Chapter 6: Cadmium 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

Cadmium is extensively used as an anticorrosive agent in the plating of metals and other alloys that are valuable in industry and, also as cadmium oxide in Ni-Cd dry cell batteries. Wider industrial applications of this metal have now resulted in Cd contamination of food and the environ. Environmental, dietary or occupational exposure to cadmium can lead to its bioaccumulation in humans as well as in cadmium exposed experimental animals. Many organs, including testis are susceptible to Cd accumulation consequent to an exposure to it (Draper and Timbrel, 1996). Further, the havoc caused by this metal is due to its longer biological half life which can result in significant tissue damage in both humans and experimentally exposed animals (Robards and Worsfold, 1991).

Following its entry into the animal body, Cd rapidly gets distributed in various animal tissues. Inside the cells, Cd enhances lipid peroxidation and negatively interferes with the functions of both enzymatic and non-enzymatic antioxidants and, also mediates altered structural conformations. This is pertinently seen in its ability to replace Zn from Cu-Zn-SOD and form Cu-Cd-SOD and also in its inactivation of GPx by removing Se from the enzyme. A realistic and environmentally simulating dosage of cadmium has been previously shown to markedly increase lipid peroxidation in both testis and epididymis and weaken the antioxidant defense system (Chapter: 2).

Testis is a steroidogenically active organ which generates great amount of ROS during normal metabolic activities within it as well as during steroidogenesis. However, the weak antioxidant machinery of the steroidogenic cells is able to handle these ROS keeping an effective check on oxidative stress (OS). Entry of a xenobiotic compound can further enhance lipid peroxidation and oxidative stress of the steroidogenic organs. Moreover, it is now well established that increased lipid peroxidation can alter activity of various enzymes including enzymes involved in steroidogenesis such as 3-β HSD and 17-β HSD (Rajeswary *et al.*, 2007). Cadmium can hamper the vital processes like spermatogenesis, steroidognesis as well as spermiation (Hew *et al.*, 1993; Yang *et al.*, 2006).

Though the deleterious effect of Cd on different organs has been reported in various animals models, only few studies are available related to male reproductive toxicity of the metal. Moreover, most of the studies are basically on single dose of acute Cd exposure or for short duration and are physiologically least relevant to humans. Need of the hour is to assess the after effects of environmentally relevant realistic dosage on various organ systems including reproductive system with the help of suitable anima model so that the results could be extrapolated to humans. Further, going through the literature survey, there is no study available with reference to *In Vitro* exposure of Cd to isolated rat Leydig cell and protective role of antioxidants. In the present investigation, an attempt is therefore made to evaluate the duration dependent (15, 30 and 60 days of treatment) effect of Cd on steroidogenic enzyme activity, serum testosterone and estradiol titre, semen parameters (sperm count, sperm motility and sperm abnormality) along with the effects on cell viability and testosterone production under basal as well as stimulated conditions by Leydig cell

primary culture. Concurrently, the putative protective role of melatonin on the above mentioned parameters, both *In Vivo* and *In Vitro,* has also been assessed.

Material and Methods:

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

Results:

3-β HSD activity:

Cadmium exposure recorded significant inhibition of testicular 3-β HSD activity which tended to decrease progressively with increasing duration. Simultaneous melatonin treatment afforded significant protection against Cd induced inhibition of the 3-β HSD activity (Table: 6.1, Figure: 6.1).

17-β HSD:

Decreasing inhibition of 17-β HSD activity with increasing duration was observed in cadmium treated animals. Maximal inhibition of testicular 17-β HSD was seen in the shorter duration exposure. Co-administration of melatonin showed marked protection against Cd induced decrement in testicular 17-β HSD activity (Table: 6.2, Figure: 6.2).

Serum Testosterone:

Cadmium treatment exerted a marked decrement in serum testosterone titre compared to the control animals. Interestingly, decrement in the serum testosterone titre was maximal in shorter duration of exposure and minimal in the longer duration of exposure. Melatonin exerted significant protection when simultaneously administered with Cd. These changes in serum T level are represented in Table: 6.3 and Figure: 6.3.

Serum Estradiol:

Cadmium treatment resulted in marked depletion of serum estradiol titre. This depletion was more pronounced at shorter duration of exposure compared to longer duration of exposure. Simultaneous treatment with melatonin exerted significant

protection against the Cd induced depletion in serum estradiol titre (Table: 6.4, Figure: 6.4).

Cauda Epididymal Sperm Count:

Cadmium treatment exerted duration dependent linear decrease in cauda epididymal sperm count in rats. The protective role of melatonin on cauda epididymal sperm number when co-administered with Cd was found to be better in short duration exposure (Table: 6.5, Figure: 6.5).

Sperm motility:

Significant linear reduction in number of motile sperm was observed in Cd administered animals. Minimum number of motile sperms was observed in longer duration of Cd exposure. Co-administration of melatonin afforded marked protection against the Cd induced decline in the number of motile sperms (Table: 6.6, Figure: 6.6).

Sperm Abnormality:

Abnormal sperm numbers increased with increasing in duration of Cd exposure with maximum abnormal sperm being seen in longer duration of Cd treatment. Concurrently, simultaneous melatonin administration showed marked protection against the Cd induced sperm abnormality with pronounced protective effect in longer duration treatment (Table: 6.7, Figure: 6.7).

In Vitro Cell Viability

Cd treatment markedly increased cytolethality of isolated Leydig cells in a temporal manner (3 – 12 hrs). Simultaneous presence of melatonin along with Cd prevented the cytolethality caused due to Cd treatment (Table: 6.10).

In Vitro Testosterone(T) Production:

Though in general, melatonin treatment decreased the basal release of testosterone from isolated Leydig cells, this effect was more pronounced when stimulated with hCG. Cadmium exposure tended to show a marked decrease in both basal and hCG stimulated testosterone secretion from Leydig cell(Table: 6.11).

Histological observations:

Testis:

The testis of animal treated with Cd (Plate: 1, Fig. T1 and T2) showed no distortion of tubules except for absence of sperm and tubules. Spermatogenesis seem to be progressing up to spermatid stage though the germinal epithelium appears to be intact, late spermatid seems to be getting detached leading to absence of sperm in most of the tubules. Co-administrations of melatonin (Plate: 1, Fig. T3 and T4) with Cd seems to have a protective effect with spermatogenesis gaining to completion in most of the tubules the density of germ cell however appear to be less.

Epididymis:

Epididymis of Cd treated (Plate: 2, Fig. E1 and E2) animals show significantly hypotrophied epithelium. Though many of the tubules were empty some contains sperms while others contain degenerative germ cells. Simultaneous treatment with melatonin (Plate: 2, Fig. E3 and E4) seems to be highly protective with the epididymis

epithelium appear to be quite thick and well formed tubule lumen contains sperms, spermatids and some degenerative cells.

Plate: 1

Figure: T_1: Photomicrograph of **Cadmium** treated testis showing seminiferous tubules and Leydig cells (100X). The tubules show very few sperms

S: Seminiferous tubules; L: Leydig cells

Figure: T_2: Photomicrograph of **Cadmium** treated testis showing a semiiniferous tubules with absence of sperm in the lumen (lu) (400X). Note degenerating spermatids and spermatocytes in the tubules and engorged blood vessels (bv) in the interstitum

Figure: T_3 : Photomicrograph of testis treated with **Cadmium** and Melatonin showing seminiferous tubules with progressive stages of spermatogenesis (100X). Leydig cells and slightly engorged blood vessels (bv) can be seen in the interstitum

S: Seminiferous tubules; L: Leydig cells

Figure: T_4: Photomicrograph of testis treated with **Cadmium** and Melatonin showing a tubule with thinner population of sperms (400X)

Sp:Sperm; L: Leydig cells

Plate: 1

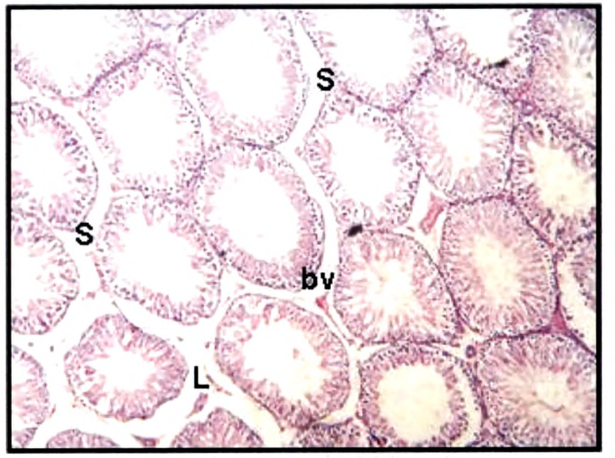


Fig. T1: Cd (100x)

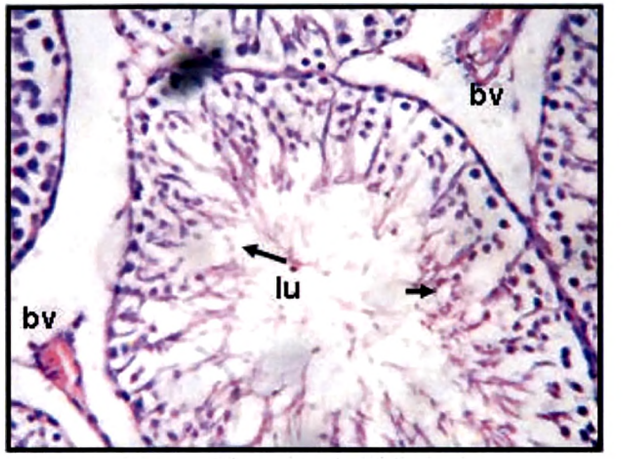


Fig. T2: Cd (400x)

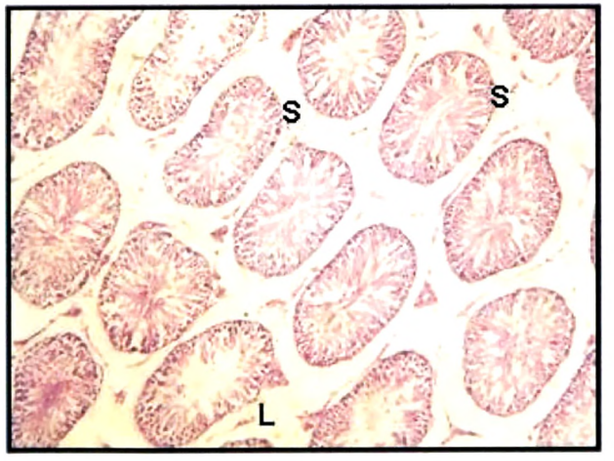


Fig. T3: Cd + Mel (100x)

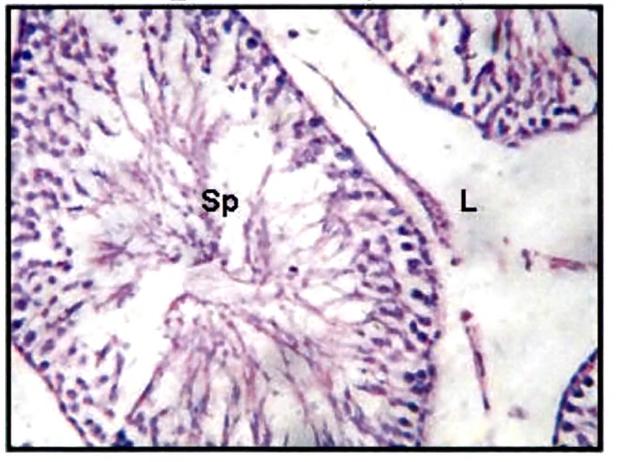


Fig. T4: Cd+ Mel (400x)

Plate: 2

Figure: E_1: Photomicrograph of **Cadmium** treated epididymis showing cauda tubules with most of the lumen empty (100X)

lu: Lumen; Sp: Sperms

Figure: E_2: Photomicrograph of **Cadmium** treated epididymis with ypotrophied epithelium (400X). Note both empty tubules and some tubules containing little sperms.

lu: Lumen; Sp: Sperms

Figure: E_3: Photomicrograph of epididymis treated with **Cadmium** and melatonin (100X). Note the presences of sperms in the lumen of most of tubules.

lu: Lumen; Sp: Sperms

Figure: E_4: Photomicrograph of epididymis treated with **Cadmium** and melatonin (400X). Note the well formed epithelium with presences of mixture of sperms and degenerating germ cells in the lumen.

Plate: 2

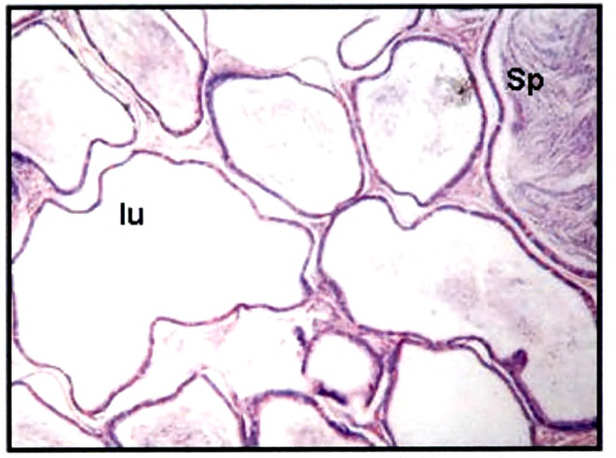


Fig. E1: Cd (100x)

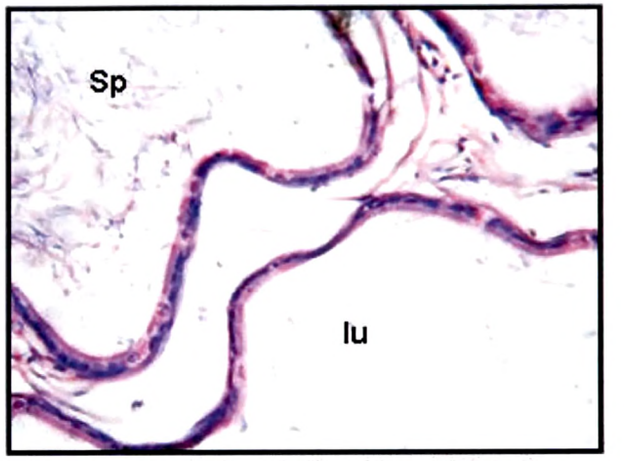


Fig. E2: Cd (400x)

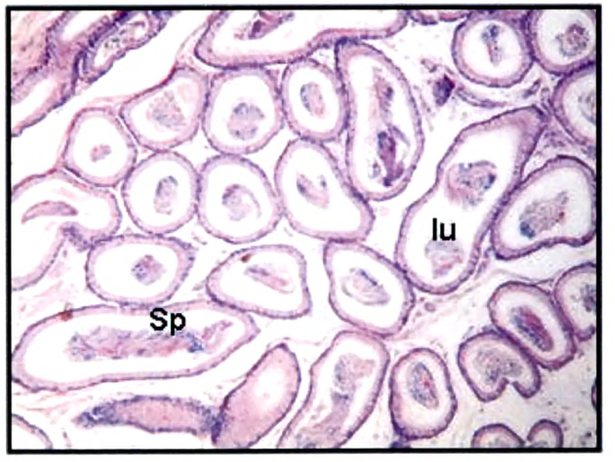


Fig. E3: Cd + Mel (100x)

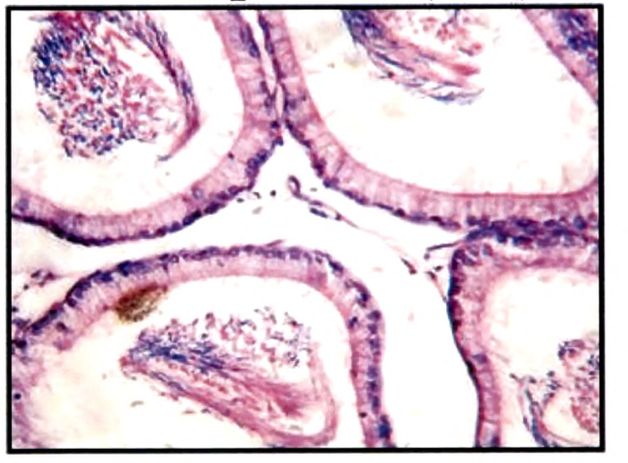
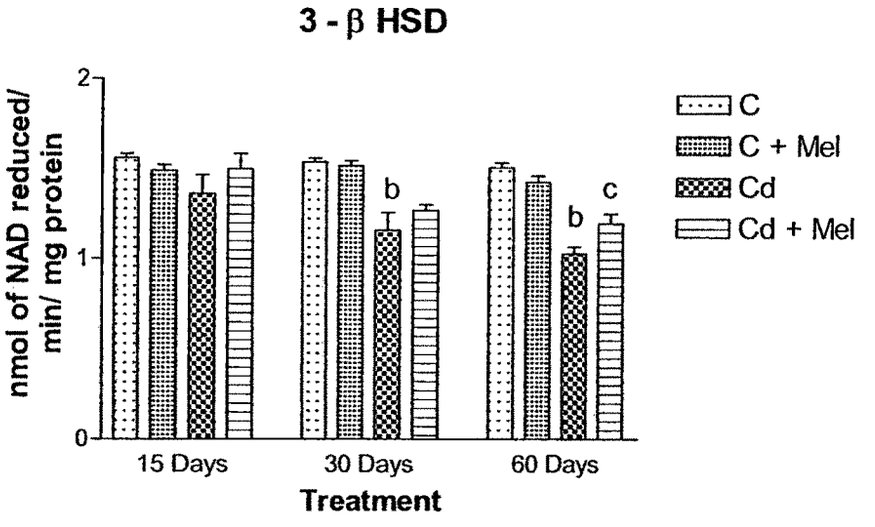


Fig. E4: Cd + Mel (400x)

Figure 6.1: Cadmium induced changes in 3 – β HSD in testis with or without Melatonin.



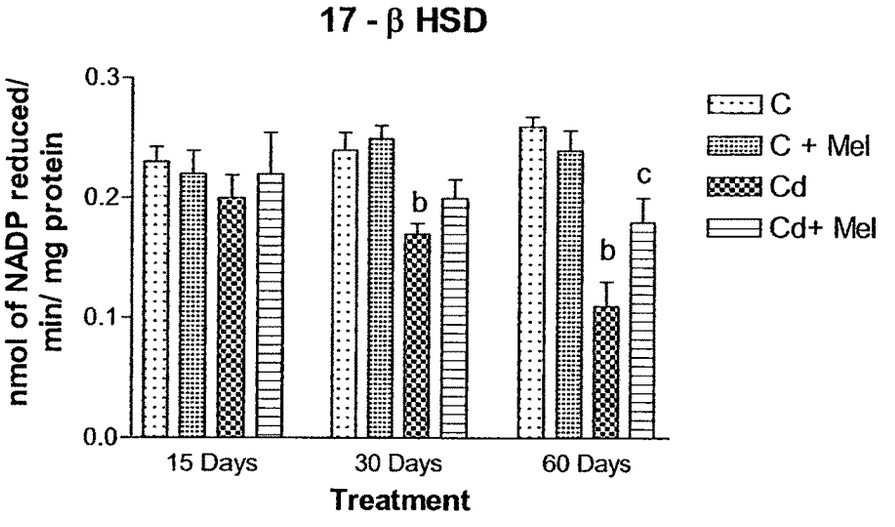
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.1: Cadmium induced changes in 3 – β HSD activity (nmol of NAD reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	1.56 ± 0.02	1.49 ± 0.03	1.36 ± 0.10	1.50 ± 0.08
30 Days	1.54 ± 0.02	1.52 ± 0.02	1.16 ± 0.1^{b}	1.27 ± 0.03^{c}
60 Days	1.51 ± 0.02	1.43 ± 0.03	1.03 ± 0.04^{b}	1.20 ± 0.05^{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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.2 : Cadmium induced changes in 17 – β HSD activity in testis with or without Melatonin.



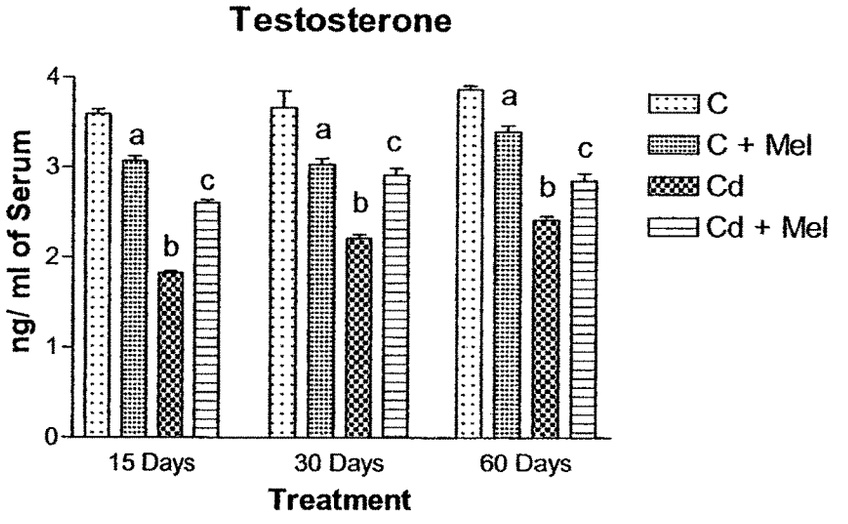
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.2: Cadmium induced changes in 17 – β HSD activity (nmol of NADP reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	0.23 ± 0.01	0.22 ± 0.01	0.20 ± 0.01	0.22 ± 0.03
30 Days	0.24 ± 0.01	0.25 ± 0.01	0.17 ± 0.01^{b}	0.20 ± 0.01
60 Days	0.26 ± 0.01	0.24 ± 0.01	0.11 ± 0.02^{b}	0.18 ± 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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.3: Cadmium induced changes in serum Testosterone (T) level with or without Melatonin.



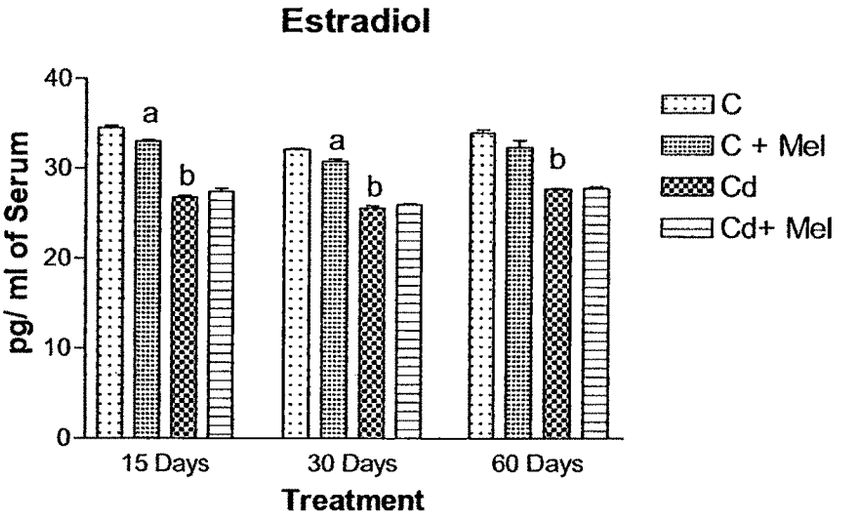
Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; Cr(VI) – Chromium;
Cr(VI) + Mel – Chromium + Melatonin

Table 6.3: Cadmium induced changes in serum Testosterone (T) level (ng/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	3.59 ± 0.05	3.07 ± 0.05^{a}	1.83 ± 0.01^{b}	2.61 ± 0.02^{c}
30 Days	3.67 ± 0.18	3.04 ± 0.06^{a}	2.22 ± 0.03^{b}	2.92 ± 0.08^{c}
60 Days	3.88 ± 0.04	3.41 ± 0.06^{a}	2.42 ± 0.04^{b}	2.86 ± 0.08^{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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.4: Cadmium induced changes in serum Estradiol (E_2) level with or without Melatonin.



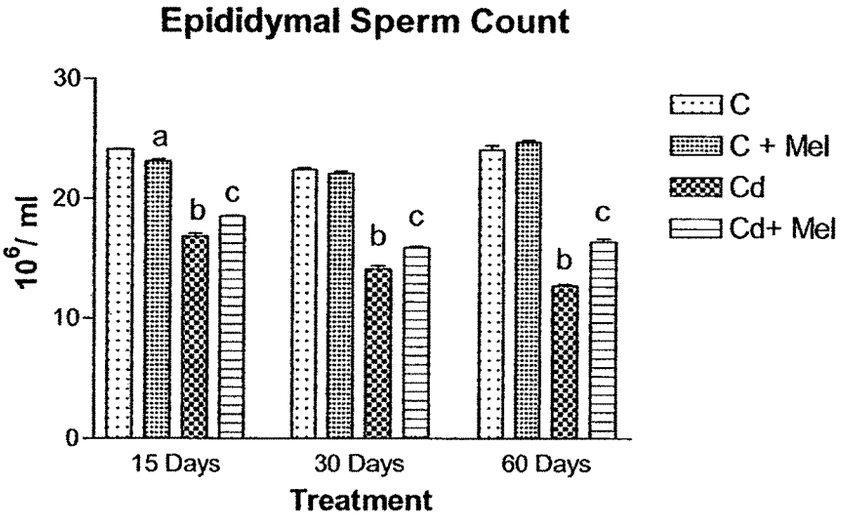
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.4: Cadmium induced changes in serum Estradiol (E_2) level (pg/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	34.5 ± 0.22	33.01 ± 0.12[a]	26.8 ± 0.19[b]	27.45 ± 0.34
30 Days	32.15 ± 0.13	30.83 ± 0.24[a]	25.6 ± 0.27[b]	26.03 ± 0.11
60 Days	34.01 ± 0.41	32.43 ± 0.73	27.79 ± 0.05[b]	27.86 ± 0.16

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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.5: Cadmium induced changes in epididymal sperm count with or without Melatonin.



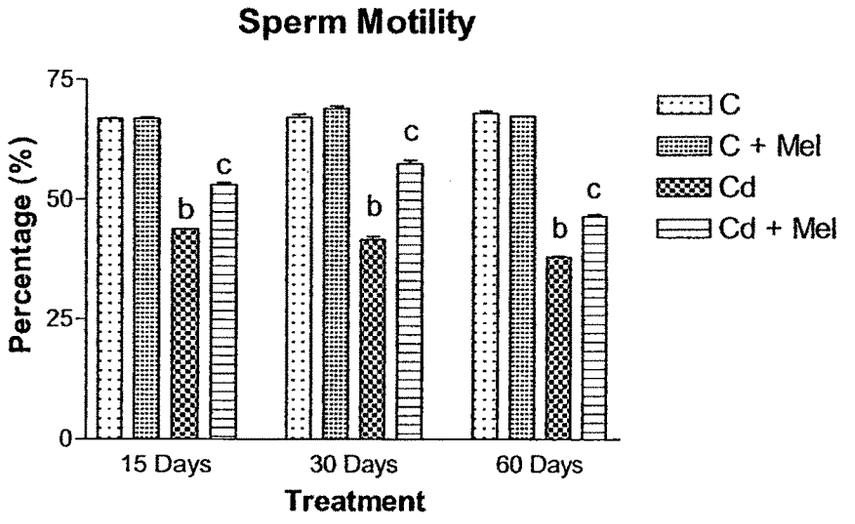
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.5: Cadmium induced changes in epididymal sperm count (10^6/ml) with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	24.10 ± 0.07	23.14 ± 0.17[a]	16.83 ± 0.26[b]	18.5 ± 0.07[c]
30 Days	22.44 ± 0.13	22.10 ± 0.19	14.12 ± 0.29[b]	15.9 ± 0.14[c]
60 Days	24.08 ± 0.39	24.73 ± 0.19	12.76 ± 0.12[b]	16.45 ± 0.22[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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.6: Cadmium induced changes in epididymal sperm motility with or without Melatonin.



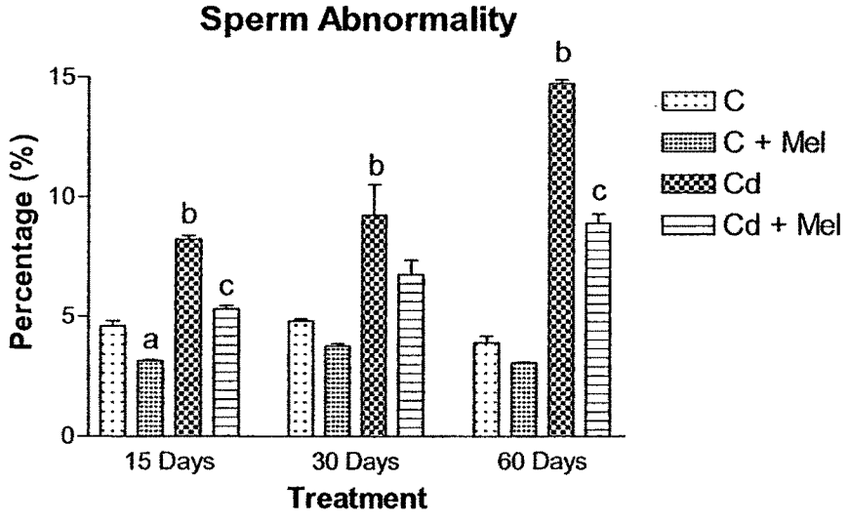
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.6: Cadmium induced changes in epididymal sperm motility (percentage) with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	66.87 ± 0.12	66.92 ± 0.27	43.83 ± 0.07^{b}	53.11 ± 0.46^{c}
30 Days	67.16 ± 0.67	69.06 ± 0.46	41.63 ± 0.72^{b}	57.48 ± 0.81^{c}
60 Days	68.12 ± 0.50	67.44 ± 0.04	38.00 ± 0.22^{b}	46.57 ± 0.44^{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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Figure 6.7: Cadmium induced changes in epididymal sperm abnormality 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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.7: Cadmium induced changes in epididymal sperm abnormality (percentage) with or without Melatonin.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	4.60 ± 0.20	3.14 ± 0.03[a]	8.23 ± 0.15[b]	5.30 ± 0.17[c]
30 Days	4.80 ± 0.09	3.76 ± 0.10	9.23 ± 1.2[b]	6.76 ± 0.60
60 Days	3.90 ± 0.28	3.05 ± 0,04	14.73 ± 0.15[b]	8.89 ± 0.38[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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.8: Cadmium induced percentage changes in cadmium content of testis and epididymis with or without melatonin.

Treatment	Testis		Epididymis	
	Cd	Cd + Mel	Cd	Cd + Mel
15 Days	26.32	7.14	23.92	48.39
30 Days	15.56	-4.26	21.33	27.39
60 Days	30.33	21.85	16.13	18.99

C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.9: Cadmium induced changes in the serum titre of melatonin (pg/ ml) in control and experimental rats.

Treatment	C	C + Mel	Cd	Cd + Mel
15 Days	121.00 ± 0.36	140.00 ± 0.60[a]	59.00 ± 0.25[b]	104.00 ± 0.51[c]
30 Days	112.00 ± 0.24	143.00 ± 0.17[a]	52.00 ± 1.24[b]	106.00 ± 0.63[c]
60 Days	93.00 ± 0.05	126.00 ± 0.14[a]	26.00 ± 1.47[b]	88.00 ± 0.84[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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.10: Chromium(VI) induced changes in Leydig cell viability (in percentage) with or without melatonin.

Treatment	Cell Viability			
	C	C + Mel	Cd	Cd + Mel
3 Hours	93.81 ± 0.06	94.10 ± 0.11	79.56 ± 0.13 [b]	87.54 ± 0.32 [c]
6 Hours	93.42 ± 0.23	93.96 ± 0.24	76.45 ± 0.24b	85.48 ± 0.14
12 Hours	92.09 ± 0.07	94.33 ± 0.04	69.66 ± 0.10 [b]	80.21 ± 0.35 [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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Table 6.11: 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	Cd	Cd + Mel	C	C + M	Cd	Cd + Mel
3 Hours	4.87 ± 0.33	4.17 ± 0.16	2.93 ± 0.37 [b]	3.49 ± 0.04 [c]	7.58 ± 0.45	5.83 ± 0.04 [a]	3.81 ± 0.08 [b]	5.17 ± 0.03 [c]
6 Hours	4.55 ± 0.16	4.06 ± 0.20	2.38 ± 0.40 [b]	3.78 ± 0.06 [c]	7.94 ± 0.04	5.93 ± 0.11	3.58 ± 0.11 [b]	5.02 ± 0.14 [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 Cadmium
C – Control; C + Mel – Control + Melatonin; Cd – Cadmium;
Cd + Mel – Cadmium + Melatonin

Discussion:

Previous studies on Cd had shown duration dependent increase in oxidative stress in both testis and epididymis, comparatively more in the latter (Chapter 2). The present study in continuation reveals deleterious effects on spermatogenesis and steroidogenesis in the testis and, on functional competence of the epididymis. Though there are no reports on histoarchitectural alteration in the epididymis, some investigations on testis have indicated disruption in the micro vasculature with leakage of fluids and electrolytes and discontinuities in the endothelium (Aoki and Hoffer, 1978), failure of spermiation with spermatogenic stage specific effects starting from stage VIII to IX (Hew *et al.*, 1993), increasing necrosis of germ and Sertoli cells with increasing dosage coupled with a decrease in interstitial cells and abundance of inflammatory cells (Yang *et al.*, 2006) and severe necrosis and lack of spermatogenesis (Kara *et al.*, 2007). The first two studies were based on single injection of Cd salt and studied at various hours thereafter while, the latter two were of one week and one month of treatment duration respectively. Due to the differing experimental protocols, valid comparisons are well neigh impossible. The vascular effects seem to be quite acute in the immediate periods after Cd administration. The other study on single injection of Cd seems to suggest an immediate inhibition of spermiation. Both of these happenings may be discounted on extended treatment with Cd as, the one week and one month treatment studies failed to record such changes. The one week treatment study suggests ischemic necrosis with ischemia in the interstitium and necrosis within tubules while the one month treatment protocol has suggested severe necrosis with total lack of spermatogenesis in the tubules. The present study involving a higher realistic dosage and duration of treatment extending

from 2 weeks to 2 months has failed to record the highly deleterious changes reported by the earlier workers. Strain difference (four different strains used by 4 groups of workers) in response to Cd can be over-ruled as, the strain of rat used in the present study and the one month exposure study is the same. Apparently, the only valid explanation that could be offered is that, the vascular changes and failure of spermiation effects are the immediate testicular response to Cd and that, their deleterious effects are overcome with the passing of time irrespective of continued exposure. Since, histological observations were not made in the 15 day and 30 day Cd exposed testis in the present study, it is likely that such changes might have been noticeable or in action latest by the 15^{th} day schedule. Since in the present study, only some degree of inhibition on spermatogenesis (as seen by the thinner population of mature germ cells in the tubules) could be envisaged, the severe necrosis and spermatogenic arrest reported by Yang *et al.* (2006) and Kara *et al.* (2008) seem improbable and may suggest of some adaptive mechanism of recovery by the testis on continued exposure to Cd. The lesser density of germ cells including sperms seen herein would justify some degree of germ cell apoptosis/ necrosis, though not severe enough to disrupt spermatogenesis completely. Persistent spermatogenic process in the testis of Cd treated animals, though with a reduced degree, is confirmed by the presence of sperms in the epididymal tubules. Some degree of interference in epididymal function can be deduced from the herein observed shrinkage or hypotrophy of the tubular epithelium.

Apart from the above cited reports of apoptosis and/ or necrosis of germ cells, even Zhou *et al.* (1999) have documented increased apoptosis with low dose and

necrosis with high dose of Cd along with alterations in p53 and c-jun gene expression. Unfortunately, even this study was with a single dose injection of Cd like most studies in metal toxicity. However, the present study on long duration exposure has failed to record any dramatic increase in apoptosis or necrosis to fully compromise the continuity of spermatogenesis. A compromise explanation that could be put forth in this context is that, in all probability the initial effect of Cd could be promotion of increased blood flow, followed by vascular damage and interstitial fluid pressure resulting in anoxia and germ cell necrosis, as has also been inferred by Johnson and Turner (1972), which get completely nullified on prolonged exposure, by the induction of some adaptive mechanism as part of habituation. This adaptive mechanism may involve the induction and increased synthesis of metallothionein (MT) under prolonged Cd stress. This is understandable in the light of the known potential of Cd to induce MT synthesis and the presently observed increase in total MT content of testis and epididymis with increasing duration of Cd exposure. Supportive evidence comes from the reported Cd induced MT synthesis and accumulates in testis by Kusakabe *et al.* (2007) and Amara *et al.* (2008). Many of the single injection treatments with Cd failing to find significant increase in MT expression in testis or epididymis, more so in the latter when arrayed against the present chronic long term exposure suggests that, significant MT expression under Cd intoxication is duration dependent. To this end, a study on long duration exposure for 10 days has documented increase in MT expression in the caudal epididymis (Zilure, 2007). The relative resistance to necrosis of germ cells and Sertoli cells seen in the present long term exposure study (as against the earlier cited single exposure studies) and the persistence of spermatogenesis though with less density of germ cells, can be easily justified by the reported presence and induction of MT in spermatogonia,

spermatocytes and Sertoli cells (Nishimira *et al.*, 1990; Tohyama *et al.*, 1994; Kusakabe *et al.*, 2007). Since no visible destruction of Leydig cells was noticeable in Cd treated testis in the present study, it is likely that part of MT expression occurring in the testis could be related with Leydig cells. In this context, localization of MT in Leydig cells has been reported (Danielson *et al.*, 1982). The herein demonstrated increases in MT as well as progressive reduction in tissue Cd load are pointers to the adaptive mechanism against Cd gaining maturity. Despite the protective effect afforded by MT, reduced number of germ cells and lighter intensity of spermatogenesis in Cd exposed rats are indications of some degree of disruptive effect of Cd. This is well reflected in the recorded decreased activity of 3β and 17 β HSD, and lowered testosterone and estrogen titres. In accordance with these, is the observation of the significantly decreased epididymal sperm count. Even other workers have reported decrease in 3β and 17 β HSD activities with lowered testosterone level and reduced sperm count on induced Cd toxicity (Biswas *et al.*, 2001; Manna *et al.*, 2008; Amara *et al.*, 2008). Decreased sperm count amply illustrates a qualitative effect on testicular spermatogenesis as, Cd must be inducing oxidative stress mediated germ cell damage. Increased oxidative stress has already been reported on chronic exposure to Cd (Chapter -2). Many studies have related Cd toxicity with increased generation of ROS and LPO and decrease in non-enzymaitc and enzymatic anti-oxidants (Wang *et al.*, 2004; Cronte *et al.*, 2005) with necrosis and apoptosis (Koizum and Li *et al.*, 1992; Sarkar *et al.*, 1998; Oteiza *et al.*, 1999) and testicular damage (Kusakaba *et al.*, 2007). The severity of damage can be expected to be higher at the earlier time periods of Cd exposure (15 days in this study), whereas the cumulative effect of a chain of events could precipitate higher tissue damage one of the mechanisms by with Cd can cause oxidative damage is by

displacing iron from iron bound proteins and increasing tissue iron content which in turn generates ROS by Fenton reaction. Necrosis/ apoptosis of germ cells and Sertoli-Sertoli tight junction (TJ) damage and breakdown of blood-testis-barrier (BTB) can all be accredited to iron(Fe) build up within the seminiferous tubule (Chung and Cheng *et al.*, 2001; Wong *et al.*, 2004; Fraga and Oteiza, 2007). Incidentally, Kusakabae *et al.* (2007) have demonstrated increased Cd and Fe contents in testis of Cd treated animals. However increase in Zn content (Kusakabe *et al.*, 2007) and MT induction in the testis on chronic exposure as in the present study contribute greatly towards development of resistance against the initiated changes and even reverse the damages to make the testis appear much like normal by about 60 days. This is the systemic habituation induced adaptive mechanism which makes testis less vulnerable to Cd toxicity. These circumstantial deductions stand vindieated by the herein recorded progressive improvement in the activities of steroid dehydrogenases and serum testosterone titre from 15 to 6o days of Cd exposure. Since testosterone and an optimal T/E_2 ratio are important for maintenance of spermatogenesis, in the present study, maximal negative effect could expectedly be at 15 days as, both serum testosterone level and testosterone/ estrogen ratio were lowest. Between 30 and 60, days there is substantial recovery in testosterone level as well as a decrease in testosterone/estrogen ratio which could account for the mere quantitative effect on spermatogenesis as attested to, by the sperm count. The increasing testosterone titre and the induction of MT in epididymis (also androgen dependent) seen to benefit greatly in improving the earlier compromised functional competence of the organ as indicated by currently observed improvement in sperm motility. However, the increasing percentage of sperm abnormalities despite improving motility may indicate extreme vulnerability of mature sperms within the testis and epididymis to the direct

or indirect modes of Cd induced oxidative stress. Recent reviews on the effect of oxidative stress on sperm structure and function clearly elucidate how ROS and oxidative stress can interact with sperms to cause abnormality. The possibility of a Cd linked with infertility may have to be viewed in this context and a recent study with very high doses of Cd (similar to that found in a lake in Iran) in mice has demonstrated certain degree of infertility (Monsefi *et al.*, 2009).

Though melatonin has a slight lowering effect on serum testosterone and testicular 3β and 17β HSDH activities in control animals, there was nevertheless favorable influence on sperm motility and sperm abnormality. But when given along with Cd, it has shown substantial protective effect on enzyme activity, steroid hormone levels, sperm count, motility and abnormality. The effect of Cd is still discernable suggesting that even at the dose of 10mg/kg, melatonin is not fully effective in checking the manifestations of metal toxicity. Earlier, melatonin was shown to have pronounced protective effect against Cd induced oxidative stress (Chapter-2). It is inferable that, while melatonin is effective in minimizing oxidative stress, it is incapable by itself to resist totally the influence on biochemical and functional features of testis and epididymis. The significant decrement in serum titre of melatonin seen between 15 to 60 days of Cd exposure implies its involvement in resisting Cd induced oxidative stress and differential tissue effects (Bareyze, 2009). In this context, α-tocopherol was found to be effective is resisting Cd toxicity only at lower doses of Cd and not at higher doses (Yang *et al.*, 2006). Kara *et al.* (2007) on the other hand have shown efficacy of melatonin against Cd induced oxidative damage in the rat testis. However, they report that, the efficacy of melatonin was near complete when used in combination with vitamin E and selenium. Such a suggestion was made earlier with

reference to Cr induced reproductive toxicity (Chapter-5). Therefore, to combat metal induced oxidative stress and consequent structural and functional damage to reproductive organs, a combination therapy involving melatonin supplementation with an optimal dosage of Co -supplements with vitamins E, C and α-tocopherol could be the choicest anti-infertility therapeutic for offsetting the metal induced oxidative damage and reproductive toxicity.

The observed *In Vivo* effects of Cd on Leydig cell steroidogenesis was further assayed in an *In Vitro* system of cultured Leydig cells along with cell viability. The assay shows that there is increasing cytolethality with increasing duration of Cd exposure (20%-30% from 3-12 hours). Concurrent presence of melatonin afforded significant protection against Cd induced cytoletahlity and the percentage of viable cells is significantly higher. Release of testosterone from cultured Leydig cells is significantly affected by the presence of Cd as under basal conditions testosterone secretion decreased by 40% at 3 hrs to about 48% at 6 hours. Even under hCG stimulation, Cd decreased testosterone secretion by 50% at 3 hours to 55% at 6 hours. Obviously, an immediate consequence to Cd toxicity is significantly reduced testosterone secretion probably attributable to the metal induced oxidative stress. This confirms the earlier inferred heightened Cd toxicity in the immediate periods or on short term exposure to Cd based on the *In Vivo* observations. The acute inhibitory effect of Cd is further substantiated by the lower testosterone secretion under basal to hCG stimulation from 30% at 3 hours to only 50% at 6 hours. As against this, control Leydig dells not exposed to Cd increased testosterone secretion from 56% at 3 hours to 75% at 6 hours. Stimulation of Leydig cells by hCG increased testosterone secretion by 5% from 3 to 6 hours while in presence of Cd, the same decreased by

6%. All dynamics of testosterone secretion in terms of basal to stimulated or 3 to 6 hours, provide compelling evidence for Cd induced inhibition of testosterone secretion. There are also a couple of other reports which corroborate the present finding (Laskey and Phelps, 1991; Yang *et al.*, 2003). Laskey and Phelps (1991) based on their studies had opined that the cellular sites of inhibitory action of Cd and other metals to be subsequent to membrane receptor and cAMP formation and prior to the cholesterol side chain cleaving enzyme in the mitochondria. Based on the known molecular pathway of LH/ hCG mediated signal transduction and steroidogenesis, the possible link affected by Cd toxicity would be the Steroid Acute Regulatory protein (StAR) needed for transport of cholesterol to the mitochondrion. Only further studies can reveal whether the Cd effect is by way of reduced expression of StAR protein. The present study again provides support to the known inhibitory effect of melatonin on testosterone secretion as seen by the average 12% decrease under basal conditions and an average 24% decline under hCG stimulation. However, under Cd stress, melatonin appears to have a favorable influence in promoting steroidogenesis as there is significant increment in testosterone secretion in Cd + melatonin set up, with an average of 40% increment under basal condition and 38% under hCG stimulation. If the purported site of action of Cd inhibition of steroidogenesis is at the level of StAR protein, the question then is "Can melatonin negate the effect of Cd on expression of StAR protein and even bring about its over expression?" The dynamics of melatonin action on Leydig cells under normal or (metal) stressed condition seems very intriguingly different and needs to be ascertained by appropriate experimental evaluations. In conclusion, the present *In Vivo* and *In Vitro* studies suggest differential acute versus chronic response to Cd with heightened testicular and epididymal damage on a short term basis and/an adaptive mechanism resisting and reverting the

damages in a qualitative sense on a long term basis. Melatonin is able to protect to a great extent the alterations induced by Cd and a therapeutic combination of melatonin supplemented with other vitamins may prove to be the mode of combating Cd induced reproductive toxicity. Further, it is also revealed that melatonin dynamics on steroidogenesis in the Leydig cells are different and it seems that melatonin has a positive favorable influence under metal stressed state.