Chapter 5: Chromium (VI) toxicity and protective effect of melatonin: *In vivo* structural and functional alterations of testis and epididymis and epididymal sperms and *In vitro* alterations in cell viability and testosterone secretion

Introduction:

The incidences of hormonal imbalance, oligozoospermia, azoospermia, altered spermatogenesis, cryptorchidism and testicular cancer leading to poor reproductive performance of human males are ever increasing. In this context, a report of World Health Organization (WHO, 1999) has highlighted the fact that the sperm count of human males has been decreased by fifty percent in the last four decades. Among the various factors held responsible for inferior performance of male reproductive organs, transition elements are now getting their due recognition in male reproductive research (Agarwal *et al.*, 2008; Acharya *et al.*, 2006). Human males are by and far getting exposed to chromium compounds by way of occupational compulsion or through diet.

Chromium(VI) is known to cause oxidative stress mediated tissue damage and, the most possible reason behind this is the fact that, hexavalant chromium can enter redox-cycling processes and generate notorious ROS in tissues leading to impaired organ functioning (Stohs and Bagchi, 1995; Stohs *et al.*, 2001). Following its exposure hexavalant chromium compounds can cause increased lipid peroxidation and disturbed antioxidant – prooxidant equilibrium in cells resulting in enhanced

oxidative stress. Though, there are many reports available related to Cr(VI) induced oxidative stress leading to under performance of the organs in various systems, studies related to Cr(VI) induced changes in spermatogenesis, steroidogenesis, semen parameters and serum hormone profile are very limited (Li *et al.*, 2001; Achary *et al.*, 2004; Achary *et al.*, 2006). Semenological investigations have revealed low sperm count, decreased sperm motility, higher incidences of abnormal sperms, elevated levels of ROS and MDA (MDA, a product of lipid peroxidation), and following Cr administration to experimental animals as well as in occupationally exposed human subjects (Earnst, 1990; Bonde, 1993; Li *et al.*, 2001).

Interestingly, the available literature regarding the effects of chromium administration on male reproductive parameters is based on acute exposure to Cr and non relevant route of exposure. Further, the doses of chromium used are based on LD_{50} values and, studies are also based on standard toxicological procedures, results of which are not extrapolatable to humans as, humans never encounter such a dose of chromium nor get exposed to by such modes. The present study is therefore initiated to assess the effects of Cr when given in a physiologically relevant realistic dose based on environmental contamination and dietary intake. Exposure to such a dosage of Cr given through drinking water has shown increased lipid peroxidation and depleted levels of both enzymatic and non-enzymatic antioxidants in both testis and epididymis of w*istar* rats (Chapter 1). Production of male gametes and androgens *i.e.* spermatogenesis and steroidogenesis respectively, occurs in the testis while the process of sperm maturation occurs in the epididymis. Moreover, testicular steroidogenic enzymes 3-β HSD and 17-β HSD play a key role in testicular androgenesis (Jana *et al.*, 2006) and any alteration in their activity can lead to

disturbed serum as well as intratesticular testosterone titre. The aim of the present study was to understand the after effects of increased oxidative stress following Cr(VI) exposure in both testis and epididymis in terms of steroidogenic enzyme activity, serum steroid levels, semen parameters (sperm count, sperm motility and sperm abnormality) and organ histoachitecture.

Previously, simultaneous administration of melatonin along with Cr(VI) was shown to have significant protective effect on oxidative stress in both testis and epididymis of rats (Chapter 1). Co-administration of melatonin along with Cr(VI) has been attempted in the present study too, to fathom whether co-administration of melatonin can protect against Cr(VI) induced possible functional alterations in testis and epididymis, as has been shown against oxidative stress previously (Chapter 1). Accordingly, in the present study, attempt has also been made to evaluate the effect of *In vitro* exposure to hexavalant chromium with or without melatonin in isolated rat Leydig cells in terms of cytolethality and testosterone production and release.

Material and Methods:

For treatment, methodology employed and protocols refer material and methods section (Page no. 17)

Results:

3-β HSD activity:

Hexavalant chromium induced 3-β HSD inhibition could be observed in a duration dependent manner. Melatonin administration afforded significant protection against Cr(VI) induced inhibition of 3-β HSD inhibition (Table: 5.1, Figure: 5.1).

17-β HSD activity:

Significant linear duration dependent inhibition in testicular 17-β HSD activity could be discerned in Cr(VI) treated rats compared to their respective controls. Conversely, the protective effect of co-administered melatonin was found to be much better in the long exposure experimental animals than in the short duration exposed ones (Table: 5.2, Figure: 5.2).

Serum Testosterone (T):

Serum testosterone titre showed marked duration dependent reduction following Cr(VI) administration. Though melatonin showed a tendency to marginally decrease serum T level in control rats, the protective effect of melatonin when co-administered with Cr(VI) in preventing against Cr(VI) induced decrement was found to be progressively better with increasing duration of exposure. These changes in serum T level are represented in Table: 5.3 and Figure: 5.3.

Serum Estradiol (E_2):

Serum of rats treated with Cr(VI) showed significant but duration independent decrement in estradiol titre. Co-treatment with melatonin exerted a protective effect on the observed depletion in serum estradiol titre (Table: 5.4, Figure: 5.4).

Cauda Epididymal Sperm Count:

Duration dependent linear decrease in cauda epididymal sperm count was the feature in hexavalant chromium treated rats. Melatonin exerted significant protection when co-administered along with Cr(VI) against the chromium induced reduction in cauda epididymal sperm count (Table: 5.5, Figure: 5.5).

Sperm motility:

Sperm motility evaluation showed a duration independent significant reduction in the number of motile sperm due to Cr(VI) exposure with maximal effect being seen at the shorter duration exposure. The protective effect of melatonin was also quite significant with maximal protection being seen at longer duration exposure (Table: 5.6, Figure: 5.6).

Sperm Abnormality:

Hexavalent chromium treatment induced a significant increase in the sperm numbers based on the duration of exposure and the maximum abnormal sperm found at longer duration. Simultaneous, melatonin exposure exerted significant protection against Cr(VI) induced deleterious effects. (Table: 5.7, Figure: 5.7)

***In vitro* Cell Viability:**

Effect of Cr(VI) added to a culture of isolated Leydig cells showed time dependent decrease in cell viability (3 – 12 hours). Simultaneous presence of melatonin along with Cr(VI) significantly improved cell viability. These changes are depicted in Table: 5.10

***In vitro* Testosterone (T) Production:**

Testosterone release evaluated in a culture of isolated Leydig cells under basal and hCG stimulated conditions showed marked decrease in presence of hexavalant chromium which was rectified to some extent by simultaneous presence of melatonin. Increasing duration of exposure to Cr(VI) tended to show a tremendous decrease in hCG mediated release and melatonin proved to be equally effective at both 3 and 6 hours of exposure. Table: 5.11 depict these changes in *In vitro* T release.

Histological Observations:

Testis: Control testis (Plate: 1, Fig. T1 and T2) sections revealed well formed seminiferous tubules with all stages of spermatogonia represented. Testis of melatonin (Plate: 1, Fig. T3 and T4) treated animal showed a quantitative reaction in the germ cell population of tubules with no qualitative effect on spermatogenesis. The testis of Cr(VI) (Plate: 2, Fig. T5 and T6) exposed animals shows distorted seminiferous tubules with most of the tubules denuded of sperm. The basement membrane was also affected and it could be seen lifting from the tubules. Spermatids and spermatocytes seem to be exfoliated. Testis of animals treated with both Cr(VI) and melatonin (Plate: 2, Fig. T7 and T8) showed better organized tubules with intact basement

membrane. Though there was quantitative effect on spermatogenesis, there was no qualitative effect on spermatogenesis that could be seen occurring in many tubules.

Epididymis:

Whereas melatonin (Plate: 3, Fig. E3 and E4) showed cytoprotective effect on epididymis epithelium, Cr(VI) (Plate: 4, Fig. E5 and E6) exposure showed hypotrophied epithelium with many vacuolated structures within the lumen of the tubules showed hardly any sperm with occasional presence of degenerative sperm cells. Epididymis of animals treated with Cr(VI) and melatonin (Plate: 4, Fig. E7 and E8) showed presence of sperms within the lumen but epithelium was found to be hypotrophied.

Plate: 1

Figure: T_1: Photomicrograph of control testis showing Seminiferous tubules and Leydig cells (100X)

S:Seminiferous tubules, L: Leydig cells

Figure: T_2: Photomicrograph of control testis showing sperms within a tubule and Leydig cells (400X)

Sp: Spermatozoa, L: Leydig cells

Figure: T_3: Photomicrograph of testis treated with **Melatonin** showing Seminiferous tubules and Leydig cells (100X)

S:Seminiferous tubules, L: Leydig cells

Figure: T_4 : Photomicrograph of testis treated with **Melatonin** showing Seminiferous tubules with sperms and Leydig cells (400X)

Sp: Spermatozoa, L: Leydig cells

Plate: 1

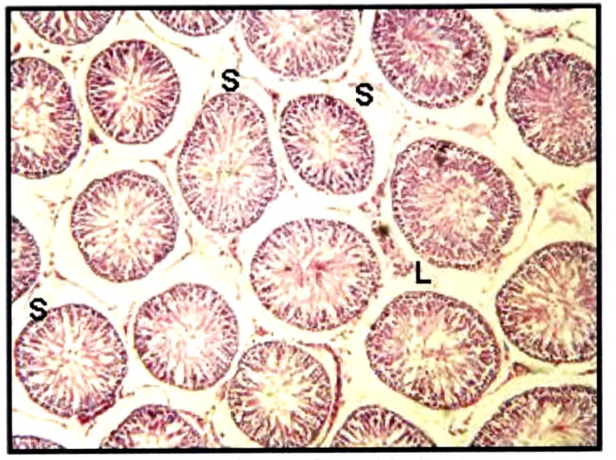


Fig. T1: C (100x)

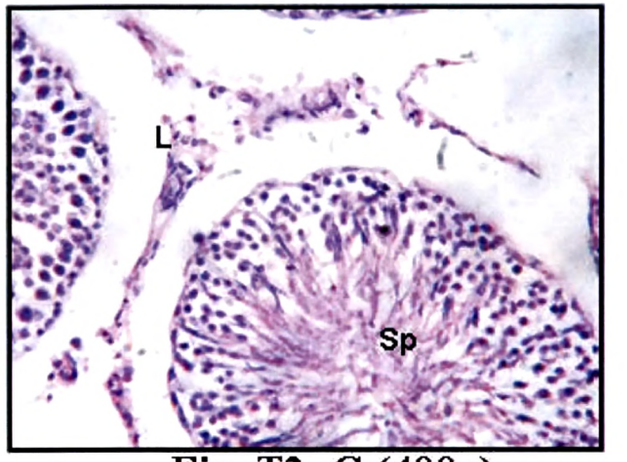


Fig. T2: C (400x)

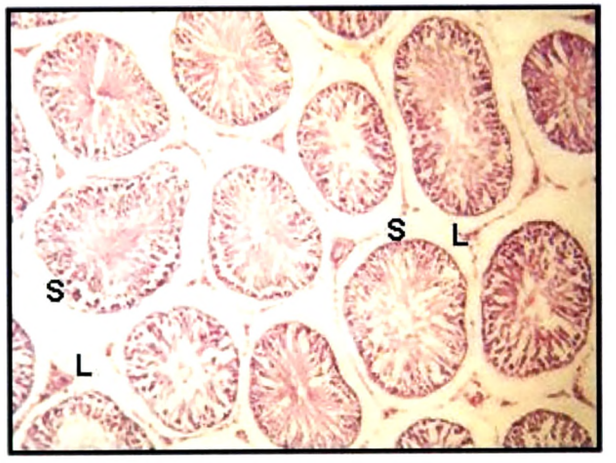


Fig. T3 : C + Mel (100x)

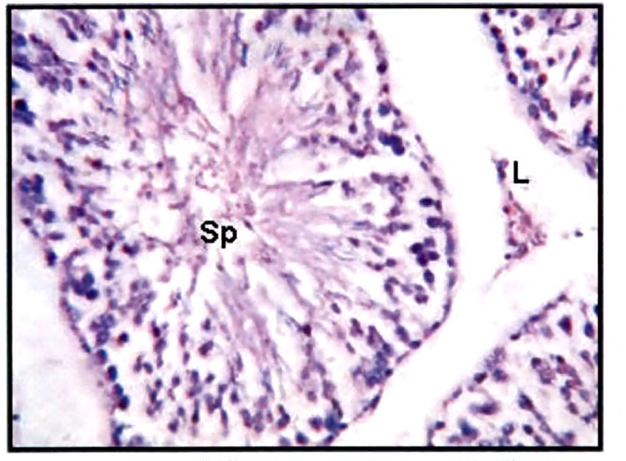


Fig. T4: C + Mel (400x)

Plate: 2

Figure: T_5: Photomicrograph of **Chromium (VI)** treated testis showing distorted basement membrane of seminiferous tubules and tubular lumen devoid of sperms (100X).

lu: Lumen; S: Seminiferous tubules; L: Leydig cells

Figure: T_6: Photomicrograph of **Chromium (VI)** treated testis showing a semiiniferous tubules with distorted basement membrane and lumen devoid of sperms (400X). note also the degenerating spermatids and post meotic spermatocytes (Arrows)

lu: Lumen; S: Seminiferous tubules

Figure: T_7: Photomicrograph of testis treated with **Chromium (VI)** and Melatonin showing better seminiferous tubules with minor distortions of basement membrane but with sperms in the lumen (100X)

S: Seminiferous tubules; L: Leydig cells

Figure: T_8: Photomicrograph of testis treated with **Chromium (VI)** and Melatonin showing a seminiferous tubule with minor distortions of the basement membrane and sperms in the lumen (400X)

S:Seminiferous tubules, Sp: Spermatozoa

Plate: 2

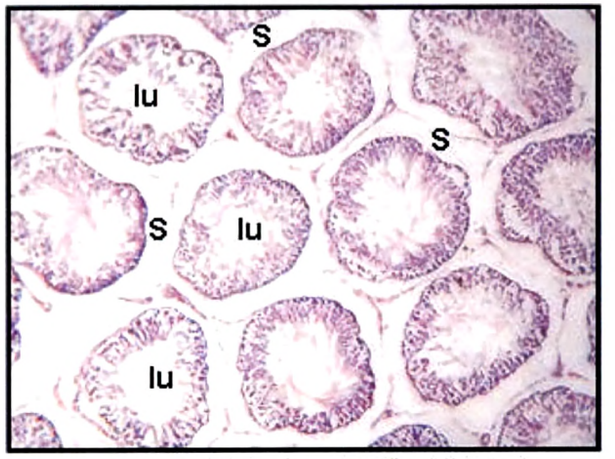


Fig. T5: Cr(VI) (100x)

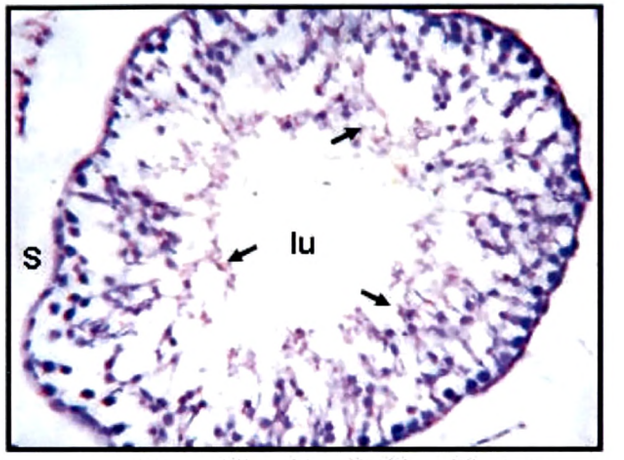


Fig. T6: Cr(VI) (400x)

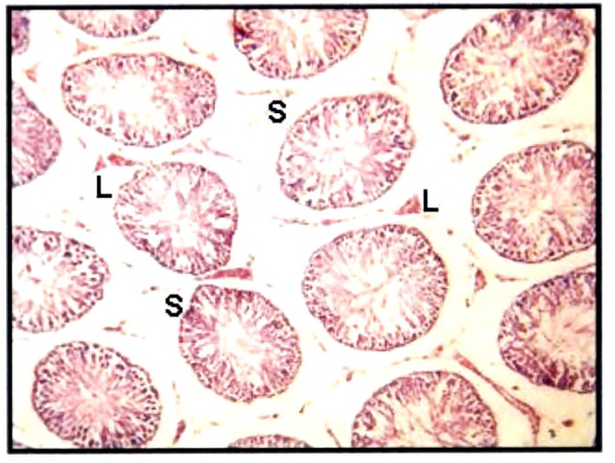


Fig. T7: Cr(VI) + Mel (100x)

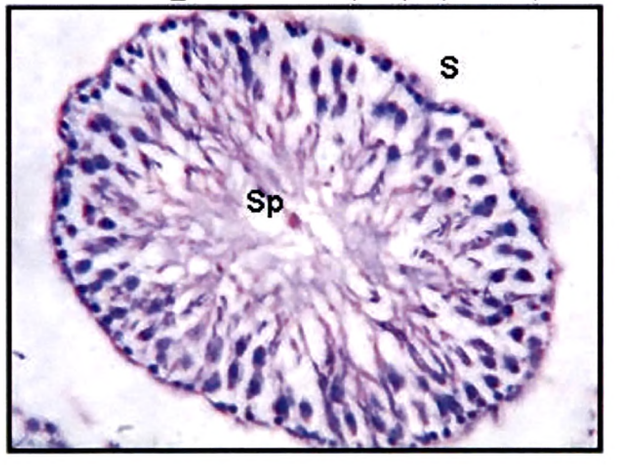


Fig. T8: Cr(VI)+ Mel (400x)

Plate: 3

Figure: E_1: Photomicrograph of control epididymis showing cauda tubules with Sperms in the lumen (100X)

lu: Lumen; Sp: Sperms

Figure: E_2: Photomicrograph of control epididymis showing cauda tubules with Sperms in the lumen (400X)

Sp: Sperms

Figure: E_3: Photomicrograph of epididymis treated with melatonin showing cauda tubules with sperm in the lumen (100X)

Sp: Sperms

Figure: E_4: Photomicrograph of epididymis treated with melatonin showing cauda tubules with sperm in the lumen (400X)

Sp: Sperms

Plate: 3

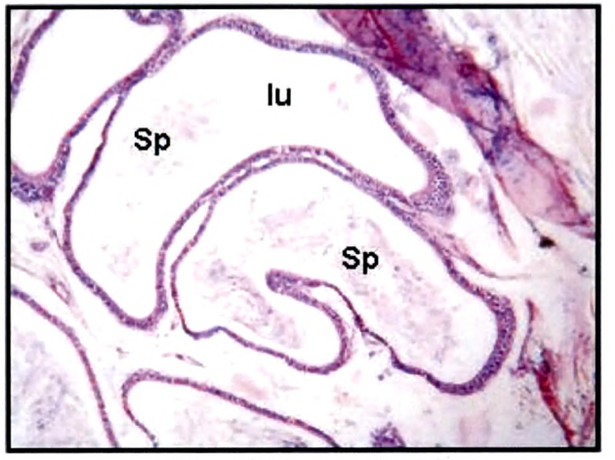


Fig. E1: C (100x)

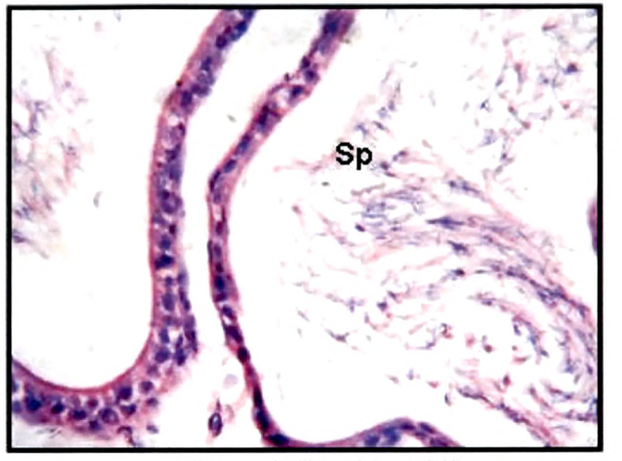


Fig. E2: C (400x)

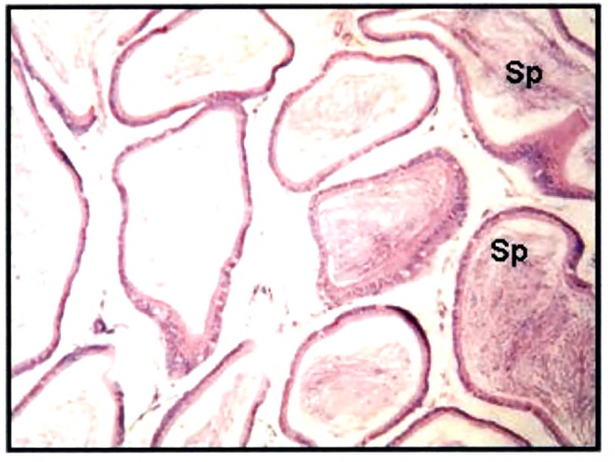


Fig. E3: C + Mel (100x)

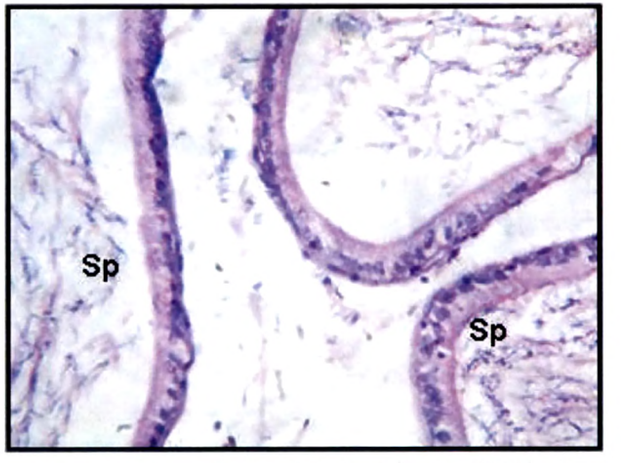


Fig. E4: C + Mel (400x)

Plate: 4

Figure: E_5: Photomicrograph of epididymis treated with **Chromium (VI)** showing cauda tubules mostly empty and a few showing sperms (100X)

lu: Lumen; Sp: Sperms

Figure: E_6: Photomicrograph of epididymis treated with **Chromium (VI)** showing cauda tubules with hypotrophied epithelium with vacuolar structures (arrows) and empty lumen (400X)

lu: Lumen; HE: hypotrophied epithelium

Figure: E_7: Photomicrograph of epididymis treated with **Chromium (VI)** and melatonin showing cauda tubules with sperms in the lumen (100X)

lu: Lumen; Sp: Sperms

Figure: E_7: Photomicrograph of epididymis treated with melatonin showing cauda tubules with sperms in the lumen (400X). Note the epithelium is hypotrophide.

Plate: 4

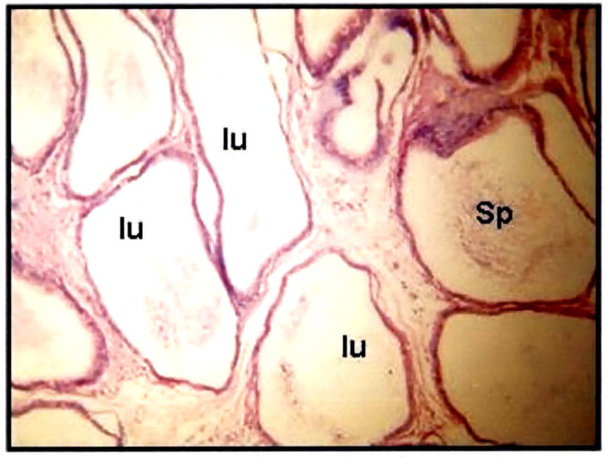


Fig. E5: Cr(VI) (100x)

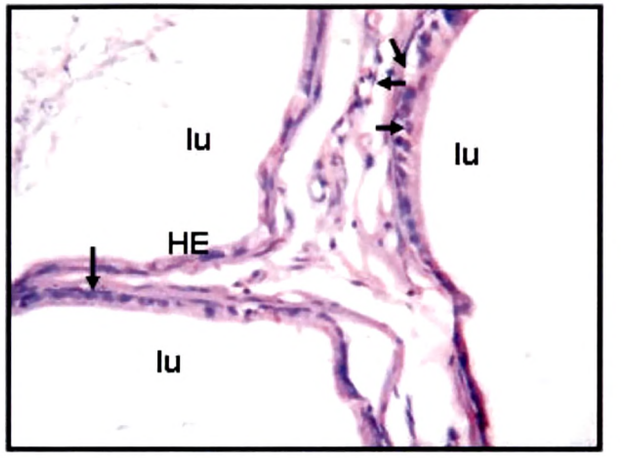


Fig. E6: Cr(VI) (400x)

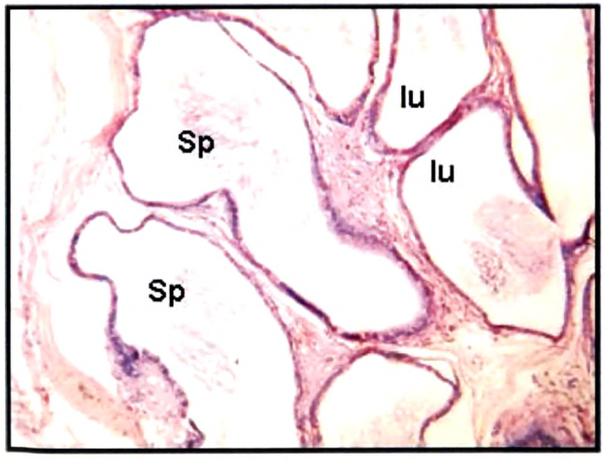


Fig. E7: Cr(VI) + Mel (100x)

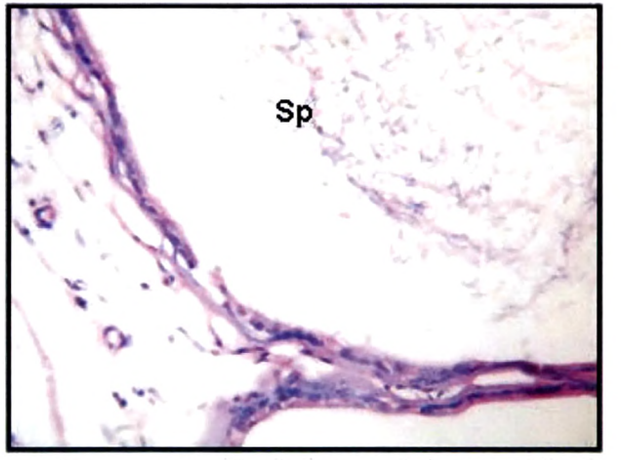
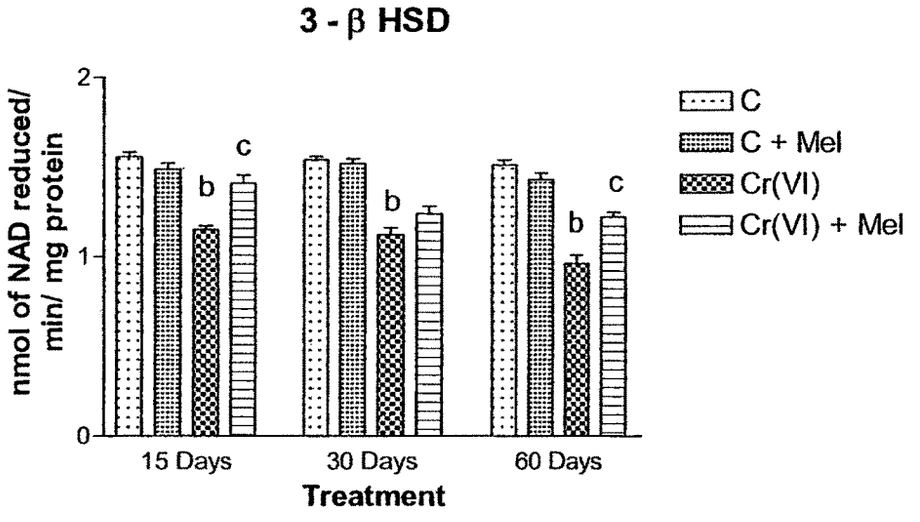


Fig. E8: Cr(VI) + Mel (400x)

Figure 5.1 : Chromium(VI) induced changes in 3 – β HSD activity in testis with or without Melatonin.



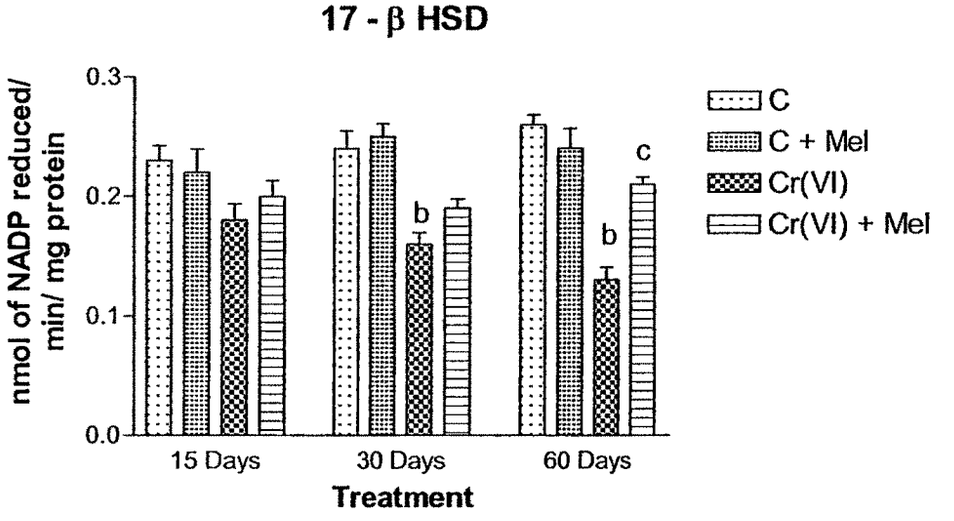
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.1: Chromium(VI) induced changes in 3 – β HSD activity (nmol of NAD reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	1.56 ± 0.02	1.49 ± 0.03	1.15 ± 0.02^{b}	1.41 ± 0.04^{c}
30 Days	1.54 ± 0.02	1.52 ± 0.02	1.12 ± 0.04^{b}	1.24 ± 0.04
60 Days	1.51 ± 0.02	1.43 ± 0.03	0.96 ± 0.04^{b}	1.22 ± 0.02^{c}

Values expressed as Mean ± SEM of 6 animals per group..
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.2 : Chromium(VI) induced changes in 17 – β HSD activity in testis with or without Melatonin.



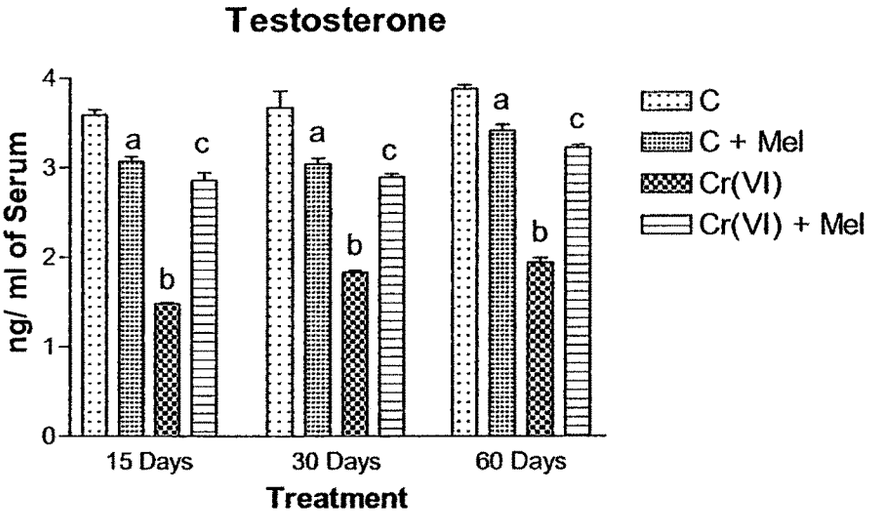
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.2 : Chromium(VI) induced changes in 17 – β HSD activity (nmol of NADP reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	0.23 ± 0.01	0.22 ± 0.01	0.18 ± 0.01	0.20 ± 0.01
30 Days	0.24 ± 0.01	0.25 ± 0.01	0.16 ± 0.01[b]	0.19 ± 0.01
60 Days	0.26 ± 0.01	0.24 ± 0.01	0.13 ± 0.01[b]	0.21 ± 0.01[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.3: Chromium(VI) induced changes in serum Testosterone(T) level with or without Melatonin.



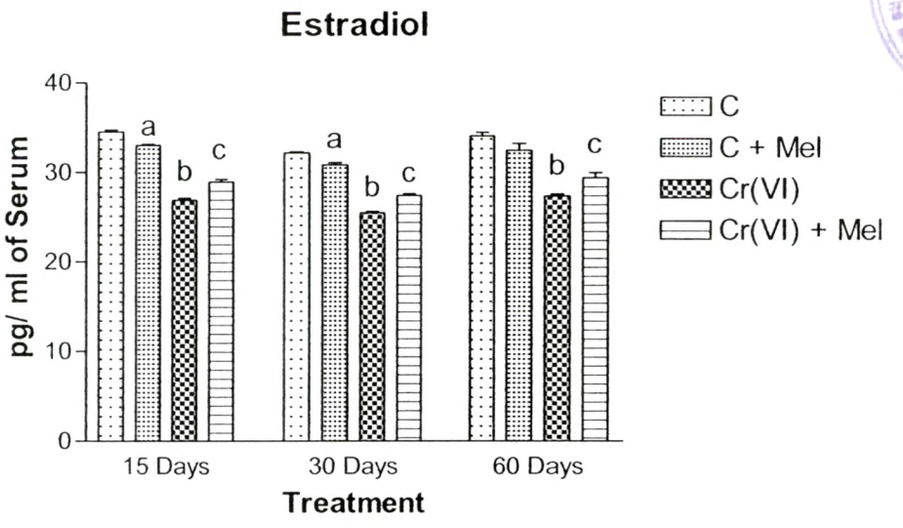
Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.3: Chromium(VI) induced changes in serum Testosterone(T) level (ng/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	3.59 ± 0.05	3.07 ± 0.05[a]	1.48 ± 0.01[b]	2.86 ± 0.08[c]
30 Days	3.21 ± 0.18	3.04 ± 0.06[a]	1.94 ± 0.02[b]	2.89 ± 0.03[c]
60 Days	3.88 ± 0.04	3.41 ± 0.06[a]	1.94 ± 0.04[b]	3.22 ± 0.02[c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.4: Chromium(VI) induced changes in serum Estradiol(E_2) level with or without Melatonin.



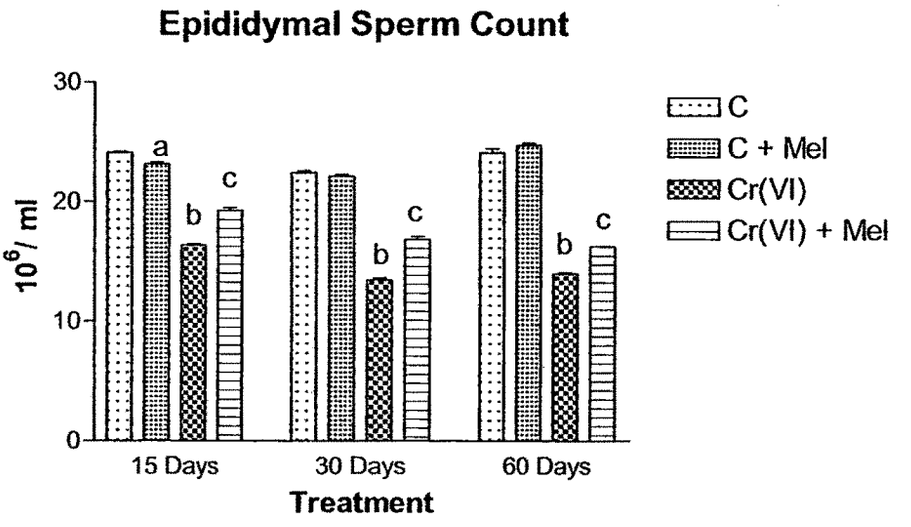
Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.4: Chromium(VI) induced changes in serum Estradiol(E_2) level (pg/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	34.51 ± 0.22	33.01 ± 0.12[a]	26.88 ± 0.24[b]	28.92 ± 0.31[c]
30 Days	32.15 ± 0.13	30.83 ± 0.24[a]	25.45 ± 0.17[b]	27.30 ± 0.21[c]
60 Days	34.01 ± 0.41	32.43 ± 0.73	27.33 ± 0.20[b]	29.36 ± 0.54[c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.5: Chromium(VI) induced changes in epididymal sperm count with or without Melatonin.



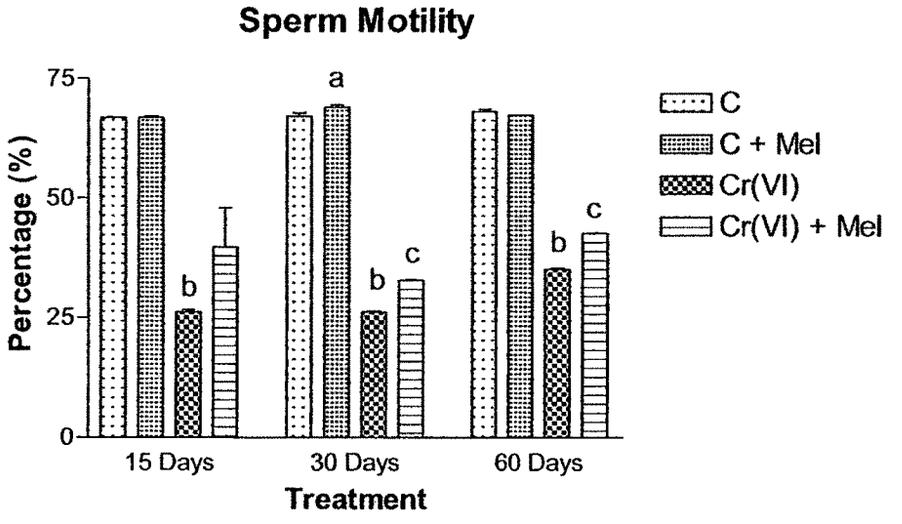
Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.5: Chromium(VI) induced changes in epididymal sperm count (10^6/ml) with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	24.10 ± 0.07	23.14 ± 0.17[a]	16.35 ± 0.11[b]	19.26 ± 0.24[c]
30 Days	22.44 ± 0.13	22.10 ± 0.19	13.44 ± 0.16[b]	16.85 ± 0.24[c]
60 Days	24.08 ± 0.39	24.73 ± 0.19	13.95 ± 0.11[b]	16.23 ± 0.06[c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.6: Chromium(VI) induced changes in epididymal sperm motility with or without Melatonin.



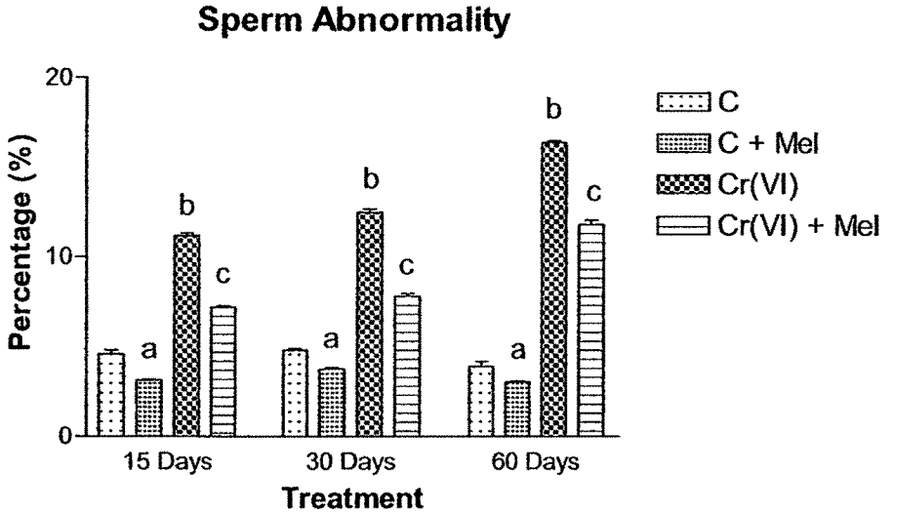
Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.6: Chromium(VI) induced changes in epididymal sperm motility (percentage) with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	66.87 ± 0.12	66.92 ± 0.27	26.15 ± 0.53^{b}	39.75 ± 8.1
30 Days	67.16 ± 0.67	69.06 ± 0.46^{a}	26.14 ± 0.27^{b}	32.77 ± 0.11^{c}
60 Days	68.12 ± 0.50	67.44 ± 0.05	35.17 ± 0.24^{b}	42.67 ± 0.11^{c}

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Figure 5.7: Chromium(VI) induced changes in epididymal sperm abnormality with or without Melatonin.



Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.7: Chromium(VI) induced changes in epididymal sperm abnormality (percentage) with or without Melatonin.

Treatment	C	C + Mel	Cr (VI)	Cr(VI) + Mel
15 Days	4.60 ± 0.20	3.14 ± 0.03[a]	11.20 ± 0.11[b]	7.20 ± 0.06[c]
30 Days	4.80 ± 0.09	3.76 ± 0.10[a]	12.50 ± 0.17[b]	7.80 ± 0.15[c]
60 Days	3.90 ± 0.28	3.05 ± 0.04[a]	16.40 ± 0.09[b]	11.80 ± 0.25[c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.8: Chromium(VI) induced percentage changes in chromium content of testis and epididymis with or without melatonin.

Treatment	Testis		Epididymis	
	Cr(VI)	Cr(VI) + Mel	Cr(VI)	Cr(VI) + Mel
15 Days	57.50	33.33	9.59	10.32
30 Days	50.00	26.19	13.33	12.21
60 Days	106.98	88.89	10.90	15.20

C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.9: Chromium(VI) induced changes in the serum titre of melatonin(pg/ ml) in control and experimental rats.

Treatment	C	C + Mel	Cr(VI)	Cr(VI) + Mel
15 Days	121.00 ± 0.36	140.00 ± 0.60[a]	67.00 ± 1.36[b]	113.00 ± 1.05[c]
30 Days	112.00 ± 0.24	143.00 ± 0.17[a]	75.00 ±1.08[b]	109.00 ± 0.35[c]
60 Days	93.00 ± 0.05	126.00 ± 0.14[a]	26.00 ± 0.69[b]	83.00 ± 0.84[c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.10: Chromium(VI) induced changes in Leydig cell viability(in percentage) with or without melatonin.

Treatment	Cell Viability			
	C	C + Mel	Cr(VI)	Cr(VI) + Mel
3 Hours	93.81 ± 0.06	94.10 ± 0.11	94.00 ± 0.14	72.50 ± 0.06 [c]
6 Hours	93.42 ± 0.23	93.96 ± 0.24	66.51 ± 0.24 [b]	68.21 ± 0.11 [c]
12 Hours	92.09 ± 0.07	94.33 ± 0.04	52.65 ± 0.08 [b]	60.17 ± 0.23 [c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 5.11: Chromium(VI) induced changes in Testosterone (ng/ 10^6 cells) secretion in isolated Leydig cell under basal and hCG stimulated condition.

Treatment	Testosterone Secretion (ng/ 10^6 cells)							
	Basal				hCG stimulated			
	C	**C + Mel**	**Cr(VI)**	**Cr(VI) + Mel**	**C**	**C + M**	**Cr(VI)**	**Cr(VI) + Mel**
3 Hours	4.87 ± 0.33	4.17 ± 0.16	3.04 ± 0.53 [b]	3.47 ± 0.02 [c]	7.58 ± 0.45	5.83 ± 0.04 [a]	4.72 ± 0.23	5.39 ± 0.14 [c]
6 Hours	4.55 ± 0.16	4.06 ± 0.20	3.36 ± 0.24 [b]	3.89 ± 0.72	7.94 ± 0.04	5.93 ± 0.11	4.19 ± 0.07 [b]	5.61 ± 0.3 [c]

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Discussion:

Chromium (VI) is a major metal pollutant whose systemic toxicity has been studied to a greater extent. Reproductive toxicity to the male system has however received much lesser attention compared to organs like liver, kidney and lung. Unfortunately, most of the studies to date are mostly based on standard toxicological procedures of LD_{50} value, acute or sub acute, NOEL, LOEL etc. Acute studies involving a single exposure or for a few days with varying modes of exposure like intraperitoneal, subcutaneous or by inhalation, have been the choice of experimenters. In practical terms, from human point of view, such studies are of minimal relevance and of pristine academic interest. It was in this context, reproductive toxicity studies on chromium in terms of oxidative stress using a realistic dosage based on environmental concentration and the quantum of systemic entry into humans through dietary components, was undertaken previously (Chapter- 1). In continuation, the present evaluation assesses the impact of such realistic dosage of Cr (VI) on structural and functional alteration in testis and epididymis. The *In vivo* findings have been logistically followed up with *In vitro* studies to gain further credence. Clearly, the realistic dosage employed in the present study has shown structurally and functionally correlatable disruption of spermatogenesis and steroidogenesis in the testis and qualitative and quantitative changes in the structure of epididymis and its store of sperm.

The observed changes in testis involve a progressive disruption in spermatogenesis marked by initial sloughing off of spermatozoa, followed by gradual loss of spermatids and post pachytene spermatocytes. Such a progressive of loss of

germ cells lumeno-peripheraed occurred between 15 and 60 days of chromium exposure and the seminiferous tubules of 60 day chromium exposed testis showed only the cells of the basal compartment with visible shrinkage of the tubules. The interstitial cells appeared to be better protected and tended to show milder hyperplasia. Similar changes have been reported even in the case of chromium exposed Bonnet monkey (Aruldhas *et al.*, 2005). A short term treatment with $[Cr^{V}$-BT$]^{2-}$ in mice has also noticed exfoliation of mature sperm into the lumen of seminiferous tubules and alterations in the structure of Sertoli cells (Pereira *et al.*, 2005). Based on the appearance of injected tracer HRP within the junctional complexes between adjoining Sertoli cells and even within Sertoli cells and spermatids, Pereira *et al.* (2002, 2004) had demonstrated disruption of the blood testis barrier.

There are hardly and studies on epididymal structure and function in relation to Cr toxicity. The present study on chronic chromium exposure has revealed marked structural disorganization of the cauda epididymis. Not only are there complete disappearance of spermatozoa from the lumen of epididymal tubules (with only presence of damaged apoptotic or necrotic germ cells) but also tubular epithelial disruption and appearance of vacuole like structures within the epithelium. Since such structures are characteristics of Cr (VI) treated epididymis only, they appear to be a specific chromium induced toxicity response of the epididymis. Interestingly, based on elegant and incisive light and ultra microscopic studies of Agnes and Akbarsha (2001) and Aruldhas *et al.* (2004), similar structures have been described in Cr exposed epididymis of monkey and aflatoxin treated mouse epididymis respectively. Extensive analysis of such structures at length led Agnes and Akbarsha (2001) to

propose these to be basal cell derived pale vacuolated epithelial cells (PVEC) which would enclose the damaged and necrotic spermatozoa entering the epididymal epithelium through breaches in the principal cells, preventing their extravasation and possible auto immune response. Similar type of structures seen in the monkey epididymis was termed microcanals performing almost similar function, with the only difference being that while PVEC of mouse epididymis is a single basal cell modification, the microcanal of monkey epididymis is a collective formation by four or five basal cells. Apparently, the currently observed vacuole like structures within the epididymal epithelium of Cr (VI) treated rats could be homologous to the PVEC of mouse or the microcanal of monkey. Only more probing and specific histological and ultrastructual studies only would resolve it conclusively. But it can be safely said that such modification of epididymal epithelium are adaptive responses to toxic challenges by xenobiotics.

Apart from the observed structural alterations in epididymis and in the testis causing disruption in spermatogenesis, the steroidogenic potential of the testis was also compromised. From the observations and recorded data, it becomes clear that Cr (VI) exposure leads to progressive decrease in 3β HSD and 17 β HSD activities and fall in serum testosterone and estrogen titres. Pertinently other investigators have also shown decrease in serum LH titre and increase in FSH (Aruldhas, 2000; Li *et al.*, 2001; Yousef *et al.*, 2005; Chandra *et al.*, 2007). Despite the increasing inhibition of steroid dehydrogenases, the circulating titre of testosterone is maintained steady at 50% of the control level. The histologically observable hyperplasia of Leydig cells in the interstitium of Cr(VI) treated testis may account for this. The possibility of decreased metabolic clearance of testosterone can also be considered as a possible

alteration due to Cr (VI) exposure. The disruption of the spermatogenic process and the epididymal structural alteration could also be related with the decline in testosterone as, both the process of spermatogenesis and, epididymis are androgen dependent. The Cr(VI) induced toxicity effects on testis and epididymis are well reflected in the quantitative changes affecting the sperm within the reproductive tract. Whereas the total epididymal sperm count decreased by 42%, sperm motility test showed 35% increase immotile sperms with about 12% increase in the occurrence of abnormal sperms. Even other workers in their acute single dose or short duration exposure to Cr have also shown a decline in sperm count and motility and increase in abnormality (Li *et al.*, 2001; Acharya *et al.*, 2003; 2005; Pereira *et al.*, 2005; Yousef *et al.*, 2005; Chandra *et al.*, 2007). The significant decrease in sperm count and motility and increase in the number of abnormal sperm suggest that some functional impairment is being inflicted on testes and epididymis by Cr.

The mechanism of Cr toxicity to reproductive organs is not yet clear. The multitudes of effects induced by Cr exposure seen in the present study affecting the structural and functional competence of tubular cells (Sertoli and germ) and epididymis, cannot be thought of as multiple mechanisms of Cr toxicity. There has to be a common generalized denominator and it appears to be the ability of Cr to induce oxidative stress. Previous study in this context has shown significant oxidative stress being generated by Cr in both testis and epididymis, more so in the epididymis, as noted by the degree of membrane lipid peroxidation and the compromised endogenous antioxidant status (Chapter-1). Apparently, oxidative stress generated by Cr could be considered the basal primary cause for all the observed effects on testis and epididymis and precisely, validity to this inference is provided by the observation

of changes in one or other parameters of oxidative stress like peroxidation, decrease in non enzymatic or enzymatic antioxidant or even generation of some ROS moieties in the testis by the earlier cited workers. Of late, the impact of oxidative stress and the role of ROS and RNS are being increasingly realized in reproductive functions especially on sperm structure and functions and are being seriously considered as factors of significance in male infertility(Agrawal and Prabakaran, 2005; Cocuzza *et al.*, 2007; Tremellem, 2008).

Other than the direct ability of Cr to increase oxidative stress, its indirect role by way of iron induced oxidative stress also cannot be over-ruled as, iron mediated testicular dysfunction in mouse (Thyagarajan and Muralidhara, 2008) and an actual increase in iron content in hydroperoxide induced oxidative stress in rat testis and epididymis (Kumar and Muralidhara, 2007) have been reported. The observation that mature sperm and spermatids are the first cell types which succumb to the Cr induced oxidative stress finds explanation in the reported absence of catalase and peroxidase in them (Yoganathan *et al.*, 1989). Their vulnerability in this context is understandable as peroxyl and $OH^{\cdot}$ radicals are generated greatly during Cr induced stress (Chapter-1) as also shown by Aruldhas *et al.* (2005). Decreased superoxide dismutase and GPx activity in the testis due to Cr exposure accounts for the decreased ability to remove superoxide anion and H_2O_2 (Chapter-1) and the possible Sertoli dell damage during Cr exposure in this context can be inferred from the fact that, Sertoli cells are rich in SOD and GPx (Bauche *et al.*, 1994). Similarly, the pachytene spermatocytes also become vulnerable later as they are also dependent on SOD activity but less on peroxidases (Bauche *et al.*, 1994). Reduced activities of 3β and 17β hydroxy steroid dehydrogenases and lowered testosterone secretion suggest the

possible impact of oxidative stress generated by Cr on Leydig cells. This become self evident in the reported poor anti-oxidant system of these cells (Kuhucha and Mishra,1993). The decreased titre of testosterone can also be considered to complement the oxidative stress induced effect on spermatogenesis in the testis and the process of sperm maturation in the epididymis as both are androgen dependent processes. Apart form oxidative stress, another factor that can be considered to add to the decline in the production of testosterone could be corticosterone as this stress hormone has been reported to decrease LH receptor expression and testosterone production in rat Leydig cells (Rangarajan and Balasubramanian, 2007). It is also relevant to note in this connection that Chandra *et al.* (2007) have shown an increase in corticosterone level following Cr exposure in rats. All the attendant affects on testis due to Cr induced oxidative stress become fathomable in the context of increasing testicular Cr load to the extend of 50-100% in chromium exposed animals. This is much higher than the testis load of Cd or Ni seen in Cd or Ni exposed animals (Chapter 6, 7). In contrast, epididymal accumulation of Cr is much lesser (9-13), though the absolute load is much higher than in testis. However even the lesser increase in Cr in epididymis seems to cause much higher oxidative stress as stated above.

Adequate strategies need to be devised to combat oxidative stress induced malfunctioning of reproductive organs and male infertility. To protect humans from oxidative stress induced male infertility concerted efforts are needed to develop an effective therapy in the form of antioxidant supplements essentially to protect sperm from oxidative related DNA damage and to boost pregnancy rates. Number of natural antioxidants like folic acids (Yousef *et al.*, 2006) and vitamins C and E (Acharya *et*

al., 2004) has been tried in combating Cr induced negative effects on reproductive performance and spermatogenesis. Though these studies have shown the usefulness in minimizing the impact of the various changes caused by Cr, their efficacy in all parameters are not to the same extent. Melatonin tried out in the present study is a natural antioxidant hormone of the vertebrate system with dose and duration dependent effects on reproductive functions. Its role as a powerful antioxidant in general and particularly as a scavenger of peroxyl and hydroxyl radicals has already been highlighted previously (Chapter 3-6). Even in the present evaluation, melatonin has been found to afford protection against almost all the parameters under study with the protective effect ranging from a minimal 50% to a maximal close to 100% efficacy. The purported importance of melatonin in competing Cr induced reproductive toxicity recorded herein gains credence in the simultaneously noticeable decrease in the circulating titre of melatonin which becomes progressively greater with increasing duration of Cr exposure. No doubt, melatonin can be considered as the principal antioxidant supplement and can be developed as an antioxidant and anti-infertility therapeutic in combination with Vit. A, C and/ or E, as these vitamins are known to be important in testicular and epididymal functions. An acceptable antioxidant combination supplement needs to worked out and an optimal proportion of vitamin A, C and E administered along with melatonin could prove to be the most efficient and an effective mode of combating growing male infertility caused due to life style or due to unfavorable exposure to environmental contaminator.

To confirm and strengthen the *In vivo* observations, *In vitro* studies on cell viability, basal and hCG stimulated testosterone secretion have been assessed in isolated Leydig cells. The results form these assays clearly reveal increasing

cytolethality in temporally increasing course of Cr exposure with about 48% lethality within 12 hours. This is much higher than that seen with cadmium or nickel (Chapters 6, 7). The evaluation of testosterone secretion under basal and stimulated conditions show an increase in testosterone secretion from 55% at 3 hours to 75% at 6 hours of hCG stimulation in control Leydig cells compared to the corresponding release under basal condition. Interestingly, in Cr exposed experimental Leydig cells, the increase in testosterone secretion upon hCG stimulation showed a decrement from 55% at 3 hours to 25% at 6 hours. Apparently, this suggests 11% reduction in testosterone secretion at 6 hours of hCG stimulation compared to the control Leydig cells. This decrease in testosterone from 3 to 6 hours of hCG stimulation from Cr exposed Leydig cells could be due to a 34% cytolethality seen in the viability assay at the same time period. This may suggest that the observed *In vivo* reduction in circulating testosterone titre could be essentially due to an actual reduction in the number of Leydig cells due to oxidative stress rather than a decrease in absolute LH sensitivity of a single Leydig cell. This becomes clear when viewed in the context of directly mentioned minimal antioxidant ability of Leydig cells compared to tubular Sertoli and germ cells (Kuhucha and Mishra, 1993).

In the above context, the inability of melatonin to prevent cell lethality to a greater degree (only 4-8% from 3-12 hours) indicates that melatonin in the absence of endogenous antioxidant machinery is not adept in combating Cr induced oxidative stress in totality. Reversal or prevention of Cr induced oxidative stress by melatonin obviously occurs by a combinations of direct scavenging of free radicals as well as by an indirect boosting or activation of antioxidant system especially by bringing about transcriptional activation of the concerned genes (Pandi-Perumal *et al.*, 2006).

Obviously, in the absence of an endogenous antioxidant machinery in Leydig cells, the protective ability of melatonin is restricted to only direct quenching of free radicals to minimally reduce oxidative stress. It can be presumed from these that, melatonin's potential to combat oxidative stress is best expressed when both its direct and indirect functions can complement each other. Pertinently, Liu *et al.* (2001) and Chang *et al.* (2003) have provided *In vitro* evidence for lead and manganese induced inhibition in steroidogenesis in Leydig cells to be due to disruption in the expression of steroidogenic acute regulatory protein (StAR), $P450_{sec}$ and 3 β-HSD. The possibility of Cr also bringing about such a change can be hypothesized and would need experimental validation. In the present study, though melatonin was not found to be very effective in protecting against Cr induced cytolethality, it nevertheless showed a relatively better ability to protect against Cr induced decrease in testosterone secretion. There are contradictory reports on melatonin induced testosterone secretion from Leydig cells. The *In vitro* studies of Valunti *et al.* (1995, 1999) suggest inhibition of testosterone secretion from Leydig cells by different mechanisms involving cAMP and Ca^{++} respectively. In the present study also, though melatonin has shown an inhibitory effect on testosterone secretion from control Leydig cells to the tune of average 12% under basal condition and 24% under hCG stimulation, in contrast, Cr exposed Leydig cells recorded an increased testosterone secretion in presence of melatonin averaging 15% under basal condition and 24% under hCG stimulation. Again, Cr exposed cells in presence of melatonin showed a 4% increase from 3-6 hours under hCG stimulation compared to 11% decrease without melatonin. This 4% increase in Cr + melatonin cells is very much identical to the increase shown by control Leydig cells under hCG stimulation from 3-6 hours. This is probably the first report bringing out the new dynamics of testosterone secretion influenced by

melatonin in chromium stressed Leydig cells. The melatonin dynamics of testosterone secretion from the Leydig cells therefore appears to be distinctly different under normal and stressed conditions. The possibilities of melatonin up regulating LH receptors and/or sensitivity, increased expression of StAR protein and activity of P450sec and steroid dehydrogenases as well as increased cAMP production, all need to be ascertained to gauge the mechanism of action of melatonin under chromium stress. Overall, it can be concluded that, Cr(VI) inflicts testicular and epididymal damage by way of oxidative stress leading to negative impact in seminal quality and sperm parameters and that melatonin is by and large potent in protecting the deleterious effects of Cr. A combination supplemental therapy with other antioxidant vitamins would be more efficient in preventing male infertility. Finally, a novel finding that comes out of this study is that the dynamics of testosterone secretion by melatonin under Cr toxicity or stress is quite different from the non-stressed condition and that mechanics of this salient observation need to be unraveled by future studies.