Chapter 7: Nickel 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:

Industrialization has contaminated the human environ with many hazardous pollutants. Humans are exposed to such hazardous pollutants due to occupational exposure or due to their presence in the food chain. These hazardous pollutants enter the human food chain due to contaminated soil, water or anthropogenic activity. The list of such hazardous compounds includes pesticides, industrial waste containing variety of chemicals, synthetic compounds, heavy metals, etc. One such heavy metal to which even non smoker and non-occupationally linked human population gets exposed is Nickel. Nickel is an essential element and plays an important role of a cofactor in facilitating the intestinal absorption of ferric ion which is required for hemopoiesis (Nielsen *et al.,* 1984). It is said that "anything in access is dangerous" and so higher exposure to any essential metal including Nickel may prove to be hazardous to human health.

Despite its status as an essential metal, nickel is a well known potential toxic metal and, toxic and carcinogenic manifestations of this essential element are well documented (Das and Dasgupta, 1997; WHO, 1998). Available human and animal data suggest that nickel compounds can cause hematoxicity, immunotoxicty, hepatotoxicity, pulmonary toxicity and nephrotoxicty depending upon the route of

exposure, amount of dosage and duration of treatment (Das and Buchner, 2007). Though, the reported deleterious effects of this metal, on various organ systems, are increased lipid peroxidation and generation of ROS, the mechanism of induction of oxidative stress by Ni is still unclear. Further, compared to all other organ systems, nickel toxicity to male reproductive system is poorly defined. Das and Dasgupta (2002) have suggested altered steroidogenesis following nickel exposure. Nickel administration can lead to structural and biochemical changes in mice including retarded antioxidant defense (Doreswamy *et al.,* 2004; Massanyi *et al.,* 2007). The major point of concern about available literature is in the route of exposure (subcutaneous, intradermal, intraperitoneal), dosage (single low or very low dose) and duration of treatment (acute treatment) employed in some of the studies which have hardly any physiological relevance to human.

Past study from this laboratory on the quantum of various metals present in food grains and vegetables grown in and around Vadodara has suggested high content of Ni in them and to which the population of the city is labeling to be exposed through dietary intake (Sharma, 1996). Considering the mentioned drawbacks in terms of dosage and mode of exposure and high content of Ni in the dietary sources of Vadodara population, an attempt has been made in the present investigation to evaluate the effect of natural route of exposure (oral through drinking water) of an environmentally realistic dosage of Ni given for increasing duration of 15-60 days. Toxicity to male reproductive system of a physiologically relevant animal model has been assessed in terms of serum titres of steroids, activity of seroidogenic enzymes and qualitative and quantitative effects on sperm by studies were also extended to

assess the effect of Ni on *In Vitro* testosterone production by isolated rat Leydig cells under basal and stimulated conditions.

Accepting the fact that entry of xenobiotic chemicals, including metals in to the human system is unavoidable, the use of efficacious natural antioxidants may help in preventing the deleterious effects caused by these toxic agents. One such natural antioxidant which has gained its due importance, is melatonin and, this indoleamine antioxidant has been used as a supplement to check the toxic effects of Ni.

Material and Methods:

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

Results:

3-β HSD activity:

Nickel treatment brought about marked duration dependent increasing inhibition of 3-β HSD activity in the testis, the rate of inhibition was more pronounced at 60 days. Simultaneous melatonin administration along with Ni afforded significant protection against Ni induced inhibition of 3-β HSD activity (Table: 7.1, Figure: 7.1).

17-β HSD:

Marked linear duration dependant decrement in inhibition of 17-β HSD activity was observed in testis of Ni treated animals with maximal inhibition at 15 days and minimal inhibition at 60 days. Conversely, the co-administered melatonin along with Ni showed marked protection in 17-β HSD activity and interestingly showed significantly better protection with increase in duration (Table: 7.2, Figure: 7.2).

Serum Testosterone:

Serum testosterone tire showed marked duration dependent marked decrement in serum testosterone titre in nickel treated rats when compared control. The noted decrement in the serum testosterone titre was maximal in longer duration of nickel exposure. Co-exposure of melatonin exerted marked protection against Ni induced decrement in the serum testosterone titre. Table: 7.3 and Figure: 7.3.

Serum Estradiole:

Serum of animals exposed to Cd showed marked gradual decreased progressive depletion in estradiol titre. Co-exposure of melatonin exerted marginal protective effect on the depletion of serum estradiol titre (Table: 7.4, Figure: 7.4).

Cauda Epididymal Sperm Count:

Nickel treated animals showed marked decline in cauda epididymal sperm count in a duration dependent manner. The protective effect of melatonin was markedly significant when co-administered along with Ni. Protective effect of melatonin was more pronounced at longer duration (Table: 7.5, Figure: 7.5).

Sperm motility:

Results of the sperm motility evaluation suggested marked reduction in the number of motile sperm following Ni exposure. Melatonin exerted marked protection when simultaneously administered along with nickel (Table: 7.6, Figure: 7.6).

Sperm Abnormality:

Though nickel administration exerted significant duration dependent increment in number of abnormal sperm, the effect was more pronounced at 60 days of nickel administration. Conversely, the protective effect of co-administered melatonin was found to be almost similar without any duration dependent effect against nickel exposure (Table: 7.7, Figure: 7.7).

In Vitro Cell Viability

Effect of Ni added to a culture of isolated Leydig cell showed time dependent increment in cytolethality (3 – 12 hrs.). Simultaneous presence of melatonin along with Ni markedly decreased cytolethality caused due to presence of Ni. (Table: 7.10)

In Vitro Testosterone (T) Production:

Testosterone secretion assessment in a culture of isolated Leydig cells under basal and hCG stimulated condition revealed significant decrement in presence of Ni which was protected partly by simultaneous presence of melatonin. Increasing duration of exposure to Ni showed a marked decreased in hCG stimulated release and melatonin proved to be equally effective at both 3 and 6 hours of exposure. Table: 7.11 depict these changes in *in vitro* testosterone release.

Histological Observations:

Testis:

Testis of animal exposed to Ni (Plate: 1, Fig. T1 and T2) showed more or less intact seminiferous tubules with the thick layer at germ cells. However, many of the tubules showed absence of sperm cells while some showed few cells. Loss of advanced germ cell seems to be a feature of presence of melatonin along with Ni (Plate: 1, Fig. T3 and T4) tended to protect against Ni induced loss of mature germ cells. Spermtogonia found to be progressing with quantitatively lesser number of germ cells.

Epididymis:

Epididymis of Ni (Plate: 2, Fig. E1 and E2) exposed animal showed shrinkage of epididymis tubular epithelium and weakening of the walls. The lumen of the tubules showed sparse population of germ cells. Melatonin along with Ni (Plate: 2, Fig. E3

and E4) was found to maintain the structural integrity of seminiferous tubules with the reasonably thick epithelium and presence of lumen filled with sperms.

Plate: 1

Figure: T_1: Photomicrograph of testis treated with **Nickel** showing seminiferous tubules (100X). Note the absence of mature germ cells in most of the tubules

S: Seminiferous tubules; L: Leydig cells

Figure: T_2: Photomicrograph of testis treated with **Nickel** showing seminiferous tubules (400X).Note the absence of sperm and clump of degenerated cells in the lumen. Exfoliation of Spermatids from the germinal epithelium can also be seen (arrows)

Figure: T_3 : Photomicrograph of testis treated with **Nickel** and Melatonin showing seminiferous tubules with progressive stages of spermatogenesis (100X).

S: Seminiferous tubules; L: Leydig cells

Figure: T_4:

Plate: 1

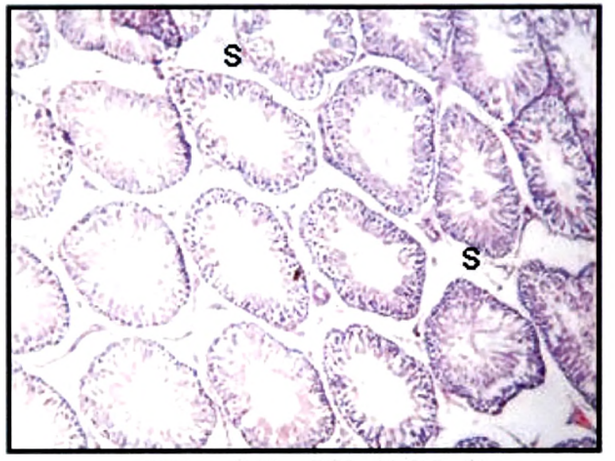


Fig. T1: Ni (100x)

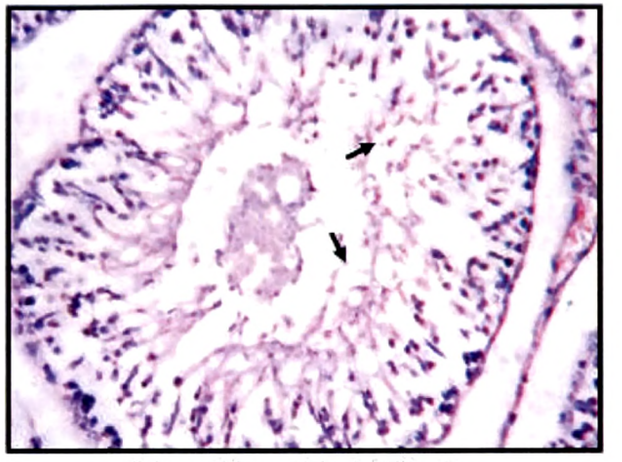


Fig. T2: Ni (400x)

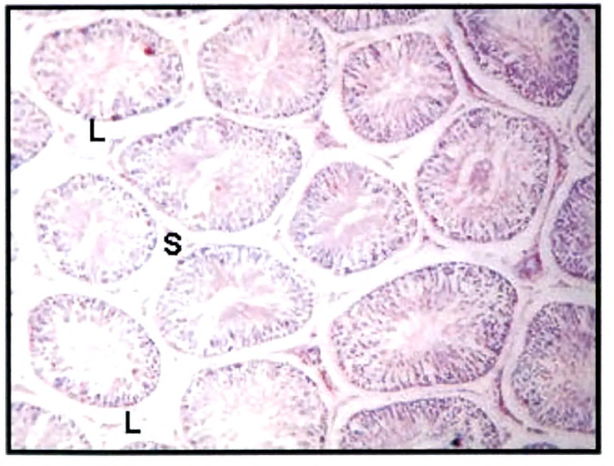


Fig. T3: Ni + Mel (100x)

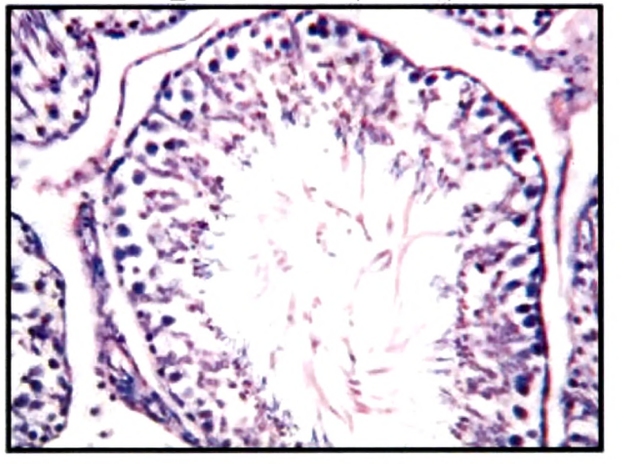


Fig. T4: Ni + Mel (400x)

Plate: 2

Figure: E_1: Photomicrograph of epididymis treated with **Nickel** showing cauda tubules with sparse sperms in the lumen (100X)

lu: Lumen; Sp: Sperms

Figure: E_2: Photomicrograph of epididymis treated with **Nickel** showing cauda tubules with hypotrophied epithelium and devoid of sperms in the lumen

lu: Lumen; Sp: Sperms

Figure: E_3: Photomicrograph of epididymis treated with **Nickel** and melatonin showing well formed cauda tubules filled with sperms (100X)

lu: Lumen; Sp: Sperms

Figure: E_4: Photomicrograph of epididymis treated with **Nickel** and melatonin showing a cauda tubule with a better looking epithelium and lumen filled with sperms (400X).

lu: Lumen; Sp: Sperms

Plate: 2

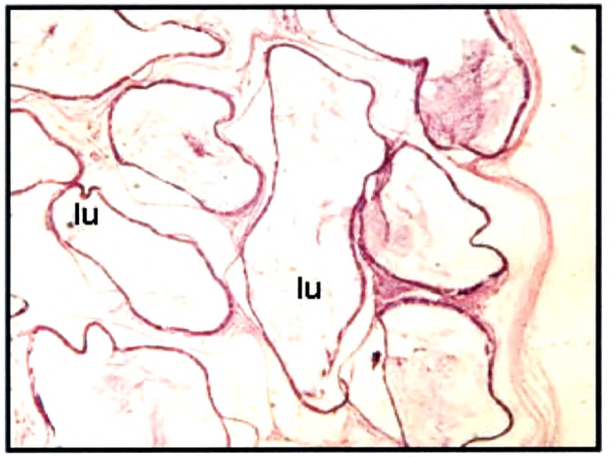


Fig. E1: Ni (100x)

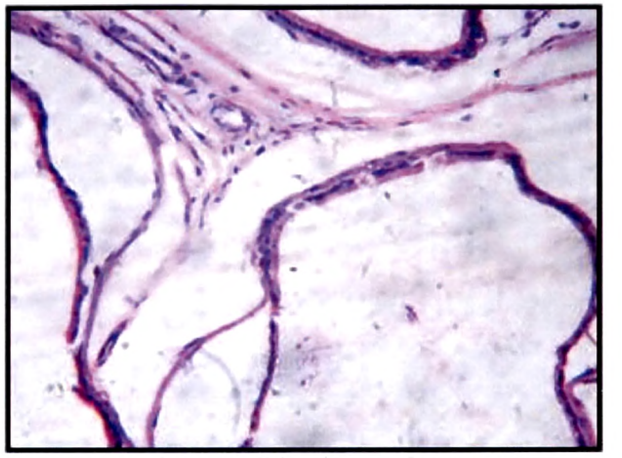


Fig. E2: Ni (400x)

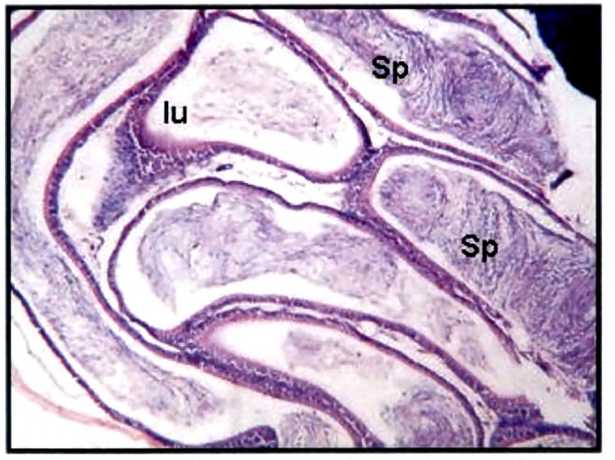


Fig. E3: Ni + Mel (100x)

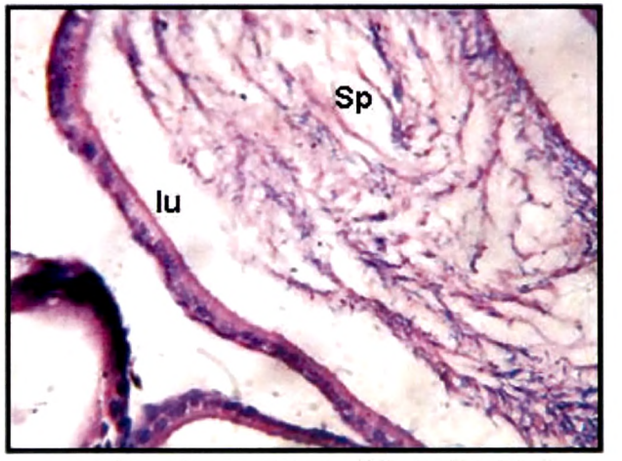
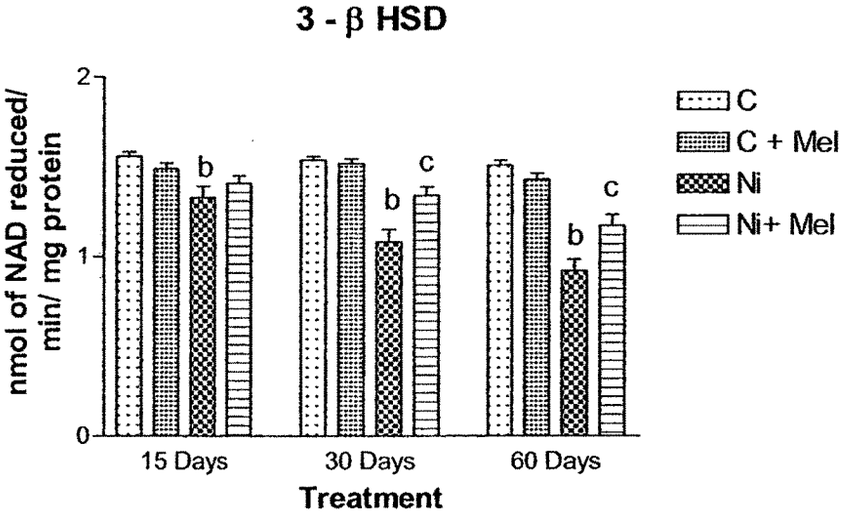


Fig. E4: Ni + Mel (400x)

Figure 7.1: Nickel induced changes in 3 – β HSD activity in testis with or without Melatonin.



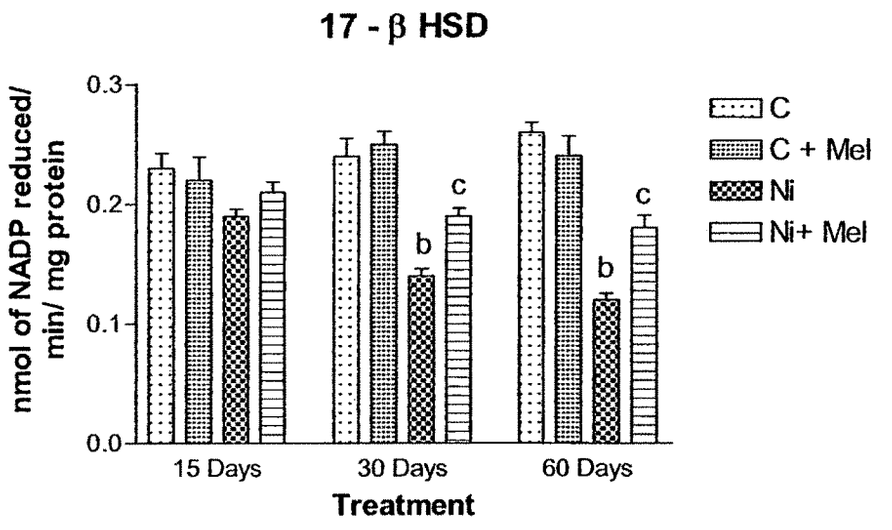
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.1: Nickel induced changes in 3 – β HSD activity (nmol of NAD reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	1.56 ± 0.02	1.49 ± 0.03	1.33 ± 0.06	1.41 ± 0.04
30 Days	1.54 ± 0.02	1.52 ± 0.02^{b}	1.08 ± 0.07^{b}	1.44 ± 0.04^{c}
60 Days	1.51 ± 0.02	1.43 ± 0.03^{b}	0.92 ± 0.06^{b}	1.17 ± 0.46^{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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.2: Nickel induced changes in 17 – β HSD activity in testis with or without Melatonin.



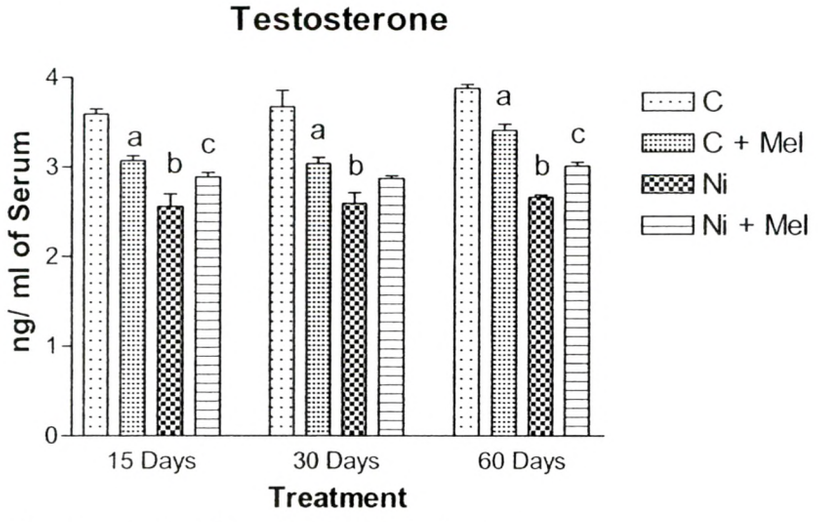
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.2: Nickel induced changes in 17 – β HSD activity (nmol of NADP reduced/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	0.23 ± 0.01	0.22 ± 0.01	0.19 ± 0.005	0.21 ± 0.01
30 Days	0.24 ± 0.01	0.25 ± 0.01	0.14 ± 0.005^{b}	0.16 ± 0.01^{c}
60 Days	0.26 ± 0.01	0.24 ± 0.01	0.12 ± 0.005^{b}	0.18 ± 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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.3: Nickel induced changes in serum Testosterone(T) level with or without Melatonin.



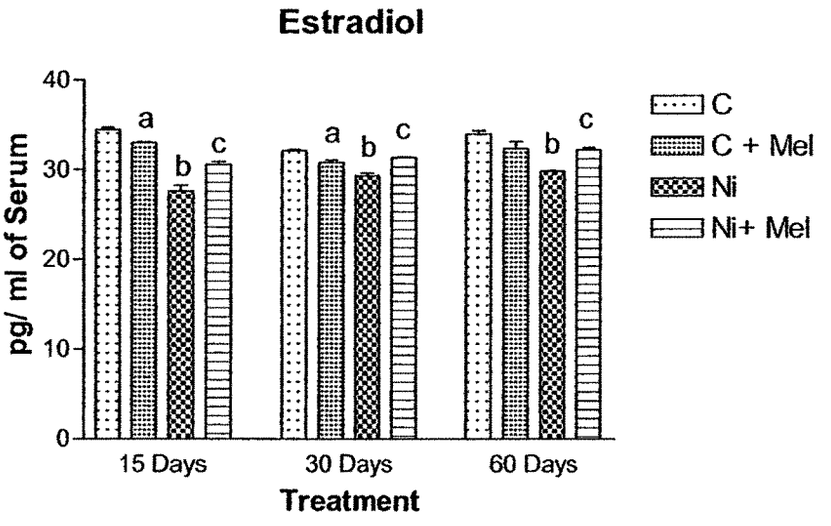
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.3: Nickel induced changes in serum Testosterone(T) level (ng/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	3.59 ± 0.05	3.07 ± 0.05[a]	2.56 ± 0.13[b]	2.89 ± 0.04[c]
30 Days	3.67 ± 0.18	3.04 ± 0.06[a]	2.59 ± 0.12[b]	2.87 ± 0.03
60 Days	3.88 ± 0.04	3.41 ± 0.06[a]	2.66 ± 0.02[b]	3.01 ± 0.004[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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.4: Nickel induced changes in serum Estradiol(E_2) level with or without Melatonin.



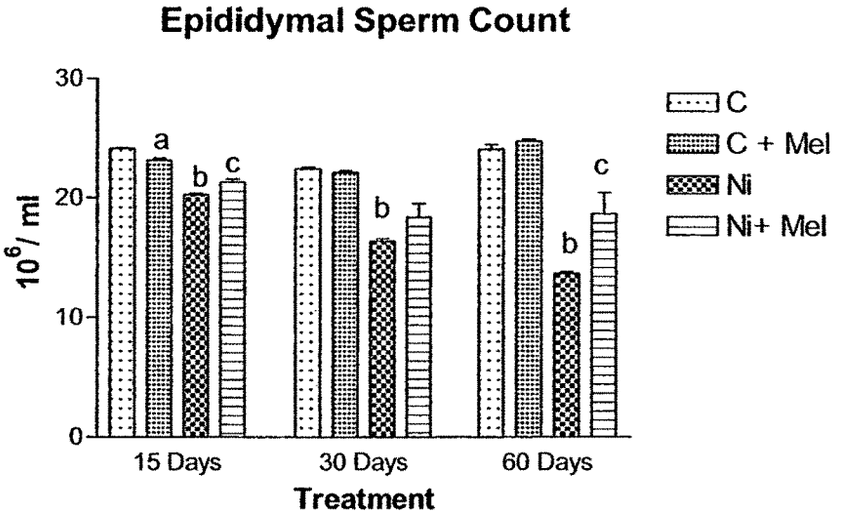
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.4: Nickel induced changes in serum Estradiol(E_2) level (pg/ ml of serum) with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	34.51 ± 0.22	33.01 ± 0.12[a]	27.63 ± 0.64[b]	30.63 ± 0.27[c]
30 Days	32.15 ± 0.13	30.83 ± 0.24[a]	29.35 ± 0.29[b]	31.35 ± 0.12[c]
60 Days	34.01 ± 0.41	32.43 ± 0.73	29.89 ± 0.01[b]	32.29 ± 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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.5: Nickel induced changes in epididymal sperm count with or without Melatonin.



Values expressed as Mean ± SEM of 6 animals per group.
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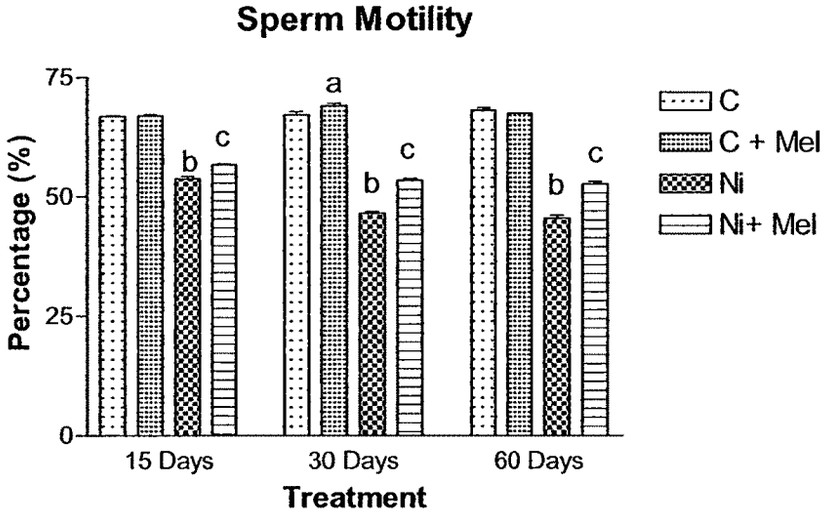
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.5: Nickel induced changes in epididymal sperm count (10^6/ml) with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	24.10 ± 0.07	23.14 ± 0.17[a]	20.26 ± 0.11[b]	21.32 ± 0.27[c]
30 Days	22.44 ± 0.13	22.10 ± 0.19	16.39 ± 0.18[b]	18.36 ± 1.1
60 Days	24.08 ± 0.39	24.73 ± 0.19	13.67 ± 0.14[b]	18.65 ± 1.7[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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.6: Nickel induced changes in epididymal sperm motility with or without Melatonin.



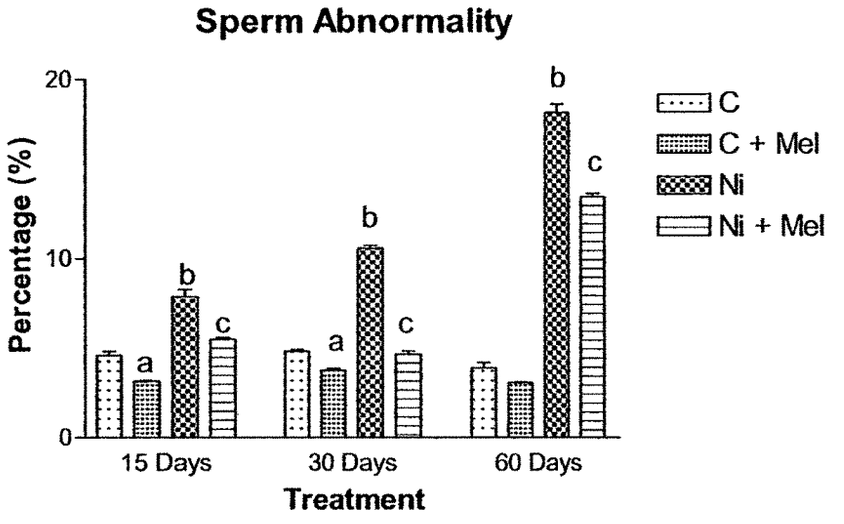
Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.6: Nickel induced changes in epididymal sperm motility (percentage) with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	66.87 ± 0.12	66.92 ± 0.2	53.70 ± 0.49^{b}	56.64 ± 0.27^{c}
30 Days	67.16 ± 0.67	69.06 ± 0.4^{a}	46.49 ± 0.30^{b}	53.36 ± 0.38^{c}
60 Days	68.12 ± 0.50	67.44 ± 0.04	45.45 ± 0.58^{b}	52.60 ± 0.47^{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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Figure 7.7: Nickel 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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.7: Nickel induced changes in epididymal sperm abnormality (percentage) with or without Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	4.60 ± 0.20	3.14 ± 0.03[a]	7.87 ± 0.38[b]	5.47 ± 0.11[c]
30 Days	4.80 ± 0.09	3.76 ± 0.10[a]	10.58 ± 0.14[b]	4.65 ± 0.16[c]
60 Days	3.90 ± 0.28	3.05 ± 0.04	18.13 ± 0.47[b]	13.42 ± 0.18[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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.8: Nickel induced percentage changes in nickel content of testis and epididymis with or without Melatonin.

Treatment	Testis		Epididymis	
	Ni	Ni + Mel	Ni	Ni + Mel
15 Days	20.41	7.75	37.79	35.63
30 Days	16.56	5.92	20.72	32.41
60 Days	19.90	25.00	31.35	34.68

C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.9: Nickel induced changes in the serum titre of Melatonin(pg/ ml) with or with out Melatonin.

Treatment	C	C + Mel	Ni	Ni + Mel
15 Days	121.00 ± 0.36	140.00 ± 0.60[a]	71.00 ± 0.43[b]	116.00 ± 0.09[b]
30 Days	112.00 ± 0.24	143.00 ± 0.17[a]	78.00 ± 0.14[b]	104.00 ± 0.36[b]
60 Days	93.00 ± 0.05	126.00 ± 0.14[a]	31.00 ± 0.61[b]	84.00 ± 0.74[b]

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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.10: Chromium(VI) induced changes in Leydig cell viability(in percentage) with or without melatonin.

Treatment	Cell Viability			
	C	C + Mel	Ni	Ni + Mel
3 Hours	93.81 ± 0.06	94.10 ± 0.11	86.90 ± 0.19 [b]	88.47 ± 0.24
6 Hours	93.42 ± 0.23	93.96 ± 0.24	81.00 ± 0.32 [b]	84.70 ± 0.26
12 Hours	92.09 ± 0.07	94.33 ± 0.04	75.59 ± 0.07 [b]	81.46 ± 0.37 [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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Table 7.11: 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	Ni	Ni + Mel	C	C + M	Ni	Ni + Mel
3 Hours	4.87 ± 0.33	4.17 ± 0.16	4.07 ± 0.03	4.11 ± 0.06	7.58 ± 0.45	5.83 ± 0.04	6.74 ± 0.15	7.22 ± 0.25
6 Hours	4.55 ± 0.16	4.06 ± 0.20	3.94 0± 0.08 [b]	4.06 ± 0.07	7.94 ± 0.04	5.93 ± 0.11	6.49 ± 0.31	7.08 ± 0.14

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 Nickel
C – Control; C + Mel – Control + Melatonin; Ni – Nickel;
Ni + Mel – Nickel + Melatonin

Discussion:

Male reproductive toxicity of Ni is evident from the herein recorded changes in steroid dehydrogenases, serum T and E_2 levels, sperm count, sperm motility, sperm abnormality and histologically noticeable lesions in testis and epididymis. Disruption in spermatogenesis is marked by loss of sperms and spermatids in most of the tubules. Post-meiotic germ cells seem to manifest their vulnerability towards Ni. This observation is supported by similar findings by Doreswamy *et al.* (2004) in the mouse. Different dose and treatment schedules of Ni have been also shown to have differential deleterious effects on spermatogenesis in both rat and mouse (Kakela *et al.*, 1999; Pandey *et al.*, 1999; Das and Dasgupta, 2002; Massanyi *et al.*, 2007). Apart from its effect on seminiferous tubules, Ni also seems to affect the Leydig cells and the process of steroidogenesis as, the activities of 3-β and 17-β HSDs and serum T and E_2 levels are all significantly compromised. A fall in serum and intratesticular T levels together can also contribute to arrest in progession of spermatogenesis as some of the stages are vitally androgen dependent. Higher degree of inhibition of the steroidogenic enzymes occurs during the longer durations of exposure (30 and 60 days) indicating the increasing toxicity of Ni on the biochemical make-up of Leydig cells. Ni-induced decrease in testicular steroid dehydrogenases has also been reported by Das and Dasgupta (2002) in their short duration exposure studies. The decrease in activities of steroid dehydrogenases is apparently reflected on the lower circulating titre of T, as has also been reported by others (Ng and Liu., 1990; Ray *et al.*, 1998; Forgaes *et al.*, 1998; Das and Dasgupta, 2002). A finer point to be noted is that, though Ni caused significant inhibition of steroid dehydrogenases like caused by Cr and Cd (Chapters 5 and 6), on a comparative basis, the degree of decrement in T

levels brought about by Ni was lesser than that induced by Cr and Cd. It is worth pondering in this context, about the possible negative effect of Ni on T clearance. Could the steroid metabolizing enzymes also be affected significantly?

The mechanism of Ni induced negative effects on both steroidogenesis and spermatogenesis can be probably sought in the metal induced free radical generation and consequent oxidative stress. This is substantiated by the recorded average 19% increase in testicular Ni load across exposure durations (15-60 days) and the previously shown alterations in endogenous antioxidants leading to increased pro to antioxidant ratio and LPO in testis and epididymis of Ni exposed rats (Chapter-3). Similar sentiments have been echoed by even other investigators (Doreswamy *et al.*, 2004; Massanyi *et al.*, 2007). In this context, oxidative damage has been considered as a major factor in reproductive organ dysfunctions and male sterility in recent times and, the potent ability of testis to generate ROS and RON has also been clearly elucidated (Middendorf *et al.*, 1997; Turner and Lysiak., 2008). The occurrence of germ cell apoptosis in testis tubules by way of DNA damage has been documented recently (Doreswamy *et al.*, 2004). Major ROS which can cause testicular damage is $OH^{\cdot}$, a powerful and rogue free radical which has been purported to be generated by Ni mediated Fenton reaction (Chapter 3). In the above study, it has been highlighted that, Ni induced $OH^{\cdot}$ radical generation by Fenton reaction is by way of Fe accumulation as; Ni has the ability to dissociate Fe from proteins and lead to its accumulation. Earlier study (Chapter 3) had also pointed out that, Ni induced Fe increase in partly possible by way of displacement of Fe from cytosolic guanylate cyclase by Ni binding and as such, an increase in Fe content in tissues under Ni toxicity has been reported (Xu *et al.*, 1985; Arthur *et al.*, 1987; Doreswamy *et al.*,

2001). Higher levels of Fe are known to increase oxidative damage and deplete antioxidants in testis (Lucesoli and Fraga, 1995; Wellejus *et al.*, 2000). Multiple doses of Ni in this context have been shown to increase oxidative stress in testis and cause genotoxic effects and DNA damage leading to apoptotic cell death (Doreswamy *et al.*, 2004). Since, ROS and RNS are known to be routinely generated during testicular steroidogenesis (Middendorf *et al.*, 1997; Turner and Lysiak, 2008), Ni induced hypergeneration of both are likely to contribute to a higher degree of oxidative stress resulting in Leydig cell damage and consequent hyposecretion of testosterone (Del Punta *et al.*, 1996; Kostic *et al.*, 1998; Chen and Zirken, 1999; Luo *et al.*, 2006). Another mode of action of Ni in decreasing T output could be by its effect on hypothalamo-pituitary-testicular axis (Pandey *et al.*, 1999) and decreased circulating level of LH (Lafuente *et al.*, 2001). The blood testis barrier (BTB) formed by the inter-Sertoli tight junctions is known to be permeable to Ni to facilitate its entry as, an optimum level of Ni is required for normal spermatogenesis (Dixon, 1986; King *et al.*, 2000; Yokoi *et al.*, 2003). Since, both and excessive and deficient dietary Ni can impair a normal process of spermatogenesis (Yokoi *et al.*, 2003), increased Ni load is likely to disrupt the Sertoli junctions as well as affect the function of Sertoli cells and germ cells leading to apoptotic/necrotic cell death (Pandey *et al.*, 1999).

Whereas the observed decrease in sperm count in Ni exposed animals could be related with the deleterious effects of Ni on the process of spermatogenesis and steroidogenesis in the testis, the significantly decreased sperm motility and morphological abnormality could be related with the toxic effects of the metal on epididymal structure and functions Pertinently, a much higher Ni load in epididymis compared to testis (30% vs 19%) and a higher oxidative stress in the epididymis

induced by Ni (Chapter 3) are points of relevance in this context. This higher uptake of Ni by epididymis may not only affect the epididymal epithelium which in turn could affect its role in sperm maturation and motility function (Hoey, 1966; Pandey *et al.*, 1999). The increased Ni load of epididymal cells as well as epithelial damages induced by the metal could lead to higher (supraoptimal) Ni content in the lumen of the cauda epididymis. It is suggested that Ni in optimal level has a role in spermatozoan transit time and motility in the epididymis through its optimal action in the regulated closing of cyclic nucleotide gated cationic channels (CNG) (Hansen and Jones, 1996). As CNG channels are present on both epididymis and sperm, cGMP can regulate the degree of closing of CNG channels, thus influencing epididymal absorptive and sperm motility functions (Yokoi *et al.*, 2003). Apparently, it can be surmised from the projected facts in the above studies that, excess (supranormal) level of Ni in epididymal epithelium and epididymal lumen can cause GMP gated maximal closing of CNG channels leading to decreased absorption of Na^+ ions and fluid from the epididymal lumen contributing to higher fluid content and reduced sperm transit time and attendant poor sperm maturation. Simultaneously, closing of the cGMP gated ion channels on the sperm flagellar surface could decrease Ca^{2+} entry and thereby affect sperm motility. These purported effects of Ni on epididymis and spermatozoa could be held responsible by the presently observed decreased sperm motility and increased sperm abnormality. Since, epididymis is an androgen target tissue, the reduced testosterone levels could compound the above effects on epididymal functions.

Given the potent role of melatonin as a powerful antioxidant and regulator of various physiological functions the recorded significant duration dependent decrement in serum Melatonin becomes self-explanatory. Exogenous melatonin administration

tried out as a protectant antidote against Ni toxicity has revealed its favorable effect on almost all the parameters affected by Ni. Though it has been found to exert varying degrees of protective effect on recorded alterations, by far, this indoleamine appears quite adept in minimizing the impact of metal toxicity on the functional features of testis and epididymis. Its cytoprotective effects, probably by means of its competence to scavenge free radicals and stimulate the endogenous antioxidant machinery, is well reflected in the documented histological appearance of testis and epididymis. Presumably, these cytoprotective effects could involve prevention of damage to the BTB and apoptosis of germ cells together with maintenance of the functional integrity of Leydig cells. To the best of our knowledge, there are no reports on the use of melatonin as a supplemental therapy for Ni toxicity. Apart from its role as an effective antioxidant, melatonin may also be considered to exert multitudes of protective effects acting at different molecular sites. The observed favorable influence of melatonin, though remarkably effective in protecting against Ni induced alterations, it is nevertheless not able to completely prevent the toxic manifestation of the metal. This may suggest the need to employ a combination therapy of melatonin supplemented with optimal concentrations of vitamin A, C and E. Melatonin as a principal component of this combination therapy is substantiated by the fact that usage of higher doses of vitamins is precluded due to their toxic manifestations of hypervitaminoses.

The observed *In Vivo* effects on steroidogenesis have been tested on an *In Vitro* systems of cultured Leydig cells. Cell viability assessed at the end of 3, 6 and 12 hours of Ni exposure of shows a gradually increasing cytotoxicity of 13% at 3 hours, 19% at 6 hours and 24% at 12 hours. Compared to the earlier observations on Cr and Cd (Chapters 5, 6), Ni seems to be less cytotoxic. This lesser degree of

cytotoxicity is paralleled by a lesser degree of inhibition of testosterone secretion by Ni under basal and hCG stimulated conditioned as compared to the earlier observed effects of Cr and Cd. The present *In Vitro* observations corroborates the earlier discussed effects of Ni in inducing lesser reduction in testosterone secretion under *In Vivo* condition despite the greater inhibitions of steroid dehydrogenases. Based on the earlier studies on the inhibitory effects Cr and Cd on testosterone secretion and also based on the In-Vitro study (Laskey and Phelps, 1991), the site of action of the heavy metals was suggested to be between post receptor second messenger generation and cholesterol transport into mitochondria. The possibility of heavy metals interfering with the expression of steroid acute regulatory protein (StAR) was envisaged. Viewed in this context, Ni might be considered to have no significant inhibitory effect on StAR protein expression. The minimal inhibition in testosterone secretion seen in this study with Ni may therefore suggest inhibition in steroidogenesis at other sites mediated by Ni generated free radicals (ROS and RNS). The degree of inhibition in testosterone secretion under both basal well as hCG stimulated conditions in Ni exposed Leydig cells compared to control Leydig cells, is uniform 15%, viz much lesser compared to the Cr and Cd induced inhibition. It is also of interest to note that the hCG stimulated testosterone secretion over that of the basal secretion in Ni exposed Leydig cells is an identical 65% as obtained for the control Leydig cells. All these emphasize, the relatively lesser inhibitory effect of Ni on Leydig cell steroidogenesis compared to other heavy metals studied. In the case of Cd and Cr, though melatonin was found to have an inhibitory effect on testosterone secretion from control Leydig cells, Ni exposed Leydig cells in presence of Melatonin not only nullified this inhibition but also brought about a much higher 'T' secretion upon hCG stimulation than the control Leydig cells. The increment in T secretion brought about

by melatonin in Ni exposed Leydig cells is only a mere 2% under basal conditions and 8% under hCG stimulation. This is much less compared to 15% and 24% seem with Cr and 40% and 38% obtained for Cd. This would also suggest a minimal effect of melatonin in bringing about 'T' secretion from stressed Leydig cells. Apparently the dynamics of 'T' secretion by melatonin under stress conditions is quite different for Ni compared to Cd and Cr (Chapters 5, 6). It is likely that melatonin induced 'T' secretion from Ni stressed Leydig cell could be only by way of overcoming the inhibitory influence of ROS and RNS. The presently observed minimal effect of Ni on 'T' secretion from cultured Leydig cells is quite different form the higher degree of inhibition reported by Laskey and Phelps (1991). However the dosage of 1000μM and 5000μM of Ni used in that study is 10 to 50 times higher than the Ni dose used in the present study. Further studies are needed to elucidate the differential molecular intricacies between Ni and Cd or Cr induced inhibition in 'T' secretion and the effect of melatonin.

In conclusion, the present study has shown Ni induced oxidative stress and deleterious effects on steroidogenesis and spermatogenesis as well as on epididymal functions and quantitative and qualitative aspects of sperm. Melatonin has been found to be effective in preventing these effects though not completely. The dynamics of 'T' secretion by Ni exposed Leydig cells seem to be quite different from that of Cd and Cr and need to be studied at length.