

5. Statistical Analysis

All the quantitative data from this study were analyzed statistically. The results were expressed as the mean ± standard deviation (SD). Differences between groups were assessed by one way analysis of variance (ANOVA) according to Peterson (1985). Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test. A P-value < 0.05 was considered significant. Statistical significance at P-values <0.001, <0.05 have been given respective symbols in the tables.

RESULTS

I. Histological Findings

The normal testicular tissue in control group appeared to be formed of regular rounded or oval seminiferous tubules and interstitial tissue in-between. Each tubule was surrounded by regular basement membrane and lined by organized germinal epithelium and Sertoli cells, which radially arranged in-between (Fig. 1A). The CP treated testis revealed atrophic shrunken seminiferous tubules with markedly distorted shape and thickened irregular basement membrane with impaired spermatogenesis and multiple degenerating vacuoles, mostly towards the basement membrane (Fig. 1B). The germinal epithelium showed marked dispersion, disorganization and loss integrity of its cellular components. The germinal cell populations exhibited signs of degeneration and vaculations in their cytoplasm and nuclei, meanwhile other germ cells were completely destroyed and replaced by degenerative vacuoles (Fig. 1B). Spermatogonia cells appeared to be more affected by CP toxicity, as most of them disappeared and replaced by degenerative vacuoles near the basement membrane. Both primary spermatocytes and round spermatids showed degenerative changes as well, however, the Sertoli and Leydig cells were apparently less affected (Fig. 1B). The interstitial spaces showed a clear increase in amount and presented dilated blood vessles with marked interstitial oedema. Some seminiferous tubules contained degenerated tissues and debris in their lumen or showed absence of mature sperms. The rehabilitated testis showed an improved picture which was further substantiated by the normal histologic findings of seminiferous tubules resident with mature

sperms, restoration of normal organized spermatogenesis along with less degenerative changes in germ cells and normal interstitial tissue both in amount and components. Other tubules were still showed degenerative vacuoles (Fig. 1C).

II. Stereological Findings

1. Testicular weight:

The present study revealed that the mean testicular weight for the control, treated and rehabilitated groups was 1.70 ± 0.06 gm, 1.59 ± 0.05 gm and 1.68 ± 0.06 gm, respectively. Analysis of variance showed that the difference in the testicular weight between the three tested groups was insignificant (P< 0.24) (Table 1a). The post Hoc tests revealed non-significant differences between control and treated or between treated and rehabilitated groups (Table 1b).

2. Testicular volume:

The present study revealed that the mean testicular volume of the control, treated and rehabilitated groups was 1.47 ± 0.02 ml, 0.97 ± 0.03 ml and 1.35 ± 0.02 ml, respectively. Analysis of variance revealed highly significant difference in the testicular volume between the three tested groups (P< 0.001) (Table 2a). The post Hoc tests revealed significant differences between control and treated and between treated and rehabilitated groups (P< 0.05) (Table 2b).

3. Diameter of seminiferous tubules:

Analysis of variance test for diameter of seminiferous tubules indicates a significant difference between the diameters of seminiferous tubules of control, treated and rehabilitated groups (p < 0.05). The mean diameter of seminiferous tubules of control, treated and rehabilitated groups was 297.9μm, 260.3μm and 290.5μm respectively (Table 3a). The post Hoc tests revealed significant differences between control and treated and between treated and rehabilitated groups (P< 0.05) (Table 3b).

4. Volume proportion of seminiferous tubules:

Analysis of variance for the volume proportion of the seminiferous tubules indicates highly significant difference in the volume proportion of the seminiferous tubules between the three tested groups (p< 0.001). The mean volume proportion of seminiferous tubules of control, treated and rehabilitated group was 90.1633%, 59.5800% and 88.0867% respectively (Table 4a). The post Hoc tests revealed highly significant differences between control and treated, also between treated and rehabilitated groups (P< 0.001) (Table 4b).

5. Epithelial height within the seminiferous tubules:

Analysis of variance of the epithelial height within the seminiferous tubules indicates a significant difference between control, treated and rehabilitated groups ($P< 0.05$). The mean epithelial height within the seminiferous tubules of control, treated and rehabilitated groups was 90.8μm, 74.1μm and 75.1μm respectively (Table 5a). The post Hoc tests revealed significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.05$) (Table 5b).

6. Number of Sertoli cells per cross section of a seminiferous tubule:

The present study revealed that the mean number of sertoli cells per cross section of a seminiferous tubule in control, treated and rehabilitated groups was 16 ± 1.76, 14.8 ± 1.92 and 15.2 ± 0.3 respectively. Analysis of variance for Sertoli cell number per cross section of a seminiferous tubule reveals insignificant difference between the three tested groups ($P< 0.63$) (Table 6a). The post Hoc tests revealed insignificant differences between control and treated, also between treated and rehabilitated groups (Table 6b).

7. Number of type A spermatogonia per cross section of a seminiferous tubule:

The mean number of type A- spermatogonia per cross section of a seminiferous tubule of the control, treated and rehabilitated groups was 18.49 ± 2.4, 7.19 ± 0.79 and 14.69 ± 1.75 respectively in this study. Analysis of variance for the number of type A-spermatogonia per cross section of a seminiferous tubule revealed a highly significant difference between the three tested groups ($P<0.001$) (Table 7a). The post Hoc tests revealed significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.05$) (Table 7b).

8. Number of type B-spermatogonia per cross section of a seminiferous tubule:

The present study revealed that the mean number of type B-spermatogonia per cross section of a seminiferous tubule in control, treated and rehabilitated groups was 21 ± 59, 14.7 ± 0.26 and 17.8 ± 2.1 respectively. Analysis of variance for the number of type B-spermatogonia per cross section of a seminiferous tubule revealed significant difference among the three tested groups ($P< 0.05$) (Table 8a). The post Hoc tests revealed significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.05$) (Table 8b).

9. Number of pachytene primary spermatocytes per cross section of

a seminiferous tubule:

The mean number of pachytene primary spermatocytes per cross section of a seminiferous tubule of control, treated and rehabilitated groups was 50.8 ± 2.5, 36.1 ± 0.36 and 46 ± 2.76 respectively as revealed in the present study. Analysis of variance for this number showed a highly significant difference between the three tested groups ($P< 0.001$) (Table 9a). The post Hoc tests revealed significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.05$) (Table 9b).

10. Number of round spermatids per cross section of a seminiferous tubule:

The present study revealed that the mean number of round spermatids per cross section of a seminiferous tubule in the control, treated and rehabilitated groups was 155.5 ± 18.6, 115.2 ± 5.7 and 136.6 ± 20.6 respectively. Analysis of variance for the number of round spermatids per cross section of a seminiferous tubule revealed a significant difference between the three tested groups ($P< 0.05$)

(Table 10a). The post Hoc tests revealed significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.05$) (Table 10b).

11. Volume proportion of interstitial spaces:

The present results revealed that the mean volume proportion of interstitial spaces for the control, treated and rehabilitated groups was 9.8267%, 40.4100% and 11.9033% respectively. Analysis of variance for the volume proportion of interstitial spaces of all studied animals revealed highly significant difference between the three tested groups ($P< 0.001$) (Table 11a). The post Hoc tests revealed highly significant differences between control and treated, also between treated and rehabilitated groups ($P< 0.001$) (Table 11b).

12. Leydig cell number per high power field (Magnification 1000):

The present study revealed that the mean Leydig cell number per high power field for the control, treated and rehabilitated groups was 11.75 ± 1.5, 10 ± 0.8 and 10.75 ± 1.2 respectively. Analysis of variance for the Leydig cell number per high power field of all studied animals revealed insignificant difference between the three tested groups ($P< 0.18$) (Table 12a). Also, the post Hoc tests revealed insignificant differences between control and treated as well as between treated and rehabilitated groups (Table 12b).

Table (1a): Shows means of testicular weights (gm) and their statistical

analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	1.7067	0.06506	0.423
Treated	6	1.5900	0.05508	
Rehabilitated	6	1.6867	0.06524	

Table (1b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.087
Control	Rehabilitated	0.746
Treated	Rehabilitated	0.144

P value <0.05 is significant.

Table (2a): Shows means of testicular volume (ml) and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	1.4767	0.02517	0.000**
Treated	6	0.9767	0.03547	
Rehabilitated	6	1.3567	0.02618	

Table (2b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.036*
Control	Rehabilitated	0.320
Treated	Rehabilitated	0.023*

P value <0.05 is significant. P value <0.001 is highly significant.
*Indicates significant difference. ** Indicates highly significant difference.

Table (3a): Shows means of seminiferous tubules diameter in microns

and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	297.9600	1.71415	0.016*
Treated	6	260.3133	14.39733	
Rehabilitated	6	290.5400	13.97221	

Table (3b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.041*
Control	Rehabilitated	0.779
Treated	Rehabilitated	0.035*

P value <0.05 is significant. * Indicates significant difference.

Table (4a): Shows means of volume proportions of seminiferous tubules and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	90.1633	3.12897	0.000**
Treated	6	59.5800	0.76492	
Rehabilitated	6	88.0867	0.74333	

Table (4b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.000**
Control	Rehabilitated	0.231
Treated	Rehabilitated	0.000**

P value <0.05 is significant. *Indicates significant difference.

P value <0.001 is highly significant. ** Indicates highly significant difference.

Table (5a): Shows means of seminiferous epithelial height in microns

and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	90.8175	2.06107	0.011*
Treated	6	74.1300	10.92894	
Rehabilitated	6	75.1025	3.60439	

Table (5b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.039*
Control	Rehabilitated	0.940
Treated	Rehabilitated	0.031*

P value <0.05 is significant. * Indicates significant difference.

Table (6a): Shows means of Sertoli cell numbers per cross section of a seminiferous tubule and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	16.000	1.76000	0.635
Treated	6	14.7967	1.92500	
Rehabilitated	6	15.1967	0.30501	

Table (6b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.76
Control	Rehabilitated	0.56
Treated	Rehabilitated	0.85

P value <0.05 is significant.

Table (7a): Shows means of A-spermatogonia numbers per cross

section of a seminiferous tubule and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	18.4967	2.40500	0.000**
Treated	6	7.1967	0.79501	
Rehabilitated	6	14.5967	1.75500	

Table (7b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.002*
Control	Rehabilitated	0.95
Treated	Rehabilitated	0.03*

P value <0.05 is significant. . P value <0.001 is highly significant.
*Indicates significant difference. ** Indicates highly significant difference.

Table (8a): Shows means of B-spermatogonia numbers per cross section of a seminiferous tubule and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	21.1400	0.59808	0.003*
Treated	6	14.7467	0.26388	
Rehabilitated	6	17.8267	2.13500	

Table (8b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.012*
Control	Rehabilitated	0.67
Treated	Rehabilitated	0.013*

P value <0.05 is significant. * Indicates significant difference.

Table (9a): Shows means of pachytene primary spermatocytes numbers

per cross section of a seminiferous tubule and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	50.800	2.5400	0.000**
Treated	6	36.1167	0.36501	
Rehabilitated	6	46.0067	2.76500	

Table (9b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.015*
Control	Rehabilitated	0.86
Treated	Rehabilitated	0.022*

P value <0.05 is significant. P value <0.001 is highly significant.
*Indicates significant difference. ** Indicates highly significant difference.

Table (10a): Shows means of rounded spermatid cell numbers per cross section of a seminiferous tubule and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	155.5000	18.66000	0.023*
Treated	6	115.2967	5.76500	
Rehabilitated	6	139.2433	10.10135	

Table (10b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.01*
Control	Rehabilitated	0.98
Treated	Rehabilitated	0.028*

P value <0.05 is significant. * Indicates significant difference.

Table (11a): Shows means of volume proportions of interstitial spaces

and their statistical analysis for control, cyclophosphamide treated and rehabilitated groups.

Group	Number of animals	Mean	SD	P value
Control	6	9.8267	3.12897	0.000**
Treated	6	40.4100	0.76492	
Rehabilitated	6	11.9033	0.74333	

Table (11b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.000**
Control	Rehabilitated	0.231
Treated	Rehabilitated	0.000**

P value <0.05 is significant. P value <0.001 is highly significant.
*Indicates significant difference. ** Indicates highly significant difference.

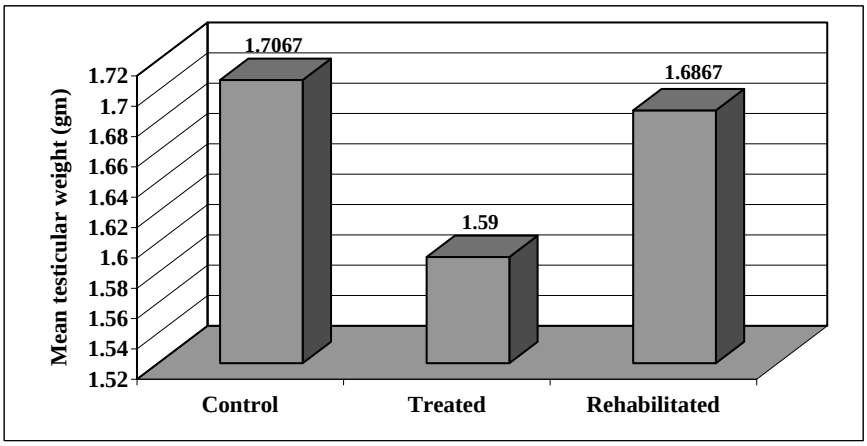
Table (12a): Means of Leydig cell number per high power field and their statistical analysis for control, cyclophosphamide treated and rehabilitated animals.

Group	Number of animals	Mean	SD	P value
Control	6	11.7500	1.50000	0.184
Treated	6	10.0000	0.81650	
Rehabilitated	6	10.7500	1.25831	

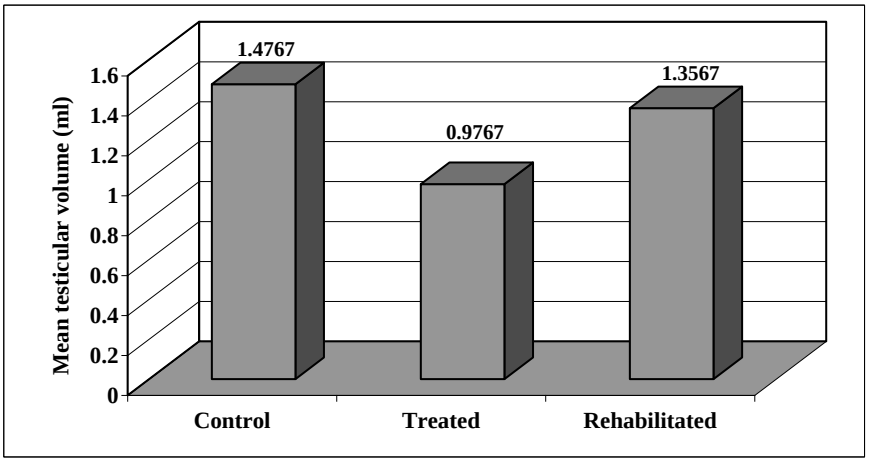
Table (12b): Shows the results of Post Hoc tests between controls, treated and rehabilitated groups.

Group	Group	P value
Control	Treated	0.074
Control	Rehabilitated	0.278
Treated	Rehabilitated	0.409

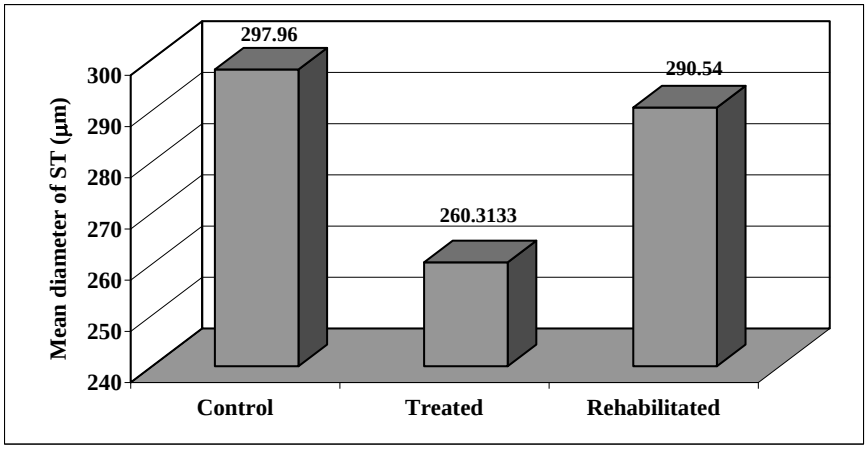
P value <0.05 is significant.


Mean testicular weight (gm)
1.72
1.7
1.68
1.66
1.64
1.62
1.6
1.58
1.56
1.54
1.52
1.7067
1.59
1.6867
Control
Treated
Rehabilitated

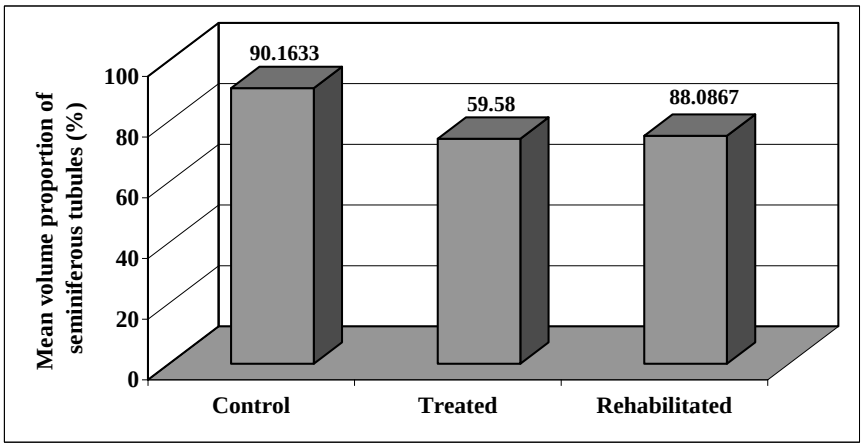
2


Mean testicular volume (ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.4767
0.9767
1.3567
Control
Treated
Rehabilitated

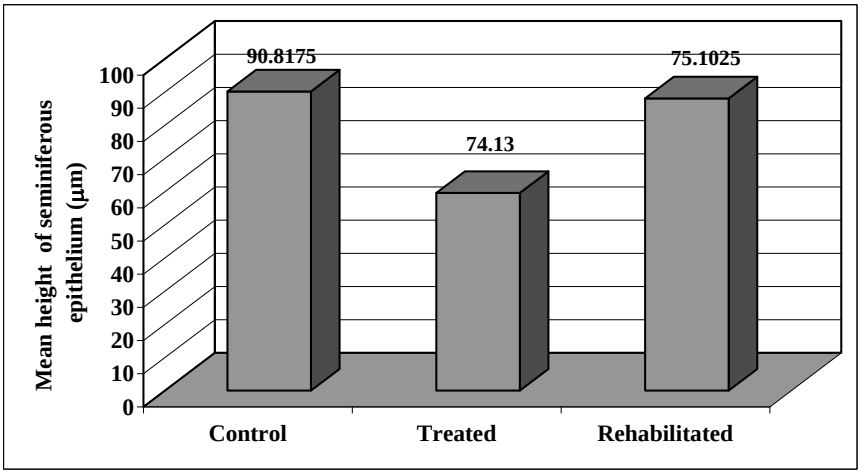
3


Mean diameter of ST (µm)
300
290
280
270
260
250
240
297.96
260.3133
290.54
Control
Treated
Rehabilitated

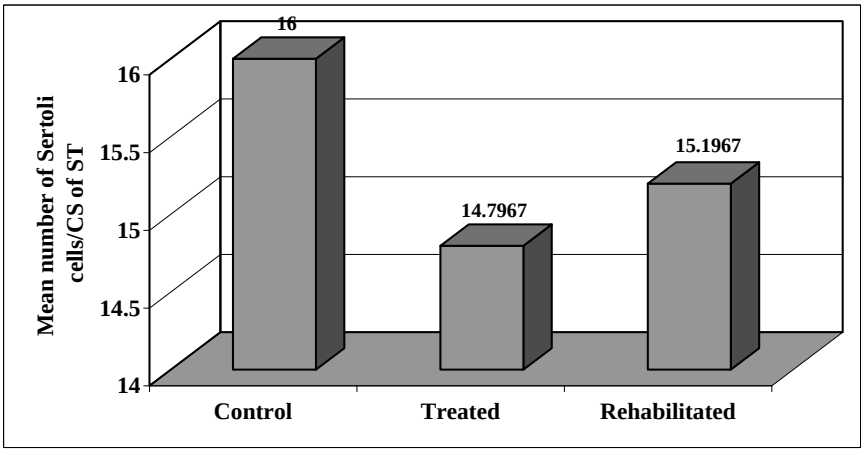
4


Mean volume proportion of seminiferous tubules (%)
100
80
60
40
20
0
90.1633
59.58
88.0867
Control
Treated
Rehabilitated

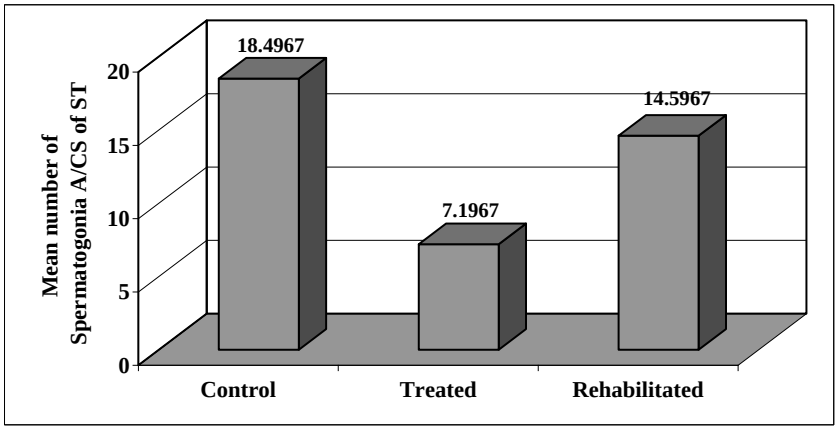
5


Mean height of seminiferous epithelium (µm)
100
90
80
70
60
50
40
30
20
10
0
90.8175
74.13
75.1025
Control
Treated
Rehabilitated

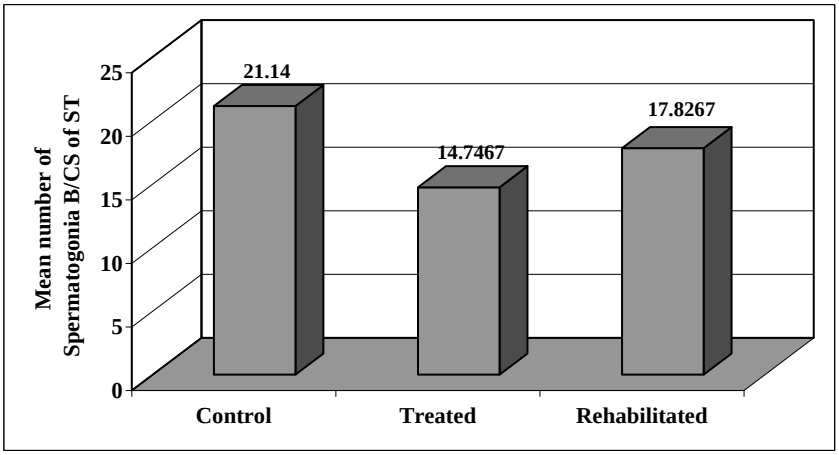
6


Mean number of Sertoli cells/CS of ST
16
15.5
15
14.5
14
16
14.7967
15.1967
Control
Treated
Rehabilitated

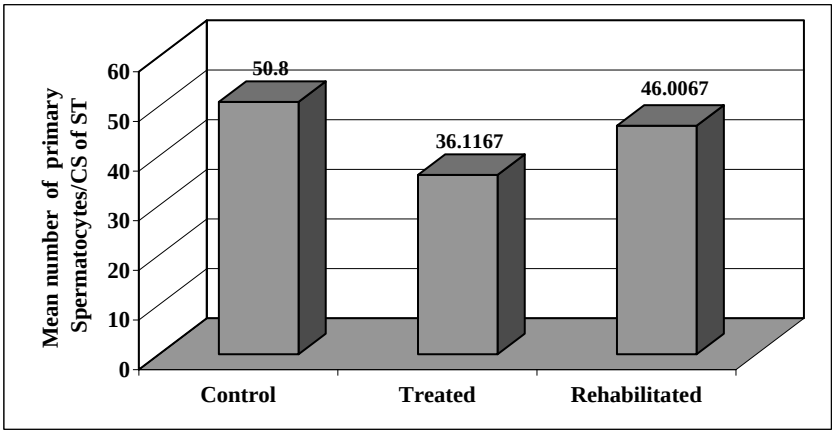
7


Mean number of Spermatogonia A/CS of ST
0
5
10
15
20
18.4967
7.1967
14.5967
Control
Treated
Rehabilitated

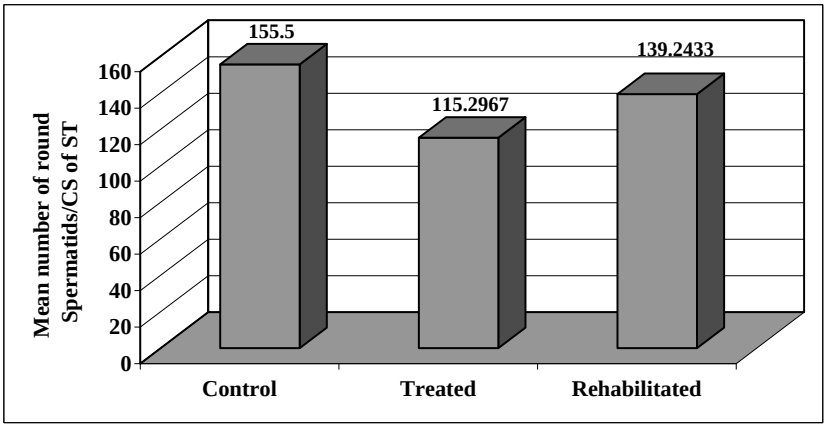
8


Mean number of Spermatogonia B/CS of ST
0
5
10
15
20
25
21.14
14.7467
17.8267
Control
Treated
Rehabilitated

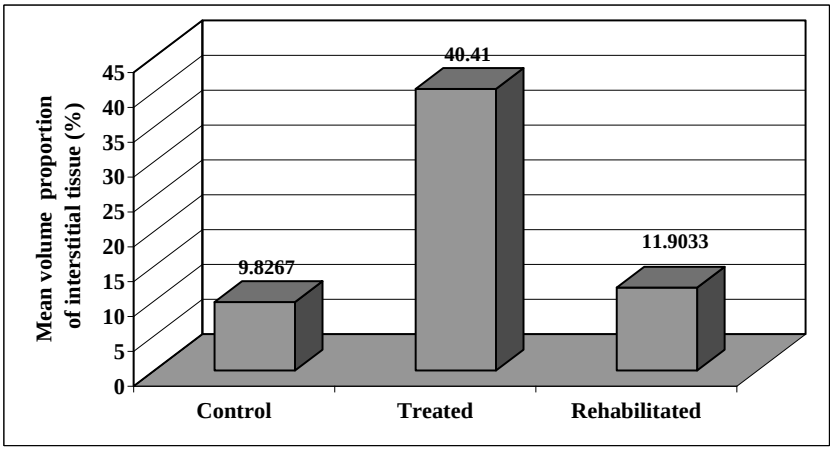
9


Mean number of primary Spermatocytes/CS of ST
0
10
20
30
40
50
60
50.8
36.1167
46.0067
Control
Treated
Rehabilitated

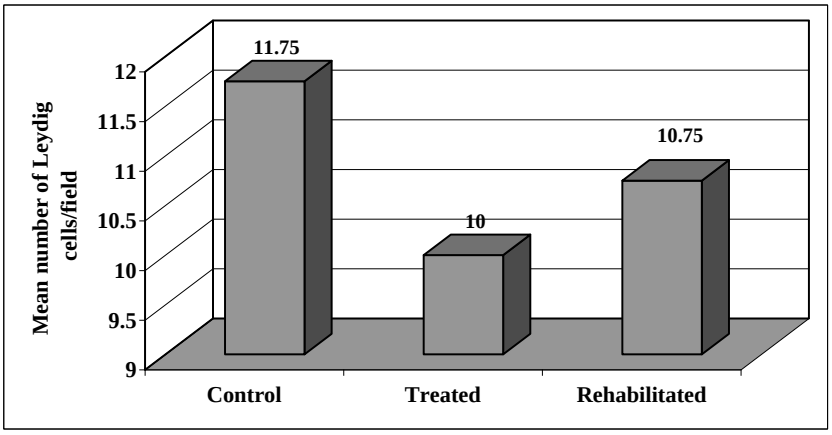
10


Mean number of round Spermatids/CS of ST
155.5
115.2967
139.2433
160
140
120
100
80
60
40
20
0
Control
Treated
Rehabilitated

11


Mean volume proportion of interstitial tissue (%)
40.41
9.8267
11.9033
45
40
35
30
25
20
15
10
5
0
Control
Treated
Rehabilitated

12


Mean number of Leydig cells/field
11.75
10
10.75
12
11.5
11
10.5
10
9.5
9
Control
Treated
Rehabilitated

13

LEGENDS:

Fig. 1: Semithin sections from testes of adult albino rats.

A: Testis from control rats showed normal features of seminiferous tubules (St), lined with Sertoli cells (S) and organized germinal epithelium at different stages of maturation and normal interstitial tissue (i) between the tubules, containing blood vessels (BV) and interstitial cells (*). Toluidine blue x 250

B: Testis from cyclophosphamide treated rats revealed irregular malformed seminiferous tubules (↑) with impaired germinal epithelium, multiple degenerative vacuoles (V) and dark degenerated cells (*) between the disorganized and dissociated germ cells. Notice the marked interstitial tissue oedema with multiple blood vessels (BV). Toluidine blue x 250

C: The testis from rehabilitated rats showed nearly normal seminiferous tubules (St) lined with normal organized epithelium and normal sized interstitial tissue in between with multiple blood vessels (BV) and Leydig cells (L). Notice irregularity of basement membrane (BM) and basal vacuoles (V) of some tubules. Toluidine blue x 250

Fig. 2: Mean of testicular weight of control animals compared to cyclophosphamide treated and rehabilitated ones.

Fig. 3: Mean of testicular volume of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 4: Mean of diameter of seminiferous tubules of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 5: Mean of volume proportions of seminiferous tubules of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 6: Mean of seminiferous epithelial height of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 7: Mean of numbers of Sertoli cells per cross section of a seminiferous tubule of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 8: Mean of numbers of type A-spermatogonia per cross section of a seminiferous tubule of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 9: Mean of numbers of type B-spermatogonia per cross of seminiferous tubule of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 10: Mean of numbers of pachytene primary spermatocytes per cross section of a seminiferous tubule of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 11: Mean of numbers of round spermatids per cross section of a seminiferous tubule of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 12: Mean of volume proportions of interstitial spaces of control animals compared to cyclophosphamide treated and rehabilitated animals.

Fig. 13: Shows mean of Leydig cell number per high power field for control animals compared to cyclophosphamide treated and rehabilitated animals.

DISSCUSION

I. General consideration

Effective cancer chemotherapy as well as immunosuppressive therapy with Cyclophosphamide (CP) is severely limited due to its reproductive toxicity in various species (Fraiser *et al.,* 1991). Cyclophosphamide, as an alkylating agent is extremely dangerous to the germinal ephitelium, whose germ cells are killed or damaged at the stage of differentiating spermatogonia (Meistrich *et al.,* 1982). High doses of this alkylating agent can cause azoospermia for more than 3 years and a cumulative dose leads to serious and sometimes irreversible damage to spermatogenesis and results in male infertility (Byrne, *et al.,* 1987; Schrader *et al.,* 2001). Long-term treatment with CP injures progeny outcomes, decreases the weight of the reproductive organs, and impairs fertility (Trasler *et al.,* 1986; Higuchi *et al.,* 1995; Oh *et al.,* 2007) due to genotoxic damage (Schimenti *et al.,* 1997; Barton *et al.* 2003) which may interfere with germ cell generation and maturation or lead to the production of non-functional spermatozoa (Iammarrone *et al.,* 2003). Apart from reduced spermatogenesis, CP treatment is associated with increased levels of follicular stimulating hormone (FSH) and low levels of testosterone (Hoorweg-Nijman *et al.,* 1992).

Cyclophosphamide is one of the most frequently used antitumor agents and an essential component of many effective drug combinations for the treatment of a broad spectrum of human cancers (Wang, *et al.,*

2007). As an alkylating agent, CP prevents cell division and proliferation due to its DNA damaging effect, primarily by cross-linking DNA strands (Crook *et al.,* 1986 & Andreson *et al.,* 1995) before pachytene stage of meiosis during spermatogenesis (Schimenti *et al.,* 1997). CP exposure affected gene expression in all cell types during spermatogenesis, but most dramatically in round spermatids and pachytene spermatocytes [Aguilar-Mahecha *et al.,* 2001 & 2002). Epididymis spermatozoa, testicular spermatids and early spermatids, respectively were the most sensitive to damage induction of CP (Preston *et al.,* 1995& Chamorro-Cevallos *et al.,* 2008).

Gonadotoxicity of most alkylating agents, especially CP, has been widely described in the literature of Shafford *et al.,* 1993; Kenney *et al.,* 2001 and Ridola *et al.,* 2009. Histological changes and quantitative reduction of spermatogenic cells in the testis of rats after repeated administration of CP (100 mg/kg/ week) for five consecutive weeks in the present study were an indication of CP drug toxicity.

2. Histological remarks

Morphological alterations were produced in the seminiferous tubules, treated with CP (Kaur *et al.,* 1997; Meistrich, 1998; Selvakumar *et al.,* 2006a; Elangovan *et al.,* 2006 & Tripathi and Jena, 2008). Histological examination, in the present study, revealed shrunken malformed seminiferous tubules with less mature sperms and thickened irregular basement membrane, disruptions of the normal spermatogenic architecture with degenerative vacuolations and impaired spermatogenesis in treated group compared to that in the control. CP induced degenerative changes in the germ cells in form of darkly stained nuclei with fragmented DNA, as well as cytoplasmic shrinkage with degenerative vacuoles and loss of cell integrity. Type A spermatogonia were predominantly affected, that they were rarely seen or replaced by degenerative basal vacuoles. Round spermatids were also damaged and showed loss of integrity, marked irregularity and intense degenerative structural changes. The interstitial spaces between the seminiferous tubules showed marked oedema with moderately dilated blood vessels and apparently less affected Leydig cells. However, Elangovan *et al.,* (2006) observed tubules with qualitatively normal spermatogenesis in mice treated with low dose of cyclophosphamide even when exposed for longer duration.

3. Quantitative studies:

3.1. Testicular weight

Trasler *et al.* (1986) reported that multiple low dose treatment with CP leads to decrease in the weight of reproductive organs. In this

sense and in agreement with the studies of (Rajinder *et al.*, 1997; Das *et al.,* 2002; Selvakumar *et al.,* 2006b; Elangovan *et al.,* 2006 & Tripathi and Jena, 2008), repeated administration of CP (100 mg/kg/week) for five consecutive weeks, in the present study decreases the testes weight and volume in the treated rat group. The testes of CREM gene –deficient mice induced by CP display a reduction of 20–25% in their weight (Oh *et al.,* 2007). CP administration (100 mg/kg body weight, for five consecutive days) showed decreased testis and cauda epididymal weight and sperm count in albino rats (Kaur *et al.,* 1997). Elangovan *et al.,* (2006) showed significant decrease in the weight of the testis with all doses of cyclophosphamide (50-200 mg/kg), 1st week after last injection, whereas, after 5th week of last injection, significant reduction was observed only in 200 mg/kg dose of cyclophosphamide in treated mice when compared to the control. The weight of the testis largely depends on the mass of the differentiated spermatogenic cells and a reduction in testis weight can be attributed due to severe decrease in spermatogenic as well as leydig cells and sperm production, as evident from flowcytometric analysis (Katoh *et al.,* 2002; Elangovan *et al.,* 2006 & Tripathi and Jena, 2008).

Testicle weight improvement observed in rehabilitated group after 6th week of last CP injection, when compared to the treated group. The recovery in testicular weight may attribute to restoration of spermatogenic cell number and reversed CP- induced gonadal damage over time. Elangovan *et al.,* (2006) found recovery in weight of the testis and epididymis after 5th week of treatment except in mice treated with high dose of cyclophosphamide (200 mg/kg) and suggested restoration of testis weight with restoration of sperm number. Chamorro-Cevallos *et al.,* (2008) attributed testicle weight improvement in Spirulina (SP) treated groups to improved sperm production. It was found that Astaxanthin (AST) treatment (Tripathi and Jena, 2008), Lipoic acid pretreatment (Selvakumar *et al.,* 2006b) substantially buffered CP induced testicular damage and improved the testis weight and sperm count.

3.2. Seminiferous tubules

Decrease in the size and diameter of seminiferous tubules provides further evidence in support of the existence of seminiferous tubule damage induced by CP. The present results, in coinciding the previous ones of Galal *et al.,* (1994); Selvakumar *et al.,* (2006a); Tripathi and Jena, (2008) demonstrated a significant reduction in the diameter and volume proportions of the seminiferous tubules in the

treated rats compared to the control. Moreover, the size and number of the seminiferous tubules decreased with increasing concentration of CP (Elangovan *et al.,* 2006; Tripathi and Jena, 2008). A marked improvement in diameter and volume proportions of the seminiferous tubules, observed in this study, in rehabilitated rats compared to treated ones may be attributed to recovery in the morphological alterations induced by CP in the seminiferous tubules during rehabilitation time.

Testicular biopsies from males exposed to cyclophosphamide showed an absence of spermatogenic cycles in their testicular tissue (Howell and Shalet, 1988) and depletion of the germinal epithelium, which is essential for normal spermatogenesis (Uderzo *et al.,* 1984; Kenney *et al.,* 2001). Supporting to these data, the present study demonstrated a significant decrease in the germinal epithelium height within the seminiferous tubule in CP treated rat group compared to control rats. These findings are confirmed by clinical investigations of men exposed to CP and show a high incidence of azoospermia (Kumar *et al.,* 1972), also male rats exposed to this drug have oligozospermia and azoospermia (Kaur *et al.,* 1997). Considerable evidence suggests that depletion of germinal epithelium leads to decrease in secretion of inhibin from Sertoli cells with subsequent elevation of serum FSH levels (Ahmad *et al.,* 2008). Decrease in the germinal epithelium height may be due to the cytotoxic and genotoxic effect of CP on the germ cells as well as reduced spermatogenesis.

3.3. Sertoli cells

Although germinal epithelium is particularly susceptible to injury by CP toxicity due to its high mitotic rate, Sertoli's cell function is often preserved due to its very low proliferation index (Ahmad *et al.,* 2008). Farily *et al.,* (1972); Etteldorf *et al.,* (1976) and Uderzo *et al.,* (1984) reported decreased spermatogenic cells with preservation of Sertoli cells in the testis of CP treated patients and observed seminiferous tubules lined only by Sertoli cells with absent germinal cells. This results altered spermatogenesis, leaving testosterone function unaffected (Fegan and Lipshultz, 1998). In line with previous studies, this literature survey revealed an insignificant decrease in number of Sertoli cells in treated group with respect to the control and nearly restored to normal levels in rehabilitated rat group. Martinova *et al.,* (2006) reported a non specific reaction in Sertoli cells induced by CP in mouse testis, while, Selvakumar *et al.,* (2006a) observed damaged Sertoli cells in rat after CP exposure; however, Galal *et al.,* (1994) revealed a significant decrease in Sertoli cell number. It was reported

that chronic cyclophosphamide treatment alters spermatogenesis (Elangovan *et al.,* 2006; Tripathi and Jena, 2008).

3.4. Spermatogonia-A

It was reported that chronic cyclophosphamide treatment alters spermatogenesis (Elangovan *et al.,* 2006; Tripathi and Jena, 2008). Spermatogenesis is the development of mature haploid spermatozoa from diploid spermatogonial stem cells. This development includes a sequence of cytological and biochemical events, including mitotic and meiotic divisions, which lead to drastic changes in cellular morphology and physiology (Russell *et al.,* 1990 and Suter *et al.,* 1997). In the testis, cyclophosphamide disrupts meiotic events during spermatogenesis before pachytene stage thus emphasizing the potential for adverse progeny outcomes due to genotoxic damage. To maintain genomic stability, eukaryotic cells respond to genetic damage by arresting or delaying cell cycle progression. Such delay, allows for the activation of DNA repair mechanisms, or when the damage is too overwhelming, of cell death pathways (Sancar *et al.,* 2004). The morphometric study of different stages in spermatogenic cycle indicates that testicular toxicity can be detected from day 7 even after a single oral administration of CP (100 mg/kg) in rat (Matsui *et al.,* 1995).

Spermatogonia are target cells of CP in the male reproductive system. Studies on rodents have shown that the damaging effects of chemotherapy occur at the level of A-spermatogonia differentiation (Meistrich *et al.,* 1998). Spermatogonia are diploid elements, small proportion of all the germinal cells involved in spermatogenesis. Cytotoxic agents and radiation effects cause blockage of spermatogenesis by killing stem cells (spermatogonia) (Clopi *et al.,* 2004). This study demonsrated that spermatogonia –A are the target cells in the testis for the damaging effect of CP as indicated by their marked degenerative changes on histological examination and the highly significant reduction in their number, morphometrically in the treated rat group compared to the control. This is in consistent with the reports of Russell and Russell (1991); Takahashi and Matsui (1993) and Matsui *et al.,* (1995) in rat and with Lu and Meistrich (1979) and Da Cuncha *et al.,* (1987) in mice, who demonstrated that spermatogonia type A is the first target cell of CP toxicity in the testis. This is most probably due to the high mitotic activity of spermatogonia –A, that make them vulnerable to the toxic effect of CP. On histological quantitative evaluation, Matsui *et al.,* (1995); Matsumoto *et al.,* (2000); fukushima *et al.,* (2005); Elangovan *et al.,* (2006) and Tripathi and Jena, (2008) reported

consistent decrease in the number of spermatogonia- A after CP exposure. Watanabe *et al.,* (2000) reported that spermatogonia type A decreased significantly in all stages of seminiferous tubules examined after CP administration. It has already been reported that CP induced apoptosis in a stage specific manner in spermatogonia and spermatocyte of rat testes (Cai *et al.,* 1997), mainly due to the increased oxidative stress which results in the lipid peroxidation and affects the membrane integrity and fluidity (Tripathi and Jena, 2008).

Elangovan *et al.,* (2006) literature revealed that some cells of spermatogonia (sperm stem cells) are resistant to damage with high dose of CP even after a period of 5 weeks. Inactivation of only a fraction of these sperm stem cells in a given tubule may have a disproportionately larger effect on spermatogenesis and hence the decrease in sperm counts. Damage to spermatogonia can lead to different degrees of oligospermia, and the death of stem spermatogonia can result in azoospermia. The duration of azoospermia appears to be related to the proportion of stem cells killed; if all stem cells are killed, the azoospermia will be irreversible (Meistrich, 1986). Azoospermia lasting only weeks to months reflects transient damage to late differentiating spermatogonia whereas a delay of years for recovery points to widespread damage of even early spermatogonia and stem cells in patients treated with CP (Ahmad *et al.,* 2008). Interestingly, an increase in external malformations and growth retardation was produced in progeny sired by germ cells first exposed to CP as spermatogonia that significantly persisted to the F2 generation (Hales *et al.,* 1992; Hales *et al.,* 2005).

The presence of stem cells is a prerequisite for being able to stimulate recovery in spermatogenesis. If certain cumulative cytostatic doses are not surpassed, these stem cells (spermatogonia- A) survive chemotherapy and form the basis for the recovery of spermatogenesis and a long time can be needed to convert them to spermatozoa (Schrader *et al.,* 2001). The present analysis revealed a significant increase in number of spermatogonia –A cells in rehabilitated group compared to the treated one. Spermatogenesis fails to recover not because stem spermatogonia are killed, but rather because surviving stem spermatogonia fail to differentiate (Kangasniemi *et al.,* 1996). The increase in the FSH levels may contribute to the inhibition of spermatogonial differentiation (McLachlan *et al.,* 2002; Elangovan *et al.,* 2006). Meistrich, (1998) subsequently achieved recovery of A-spermatogonia differentiation by administering testosterone or GnRH agonists. Tripathi and Jena, (2008) explore that the reduction in number

of spermatogonia induced by CP can be prevented by Astaxanthin (AST) treatment.

3.5. Spermatogonia-B

Type B spermatogonia rapidly destroyed by cytostatics because of high mitotic activity. Lu and Meistrich, (1979) found that types A and B spermatogonia were sensitive to CP in mice and Martinova *et al.,* (2006) showed degenerative changes in spermatogonia B induced by CP. Type B spermatogonia in the present study showed a significant reduction in number in CP treated group compared to the control and a significant recovery in number could be observed after 6 weeks of last injection (rehabilitated group). Watanabe *et al.,* (2000) revealed that spermatogonia type B were affected and decreased in number after a higher dose of CP than that of type A spermatogonia and complete spermatogenesis was seen in all tubular cross sections, 56 days after CP injection in mice.

3.6. Spermatocytes and spermatids

Other cells in the seminiferous tubules such as spermatocytes and spermatids are damaged by low dose of CP and leads to impaired spermatogenesis. Auroux and Dulioust, (1985); Aguilar- Mahecha *et al.,* (2001) and Satoh *et al.,* (2002) in their morphometric evaluation of the rat testis after CP injection demonstrated significant decrease in the number of primary spermatocytes and spermatids. Russell and Russell, (1991) reported consistent decrease in the numbers of leptotene, zygotene and early pachytene spermatocytes in the testis of rats, 15 days after two injections, 24h apart, of 65 mg/kg of CP. Matsui *et al.,* (1995) and Matsumoto *et al.,* (2000) found significant decrease in the number of spermatocytes at day 14 and day 21 after one CP injection (100mg/kg). Confirming to these previous data, this literature together with that of Elangovan *et al.,* (2006) revealed significant decrease in the number of primary spermatocytes and round spermatids in the CP treated testes compared to the control that significantly improved to normal levels after 6 weeks of last injection, in rehabilitated group.

It has already been reported that CP induced apoptosis in a stage specific manner in spermatogonia and spermatocyte of rat testes (Cai *et al.,* 1997). Aguilar- Mahecha *et al.,* (2001) and Satoh *et al.,* (2002) suggested that round spermatids were more vulnerable to the damaging effects induced by CP due to the chromatin remodeling that is takes place at this stage of spermatogenesis. A single injection of high-dose cyclophosphamide during the development of mouse spermatocytes resulted in heritable translocations (Sotomayor and Cumming, 1975) and

increased the incidence of micronuclei (Tates, 1992) as well as genotoxic effect on the genome during meiotic prophase (Schimenti *et al.,* 1997). Hales *et al.,* (2005) observed increased transcript levels for 15% of the genes in round spermatids, whereas only 3% were affected in pachytene spermatocytes during acute CP treatment, while the predominant effect of chronic CP treatment was to decrease the expression of the genes studied in pachytene spermatocytes (34%) and round spermatids (29%).

Codrington *et al.,* (2007) suggested that round spermatids attempt to compensate for DNA damage induced by CP by the expression of DNA repair genes (Aguilar-Mahecha *et al.,* 2001). It was found that Astaxanthin (AST) treatment (25 mg/kg) prevents the reduction in number of spermatocytes and spermatids induced by CP in mice (Tripathi and Jena, 2008).

3.7. Leydig cells

Testicular function is influenced by both endocrine (extra-testicular) and paracrine (intra-testicular) factors. The intra-testicular regulation of spermatogenesis is thought to be carried out by steroids, such as testosterone and oestradiol, which are synthesized by Leydig cells (Elangovan *et al.,* 2006). The inhibition of spermatogenesis may be due to low levels of plasma gonadotrophin and testosterone, which are prime regulators of spermatogenesis in rats (Chowdhury, 1979) and the stimulation of recovery of spermatogenesis by GnRH agonists appears to be related to suppression of intra-testicular testosterone (Meistrich and Kangasniemi, 1997).

Ahmad *et al.,* (2008) reported preservation of Leydig's cell function due to its very low proliferation index. Testicular biopsies from males exposed to cyclophosphamide show depletion of the germinal epithelium, which is essential for normal spermatogenesis, and morphologically normal Leydig cells, which function in steroidogenesis (Shalet *et al.,* 1981). The results of above reports corroborate well with the present findings in this study, which revealed an insignificant decrease in number of Leydig cells in CP treated rats compared to control ones. Leydig cell damage could be explained by the lipid accumulation in the cells leading to their degeneration (Flickingerand Loving, 1976). The effect of CP on the Leydig cell morphometry could be due to the effect of CP on the endogenous gonadotrophin secretion and also could be due to direct effect of CP on the Leydig cells (Fichna and Malendowicz, 1975).

The decrease of testosterone in mice was associated with the alteration of Leydig cell function. Elangovan *et al.,* (2006) observed occasionally and moderately altered Leydig cells as shown by significant decrease in serum testosterone and LH levels in CP exposed mice. Selvakumar *et al.,* (2006a) observed impaired spermatogenesis and Leydig cell hyperplasia in CP exposed rats. Severe decrease in spermatogenic as well as leydig cells can be attributed to the decrease in testis weight induced by CP (Katoh *et al.,* 2002). It was reported that children who receive high dose of CP as part of their chemotherapy are at high risk for long-term gonadal damage including subclinical Leydig cell insufficiency and infertility (Kenney *et al.,* 2001).

3.8. Interstitial tissue

Regarding the interstitial tissue, the present study revealed that the treatment of cyclophosphamide resulted in increase in the volume of interstitial spaces on the expense of seminiferous tubules. In accordance with the results of Galal *et al.,* (1994) and Dina *et al.,* (1996), the present findings revealed highly significant increase in the volume proportion of interstitial spaces in treated group animals compared to the control and a significant recovery in this volume proportion was observed in the rehabilitated group after 6 weeks of last CP injection. These changes could be attributed to the decrease in the perimeter and the volume proportion of seminiferous tubules (Tripathi and Jena, 2008) that resulting from reduction in the height of seminiferous epithelium and loss of the germinal epithelial cells under the toxic effect of CP.

4. Recovery in the spermatogenesis

Studies also have suggested that cyclophosphamide-induced gonadal damage can be reversed over time, and that gonadal damage is dose-dependent (Chamorro-Cevallos *et al.*, 2008). There was tendency of recovery in the spermatogenesis observed at lower doses of CP treated groups as compared to higher doses (Elangovan *et al.,* 2006). Recovery of spermatogenesis may be spontaneously achieved within 1 year in the most favorable cases, while in others it may take several years (Clopi *et al.,* 2004). Ahmad *et al.,* (2008) observed recovery in spermatogenesis and FSH levels to normal in patients receiving CP regimen. Meanwhile, Buchanan *et al.,* (1975) stated that permanent sterility is not a consequence of cyclophosphamide chemotherapy and the resultant azoospermia was likely to be reversible. Oh *et al.,* (2007) revealed that Yukmijihwang-tang (YJT) can protect against adverse reproductive effects of CP by inhibiting oxidative stress and enhancing spermatogenesis related with cAMP-responsive element modulator

(CREM) gene expression in rat. Chamorro-Cevallos *et al.*, (2008) demonstrated that Spirulina (SP) treatment substantially buffered CP induced testicular spermatogenic cell damage (Selvakumar *et al.*, 2006b), thereby improved sperm production. Moreover, sperm testicular extraction techniques can allow recovery of spermatogenetic cells to be used with intracytoplasmic sperm injection (ICSI) and cryopreservation of testicular tissue is also useful in prepubertal boys undergoing chemo–radiotherapy (Clopi *et al.*, 2004).

5. Conclusion remarks

Based on the results from the present study it is concluded that chronic cyclophosphamide treatment of male rats alters spermatogenesis via a direct effect on male germ cell number and differentiation or quality that may be considered as one of the mechanisms of action of CP in producing alteration in sperm number and function and hence alter fertility and progeny outcome. Marked affection in spermatogonia, spermatocytes and round spermatides reflects the susceptibility of these germ cells to CP insult during spermatogenesis in the testis. The tendency of recovery in the spermatogenesis observed in rehabilitated group over time reflects the important role of rehabilitation in differentiation of survive stem cell spermatogonia which may form the basis for this recovery.

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