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CYTOTOXIC EFFECTS OF A CHEMOTHERAPEUTIC DRUG OXALIPLATIN ON RAT TESTIS AND PLASMA TESTOSTERONE CONCENTRATION

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ABSTRACT
The toxic effect of Oxaliplatin ($C_8H_{14}N_{20}4Pt$), an analog of Cisplatin, the first successful platinum containing anticancer drug on the testis and evaluation of testosterone levels were studied by intraperitonially injecting chronic low dose (4mg/KgBW for 15 days) and high dose (8mg/KgBW for 5 days) to adult male Wistar rat, *Rattus norvegicus*. For comparing the effects the vehicle control rat was injected same amount of saline and were maintained for the same duration. Both the treatments resulted into significant suppression of plasma testosterone. As compared to vehicle-treated control group treated animals revealed a number of untoward behaviour such as low appetite, withdrawn behaviour, thickness of skin, hair fall all over the body and continuous licking of testis, oral mucositis etc. Oxaliplatin treatment resulted into dose and duration dependent histopathological changes in the testis as well as testosterone concentrations. Thus the results suggested antiandrogenic effect of Oxaliplatin.

KEYWORDS: *Oxaliplatin, testis, testosterone*

INTRODUCTION

Oxaliplatin ("cis – [C1R,ZR] -1,2 cyclohexane diamine – N,N") [Oxalato (2-)-O-O'] platinum , Eloxatin) is a novel platinum co-ordination complex used for the treatment of metastatic colorectal carcinoma in combination with fluropyrimidines. It interferes with the genetic material, or DNA, inside the cancer cells (Dunn *et al*., 1997; Soulie *et al*., 1997a; 1997b; Cvitkovic,1998; Huang *et al*.,2003). The goal of present study is to elucidate impact of Oxaliplatin treatment on male germ cells which directly play an important role in male reproductive performance of animal since scanty and fragmentary notes are available only on the testicular atrophy or gonadal dysfunction (Dunn *et al*.,1997; Pectasides *et al*., 2004; De Giorgi *et al*., 2004; 2006; Corazzeli *et al*., 2006; Chater *et al*.,2007) as well as on evaluation of the male gonadal hormone, testosterone are not well documented, therefore, in the present study main emphasis has been given on the evaluation of testosterone correlative to histopathological changes as undergone by the testis and testicular weight. In view of understanding the monotherapic action of this drug rat was used as a model.

MATERIALS AND METHODS

Drug

Oxaliplatin injection by Oplax (50mg/25ml) Marksans Pharma Ltd.

Animals and treatment

Twenty four male healthy Wistar strain albino rats (281.67 ± 6.01 - 276.00 ± 3.06) gm in weight were obtained from the Department of Biochemistry, RTM Nagpur University, Nagpur breeding colony and raised on a commercial pellet diet (Hindustan lever Ltd.) and water *ad libitum*. The animals were housed at constant temperature (28 ± 2°C) and relative humidity (60 ± 10%) with a 12 h light: 12 h dark cycle.

Histological examination

The Animals were sacrificed using chloroform 24 hours after the last day of each experiment. Immediately the testis was dissected from the surrounding tissues, weighed on an analytical balance and fixed in Bouin's fluid for 24hrs and preserved in 70% alcohol. The tissues were dehydrated by passing through graded series of alcohol, cleared in xylol and after embedding in paraffin, blocks were prepared and testis were cut in numerous parallal 5µm sections. For routine histological study the sections were stained with Ehrlich's haematoxylin and counter-stained with eosin.

Testosterone evaluation

For the determination of testosterone level in blood, rats were anesthetized by ether and 2ml of blood was drawn by cardiac puncture with 2ml sterile syringe. The blood was allowed to clot at room temperature for half an hour. The clotted blood was sent to NRPL Pathology laboratory, Nagpur for further processing by enzyme linked florescent assay (Delahunt, 1993).

TABLE 1. Experimental Design for low dose Oxaliplatin chronic treatment

Number of animals and sex	Treatment	Dose mg/Kg BW	Route	Duration
6 males (Experimental)	Oxaliplatin	4mg daily	I.P.	15 days
6 males (control)	Saline	E.V.	I.P.	15 days

TABLE 2.: Experimental Design for high dose Oxaliplatin chronic treatment

Number of animals and sex	Treatment	Dose mg/Kg BW	Route	Duration
6 males (Experimental)	Oxaliplatin	8 mg daily	I.P.	5 days
6 males (control)	Saline	E.V.	I.P.	5 days

Abbreviations : E.V. = Equal volume, I.P. = Intraperitoneal, B.W. = Body weight

RESULTS

Both the treatments resulted in the suppression of the organ weight (fig. 1) and testosterone concentration (fig.2) and also showed regressive changes in histopathological architecture.

Vehicle-treated controls

The histological sections of testis showed elongated to circular seminiferous tubules covered with moderately thick lamina propria.The germinal epithelium showed two type of cells the Sertoli and germinal. Sertoli cells formed a frame around the developing germ cells. The germ cells were stacked in 4-8 layers. All stages of spermatogenesis were observed. Leydig cells were round or polygonal with central nuclei (figs. 3 - 4).

Plate-1

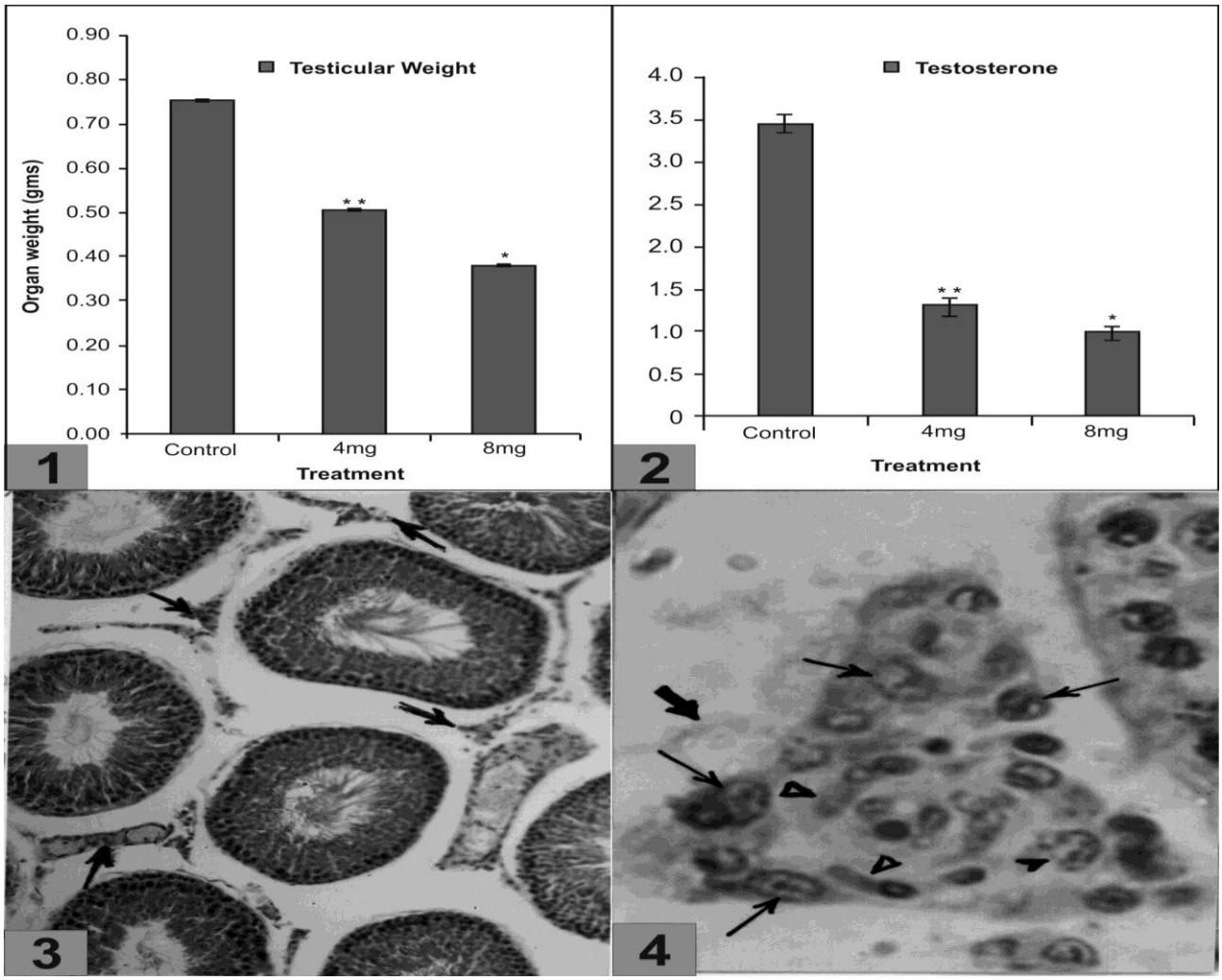

Fig. 1 : Weights of testis after 5 & 15 days of treatment with vehicle or Oxaliplatin (control, n = 6, Oxaliplatin, n= 6). There was a significant decrease in the weights of testis $p < 0.1$*, $p < 0.001$**

Fig-2 : Testosterone level changes over the course of 5 and 15 days treatments with Oxaliplatin (control, n = 6, Oxaliplatin, n = 6). There was a significant decrease in the blood serum testosterone level of rat when compared to vehicle. $P < 0.001$**, $p<0.1$*

Fig. 3 : Low power photomicrograph of vehicle-treated control rat testis. Many seminiferous tubules cut in a cross section separated from one another by a small amount of interstitial connective tissue containing the Leydig cells (arrow) X100.

Fig. 4 : Interstitial connective tissue shows presence of Leydig cells (arrow),fibroblasts (open arrow), macrophages (arrow head), lymphocytes, monocytes and mast cells and blood vessels (thick arrow). The Leydig cells were rounded or polygonal in shape with a central nucleus X 400.

Chronic low dose treatment (4mg/Kg BW Oxaliplatin for 15 days, figs. 5-16)

The low dose treatment has resulted into significant thickening of tunica albuginea, dimunation in their size as well as variability in their contour and hence noticeable increase in intertubular spaces due to their sparse distribution (fig.5). The interesting feature of the drug treatment is the remarkable development of interstitial mesenchymal tissue not only in the interstices of the seminiferous tubules but around the lamina propria of each tubule. The interstitial fibrosis or edema was found to be a little mild than the high dose experimental group. Distortion of the tunica propria appeared as loops hanging all over the tubules giving naked appearance to the tubule, however, there was simultaneous increase in the thickness of lamina propria.

Plate-2

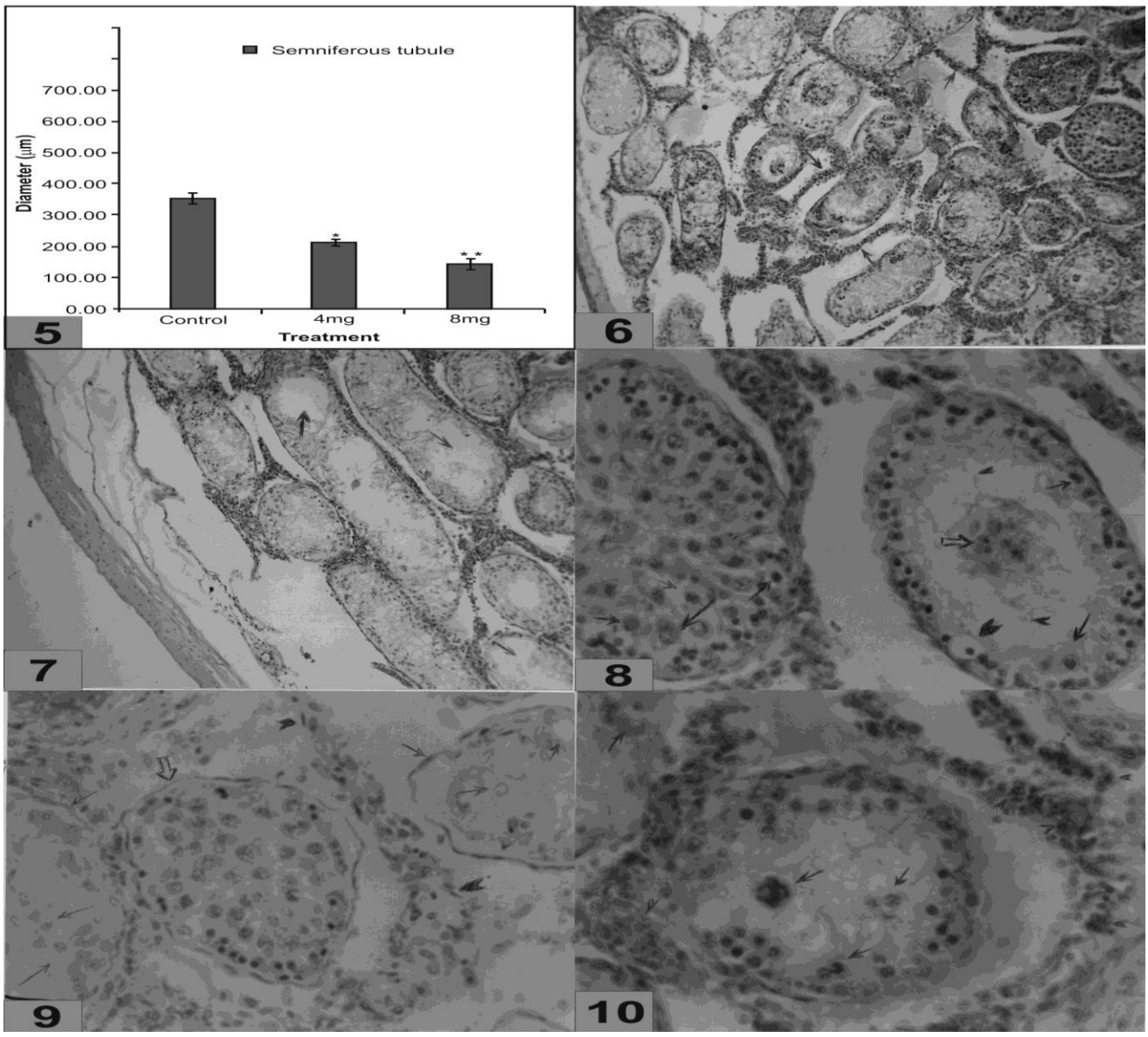

Fig 5: Effect of 4 mg and 8mg/Kg BW Oxaliplatin chronic treatment for 15 days and 5 days on seminiferous tubule diameter (control, n=6, Oxaliplatin, n=6).There was a significant decrease in the diameter when compared with the vehicle. $P < 0.005$*, $P < 0.05$**

Fig. 6 Testis from Oxaliplatin treated group (4 mg/KgBW for 15 days). Note dimunation in size of seminiferous tubules as well as variability in their contour. Peculiarity being different degrees of damage implicated all over the cross-section, as observed by "complete Sertolization" and extreme low frequency of germinal lineage cells in the peripheral tubules. However, some tubules survived the damage. Intraepithelial vacuolation is of many types sometimes leading to central aggregation of germinal lineage cells characterizing degeneration. The appearance of proliferative interstitial mesenchymal cells is similar all over (arrow) X 400.

Fig. 7 Peripheral portion of the testis photographed from chronic low dose. Note significant thickening of tunica albuginea. Seminiferous tubules are apparently shrunken, depopulation of gonial elements is

extreme, intratubular large spaces (arrow) prevails in almost 90% tubules. The degree of vacuolation and loss of germinal epithelium is variable in all the tubules X 400.

Fig. 8 Two seminiferous tubules from chronic low dose treated group. The right one seems to survive the degeneration, however, few mononucleate large giant cells appear in the basal region (arrow) but peripheral aggregation of micronuclei indicating the degeneration of gonial elements (arrow head). Note low cellular population density with corresponding enlargement of lumen diameter and intra-epithelial vacuolation (arrow head). Central aggregation of germinal lineage cell characterizing the degeneration (open arrow). Also note large vacuoles with central nucleus (chevron), uni and binucleate giant cells X 400.

Fig. 9 Few seminiferous tubules (4mg/KgBW/day) Oxaliplatin treated group. The extreme left and right convoluted tubule shows shrinkage and extreme loss of gonial cells, intraepithelial large vacuolation with cellular debris (arrow) suggesting apoptosis. The interstitial cellularity is similar throughout, however, exhibiting numerous cells of rather diverse shape. Attenuated mesenchymal cells in a peritubular location are observed (chevron). The central seminiferous tubule exhibit hypotrophic epithelium or accentuated reduction in germinal cells (open arrow) X 400.

Fig. 10 Seminiferous tubule from low dose treatment. Please note multinucleated formations of round spermatids with condensations of chromatin in two of them and vacuolation in other two remaining (arrow). Other features of apoptosis are same as described for other tubules. Highly proliferated interstitial mesenchyme reveal degenerative changes. Leydig cells appear vacuolated and partially atrophied (arrow head) X 400.

The most peculiar feature noted was hyperthermia of blood vessels. Peculiarity of the treatment being different degrees of damage implicated all over the cross-section, as observed by "Complete Sertolization" and extreme low frequency of germinal lineage cells in the peripheral tubules. However, some tubules survived the damage-but revealed obliteration of lumen by enlargement of germinal cells into large mono-nucleate or sometimes binucleate giant cells, some tubules showed low cellular population density with corresponding enlargement of lumen diameter and intraepithelial vacuolation. Intratubular large spaces prevailed in almost 90% tubules. The vacuolation observed were of three types, some tubules exhibited small central vacuoles, in others it enlarged to medium size, while in other tubules the complete tubules were occupied by a large vacuole leaving only peripheral layer of germinal cells undergoing degeneration, however, some tubules showed central aggregation of germinal lineage cells characterizing the degeneration.

Multinucleated formations of round spermatids and other rare forms of primary spermatocytes were also noted in this group with marginated condensations of chromatin in some nuclei or in several homogeneous masses, surrounding the nuclear membrane suggesting apoptosis. Some sections displayed disorganized cellular associations corresponding to different stages of the seminiferous epithelium cycle while others exhibited a large quantity of cellular debris and cells at various phases of maturation localized in the tubular lumen that is displaying quantitative alterations of the germinal lineage cells. Some tubules exhibited hypotrophic epithelium or accentuated reduction in germinal cells. The apoptosis was much more infrequent. Some degenerating spermatids were also occasionally seen. At the periphery of some seminiferous tubules a significant increase in meiotic micronuclei formation was observed indicating the degeneration of gonial elements. The interstitial cellularity appeared similar throughout, however, exhibiting numerous cells of rather diverse shape, but attenuated cells in peritubular location were common. Leydig cells appeared vacuolated and partially atrophied and low in frequency. Some tubules showed loss of Sertoli cells and if seen were extremely regressed. The spermatogonia showed pyknosis and leptotene spermatocytes with condensation of chromatin and an increase in the number of mast cells and macrophages.

Chronic high dose treatment (8mg / Kg BW Oxaliplatin for 5 days, figs.17-24**) .**

This treatment has implicated severe shrinkage and loss of regular configuration of the seminiferous tubules (fig.5). Almost all the tubules showed damage which was of the same degree and not of different types and none of the tubule survived the damage as has been described for the low dose treatment. The loosening and wavy infolding of lamina propria seemed to be significant when compared to the low dose treated group, moreover the thickening was also remarkable, decrease in the number of myoid cell was noticeable. There was an extreme proliferation of interstitial mesenchymal tissue resulting into severe edema or fibrosis, tremendous depletion of germinal epithelium, further increase in intraepithelial vacuolation, most of the tubules showed "Sertolization", tremendous loss of spermatogonia was visualized in many tubules, pyknotic nuclei of germinal lineage were observable characterizing the degeneration of the seminiferous epithelium or some other tubules exhibited hypotropic epithelium and large quantity of cellular debris, thus suggestive of progressive scarceness of spermatogonia showing repercussions on the reproductive capacity due to azoospermia. However, some of the tubules revealed obliteration of lumen by the large mononucleate giant cells, survival of some stages of germinal epithelium such as preleptotene, leptotene, pachytene and zygotene, but such conditions appeared to be rare. Even though there was extensive growth of mesenchymal tissue but Leydig cells showed atrophic changes such as decrease in their frequency, shape, vacuolated appearance, pyknosis or condensation of nuclei and an increase in mast cells and macrophages. Hyperthermia was notable in the mesenchyme .

Evaluation of testosterone

The low dose group showed significant decrease ($P < 0.1$) but the high dose treatment resulted into significant decrease ($P < 0.001$) in serum testosterone concentration compared to control values (fig-2)

Plate- 3

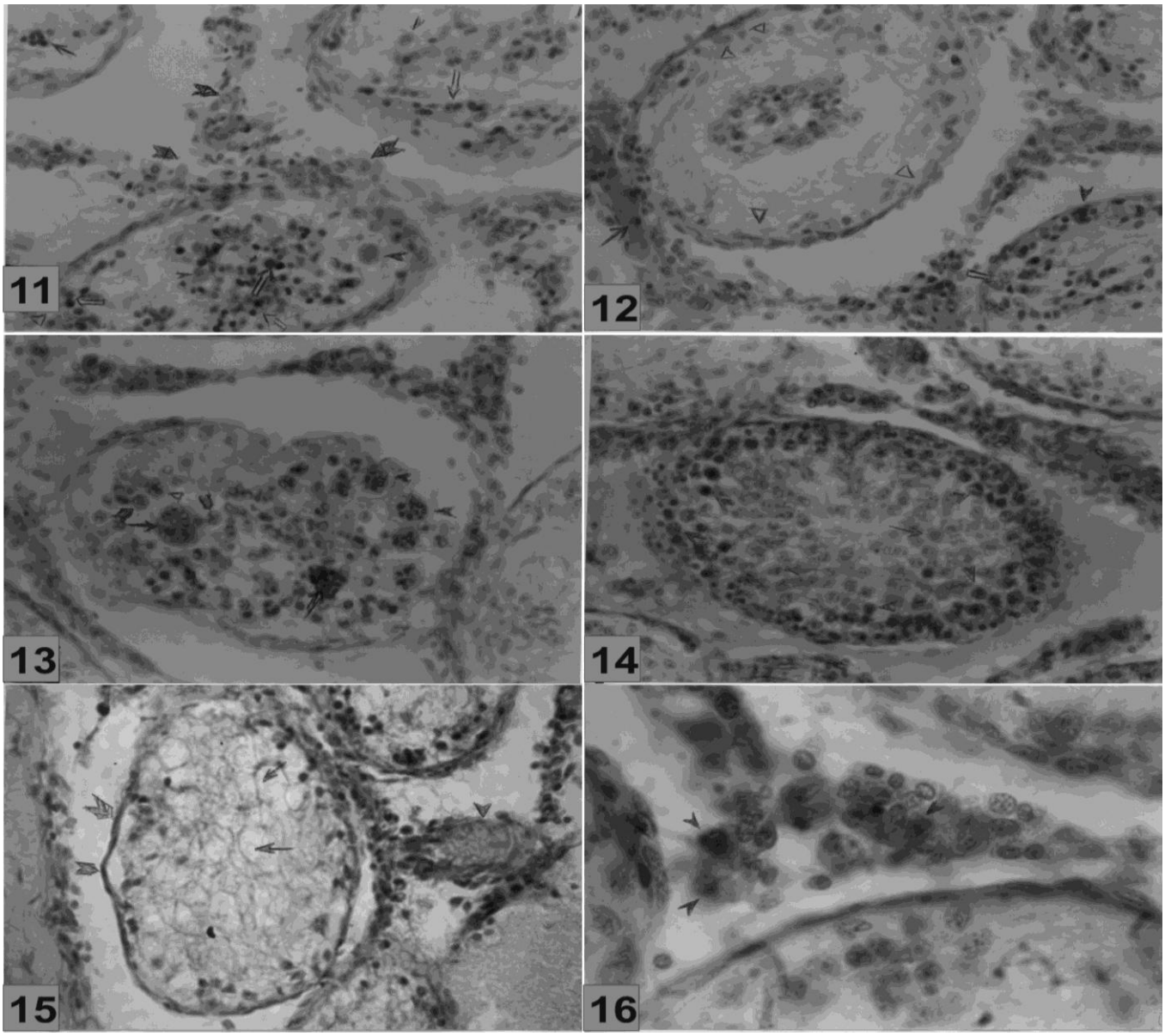

Fig. 11 Three seminiferous tubules photographed to show different degrees of damages from chronic low dose treated group. Please note multinucleated formation from primary spermatocytes with extreme condensation of chromatin material (arrow). Large mononucleate giant cells (arrow-head) and binucleate giant cells (open arrow) in the atrophied seminiferous tubule. Interstitial mesenchyme reveal low frequency of Leydig cells with similar degenerative changes (cheveron). All the tubules display disorganized cellular associations corresponding to different stages of the seminiferous epithelium cycle X 400.

Fig. 12 Few seminiferous tubules and extensive mesenchymal tissue in the interstices which is richly vascularized (arrow). Extreme lower seminiferous tubule shows multinucleate formation from primary spermatocytes (arrow head) as well as degenerating leptotene spermatocytes (open arrow). The tubule along with cells of germinal lineage show loss of Sertoli cells (triangle), and if seen are extremely regressed. Also note large quantity of cellular debris and cells at various phases of maturation localized in the tubular lumen indicating quantitative alterations of the germinal lineage cells X 400.

Fig. 13 Seminiferous tubule from low dose Oxaliplatin treated group. Please note multinucleated formation of round spermatids (arrow) and from primary spermatocytes (arrow head) with marginal condensations of chromatin in some nuclei. Also note uninucleate giant cells (chevron) and binucleate giant cells (triangle). Other stages of spermatogenic cycle elucidate apoptosis X 400.

Fig. 14 Transverse section of testis (4mg/kgBW/day) Oxaliplatin treated group .Note oedematous nature of interstitial mesenchyme and hyperthermia. The atrophy implicated by the drug seems to be less toxic as evident by obliteration of lumen by mononucleate giant cells (arrow), peripheral germinal cells undergoing apoptosis as their nuclei appear pyknotic, pre-leptotene, leptotene, pachytene stages of primary spermatocytes are noticeable (arrow head). Vacuolation also persists due to degeneration of germinal epithelial cells X 400.

Fig. 15 Apoptosis of germinal epithelium is much more frequent as evident by their remarkable loss and appearance of large intraepithelial vacuoles (arrow). The spermatogonia show pyknosis, few surviving Sertoli cells are extremely regressed and primary leptotene spermatocyte show condensed chromatin material. The most peculiar feature is hyperthermia of blood vessels (arrow head). Peritubular membrane show remarkable thickening and displacement (cheveron) X 450.

Fig. 16 Testis from low dose treatment. The interstitial fibrosis or edema seems to be milder than the high dose group (arrow). The mesenchyme development is not enormous and Leydig cell appear less atrophic (arrow head) X 1000.

Plate-4

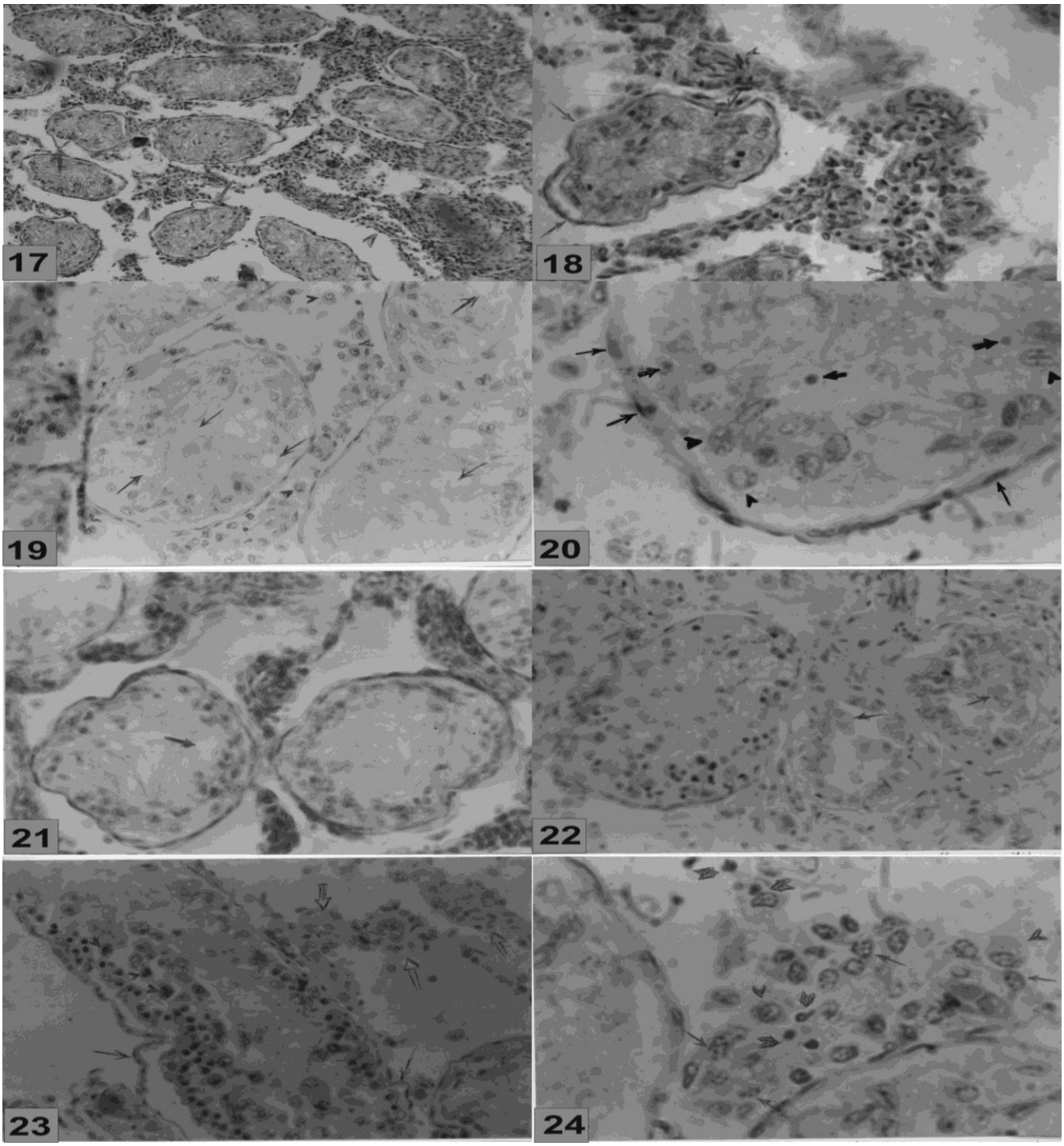

Fig. 17 A part of testis from chronic high dose Oxaliplatin treatment (8mg/KgBW for 5 days). Almost all the seminiferous tubules show damage, basement membrane show wavy lamellae and infoldings enveloping the tubule, sometimes hanging into loops (arrow). An interesting feature is the extreme proliferation of interstitial mesenchyme or severe edema (arrow head) X 100.

Fig. 18 Transverse section of testis from chronic high dose treated group. Please note significant decrease in the size of seminiferous tubule, loss of circular configuration, depletion in the germinal epithelium, remarkable thickening of lamina propria (arrow). There is extensive growth of mesenchyme (arrow head) but degeneration of Leydig cells as evident by decrease in their number, shape, vacuolated appearance, pyknosis or condensation of nuclei (short arrow), an increase in mast cells and macrophages X 450

Fig. 19 High dose treatment resulted into further increase in the intraepithelial vacuolation and depletion of germinal epithelium (arrow), Leydig cells appear further scarce and vacuolated (arrow head) X 450.

Fig. 20 A part of seminiferous tubule from high dose treated group. Note reduction in the number of myoid cells (arrow) and low cellular population density. Tubular section contained only Sertoli cells "Sertolization" of the tubule (arrow head), spermatogonia appear to be totally lost from this section, pyknotic nuclei of germinal lineage are observable (short arrow) characterizing the degeneration of the seminiferous epithelium or exhibiting hypotropic epithelium and large quantity of cellular debris, thus suggestive of progressive scarceness of spermatogonia showing repercussions on the reproductive capacity due to azoospermia X 400.

Fig. 21 The convoluted seminiferous tubules showing low cellular population density with corresponding enlargement of lumen diameter and intraepithelial vacuolation (arrow). Apoptosis is seen in all the tubules X 400.

Fig. 22 Few seminiferous tubules from 8 mg/KgBW/day Oxaliplatin treatment. The increment in dose resulted into further enhancement in the proliferation of interstitial mesenchyme matrix but actual decrease in the quantity of cells, both the mesenchymal and the Leydig. Peculiar feature of the damage is occurrence of only Sertoli cells in the tubule and depletion of sperm mother cells and other stages of spermatogenesis (arrow) X 400.

Fig. 23 Seminiferous from high dose treatment. Note wavy and displaced peritubular membrane (arrow). The spermatogenesis is restricted upto pachytene stage of primary spermatocyte (arrow head), the lumen shows intraepithelial vacuolation, cellular debris of germinal cells and hence depletion. The interstitial mesenchyme elucidate hyperthermia (open arrow) X 400.

Fig. 24 Interstitial mesenchyme from high dose treated group. Note the increase in the percentage of mast cells and macrophages (arrow). Leydig cells appear vacuolated and less in frequency (arrow head) and atrophied as their nuclei show condensation (cheveron) X 1000.

Statistical Analysis

To indicate individual variations in each corresponding region, the mean values and standard deviation (mean ± SD) for measurements from three animals were calculated. The statistical significance of differences for these values in different regions was assessed using't-test' (Delgaard, 2008). A significant level of $P<0.05$ was accepted.

DISCUSSION

Male sterility belongs to the recently recognized complications of cancer chemotherapy and has an increasing importance. Therefore, more information about the mode of action of anticancer drugs on mammalian spermatogenesis is needed. From our low and high dose experimental group it was conclusive that the action of Oxaliplatin was antigonadotrophic, antiandrogenic and antispermatogenic as evident by a number of changes such as decrease in the size of the testis, thickening of tunica albuginea, thickening of blood capillary wall, the regression and loss of regular configuration of seminiferous tubules, thickening and disruption of lamina propria (Davidoff *et al*.,1990). Similarly in the low dose treated group a very interesting and unusual occurrence of spherical masses of cytoplasm with 5-8 nuclei , large mononucleate and sometimes binucleate cells as described by Williams *et al*.,1968 ; Roosen Runge, 1973 and Freitas *et al*., 2002 either in normal condition or with drug treatment. According to them the occurrence of such multinucleated formations has been observed in testes of prepubertal normal rats (Miraglia and Hayashi, 1993), of elderly people (Holstein and Eckmann, 1966), in adverse conditions and pathological circumstances (Kaya and Harrison, 1975; Hayashi and Cedenho, 1980; Martinova *et al*., 1989; Miraglia and Hayashi, 1993; Scott *et al*., 1996; Botelho Cabral *et al*., 1997; Sasso-Cerri *et al*., 2001). Formation of such giant cell may be due to increased frequencies of hyperploidy observed in meiotic second cells (Sheu *et al*.,1992) or failures in the intercellular bridges (Holstein and Eckmann,1986).

As observed in the present study Oxaliplatin found to be a very potent inducer of apoptosis and the most sensitive cells included pachytene and dividing spermatocytes , however , Meistrich *et al*.,1982 and Russell and Russell,1991; Chater *et al*.,2007 described apoptosis of spermatogonia, and according to them which is a way to eliminate damaged germ cells as it is a controlled form of cell selection acting as a molecular control point regulating physiological process, toxicities and diseases through cell deletion (Corcoran *et al*.,1994). The apoptosis as resulted into low cellular population density (Chater *et al*., 2007), as well as 'Sertolization ' of the tubule (Freitas *et al*.,2002), and hence azoospermia. Micronuclei formation in the low dose group have been observed to some extent since it is also genotoxic (Lehdetie,1983; Lahdetie *et al*., 1994). The other interesting feature in the seminiferous tubule is hydropocity or hyalinization or accumulation of watery fluid in between the germinal epithelial cells and in the intraepithelial vacuoles. This may be due to accumulation of residual cytoplasm after the disintegration of different stages of spermatogenesis and retention of testicular fluid, a fibronoid exudates filling the lumen of a few tubule. The atrophy of Sertoli cells is a direct manifestation of decreased testosterone levels and atrophy of Leydig cells and therefore Oxaliplatin induced azoospermia since Sertoli cells are a possible mechanism to explain an elevated rate of germ

cell apoptosis and consequent infertility (Wallace *et al*.,1989; Aydiner *et al*.,1997; Monsees *et al*.,2000 and Bieber *et al*.,2006).

In the present study an interesting feature observed was hyperplasia of interstitial mesenchyme in the interstics of seminiferous tubules as well as around the peritubular membrane with partial atrophy of Leydig cells. Even though, there was a remarkable increase in the proliferation of mesenchymal cells with the high dose treatment but degeneration or necrosis of Leydig cells was partial. Such conditions have not been described in previous literature on Oxaliplatin treatment, however, interstitial fibrosis has been described in antiandrogen, Casodex (ZM176,334) treated patients (Jones *et al*.,1994); polytherapic treatment by anticancer drugs (Lendon et al., 1978; Hensle *et al*., 1984); autoimmune thyroiditis (Hoffman *et al*.,1991), in infertile men (Carmignani *et al*.,2004). In the present study regression and atrophy of Leydig cells was also associated with the decrease and significant decrease in the high dose of serum testosterone levels.Similarly damage to the germinal epithelium and a reduction in testicular volume may also affect Leydig cell function by causing structural changes within the testis or alteration in the paracrine control of Leydig cell function (Huhtaniemi and Toppari,1995;Carreau,1996).From the foregoing it is concluded that the treatment brings about structural changes as observed in the testis, probably due to a direct action of the drug, Oxaliplatin on the blood-testis barrier.

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