CHAPTER 3

Chapter 3

Chronic Cadmium exposure to a realistic dosage causes elevated hepatic oxidative stress but, decreases the same on longer exposure by adaptive mechanisms and, melatonin protects and augments the same.

Cadmium (Cd) has been recognized as one of the most toxic environmental and industrial pollutant with greater chances of occupational exposure. Cadmium is also an important byproduct during the processing of other metals and, finds extensive application in plating, alloy, pigments, catalysts, batteries etc. Soil and water may get contaminated with Cd naturally or by emission from industries and, crops grown on this contaminated soil or water take up Cd efficiently (Elinder, 1985). Humans are generally exposed to Cd via food, water, air and dust and cadmium induced hepatic, renal and pulmonary damage (Liu *et al.,* 1994) or effects on nervous system (Pal *et al.,* 1993) are dependent on duration and route of exposure. Cd is a known human carcinogen (especially for lungs, testes and prostate) and is effective as a single-dose carcinogen in rodents (IARC, 1993; Waalkes, 2000). Chronic exposure to Cd has been shown to cause hepatotoxicity and nephrotoxicity (Webb and Cain, 1982; Friberg, 1984; Dudley *et al.,* 1985) while, administration of Cd in mice results in injury to many tissues (liver, testes and others) marked by lipid peroxidation and formation of Reactive Oxygen Species (ROS) (Sharma *et al.,* 1991). The mechanism responsible for the toxic effects of Cd is not completely understood. Unlike Chromium, Cd is not able to produce free radicals directly as Cd has got only one oxidation state but indirect generation of various radicals like superoxide, hydroxyl and nitric oxide has been reported by Galan *et al.* (2001).

Oxidative damage of tissues and DNA is considered as early manifestations of Cd toxicity (Eybl *et al.,* 2006) and shifting the prooxidant-antioxidant balance in favour of the former seems to bring about oxidative stress induced by Cd (Sies, 1985). Oxidative stress plays a major role in chronic Cd induced hepatic and renal toxicity as it inhibits antioxidant defense system components (Koyu *et al.,* 2006). Accumulation of Cd primarily occurs in liver and to a lesser extent in the kidneys and is stored bound to metallothionein (MT), a heavy metal binding low-molecular weight protein as CdMT (Shaikh and Lucis, 1972; Webb, 1986).

The major route of entry of Cd in humans is the oral route through food and water. There are only few toxicity studies involving oral administration of Cd as, most of the studies have evaluated Cd toxicity by intraperitoneal (ip) or subcutaneous (sc) administration. Further, there is also dearth in studies involving long duration exposure to Cd. This becomes pertinent in the local context as, Cd has been identified as a major environmental pollutant present in high amounts in vegetables, cereals, pulses and grass grown in the highly industrialized city of Vadodara, Gujarat (Blacksmith Institute Report, 1999; Labunska *et al.,* 1999; Ramachandran 2003). This has necessitated the present study on Cd induced hepatic oxidative stress and toxicity in male *Wistar* rats. In this context, since the study is aimed at understanding the possible Cd toxicity on long term systemic entry into humans through diet and water, a realistic dosage has been worked out based on the Cd content in vegetables and food grains and an average daily food intake. Conversation factor of 6.2 (OECD, 2005) has been used for extrapolation of dosage from human to rat. Using such a dosage, a duration dependent (15, 30 and 60 days) hepatic oxidative stress and toxicity have been evaluated.

As entry of metal toxicants into the body is unavoidable due to industrialization, there is need to evaluate the role of agents which can be used as antioxidant therapeutants. Since melatonin is recognized as a powerful natural antioxidant of the body, the efficacy of the same has been tested as a protectant by co-administration along with cadmium.

Material and Methods: Same as in Chapter 1 but the metal given is Cadmium (Cd) and the dose given is 9 mg/kgBW/day.

Results:

Lipid Peroxidation (LPO): Changes in the hepatic LPO levels following Cadmium (Cd) treatment for 15, 30 and 60 days are shown in fig 3.1; table 3.1. An age dependent gradual increase in LPO could be seen in control (Con) rats. There was significant increase in LPO in the Cd treated group of animals when compared to Con animals. There was significant decrease in LPO in Con and Cd exposed rats treated with melatonin. Cadmium treatment showed duration dependent decrement in LPO with maximal level being at 15 days and minimal at 60 days. Irrespective of duration of Cd exposure, melatonin afforded same degree of protective effect.

Glutathione (GSH): Changes in hepatic glutathione levels following Cd treatment for 15, 30 and 60 days are shown in figure 3.2; table 3.2. There was significant increase in the levels of GSH in Mel group of animals and a significant decrease in Cd exposed animals compared to the Con group of animals. A gradual age dependent decrease in GSH level was the feature of Con rats. Similar degree of decrement in GSH by Cd exposure and same degree of protective effect by melatonin on co-administration with Cd irrespective of duration could also being seen.

Ascorbic Acid (Vit C): Changes in hepatic Vit C levels following Cadmium treatment for 15, 30 and 60 days are shown in figure 3.3; table 3.3. Liver being the storage organ of Vit C in rodents, has a much higher content than the synthetic organ, the kidney. There was significant decrease in Vit C levels in the Cd group when compared to Con group of animals. The degree of depletion of hepatic Vitamin C content was progressively less with increasing duration of Cd exposure and was similar to hepatic GSH levels. There was duration dependent degree of protection by melatonin with maximal protection being at 30 and 60 days in that order.

Superoxide Dismutase (SOD): Changes in hepatic SOD activity following Cd treatment for 15, 30 and 60 days are shown in figure 3.4; table 3.4. Control animals showed an age dependent decrease in SOD activity. There was significant increase in SOD activity in Mel and Cd+Mel group of animals compared to Con and Cd group of animals respectively. There was significant decrease in SOD activity in the Cd group of animals compared to the Con group of animals with, relatively and significantly lesser decrement in the short term duration of Cd exposure. The corresponding degree of protection with melatonin was also less in the 60 day Cd exposure group.

Catalase (CAT): Changes in hepatic CAT activity following Cd treatment for 15, 30 and 60 days are shown in figure 3.5; table 3.5. Hepatic CAT activity tended to show an age dependent decrement. There was significant decrease in CAT activity in Cd group of animals. Catalase activity was significantly decreased to the same degree in Cd exposed rats irrespective of duration of exposure. Degree of protective effect of melatonin was also found to be duration independent.

Glutathione Peroxidase (GPx): Changes in hepatic glutathione peroxidase (GPx) following Cd treatment for 15, 30 and 60 days are shown in figure 3.6; table 3.6. An age dependent decrement in GPx activity was the feature of Con group of rats. The Cd group of animals showed significant decrease in GPx activity but, the degree of inhibition of GPx activity and the degree of protective effect of melatonin were found to be duration independent.

Metal Load: Changes in hepatic accumulation of Cd following exposure for 15, 30 and 60 days are shown in figure 3.7; table 3.7. Cd treated rats showed significant increase in hepatic Cd load. There was decrement in Cd accumulation in animals

treated with melatonin alone or in combination with Cd. Both, Cd induced hepatic load as well as the degree of protection by melatonin were both duration independent.

Serum Parameters: Changes in serum glucose, insulin, melatonin and hepatotoxicity parameters like Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP) are shown in tables 3.8 to 3.13. There is an age dependent decrease in melatonin level in the Con group of animals. There was significant decrease in melatonin level in the Cd treated group. There was an increase in the blood glucose level and decrease in insulin titre in Cd exposed group compared to Con group of animals with melatonin being able to decrease the blood glucose level to near normal values with corresponding changes in insulin level. The hepatic toxicity parameters (ALT and ALP) showed significant increase in Cd treated groups and, significant protective effect was seen in animals co-administered with melatonin.

Histology: Changes in hepatic histology following Cd treatment for 15, 30 and 60 days are shown in figures 3.8 to 3.10. Cd induced histological changes like hypertrophy and degeneration of hepatocytes, dilation of sinusoids, disruption of hepatic cords, disruption of central vein and infiltration of leucocytes and inflammations could be seen clearly in all the duration periods of exposure. These changes were reversed and near normal histoarchitecture was observed by co-administration with melatonin. Hence, melatonin is able to protect the hepatic tissue in all the three treatment periods.

Figure 3.1: Graph showing levels of hepatic lipid peroxidation (LPO) in animals exposed to Cd for 15, 30 or 60 days

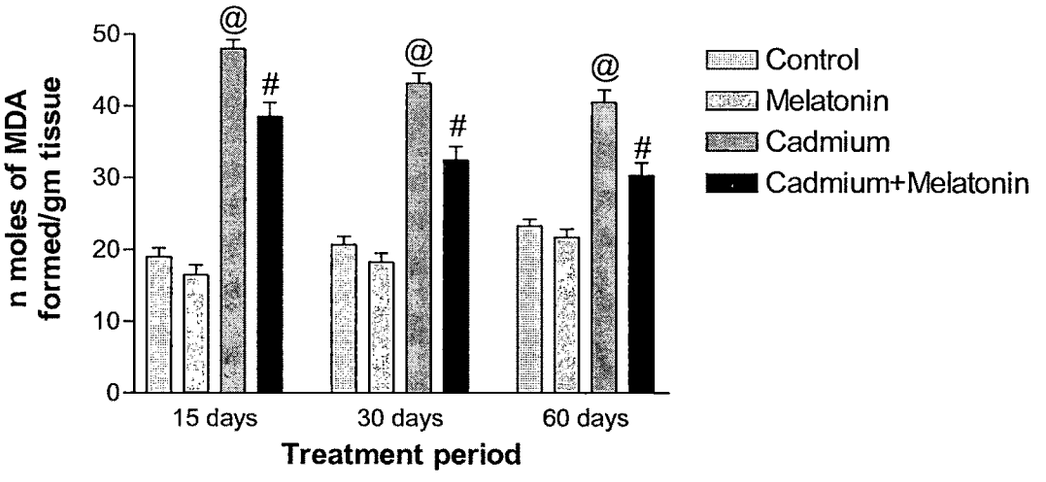

Table: 3.1 Levels of hepatic lipid peroxidation (LPO) following 15, 30 and 60 days of Cadmium exposure

	15 days	30 days	60 days
Con	19.02 ± 1.26	20.79 ± 1.080	23.30 ± 1.02
Mel	16.490 ± 1.39	18.280 ± 1.290	21.76 ± 1.17
Cd	48.02 ± 1.25@	43.27 ± 1.38 @	40.59 ± 1.78 @
Cd+Mel	38.56 ± 1.96#	32.5 ± 1.85 #	30.39 ± 1.78 #

@ p<0.05 between Con vs Cd
 # p<0.05 between Cd vs Cd+mel
***p<0.05 between Con vs Mel**

Figure 3.2: Graph showing levels of hepatic glutathione (GSH) content in animals exposed to Cd for 15, 30 or 60 days

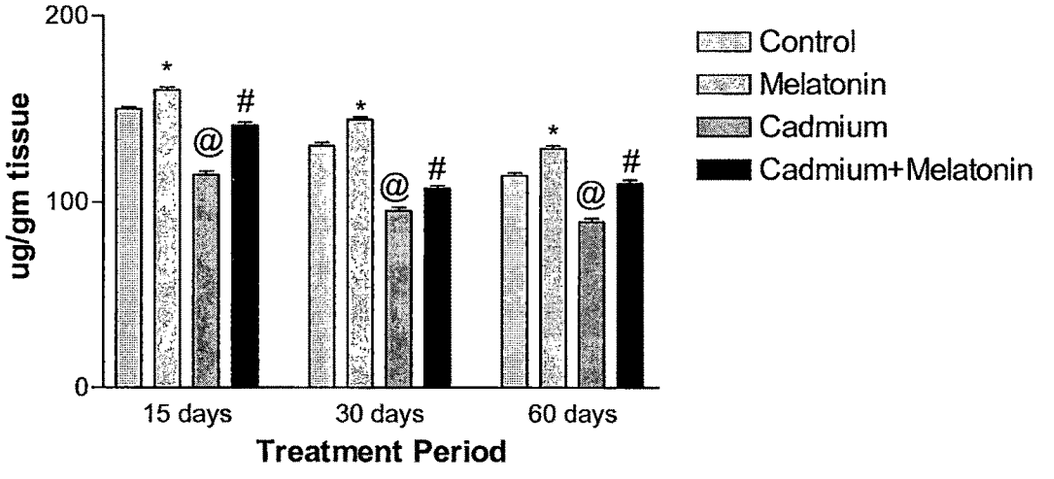

Table 3.2. Levels of hepatic glutathione (GSH) following 15, 30 and 60 days treatment with Cadmium

	15 days	30 days	60 days
Con	0.150 ± 0.0020	0.130 ± 0.007	0.114 ± 0.005
Mel	0.160± 0.002 *	0.144 ± 0.002 *	0.128±0.004 *
Cd	0.114±0.002 @	0.095 ± 0.009@	0.089± 0.008@
Cd+Mel	0.141 ± 0.008 #	0.125± 0.006 #	0.110± 0.002 #

@ $p<0.05$ between Con vs Cd
 # $p<0.05$ between Cd vs Cd+mel
***$p<0.05$ between Con vs Mel**

Figure 3.3. Graph showing levels of hepatic Ascorbic Acid (Vit C) content in animals exposed to Cd for 15, 30 or 60 days

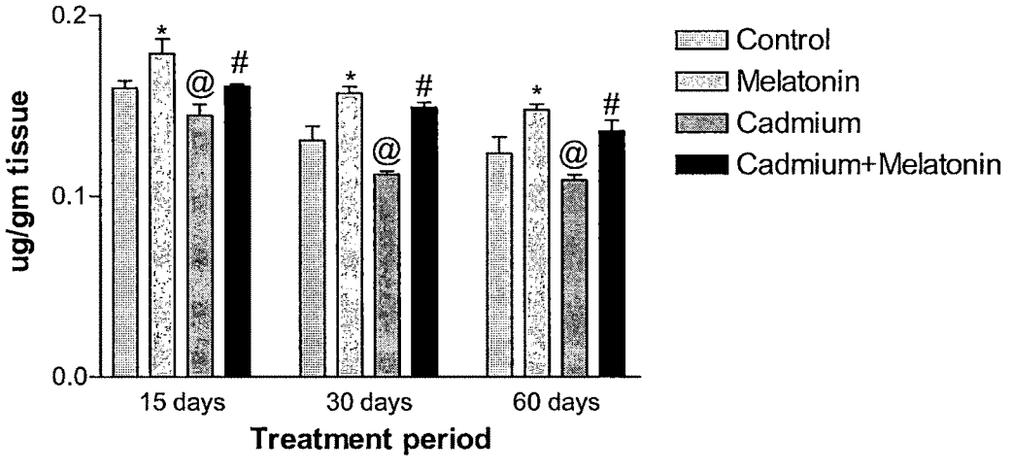

Table 3.3. Contents of hepatic Ascorbic Acid (Vit C) following 15, 30 or 60 days treatment with Cadmium

	15 days	30 days	60 days
Con	0.160±0.004	0.131 ± 0.008	0.124 ± 0.009
Mel	0.179 ± 0.008*	0.157± 0.004*	0.148± 0.002 *
Cd	0.145 ± 0.006@	0.112 ± 0.002@	0.104± 0.003@
Cd+Mel	0.161 ± 0.001#	0.139± 0.003 #	0.136 ±0.006#

@ p<0.05 between Con vs Cd
 # p<0.05 between Cd vs Cd+mel
***p<0.05 between Con vs Mel**

Figure 3.4. Graph showing hepatic Superoxide Dismutase (SOD) activity following 15, 30 or 60 days exposure with cadmium.

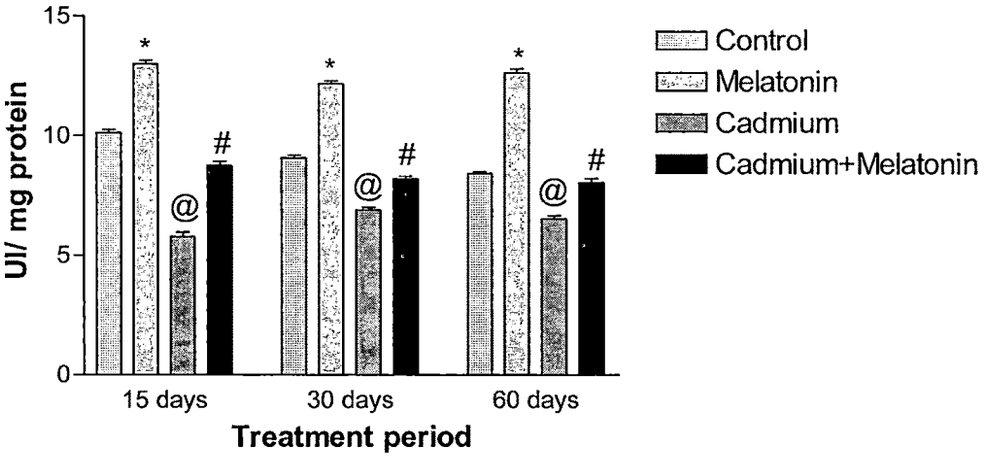

Table 3.4. Levels of hepatic Superoxide Dismutase (SOD) activity following 15, 30 and 60 days exposure with Cadmium

	15 days	30 days	60 days
Con	13.8700 ± 0.1300	12.0300 ± 0.1600	10.5200 ± 0.1270
Mel	16.0100 ± 0.1600*	14.2700 ± 0.1100 *	12.3700 ± 0.1610 *
Cd	5.78 ± 0.190 @	6.89 ± 0.102 @	6.52 ± 0.18 @
Cd+Mel	8.760 ± 0.2 #	8.19 ± 0.10 #	8.03 ± 0.20 #

@ $p<0.05$ between Con vs Cd
 # $p<0.05$ between Cd vs Cd+mel
***$p<0.05$ between Con vs Mel**

Figure 3.5. Graph showing hepatic catalase (CAT) activity following 15, 30 or 60 days exposure to cadmium.

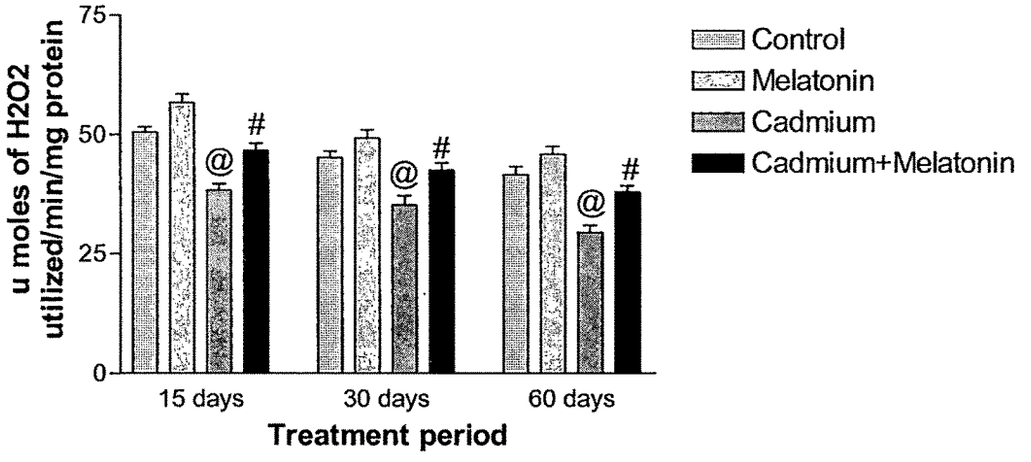

Table 3.5. Levels of hepatic catalase (CAT) activity following 15, 30 or 60 days exposure with Cadmium

	15 days	30 days	60 days
Con	50.600 ± 1.100	45.190 ± 1.390	41.630 ± 1.730
Mel	56.80 ± 1.780	49.29 ± 1.820	45.97 ± 1.620
Cd	39.52 ± 1.360 @	34.27 ± 1.920 @	29.540 ± 1.490 @
Cd+Mel	46.690± 1.580 #	42.580 ± 1.590 #	38.050 ± 1.320 #

@ $p<0.05$ between Con vs Cd
 # $p<0.05$ between Cd vs Cd+mel
***$p<0.05$ between Con vs Mel**

Figure 3.6. Graph showing hepatic Glutathione Peroxidase (GPx) activity following 15, 30 and 60 days exposure to cadmium.

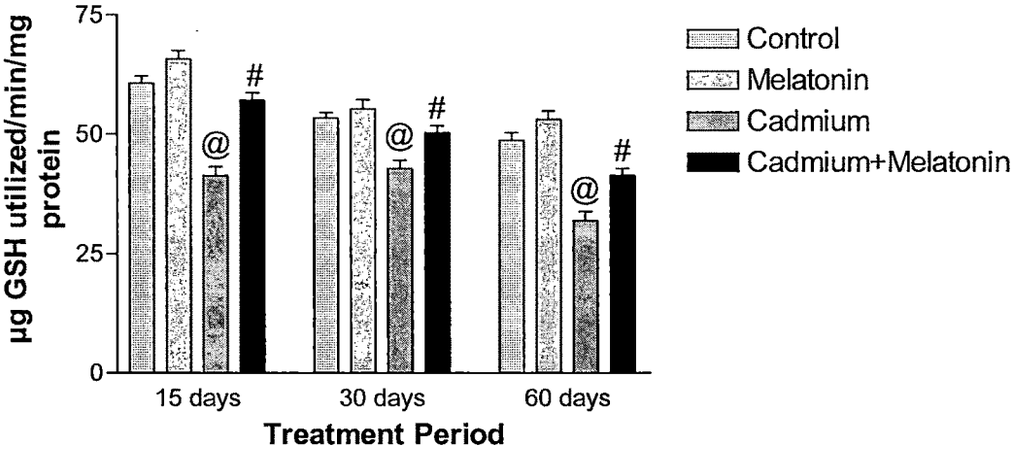

Table 3.6. Levels of hepatic Glutathione Peroxidase (GPx) activity following 15, 30 and 60 days exposure with cadmium

	15 days	30 days	60 days
Con	60.730 ± 1.5	53.38 ± 1.15	48.72 ± 1.62
Mel	65.80 ± 1.7	55.28 ± 1.91	53.02 ± 1.85
Cd	48.61± 1.86 @	39.99 ± 1.84 @	31.89 ± 1.96 @
Cd+Mel	57.10± 1.59 #	50.29 ± 1.50 #	41.32 ± 1.39 #

@ $p<0.05$ between Con vs Cd
 # $p<0.05$ between Cd vs Cd+mel
***$p<0.05$ between Con vs Mel**

Figure 3.7. Graph showing % accumulation of Cd in hepatic tissue following 15, 30 and 60 days treatment with cadmium.

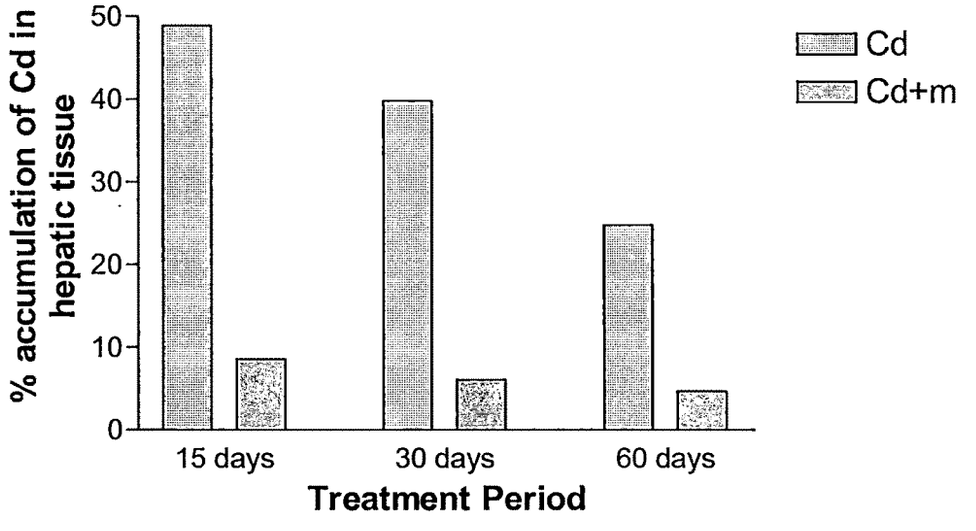

Table 3.7. % accumulation of Cd in hepatic tissue following 15, 30 and 60 days treatment with Cadmium

Treatment Period	Cd	Cd+M
15 days	48.9	8.6
30 days	39.8	6.1
60 days	24.8	4.7

Table 3.8 Changes in serum glucose, insulin and melatonin following treatment with Cr(VI) for 15, 30 and 60 days

	15 Days			30 Days			60 Days		
	Serum glucose(mg/dl)	Insulin (µg/l)	Melatonin (pg/ml)	Serum glucose(mg/dl)	Insulin (µg/l)	Melatonin (pg/ml)	Serum glucose(mg/dl)	Insulin (µg/l)	Melatonin (pg/ml)
Control (Con)	112.5±2.51	1.7±0.06	121±1.65	104.25±2.85	1.58±0.09	112±2.08	115.75±3.85	1.79±0.01	93±4.41
Melatonin (Mel)	125±3.11	0.6±0.09	140±2.65*	115.50±1.65	0.95±0.01	153±6.65*	120.5±2.65	0.81±0.01	126±5.65*
Chromium (Cr)	141.60.5±3.15	0.44±0.04@	59±3.85@	139.5±4.08@	0.49±0.09@	52±3.85@	133.50±0.65@	1.23±0.11@	26±7.01@
Cr+Melatonin (Cr+Mel)	126.75±1.85	1.1±0.06#	104±1.24#	108±3.08	1.05±0.05	106±2.85	119±0.11#	1.61±0.09#	886.85±3.0#

@ $p<0.05$ between Con vs Cr
 # $p<0.05$ between Cr vs Cr+mel
***$p<0.05$ between Con vs Mel**

Table3.9 Changes in ALP and ALT following 15, 30 and 60 days of treatment with Cadmium

	15 days		30 days		60 days	
	ALP(U/I)	**ALT(U/ml)**	**ALP(U/I)**	**ALT(U/ml)**	**ALP(U/I)**	**ALT(U/ml)**
Control (Con)	154.0.5±3.98	45.2±1.25	161±4.6	48.5±2.10	159.8±3.45	49.9±1.90
Melatonin (Mel)	119±4.80	43.1±3.40	134.6±3.92*	47.8±2.90	129.6±2.5*	51.1±3.10
Chromium (Cr)	186.9±3.45@	58.4±2.82@	198.5±2.95@	60.2±3.19@	201±4.60@	65.5±2.92@
Cr+Melatonin (Cr+Mel)	189.65±1.90#	49.51±3.10#	164.9±4.10#	51.6±4.10#	164.23±3.51#	55.5±3.59

@ $p<0.05$ between Con vs Cr
 # $p<0.05$ between Cr vs Cr+mel
***$p<0.05$ between Con vs Mel**

Table 3.10 Changes in Hemoglobin, Erythrocyte count and Packed Cell Volume following Cd treatment for 15, 30 and 60 days

	15 Days			30 Days			60 Days		
	Hemoglobin	Erythrocyte count	Packed Cell Volume (PCV)(%)	Hemoglobin	Erythrocyte count	PackedCell Volume (PCV)(%)	Hemoglobin	Erythrocyte count	Packed Cell Volume (PCV)(%)
Control (Con)	15.05±0.17	9.66±0.01	51.75±0.85	16.75±0.48	10.27±0.01	51.50±1.32	15.75±0.13	10.68±0.09	60.25±1.25
Melatonin (Mel)	15.60±0.15	10.13±0.11	56.00±0.91	16.00±1.08	9.92±0.01	56.25±1.11*	15.75±0.85	10.72±0.01	54.00±1.82
Cadmium (Cd)	8.58±0.13@	7.18±0.01@	32.43±1.04@	15.80±0.09	9.62±0.01	51.00±1.29	14.30±0.09	10.74±0.01	64.50±1.32@
Cd+Melatonin (Cd+Mel)	14.73±0.09#	9.76±0.01#	51.25±0.85#	15.58±0.09	9.79±0.01	57.50±0.65#	14.70±0.09	9.67±0.01	50.50±0.65#

@ $p<0.05$ between Con vs Cr
 # $p<0.05$ between Cr vs Cr+mel
***$p<0.05$ between Con vs Mel**

PLATE I

Fig 3.8 Photomicrograph of 15 day control liver (400X) showing a hepatic lobule. Note the cord like organization of hepatocytes and a central vein. CV- Central Vein, S- Sinusoid, K-Kupfer cells

Fig 3.9 Photomicrograph of 15 days melatonin treated liver (400X). Note the well organized hepatic cords and central vein and robustness of hepatocytes.

Fig 3.10 Photomicrograph of 1 5day cadmium exposed liver (400X). Note the disorganization of hepatic cords, irregular sinusoids and disrupted endothelium of central vein (arrow). Note the presence of apoptotic cells (mark)

Fig 3.11 Photomicrograph of liver exposed to Cd +Mel for 15 days (400X). Note the near normal organization of hepatic lobule and intact central vein.

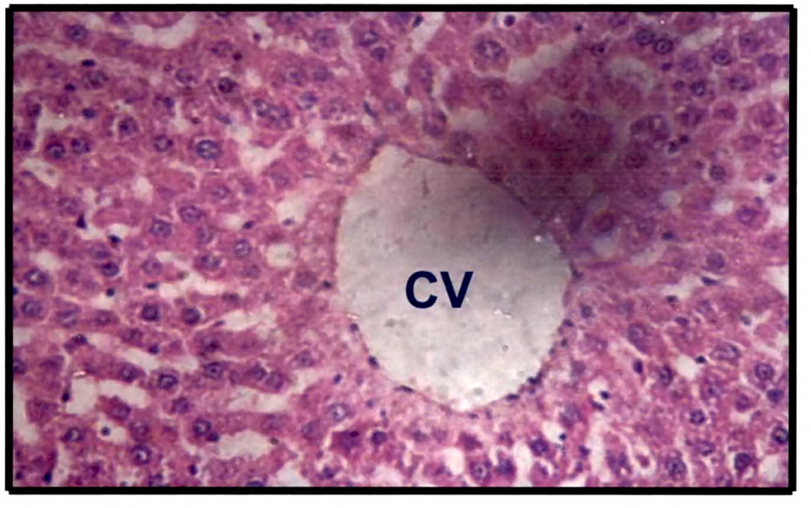

Fig 3.8 Control

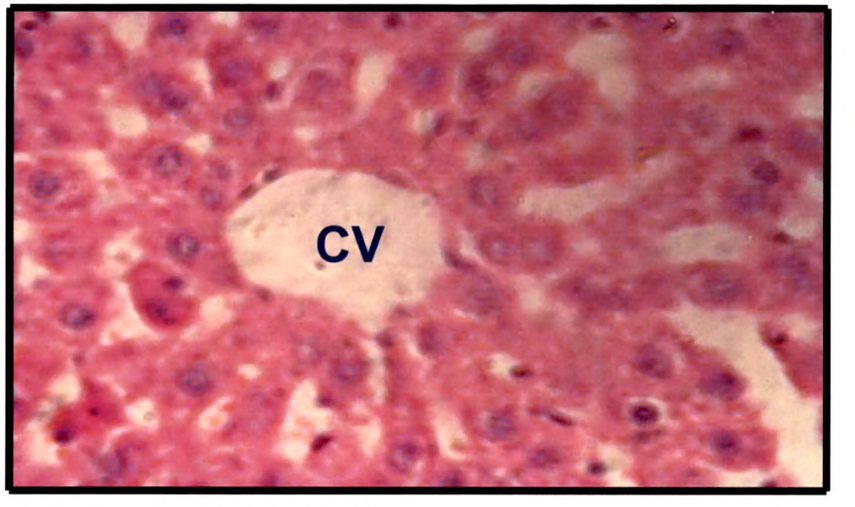

Fig 3.9 Melatonin

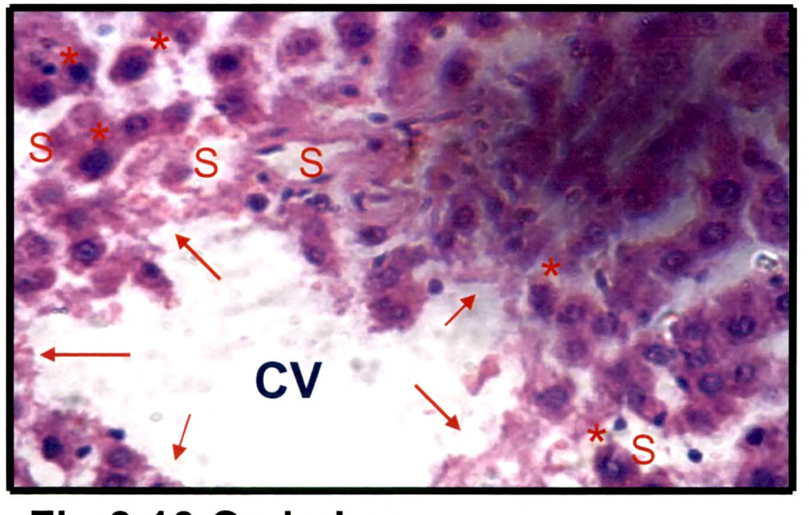

Fig 3.10 Cadmium

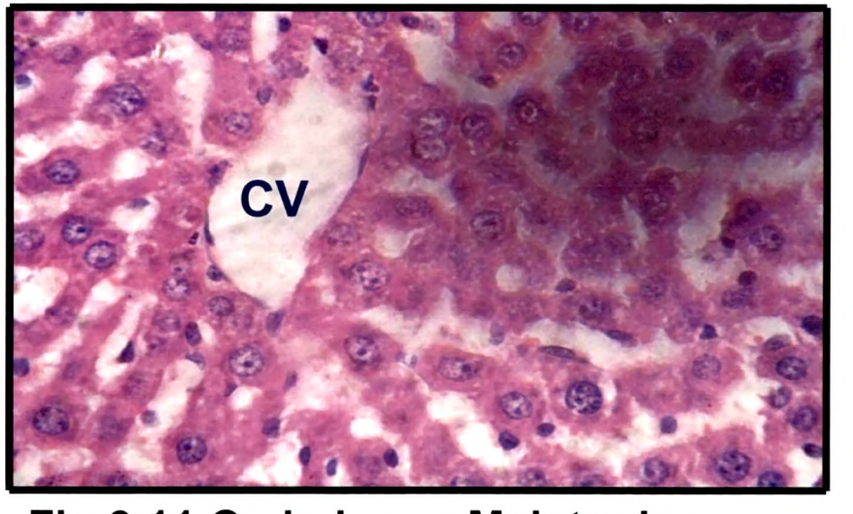

Fig 3.11 Cadmium + Melatonin

PLATE II

Fig 3.8(a) Photomicrograph of control liver showing periportal area (400X). PPA-Periportal Area

Fig 3.9(a) Photomicrograph of melatonin treated liver showing PPA (400X)

Fig 3.10(a) Photomicrograph of 15 day cadmium treated liver showing periportal area (400X). Note the congested portal vein (PV), leucocytic infiltration (arrow) and fibrotic degeneration (*).

Fig 3.11(a) Photomicrograph of liver exposed to Cd+Mel for 15 days showing periportal area (400X). Note the near normal organization.

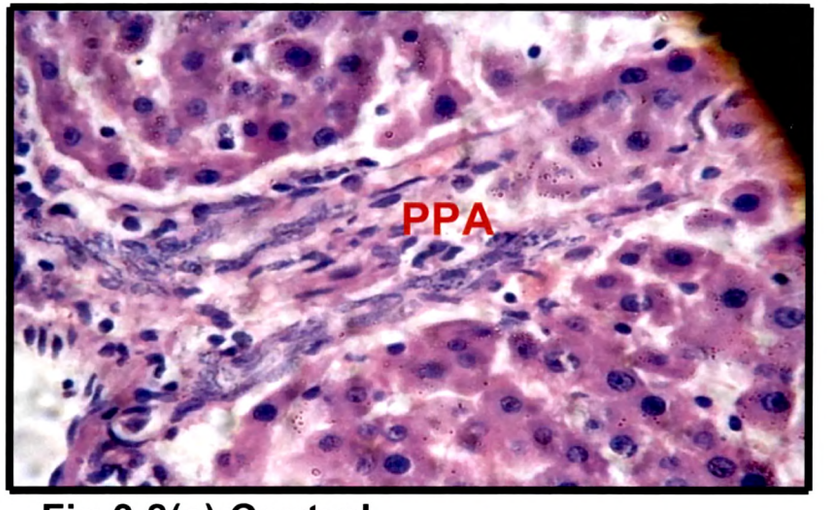

Fig 3.8(a) Control

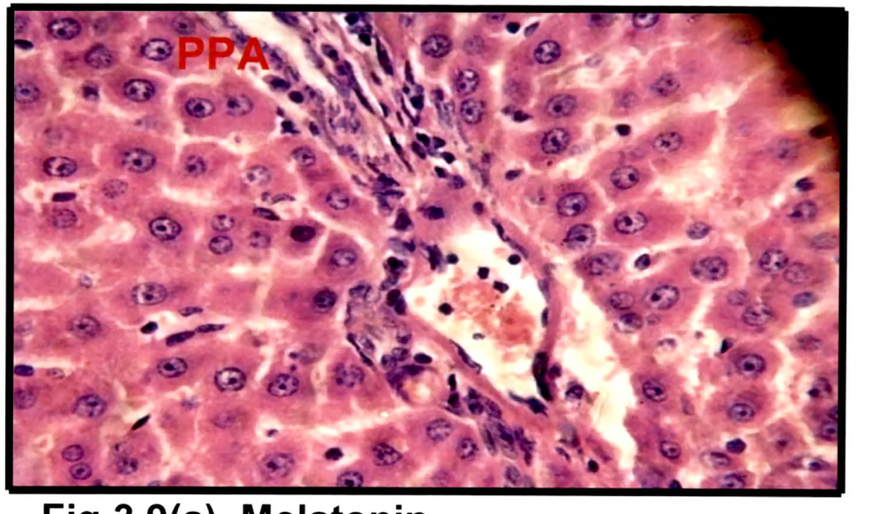

Fig 3.9(a) Melatonin

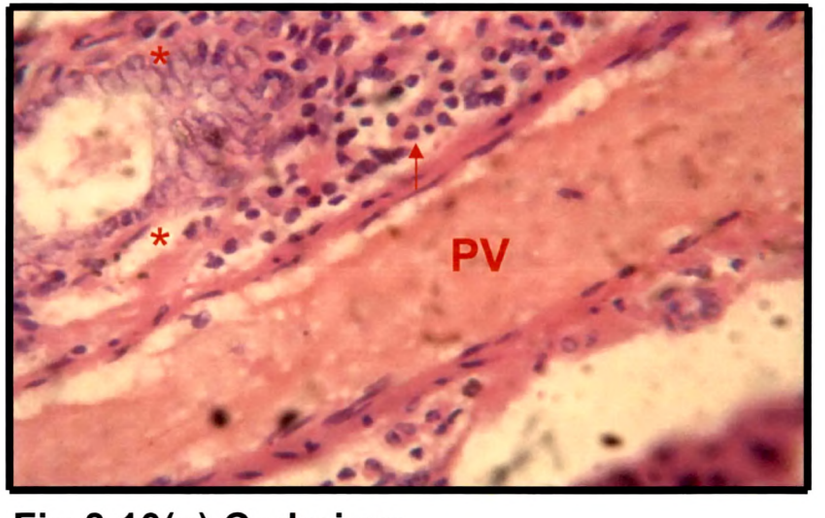

Fig 3.10(a) Cadmium

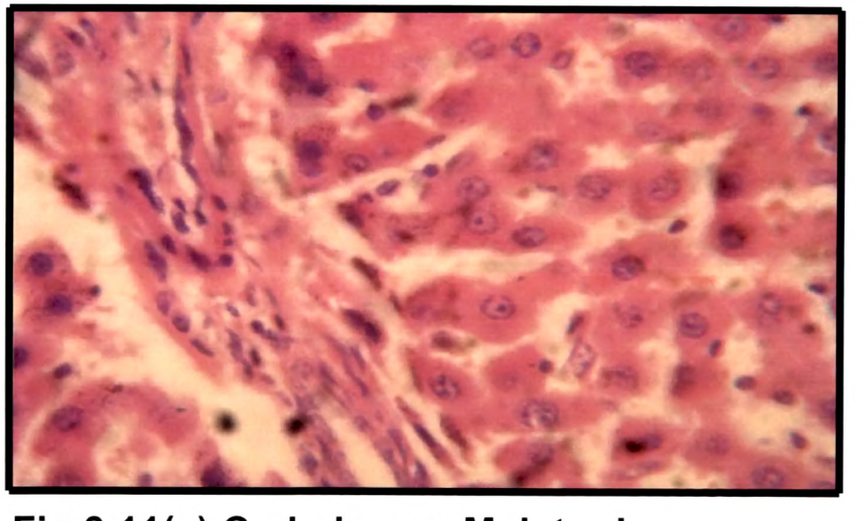

Fig 3.11(a) Cadmium + Melatonin

PLATE III

Fig 3.12 Photomicrograph of 30 day control liver (400X) showing a hepatic lobule. Note the cord like organization of hepatocytes and a central vein. CV- Central Vein, S- Sinusoid

Fig 3.13 Photomicrograph of 30 day melatonin treated liver (400X). Note the robustness of hepatocytes.

Fig 3.14 Photomicrograph of 30 day cadmium exposed liver (400X). Note the disrupted central vein (arrow) and disorganized hepatic cords and degenerating cells.

Fig 3.15 Photomicrograph of liver exposed to Cd+Mel for 30 days (400X). Intact central vein and normal appearing hepatic cords

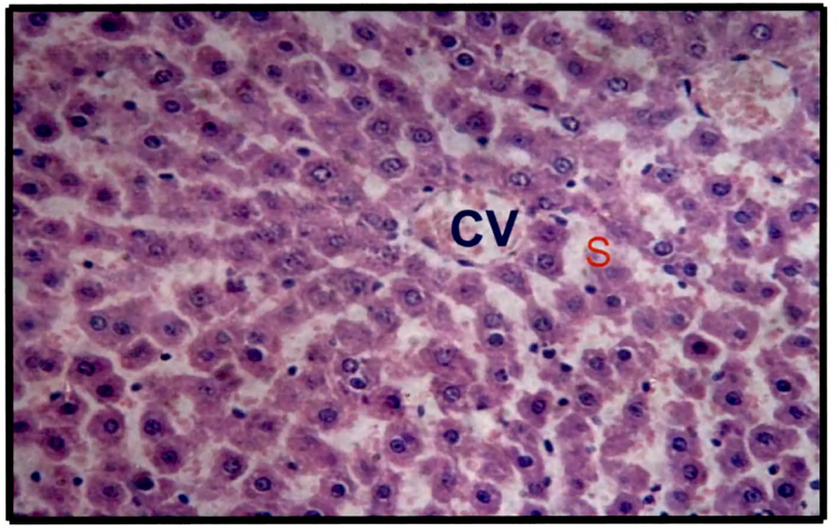

Fig 3.12 Control

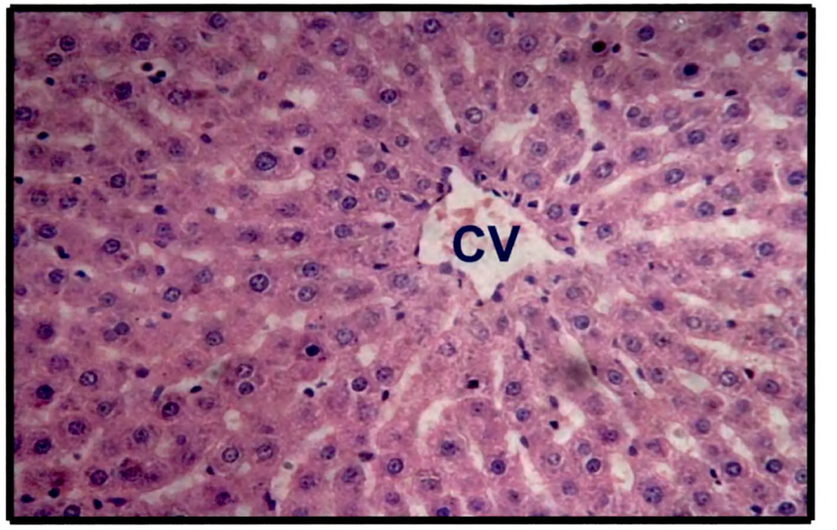

Fig 3.13 Melatonin

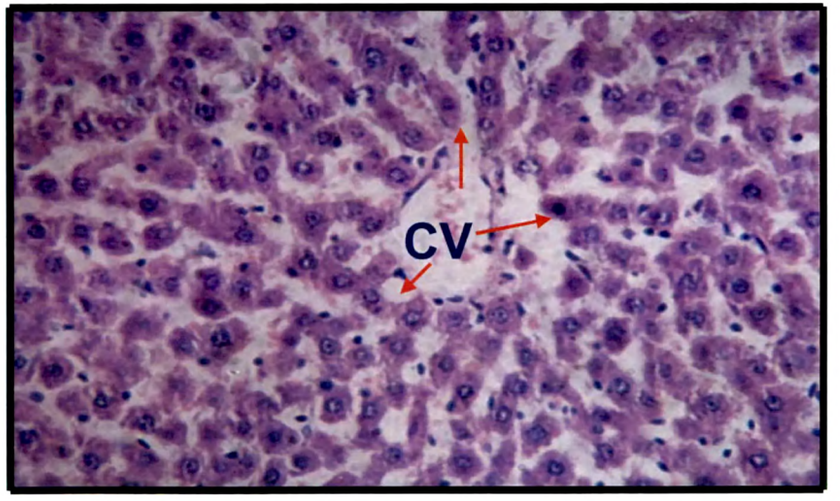

Fig 3.14 Cadmium

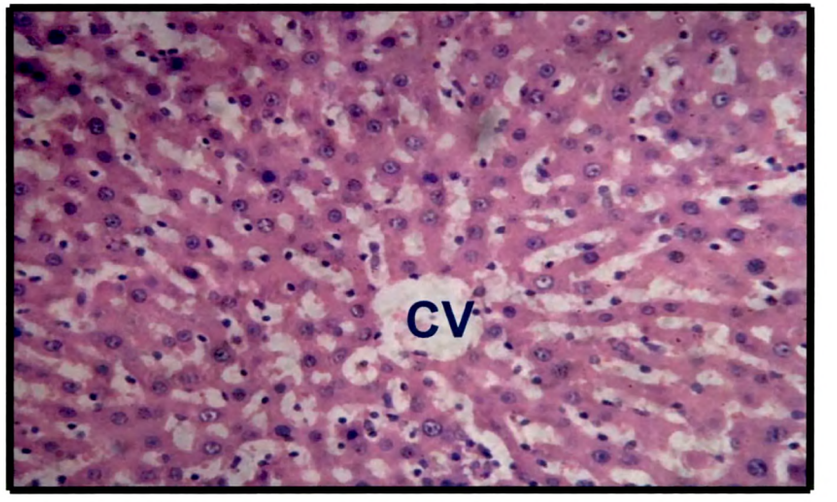

Fig 3.15 Cadmium + Melatonin

PLATE IV

Fig 3.12(a) Photomicrograph of control liver showing periportal area (400X)PPA-Periportal Area

Fig 3.13(a) Photomicrograph of melatonin treated liver showing PPA (400X); PV-portal vein HA- hepatic artery.

Fig 3.14(a) Photomicrograph of 30 day cadmium treated liver showing periportal area (400X). Note the congested portal vein and glassy degeneration of hepatocyte. PV-portal vein

Fig 3.15(a) Photomicrograph of liver exposed to Cd+Mel for 30 days showing periportal area (400X). Note the near normal organization but minor effect of glassy degeneration visible. PPA- Periportal Area.

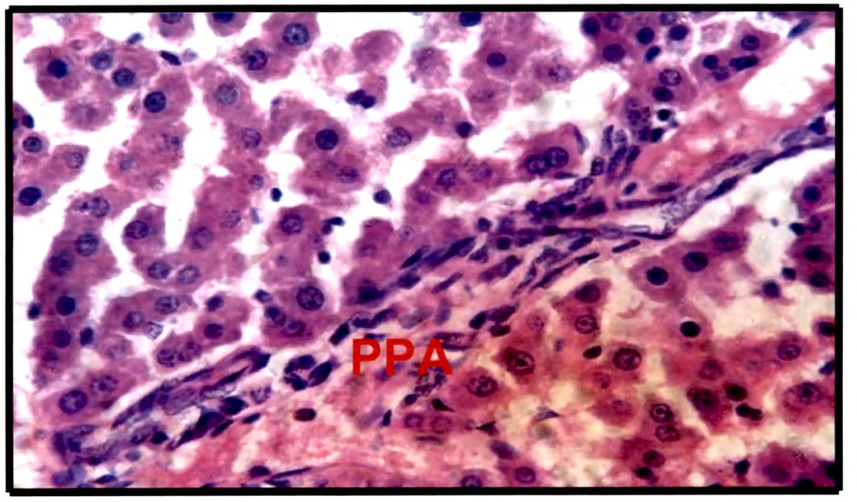

Fig 3.12 (a) Control

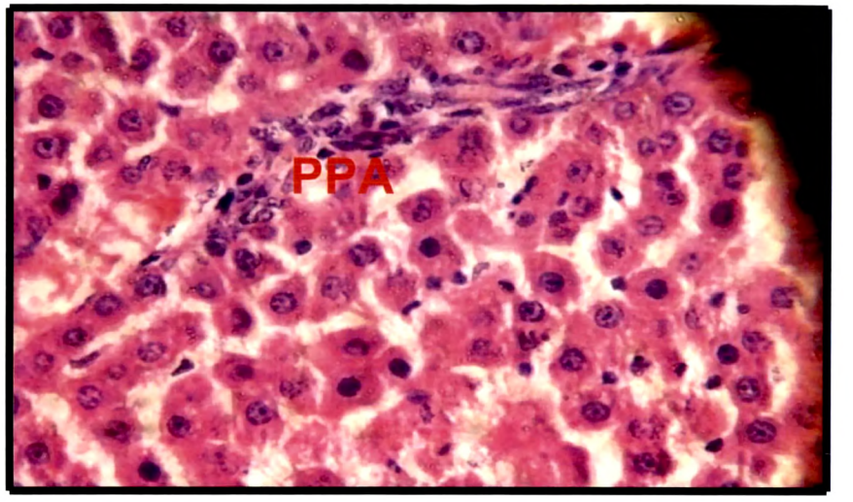

Fig 3.13(a) Melatonin

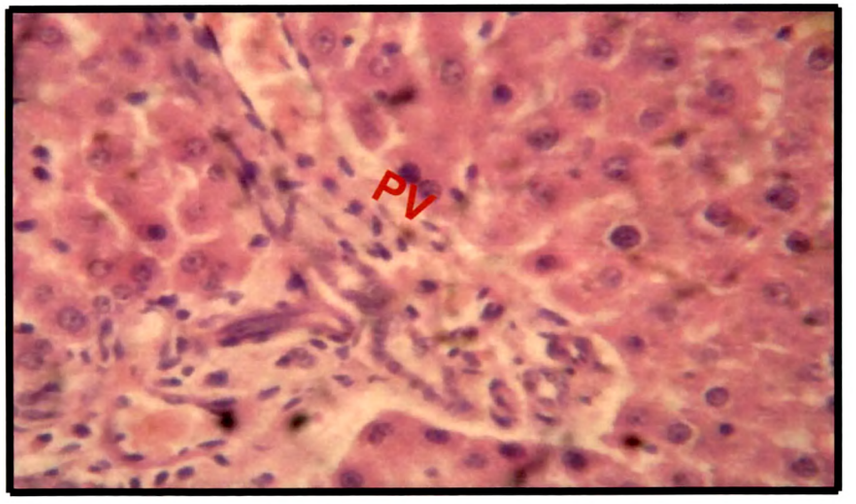

Fig 3.14(a) Cadmium

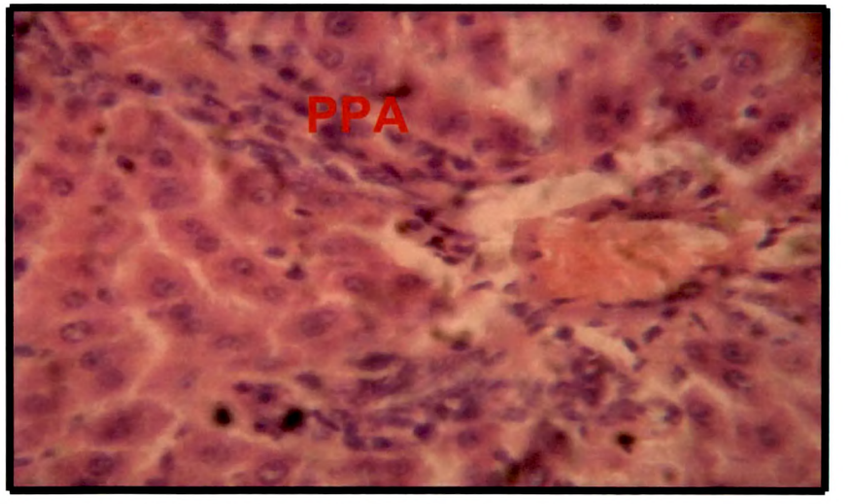

Fig 3.15(a) Cadmium + Melatonin

PLATE V

Fig 3.16 Photomicrograph of 60 day control liver(400X) showing a hepatic lobule. Note the cord like organization of hepatocytes and a central vein. CV- Central Vein

Fig 3.17 Photomicrograph of 60 day melatonin treated liver (400X). Note the robustness of hepatocytes. CV- Central Vein, S-Sinusoids

Fig 3.18 Photomicrograph of 60 days cadmium exposed liver (400X). Note the completely disruption of central vein distended sinusoids and apoptotic hepatocytes. CV- Central Vein, S-Sinusoids

Fig 3.15 Photomicrograph of liver exposed to Cd+Mel for 60 days (400X). Note the nearly intact central vein with minor disruption and minor degree of hepatocyte degeneration.

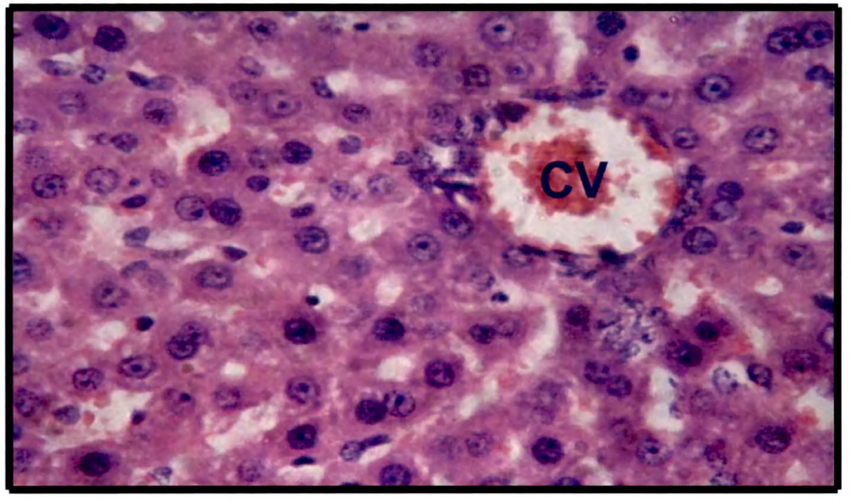

Fig 3.16 Control

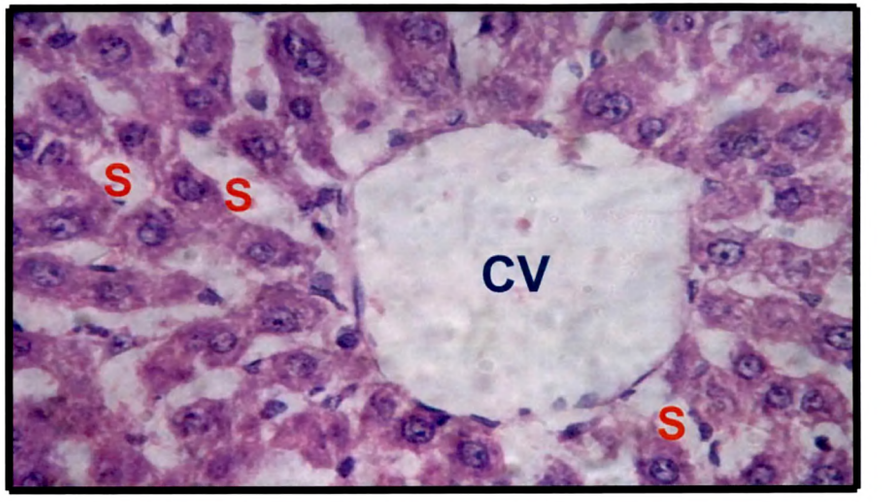

Fig 3.17 Melatonin

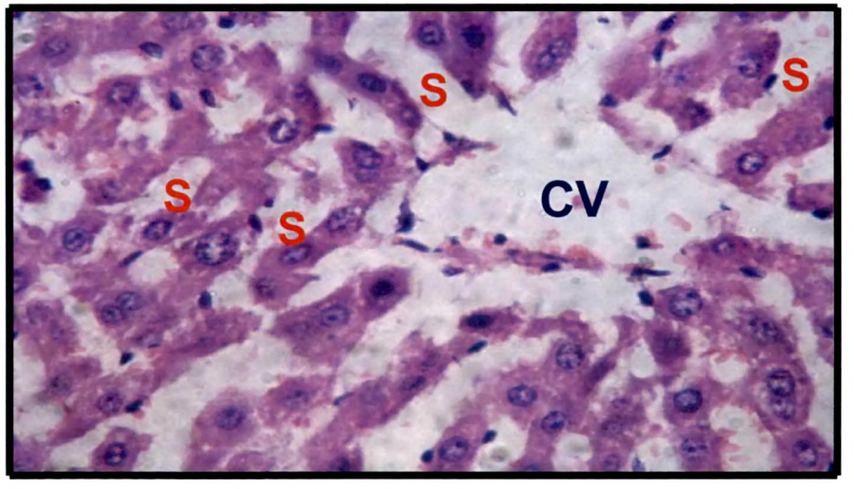

Fig 3.18 Cadmium

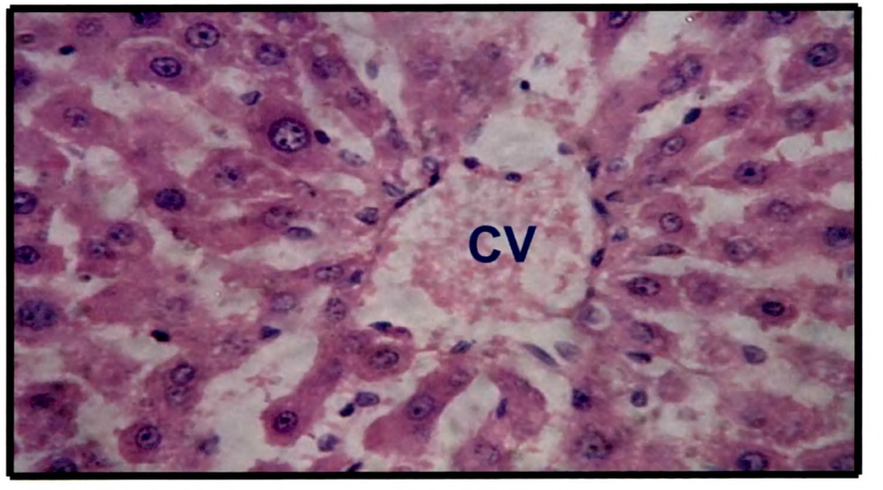

Fig 3.19 Cadmium + Melatonin

PLATE VI

Fig 3.16(a) Photomicrograph of liver showing periportal area (400X).

Fig 3.17(a) Photomicrograph of liver treated with melatonin for 60 days showing periportal area (400X)

Fig 3.18(a) Photomicrograph of liver exposed to Cd for 60 days showing disintegrated periportal area and confluencing sinusoids (400X). Many apoptotic cells can be seen.

Fig 3.19(a) Photomicrograph of liver exposed to Cd+Mel for 60 days showing periportal area (400X). Hepatocytes appear normal but some breaches in periportal area can be seen.

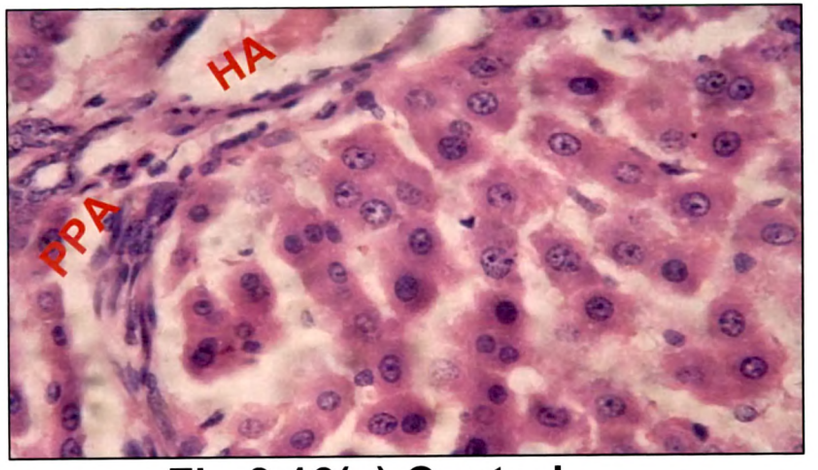

Fig 3.16(a) Control

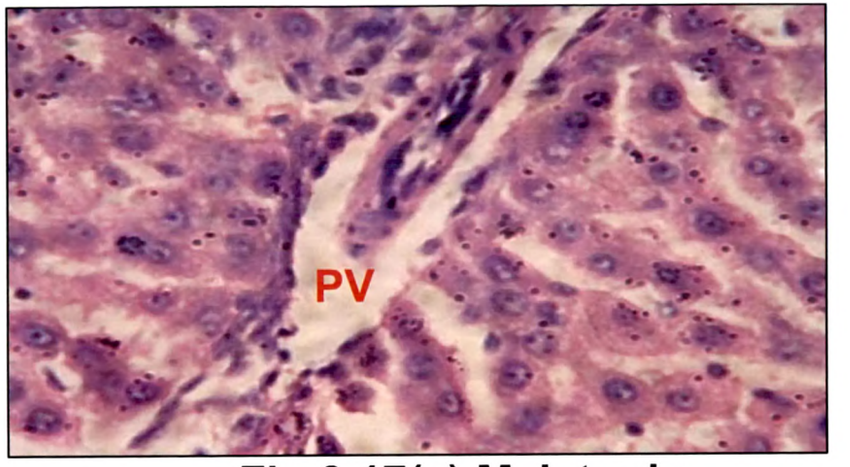

Fig 3.17(a) Melatonin

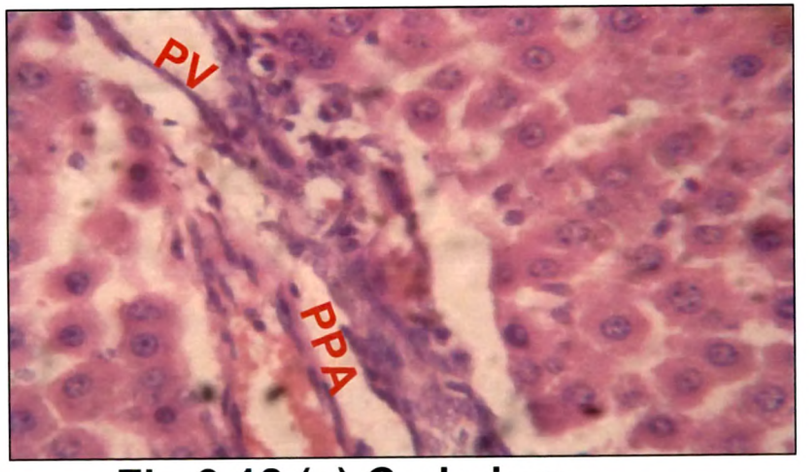

Fig 3.18 (a) Cadmium

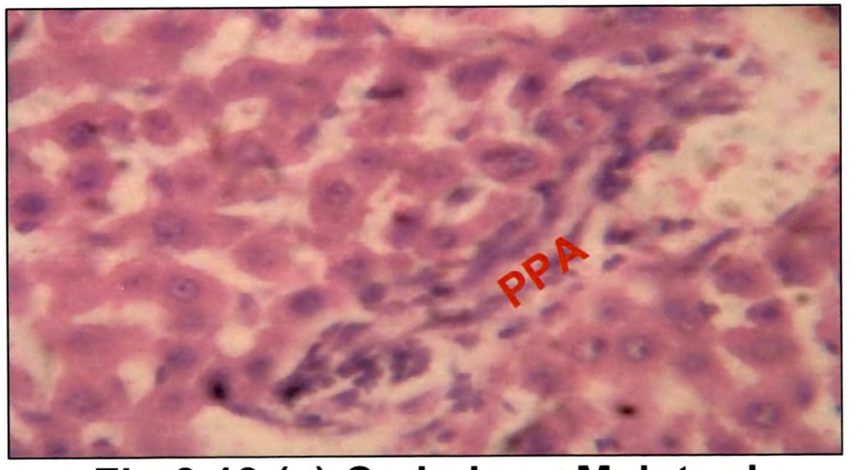

Fig 3.19 (a) Cadmium+Melatonin

Discussion:

The present study undertaken to evaluate hepatic responses to chronic exposure to Cd, has shown induction of oxidative stress and certain degree of toxic manifestations. Hepatic oxidative stress is marked by the significantly high levels of LPO and decreased contents of non-enzymatic antioxidants (GSH and Vit C) and activity of enzymatic antioxidants (SOD, CAT and GPx). Highest LPO level is noticeable at 15 compared to 30 and 60 days. The decreasing degree of LPO with increasing duration tends to suggest the induction of some adaptive/protective mechanism to stem the oxidative damage. Reduction in the levels of non-enzymatic antioxidants as well as inhibition of enzymatic antioxidants contributes to increased LPO and increased oxidative stress. Cadmium induced oxidative stress is reported in a wide variety of organs marked by increased LPO and decreased endogenous antioxidant status. However, most of the studies are on short term basis with the Cd exposure period ranging from hours to days or even a single dose acute administration (El-Maraghy *et al.,* 2001; Casalino *et al.,* 2002; Eybl *et al.,* 2004, 2006). The present study involving short (15 days), medium (30 days) and long term (60 days) duration of oral Cd exposure has revealed maximal oxidative stress, as marked by LPO to be by 15 days. The gradually decreasing hepatic LPO by 30 and 60 days of exposure, despite increasing hepatic metal load is an indication of optimal commissioning of the endogenous antioxidant machinery to resist oxidative damage to liver, the metabolic work force of vertebrate body. A steady level of depletion of GSH and Vit C and decrease in the activities of enzymatic antioxidants lead to

oxidative damage at all three durations of Cd exposure. Cadmium is an intracellular GSH depleter in some organs (Stohs *et al.,* 2000) as Cd binds the sulphydryl group present in reduced glutathione (GSH). Cadmium intoxication is marked by decrease in GSH levels at all time periods which may be accredited to the binding of the metal to sulphydryl group of GSH. Depleted GSH level and consequent lower GSH/GSSG ratio can favour production of free radicals (Leelank and Bansal, 1996) and hence the recorded higher incidence of LPO. The increased LPO could also be iron mediated as, cadmium treatment in rat and mice is shown to cause an increase in iron content by release from membranes and displacement (Koizumi and Li, 1992; Nigam *et al.,* 1989; Casalino *et al.,* 1997; Eybl *et al.,* 2004).

Though Cd by itself is not capable of generating free radicals, it can indirectly generate free radicals such as superoxide, hydroxyl and hydrogen peroxide (Stohs *et al.,* 2000). All the three enzymatic antioxidants, SOD, CAT and GPx also showed a compromised activity during the entire duration of Cd exposure. Hydroxyl radicals are effectively neutralized by catalase-glutathione reductase and/or GPx pathways. Since both CAT-GR and CAT-GPx mediated removal of hydroxyl and peroxide radicals are dependent on ready availability of NADPH (Bradberry and Vale, 1999; Dey *et al.,* 2001), it is presumable that under increasing and persistent Cd stress, hepatic tissue would be generating NADPH by the operation of HMP shunt pathway. However, as G-6-PDH, the key enzyme of shunt pathway is likely to be inactivated by Cd (Manca *et al.,* 1991; Koizumi and Li, 1992) GR and GPx enzymes are liable to be suppressed. This suppression of GR and GPx can lead to inactivation of CAT-

GR and CAT-GPx systems which can result in further build up of hydroxyl radical and contribute to more oxidative stress. The other possible mechanism for inhibition of GPx is the formation of chemical complex between Cd and Se at the site of GPx as suggested by Gambhir and Nath (1992). Apparently, there is a steady inactivation of both CAT and GPx in the Cd treated group of animals for all the treatment periods.

Superoxide radicals are effectively quenched in cells with the help of SOD. Three SODs, copper/zinc SOD (cytosolic SOD), manganese SOD (mitochondrial SOD) and extracellular SOD (ECSOD) are the major antioxidant enzymes based on cellular distribution and localization. In the present study we have evaluated total SOD i.e the cytosolic and mitochondrial in the hepatic tissue. The inactivation of SOD is probably due to Cd substituting for manganese in the manganese SOD (Hussain *et al.,* 1987). *In vitro* studies have indicated that Cu/Zn SOD activity could be strongly inhibited by cadmium as Cd can replace Zn, thereby affecting SOD activity (Bauer *et al.,* 1980; Muller, 1986; Hussain *et al.,* 1987; Kofod *et al.,* 1991). In the present study it is observed that, there is relatively greater inhibition of SOD at 15 days of Cd exposure relative to 30 and 60 days. Therefore, the greater inhibition of SOD by the above mechanism may be the contributing factor for the observed higher LPO on short term exposure by way of the unquenched superoxide.

The present study on environmentally based realistic dosage of Cd in a simulated human context of chronic duration dependent exposure has thrown up certain interesting observations hitherto not revealed by single dose or

acute studies on Cd. Whereas all the single dose/acute studies evaluating the response of liver in terms of hours or days have highlighted increased oxidative stress marked by significant depletion in GSH and Vit C and inhibition/inactivation of enzymatic antioxidants (SOD, CAT, GPx), in the present evaluation there was time dependent increase in LPO and reduced inhibition of antioxidant enzymes. Concurrently, there is also time dependent reduction in the percentage hepatic Cd load though, the absolute level was progressively increased. All this taken together, tends to emphasize the earlier suggested commissioning of an endogenous adaptive mechanism on chronic exposure to Cd over a long term period. These necessitate a closer scrutiny of the observations and understand the basis of the observed effects/changes. The maximal degree of LPO seen at 15 days together with minimal degree of inhibition/inactivation of antioxidant machinery (as most of the acute studies have shown more than 60 % decrease in enzymatic and non-enzymatic antioxidants) suggest an indirect mechanism of excessive generation of oxidative stress by Cd. In this context, it is worth recalling certain relevant reports which elucidate Cd induced generation of oxidative stress by way of release of iron (Fe) and zinc (Zn) from proteins like ferritin, hemosederine and SOD, contributing to higher tissue concentrations of iron and zinc. The free Fe and Zn in turn can generate ROS like superoxide, H_2O_2 and hydroxyl radicals by the Fenton reaction ( ). It is this short term Cd induced oxidative stress that seems to be responsible for this high degree of LPO recorded at 15 days of Cd exposure in the study. This contention is well supported by the herein recorded anemic status of animals exposed to Cd for 15 days as marked by lowered erythrocyte count, hemoglobin and packed cell

volume (PCV). Apparently, Cd induced release of Fe from storage or binding proteins lead to an apparent iron deficiency resulting in lowered hemoglobin synthesis and erythropoesis. Such observations have been made by some other workers in recent times on chronic Cd toxicity (Das *et al.,* 2007). The high degree of oxidative stress on 15 days of Cd exposure by way of Fe mediated Fenton reaction also seems to affect the pancreatic β cell functioning and glucose homeostasis. The oxidative stress induced dysregulation of β cell functioning is clearly marked by the diabetogenic induction of hypoinsulinemia and hyperglycemia. It is again interesting to note that, there are some reports showing such diabetogenic effects of Cd (Merali and Singhal,1980).

The continued exposure of Cd beyond 15 days seems to activate the adaptive machinery for controlling oxidative stress. This is understandable in the context of decreasing degree of LPO, hepatic metal load and, decreasing inhibition of antioxidant enzymes. Some of the possible components of these adaptive machinery based on available relevant information are, melatonin, metallothionein and iron binding proteins. Melatonin is potentially capable of directly or indirectly scavenging free radicals (Reiter *et al.,* 2001) and can even trigger antioxidant cellular mechanisms by transcriptional activation of genes (Rodquizes *et al.*, 2004). However, in the present study, activation of antioxidant enzymes seems to be not the mechanism of melatonin action as, there is no stimulated expression of these enzymes. The significant reduction in oxidative stress and hepatic metal load between 30 and 60 days of Cd exposure together with progressive decrease in serum titre of melatonin seem

to have a definite correlation. Since melatonin is liposoluble it can diffuse across cell membrane and facilitate quenching of free radicals (Reiter *et al.*, 2000). It is also likely that, melatonin may even help in eliminating Cd from tissues by forming a complex with melatonin (Carnata *et al.*, 1992). The decreased titre of assayed serum melatonin in the present study could be in this context considered as an adaptive mechanism of melatonin to dampen/protect organs from Cd toxicity. It is also likely that, constitutive induction of metallothionein and also of iron binding proteins on continued presence of Cd could also be pivotal in reducing Cd induced oxidative stress directly by the former mechanism and later indirectly. Support for these inferences come from our observed recovery of erythrocyte and hemoglobin content as well as reports of induction of both metallothionein and iron binding proteins on chronic Cd induced toxicity.

Though oxidative stress seems to be managed well by the endogenous biochemical antioxidant machinery, cytotoxicity is clearly indicated by the observed histopathological alterations in the hepatic tissue. Progressive deterioration of the organization of the hepatic cords with disruption of the endothelial lining of sinusoids and central vein and presence of necrotic/apoptotic cells are characteristic features. These changes are very prominently manifested in the 60 day liver sections indicating the increasing cytotoxicity due to Cd exposure. Granular and vacuolar degenerations were prominent in the Cd group of animals given 15 ppm/day for 30 days (Koyu *et al.*, 2006). Wlostowski *et al.* (2000) have reported dietary cadmium-induced liver degeneration with increased sinusoidal diameters. There was a

progressive disruption of cord like organization of hepatic parenchyma, dilation of sinusoids and very clear cut disruption in the endothelial lining of central vein and sinusoids. Many necrotic/apoptotic cells were also visible. Hepatocellular injury was produced as a result of ischemia caused by damage to endothelial cells (Rikans and Yamano, 2000). Rikans and Yamano (2000) have postulated that the secondary damage occuring due to Cd is by inflammatory processes that are initiated by the activation of Kupffer cells. These activated Kupffer cells release a number of inflammatory mediators. These inflammatory mediators then activate a cascade of events involving several types of liver cells and a large number of inflammatory and cytotoxic mediators. These cytotoxic manifestations indicating Cd induced hepatotoxictity find biochemical correlation in the observed increase of serum ALP and ALT, marker enzymes of hepatic damage.

The liver toxicity serum parameters like ALP and ALT are known to increase significantly with a single or repeated administration of Cadmium to rats (El-Maraghy *et al.,* 2001). In the present study, both the toxicity markers show a significant increase with increase in hepatic toxicity as shown by the histopathological changes in the liver. In the present study, cadmium exposure leads to a diabetogenic effect with an elevated level of serum insulin titres in all the three treatment periods. Elevated blood glucose level may be due to the enhancement of gluconeogenesis (Merali and Singhal, 1980), activation of key gluconeogenic enzymes (Chapatwala *et al.,* 1982) and a reduction in activity of glycolytic enzymes on Cd exposure (Kielan *et al.,* 1989). The other possible mechanism of increased blood glucose in the

cadmium treated animals is, the inactivation of insulin receptors in the muscle. Increased insulin levels should lead to increased glucose transporter GLUT 4 in the muscles but cadmium administration leads to a decrease in GLUT 4 translocation leading to lesser glucose uptake which is totally reversed by melatonin administration (Fickova *et al.,* 2003).

In the light of the role of melatonin as a powerful natural antioxidant, simultaneous supplementation with melatonin in Cd intoxicated animals has shown significant protection against LPO and endogenous levels of antioxidants. Melatonin is known to affect the antioxidant status directly or indirectly. It affects directly, by scavenging free radicals (Hardeland *et al.,* 1993; Allegre *et al.,* 2003) and indirectly, by enhancing the antioxidant status (Reiter *et al.,* 2000c; Rodriquez *et al.,* 2004). Melatonin is known to stimulate the synthesis of GSH (Urata *et al.,* 1999) and, this is well corroborated by the observed increase in the level of GSH in animals treated with melatonin alone or in combination with cadmium. Melatonin is known to stimulate G6PDH activity as well (Pierrefiche and Laborit, 1995), which can keep the CAT-GPx and CAT-GR pathways active, thereby reducing the generation of hydroxyl radicals. Similarly, melatonin is known to bring about transcriptional activation of SOD, CAT (Rodriquez *et al.,* 2004) and GPx (Pablos *et al.,* 1995) in tissues leading to upregulation of these enzymes and minimizing the degree of tissue oxidative stress thereat. There is a protective maintenance of all these enzymatic antioxidants when melatonin is supplemented alone or given along with the cadmium. The protective effect of melatonin is further validated by recent reports of ability to increase the induction of Cd induced

metallothionein (Carolina *et al.,* 2008). Apart from the observed effects of melatonin in reducing oxidative stress, its role in preventing cell damage and apoptosis is clearly seen by the near normal histological architecture of hepatic tissue. Apparently, melatonin supplementation along with Cd, is able to minimize to a greater degree the cytotoxic effects of the metal. This inferred role of melatonin in controlling oxidative stress and cytotoxicity is clearly emphasized by the herein observed decrement in circulating melatonin level in Cd exposed group of rats. The increasing degree of oxidative stress and cytotoxicity with increased duration of Cd exposure is paralleled by duration dependent significant decline in serum melatonin titre. Though there are reports of melatonin as a powerful antioxidant, even better than other free radical scavengers like Vit C, A, and E, against oxidative stress generated by many chemical and environmental agents including metals (Flora *et al.,* 2008), there is hardly any report on the ability of melatonin to resist Cd toxicity for differing duration of exposure. The present observation suggests that melatonin is one of the few antioxidants that has the ability to reduce the metal load of hepatic tissue while, other antioxidants have failed to show such an effect.

Overall, the present study suggests a duration dependent effect of Cd on increased hepatic oxidative stress and cytotoxicity along with alterations in serum insulin, melatonin and glucose levels. Further, melatonin is found to be very effective in counteracting the negative effects of Cadmium.

Summary of Chapter 3

The present study has been undertaken to essentially the evaluate the hepatic responses to a chronic oral exposure of cadmium (Cd) at a realistic dose of the metal (9 mg/BW/day) dissolved in drinking water in male *Wistar* rats for 15, 30 and 60 days of duration. Hepatic oxidative stress is marked by significantly high levels of LPO and decreased contents of non-enzymatic (GSH and Vit C) and activity of enzymatic antioxidants (SOD, CAT and GPx). Highest LPO level was noticed at 15 days compared to 30 and 60 days. The decreasing degree of LPO with increasing duration tends to suggest the induction of some adaptive/protective mechanism to stem the oxidative damage. The gradual decrease in hepatic LPO by 30 and 60 days of exposure, despite increasing hepatic metal load is indicative of optimal commissioning of the endogenous antioxidant machinery to resist oxidative damage to liver. A steady level of depletion of GSH and Vit C and decrease in the activities of enzymatic antioxidants lead to oxidative damage at all the three periods of study by Cd exposure. Cadmium exposed rats recorded anemic status by 15 days and also was marked by lowered erythrocyte count and packed cell volume. The oxidative stress induced by Cd leads to dysregulation of β cell functioning clearly marked by the diabetogenic induction of hypoinsulinemia and hyperglycemia. The adaptive/protective mechanism of liver against Cd induced oxidative stress may be because of induction of metallothionein, iron binding proteins and other stress proteins. Cadmium induced histopathological lesions are prominently manifested in the liver of 60 days. The histopathological findings have co-relation in the observed increase in serum ALP and ALT, markers of hepatic damage. Co-administration of melatonin along with metal tended to prevent the negative effects induced by Cd.

Overall, the present study suggests a duration dependent effect of Cd on increased hepatic oxidative stress and cytotoxicity. Further, melatonin was found to be very effective in counteracting the negative effects of Cd.