# CHAPTER 1

# Chapter 1

## Hepatic oxidative stress and toxicity due to Chromium (VI) exposure: Duration dependent study using realistic dosage and protective effect of melatonin.

Hexavalent chromium [Chromium (VI)] and trivalent chromium [Chromium (III)] are the principal forms of chromium of common occurrence in work places. Hexavalent chromium compounds are used extensively in diverse industries like steel, alloy cast iron, chrome, paints, and metal finishes. Neurotoxicity, dermatotoxicity, genotoxicity, immunotoxicity, and, carcinogenicity have been associated with hexavalent chromium, a general environmental toxicant (Von Burg and Liu, 1993; Barceloux, 1999). Adverse renal and hepatic effects on exposure to chromium have been reported (Love, 1983; Verschoor *et al.,* 1990). Functional disruption of several organs on accumulation of Cr due to long term exposure has been suggested (Nieboer and Jusys, 1988). The exact mechanism of action of chromium compounds on tissues is not extensively studied but, it is observed that, chromium can generate massive amount of reactive oxygen species (ROS) during its reduction in successive oxidation states from Cr (VI) to Cr (III), which are well known to produce toxic effects (Shi and Dalai, 1990; Sugden *et al.,* 1992; Luo *et al.,* 1996; Shi *et al.,* 1999; O'Brien *et al.,* 2003). Such excessive production of ROS leading to a state of oxidative stress can affect the functional integrity of organs by causing injury to cellular protein, lipids and DNA (Nordberg and Arner, 2001). Though Cr (III) is considered essentially as a trace element in glucoregulation and, Cr supplements have been used for combating diabetes, its ability to form cross links with DNA and protein makes it a potentially dangerous agent when present in excess amounts.

Studies in mammals have suggested harmful effects of Cr (VI). Subcutaneous administration of Cr (VI) in rats have been shown to result in progressive proteinuria, increased urea nitrogen and creatinine along with elevated serum activity level of alanine aminotransferase and hepatic lipid peroxidation (Kim and Na, 1991). Increased hepatic lipid peroxidation has been shown in mice administered with Cr (VI) intraperitoneally (i.p) (Susa *et al.,* 1989). Further, increased hepatic mitochondrial and microsomal lipid peroxidation as well as elevated excretion of urinary lipid metabolites have been documented by oral administration of Cr (VI) through drinking water (Bagchi *et al.,* 1995a, b). Moreover, evidence of Cr (VI) induced toxicity in humans is available in the form of DNA strand breaks in peripheral lymphocytes and appearance of lipid peroxidation products in urine in chromium exposed workers (Gambelunghe *et al.,* 2003; Goulart *et al.,* 2005).

The major route of entry of Cr in humans is the oral route through food and water. There are only few toxicity studies involving oral administration of Cr as, most of the studies have evaluated Cr toxicity by intraperitoneal or subcutaneous administration. Further, there is also dearth in studies involving long duration exposure to Cr. This becomes pertinent in the local context as Cr has been identified as a major environmental pollutant present in high amounts in vegetables, cereals, pulses and grass 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 Cr induced hepatic and renal oxidative stress and toxicity in male *Wistar* rats. In this context, since the study is aimed at understanding the possible Cr toxicity on long term systemic entry into humans though diet and water, a realistic dosage has been worked out based on the Cr content in vegetables and food grains and an average daily food intake. Conversion 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 chromium.

## Material and Methods:

### Animals:

Adult male albino rats of *Wistar* strain were used as the animal model for the experiments. All selected rats were of 180 days old and in the weight range of 325-375 gms. The animals were kept in the animal house under controlled conditions of ambient temperature of 23 ± $2^0$C and a photoperiod of L:D 12:12 throughout the experimental period. Food pellets manufactured by Pranav Agro Industried Ltd, E/5 MIDC Kupwad Block, Sangli – 416436, MHR, India were used with provision of water *ad libitum.* The rats were acclimatized for a period of 10 days prior to experimentation. After acclimatization, the intake of water was observed for 5-7 days and the average intake was used for arriving at the fixed dosage. All animal experiments were conducted accordance with the guidelines of CPCSEA and approved by the Departmental Ethical Committee (Approval No.827/ac/04/CPCSEA).

### Experimental Protocol:

The rats were randomly divided into four groups of five animals each. Grouping was done as follows:

**Group I: Control (Con)**

**Group II: Melatonin (Mel)**

**Group III: Chromium (Cr)**

**Group IV: Chromium + Melatonin (Cr+Mel)**

**Treatment Schedule:**

There were three treatment durations and accordingly the animals were treated for 15, 30 or 60 days. The grouping schedule was as follows:

| GROUPS | TREATMENT SCHEDULE |
|---|---|
| Control (Con) | Normal Tap Water |
| Melatonin (Mel) | Normal Tap Water + 10 mg/kgBW/day Melatonin |
| Chromium (Cr) | 20mg/kg BW/day $CrO_3$ dissolved in tap water |
| Chromium + Melatonin (Cr+Mel) | 20mg/kg BW/day $CrO_3$ dissolved in tap water + 10 mg/kgBW/day Melatonin |

The melatonin group of animals received melatonin at 1800 hrs daily according to the treatment period while the control group received saline at the same time. Following the treatment schedule of 15, 30 or 60 days, animals were sacrificed on $16^{th}$, $31^{st}$ and $61^{st}$ day. The timing of sacrifice was 0530 hrs. On completion of the treatment period, animals were weighed and sacrificed under mild anesthesia with minimal stress to the animals. Blood sample was collected by jugular vein puncture. The organs were quickly excised, cleared off of the adhering fat, blotted and, weighed after which they were processed for biochemical studies.

**Chemicals:**

Melatonin was purchased from Hi Media Laboratories, Mumbai. Chromic Acid, a chromium (VI) oxide, manufactured by Qualigens, Fine Chemicals, Glaxo India Ltd., Mumbai was used. All other chemicals and solvents used were of highest purity and procured from Sisco Research Laboratoty, SRL, Mumbai.

**Preparation of Chemicals:**

Known quantity of melatonin was dissolved in a drop of alcohol and then diluted with 0.9% saline to attain the desired concentration. Known amount of chromic acid was dissolved in tap water such that the animals got 20 mg/kg BW/day through drinking water based on the known average water consumption /animal/day.

**Biochemical Assays:**

***Oxidative Stress Parameters:***

**Lipid Peroxidation (LPO)**

Method: Beuge and Aust, 1987.

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substances (TBARS). TBARS give a characteristic pink colour that can be measured colorimetrically at 532 nm.

Procedure: Take 1 ml of 10% tissue homogenate and mix it with 1 ml of TBA reagent. Keep in boiling water bath for 20 mins, cool the tubes, centrifuge at 3000 rpm for 15 mins and read absorbance at 532 nm.

Units: nmoles of MDA formed/g tissue.

**Reduced Glutathione (g-glutamlycysteinylglycine, GSH):**

Method: Beutler *et al.*, 1969

Principle: Glutathione is a major non-protein thiol present in the tissue. The suphyhydryl group in glutathione reduces the 5,5'-dithio bis -2 nitro benzoic acid to form one mole of 5-thio-2-nitro benzoate per mole of -SH. The 5-thio-2-nitro benzoate anion has an intense yellow colour with an absorbance maximum at 412 nm and can be used to measure –SH group.

Procedure: The test system contained 100µl of tissue homogenate with 1ml of precipaitating solution, 3ml of phosphate buffer and 0.5 ml of DTNB. The solution was mixed well and the aborbance was recorded at 412 nm using spectrophotometer within 1 minute of adding DTNB.

Units: mg/gm tissue.

**Ascorbic Acid (Vitamin C):**

Method : Omaye *et al.*, 1979

Principle: Ascorbic acid is oxidized by UV to dehydro ascorbic acid and diketo-glutonic acid. The precipitates are treated with 2.4-dinitro phenyl hydrazine (DNPH). This compound in presence of sulphuric acid forms a precipitate with maximum absorbance at 520 nm.

Procedure: Take 1ml of homogenate and to it add 1 ml of ice cold 10% TCA mixed thoroughly and centrifuges for 20 min at 3500 g. 0.5 ml supernatant was taken and mixed with 0.1 ml of DTC and incubated for 3 hrs at $37^{O}C$. Then, 0.675 ml of ice cold 65% $H_2SO_4$ was added and mixed well and the solution were allowed to stand at room temperature for 30 min and read at 520 nm.

Units: mg/gm tissue.

**Superoxide Dismutase (SOD):**

Method: Marklund and Marklund, 1974.

Principle: Pyragallol auto-oxidizes at 420 nm at pH. SOD inhibits this auto oxidation of pyragallol in a rate limiting fashion. 50% inhibition of pyragallol auto oxidation is equivalent to 1 IU of enzyme.

Procedure: The final assay mixture containing 1ml of potassium phosphate buffer, 20µl of liver homogenate and 25 µl of kidney homogenate and 50 µl of pyragallol.

Control tubes required volume of distilled water was taken in place of tissue homogenate. This was used to determine the uninhibited auto oxidation of pyragallol. The reaction was started by the addition of pyragallol and the change in optical density was recorded for 180 sec at 30 sec interval. Change in absorbance/minute was calculated from the reading.

Unit: IU/mg protein

**Catalase (CAT):**

Method: Sinha *et al.,* 1972

Principle: Catalase is a heme containing enzyme, which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240nm.

Procedure: To 1 ml of tissue homogenate 10 µl of ethanol was added and these tubes incubated in ice bath for 30 min. Just before the assay 10 µl TritonX 100 and 4 ml of phosphate buffer were added. Immediately after adding hydrogen peroxide decrease in the absorbance was recorded at every 5 sec interval for 15 sec at 240 nm.

Units: µmoles of $H_2O_2$ utilized/min/mg protein.

**Glutathione Peroxidase (GPx):**

Method of Rotruck *et al.,*1973

Principle: Glutathione peroxidase catalyses the reduction of hydrogen peroxide by reduced glutathione resulting in formation of hydrogen peroxide and oxidized glutathione which is then instantly and continuously converted into glutathione by an excess of glutathione reductase using NADPH providing for a constant level of glutathione. The estimation is based on the oxidation of glutathione by 5,5' dithiobis-2-nitrobenzoic acid to measure the total glutathione content of the biological samples.

Procedure: The assay mixture containing 0.4 ml phosphate buffer, 0.1 ml sodium azide, 0.2ml of tissue homogenate was mixed well and 0.1 ml of hydrogen peroxide was added and made upto 2ml with water. The tubes were incubated at 37$^{O}$C for 10mins along with control tubes containing all components except the enzyme.

The reaction was terminated by the addition of 0.5 ml of 10% TCA which was then centrifuges at 4000 rpm for 10 min at 4 $^{O}$C. 1ml of this supernatant was taken and added to 3 ml of disodium hydrogen phosphate and 1 ml of DTNB. The colour formed was measured at 412 nm.

Units: µg GSH utilized/min/mg protein.

**Tissue Metal content:** Hepatic load of chromium was assessed by the method of Iyengar *et al.* (1998) using Inductive Couple Plasma Atomic Emission Spectrophotometer (ICP-AES) HORIBA Jopin Yvon, France Model No: ULTIMA-2 and the tissue load was expressed as µg/gm of tissue.

**Hematological Parameters:**

About 1.5 ml of whole blood was collected in a heparinised test tube. It was used to measure hemoglobin content, erythrocyte count, leucocyte count, packed cell volume (PCV), platelet count, polymorph count, lymphocyte count, eosinophil count and monocyte count using hematological analyser.

**Clinical Chemistry Parameters:**

About 3 ml of whole blood was collected in a test tube and centrifuged for about 10 mins at 3000 rpm at room temperature. The serum collected was then stored at -20$^{o}$C for further use. Serum lipid profile, urea, creatinine, alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, insulin and melatonin were measured. Except

for insulin and melatonin, all other parameters were evaluated using Merck Diagnostic kit method using Merck Microlab 300 semi autoanalyser.

**Insulin:** Insulin was measured using Mercodia Rat Insulin ELISA kit (Mercodia AB, Sweden) on a Biotek ELx800 microplate reader.

**Melatonin:** Melatonin was measured using LDN Melatonin RIA Kit (Germany) and counts were measured using EC 500 gamma counter.

**Histology:** Liver tissue was blotted free of blood and other body fluid and fixed in 10% formaldehyde. Following the routine procedure, 5-6μm thick sections were cut on a microtome and stained with Hematoxylin and Eosin. The sections were observed under Leica Image Analyser.

**Statistical Analysis:**

One way ANOVA with Bonferroni index post test was performed using GraphPad Prism version 3.00, Graph Pad Software, San Diego, California, USA, www.graphpad.com. Confidence limit was set at 95%.

## RESULTS:

**Lipid Peroxidation (LPO):** Changes in the hepatic LPO levels following chromium (Cr) treatment for 15, 30 and 60 days are shown in figure 1.1; table 1.1. An age dependent gradual increase in LPO was seen in control rats. There was significant increase in LPO in Cr treated group when compared to control (Con) animals. Significant decrease in LPO was seen in rats treated with melatonin while, there was significant resistance against the increase in LPO in Cr exposed rats co-administered with melatonin. Cr treatment was seen to show duration dependent decrement in LPO with maximal level at 15 days and minimal at 60 days. Irrespective of duration of Cr exposure, melatonin depicted same degree of protective effect.

**Glutathione (GSH):** Changes in hepatic glutathione levels following Cr treatment for 15, 30 and 60 days are shown in figure 1.2; table 1.2. There was significant increase in the levels of GSH in the melatonin (Mel) group of animals and a significant decrease in the Cr exposed animals when compared to the Con group of animals. Similar degree of decrement in GSH by the Cr exposure and same degree of protective effect by melatonin on co-administration with Cr, irrespective of duration, were the feature. A gradual age dependent decrease in GSH levels was the feature in Con rats.

**Ascorbic Acid (Vitamin C):** Liver being the storage organ of Vit C in rodents, has a much higher content than the synthetic organ, the kidney. Changes in hepatic Vit C levels following Cr treatment for 15, 30 and 60 days are shown in figure 1.3; table 1.3. There was significant decrease in Vit C level in the Cr group of animals when compared to Con. The degree of depletion of hepatic Vitamin content was

progressively less with increasing duration of Cr exposure and was also relatively lesser than that of GSH. There was duration independent similar degree of protection by melatonin at all the three time periods of Cr exposure.

**Superoxide Dismutase (SOD):** Changes in hepatic SOD activity following Cr treatment for 15, 30 and 60 days are shown in figure 1.4; table 1.4. Control animals showed an age dependent decrease in SOD activity. There was significant increase in SOD activity in the Mel and Cr+Mel group of animals when compared with corresponding Con and Cr group of animals respectively. There was significant decrease of SOD activity in the Cr group of animals compared to the Con group of animals with, relatively and significantly lesser decrement in the long duration Cr exposure group. Similarly, the corresponding degree of protective effect with melatonin was also less in the 60 day Cr exposure group.

**Catalase (CAT):** Changes in hepatic CAT activity following Cr treatment for 15, 30 and 60 days are shown in figure 1.5; table 1.5. Hepatic CAT activity tended to show an age dependent decrement. There was significant decrease in CAT activity in Cr group of animals compared to Con group of animals. Catalase activity was significantly decreased to the same degree in Cr exposed rats irrespective of duration of exposure. Degree of protective effect of melatonin was also found to be duration independent.

**Glutathione Peroxidase (GPx):** The changes in hepatic GPx activity following Cr treatment for 15, 30 and 60 days are shown in figure 1.6; table 1.6. An age dependent decrement in CAT activity was the feature of Con rats. The Cr 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 recorded to be duration independent.

**Metal Load:** Changes in hepatic accumulation of Cr following Cr treatment for 15, 30 and 60 days are shown in figure 1.7; table 1.7. Cr treated rats showed significant increment in hepatic load of Cr. There was significant decrease in Cr metal accumulation in animals treated with melatonin alone or in combination with Cr. Both Cr induced increase in hepatic load as well as the degree of protection afforded by melatonin were duration independent.

**Histology:** Changes in the hepatic histopathology following Cr treatment for 15, 30 and 60 days are shown in figures 1.8 to 1.19(a). Cr induced histological changes are seen clearly in all the duration periods. These changes are brought to near normal histoarchitecture by the simultaneous administration of melatonin. Hence, melatonin is able to protect the hepatic tissue in all the three treatment periods.

**Serum Parameters:** Changes in serum insulin, glucose and melatonin are shown in table 1.8 and serum lipid profile in table 1.9 to 1.11. Cr induced hypoinsulinemia and hyperglycemia with decrease in melatonin levels. Serum triglyceride and cholesterol levels tended to show a decrease in Cr exposed animals. Administration of melatonin along with Cr prevented the changes induced by chromium most effectively. The enzyme markers of hepatic damage ALT and ALP (table 1.7) showed an increase in Cr treated rats while they tended to remain in normal range in rats co-administered with melatonin.

**Figure 1.1. Graph showing levels of hepatic lipid peroxidation (LPO) in animals exposed to Cr for 15, 30 or 60 days**

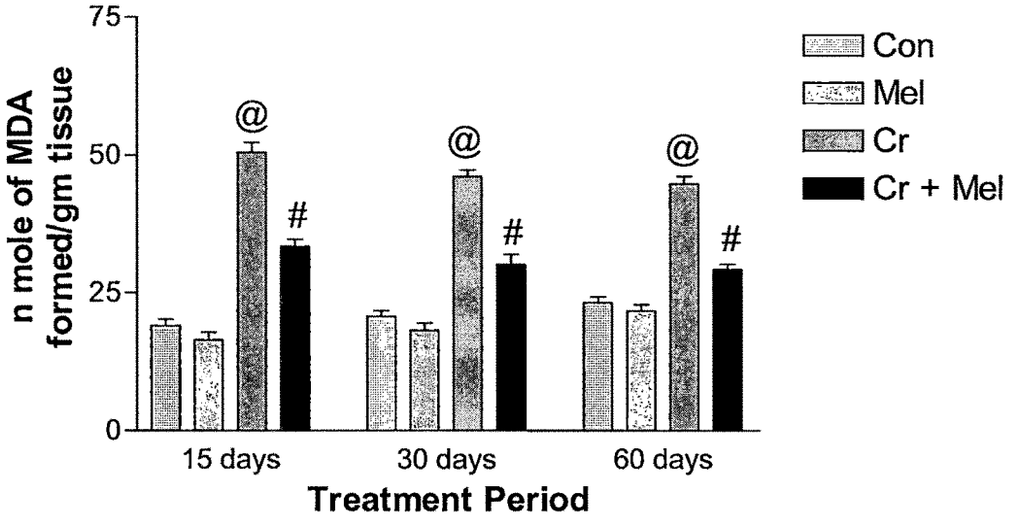


**Table 1.1. Levels of hepatic lipid peroxidation (LPO) following 15, 30 and 60 days of Chromium exposure**

| | 15 days | 30 days | 60 days |
|---|---|---|---|
| Con | 19.02± 1.26 | 20.79 ± 1.080 | 23.30 ± 1.02 |
| Mel | 16.49± 1.39 | 18.280 ± 1.290 | 21.76 ± 1.17 |
| Cr | 50.56±1.78@ | 46.190 ± 1.220 @ | 44.820 ± 1.390 @ |
| Cr+Mel | 33.45±1.36 # | 30.240 ± 1.760 # | 29.260 ± 1.020 # |

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

**Figure 1.2. Graph showing levels of hepatic glutathione (GSH) content in animals exposed to Cr for 15, 30 or 60 days**

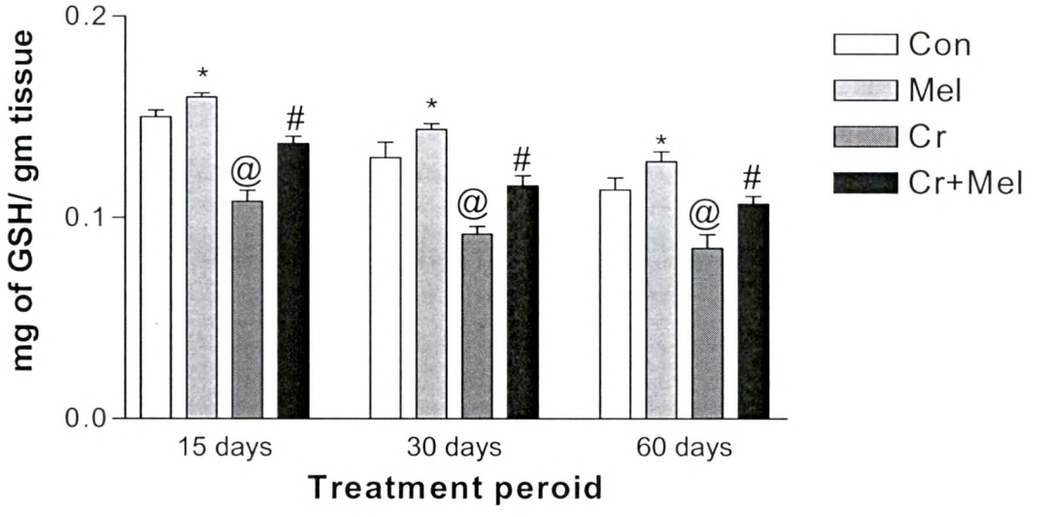


**Table 1.2. Contents of hepatic glutathione (GSH) contents following exposure to Cr for 15, 30 or 60 days**

| | 15 days | 30 days | 60 days |
|---|---|---|---|
| Con | 0.150± 0.003 | 0.130 ± 0.008 | 0.114 ± 0.006 |
| Mel | 0.160± 0.002* | 0.144± 0.003 * | 0.128± 0.005 * |
| Cr | 0.108± 0.004 @ | 0.092 ± 0.006@ | 0.085 ± 0.007 @ |
| Cr+Mel | 0.137± 0.003 # | 0.116. ± 0.005 # | 0.107± 0.004 # |

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

**Figure 1.3. Graph showing levels of hepatic Ascorbic Acid (Vit C) content in animals exposed to Cr for 15, 30 or 60 days**

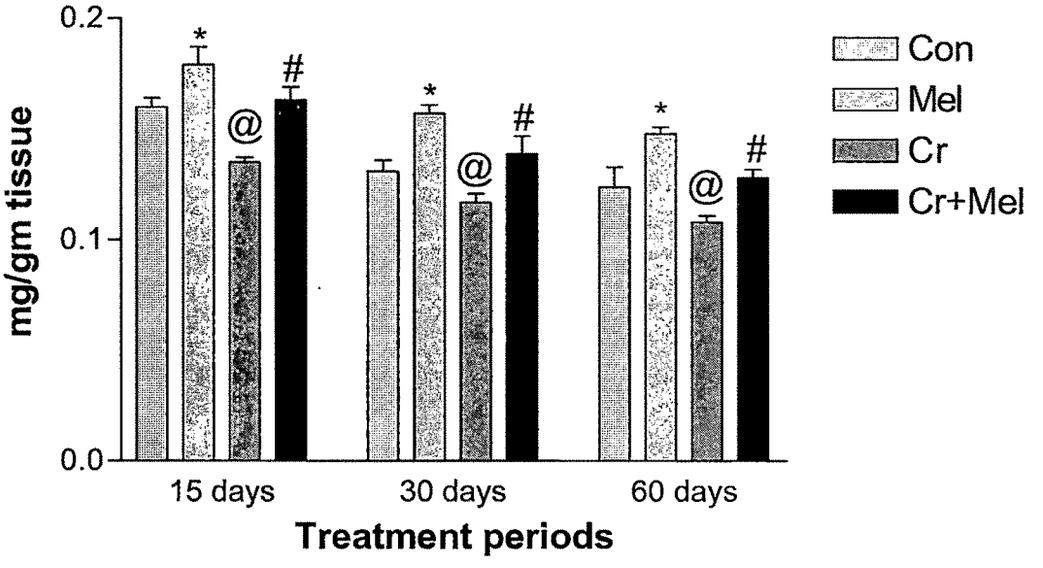


**Table 1.3. Contents of hepatic Ascorbic Acid (Vit C) following 15, 30 or 60 days treatment with Chromium**

| | 15 days | 30 days | 60 days |
|---|---|---|---|
| Con | 0.160±0.004 | 0.131± 0.004 | 0.124± 0.009 |
| Mel | 0.179± 0.008* | 0.157± 0.004* | 0.148± 0.003 * |
| Cr | 0.135±0.002@ | 0.117± 0.004 @ | 0.108± 0.003 @ |
| Cr+Mel | 0. 163± 0.006# | 0.139± 0.008 # | 0.128± 0.004# |

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

**Figure 1.4. Graph showing hepatic Superoxide Dismutase (SOD) activity following 15, 30 or 60 days exposure with chromium.**

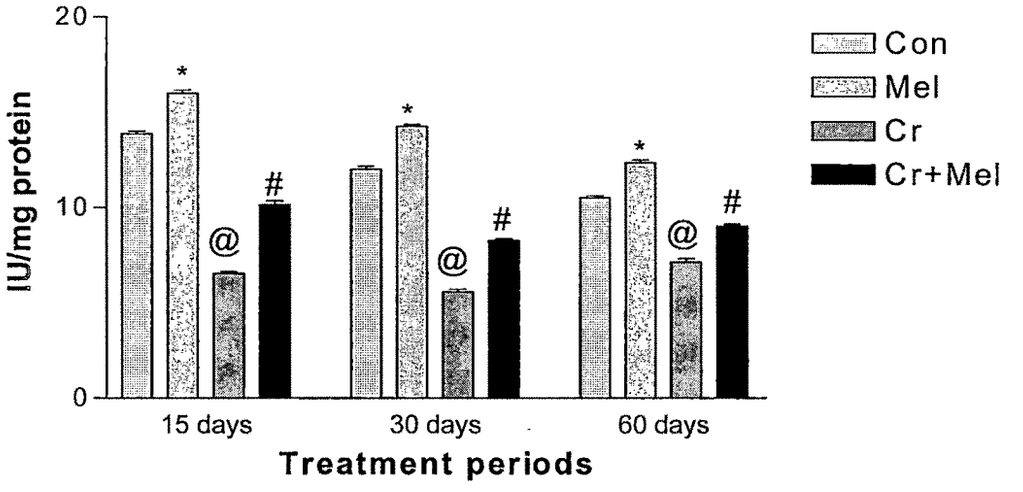


**Table 1.4. Levels of hepatic Superoxide Dismutase (SOD) activity following 15, 30 and 60 days exposure with Chromium**

| | 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 * |
| Cr | 6.5400 ± 0.0950 @ | 5.5800 ± 0.1500 @ | 7.1600 ± 0.1850 @ |
| Cr+Mel | 10.1800 ± 0.1800 # | 8.2900 ± 0.0880 # | 9.0400 ± 0.0960 # |

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

**Figure 1.5. Graph showing hepatic catalase (CAT) activity following 15, 30 or 60 days exposure to chromium.**

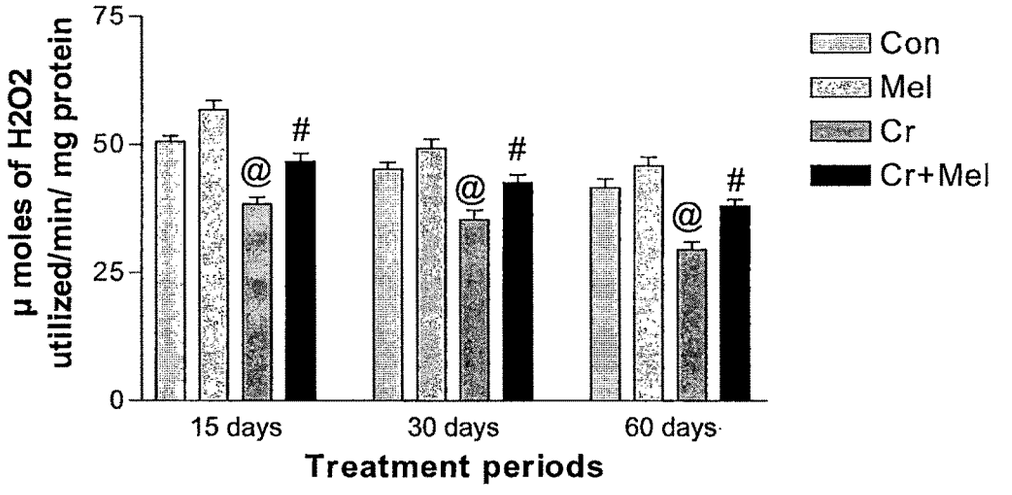


**Table 1.5. Levels of hepatic catalase (CAT) activity following 15, 30 or 60 days exposure with Chromium**

| | 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 |
| Cr | 38.360 ± 1.360 @ | 35.290 ± 1.920 @ | 29.540 ± 1.490 @ |
| Cr+Mel | 46.690± 1.580 # | 42.580 ± 1.590 # | 38.050 ± 1.320 # |

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

**Figure 1.6. Graph showing hepatic Glutathione Peroxidase (GPx) activity following 15, 30 and 60 days exposure to chromium.**

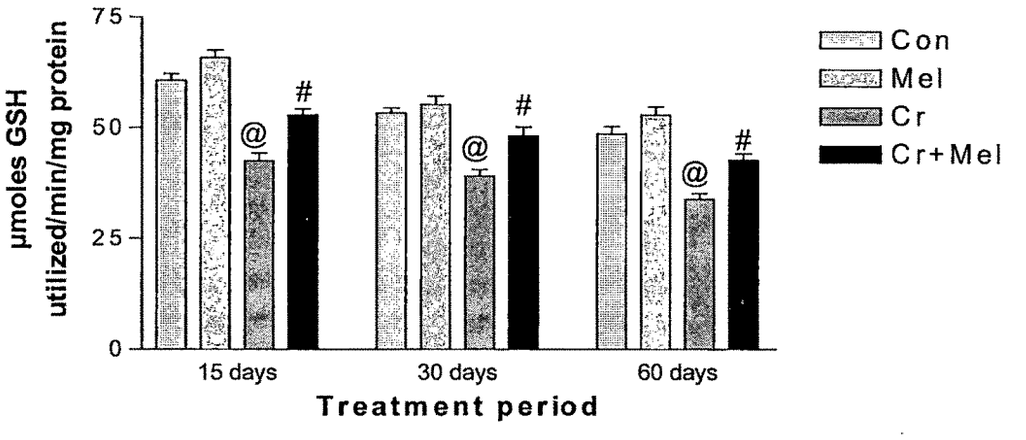


**Table 1.6. Levels of hepatic Glutathione Peroxidase (GPx) activity following 15, 30 and 60 days exposure with Chromium**

| | 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 |
| Cr | 42.54 ± 1.74 @ | 39.10 ± 1.52 @ | 33.91 ± 1.29 @ |
| Cr+Mel | 52.92± 1.58 # | 48.29 ± 1.59 # | 42.72 ± 1.32 # |

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

**Figure 1.7. Graph showing % accumulation of Cr in hepatic tissue following 15, 30 or 60 days exposure to chromium.**

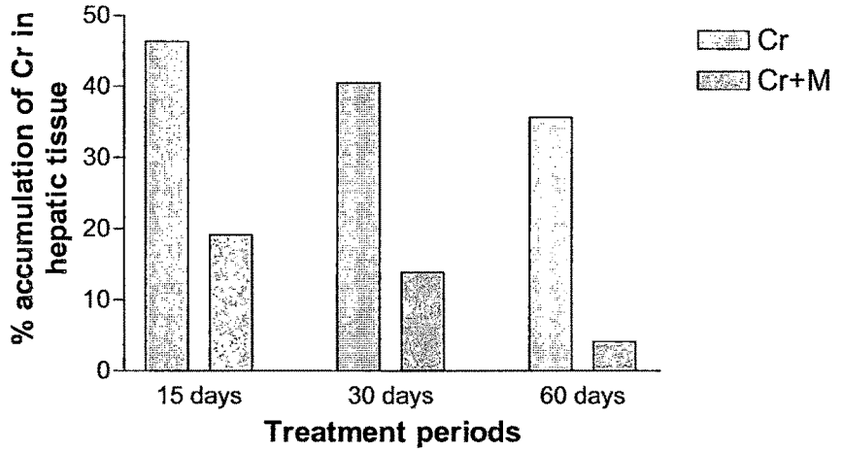


**Table 1.7. % accumulation of Cr in hepatic tissue following 15, 30 or 60 days exposure to Chromium**

| Treatment Period | Cr(VI) | Cr+M |
|---|---|---|
| **15 days** | 46.3 | 19.15 |
| **30 days** | 40.57 | 13.84 |
| **60 days** | 35.74 | 4.15 |

**Table 1.8. Changes in serum ALP and ALT activities following 15, 30 or 60 exposed to Cr**

| | 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)** | 190±3.60@ | 68.71±1.98@ | 201.3±4.75@ | 73.2±3.09@ | 211.90±2.80@ | 80.09±3.20@ |
| **Cr+Melatonin (Cr+Mel)** | 139.34±2.10# | 49.51±2.50# | 167.9±3.76# | 51.24±2.10# | 168.23±3.05# | 58.32±3.1 |

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

**Table 1.9 Changes in serum glucose, insulin and melatonin levels following 15, 30 or 60 days treatment with chromium.**

| | 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±0.05 | 126±5.65* |
| **Chromium (Cr)** | 132.5±1.04 | 0.43±0.01@ | 67±4.65@ | 159.62±4.13@ | 0.60±0.09@ | 75±4.09@ | 165.75±2.85@ | 0.46±0.01@ | 26±2.01@ |
| **Cr+Melatonin (Cr+Mel)** | 128.58±2.15 | 0.94±0.01# | 113±1.85# | 129.52±3.09 | 1.50±0.01 | 109±4.91 | 121±2.06# | 1.61±0.03# | 83.22±3.0# |

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

**Table 1.10 Changes in serum lipid profile following 15 days of exposure with chromium (Units expressed as mg/dl)**

| | Cholesterol | Triglyceride | H DL | VLDL | LDL |
|---|---|---|---|---|---|
| **Control (Con)** | 103.50±1.04 | 119.25±0.85 | 64.25±0.85 | 24.25±0.85 | 25.16±1.11 |
| **Melatonin (Mel)** | 91.75± 0.63* | 132.00±1.08* | 55.00±1.47 | 26.00±1.08 | 11.75±0.85* |
| **Chromium (Cr)** | 93.75± 1.95@ | 134.50±1.32@ | 49.00±1.08@ | 25.75±0.85 | 19.50±1.04@ |
| **Chromium+Melatonin(Cr+M)** | 106.75± 1.85# | 132.25±0.85# | 59.00±0.91 | 26.50±1.04 | 23.25±0.85 |

**Table 1.11 Changes in serum lipid profile following 30 days of exposure with chromium (Units expressed as mg/dl)**

| | Cholesterol | Triglyceride | H DL | VLDL | LDL |
|---|---|---|---|---|---|
| **Control (Con)** | 122.75±1.85 | 127.75±1.11 | 54.50±1.04 | 28.50±1.19 | 33.75±0.85 |
| **Melatonin (Mel)** | 105±1.48 | 134.50±1.65 | 55.50±1.04 | 27.50±0.65 | 34.00±1.08 |
| **Chromium (Cr)** | 105.50±1.04 | 115.50±2.65 | 49.75±1.48 | 23.50±0.65 | 24.75±1.11@ |
| **Chromium+Melatonin(Cr+M)** | 108.00±1.08 | 116.75±2.11 | 58.25±2.25 | 28.50±0.65 | 21.75±0.85 |

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

**Table 1.12 Changes in serum lipid profile following 60 days of exposure with chromium (Units expressed as mg/dl)**

| | Cholesterol | Triglyceride | H DL | VLDL | LDL |
|---|---|---|---|---|---|
| **Control (Con)** | 117.75±1.11 | 139.25±1.5 | 57.50±1.04 | 29.00±1.30 | 32.00±1.08 |
| **Melatonin (Mel)** | 111.75±2.85 | 135.75±2.5 | 59.00±1.08 | 27.25±0.85 | 25.25±0.85 |
| **Chromium (Cr)** | 108.25±1.65 | 115.50±2.5@ | 48.75±1.11 | 22.75±0.85 | 36.75±0.85 |
| **Chromium+Melatonin(Cr+M)** | 92.00±0.91 | 112.25±1.8 | 39.50±1.04 | 22.00±1.08 | 28.25±0.85 |

**@ 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 1.8** Photomicrograph of control liver showing a hepatic lobule(400X). Note the cord like organization of hepatocytes and a central vein(CV). CV- Central Vein; S- Sinusoid; K-Kupfer cells.

**Fig 1.9** Photomicrograph of 15 day melatonin treated liver (400X). Note the well organized hepatic cords and central vein and robustness of hepatocytes. S- Sinusoid ; K- Kupfer cells

**Fig 1.10** Photomicrograph of 15 day chromium exposed liver (400X). Note the beginning of disorganization of hepatic cords and endothelial rupture of central vein (arrows). Note the many apoptotic cells (* mark)

**Fig 1.11** Photomicrograph of liver exposed to Cr+Mel for 15 days (400X). Note the near normal organization of hepatic lobule and intact central vein.

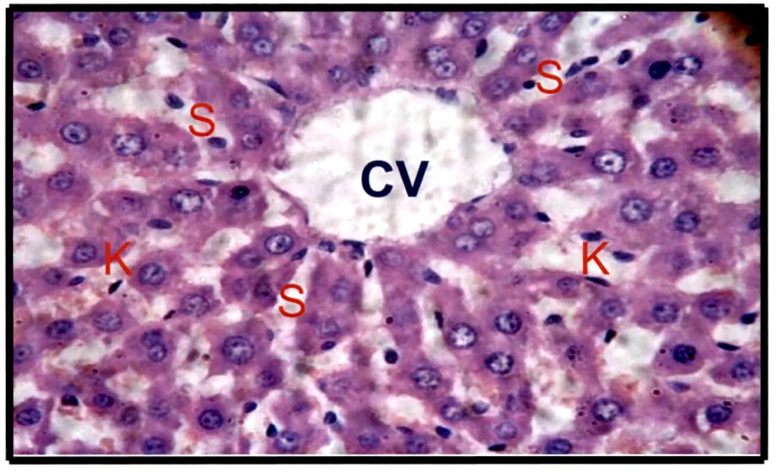


Fig1.8.Control

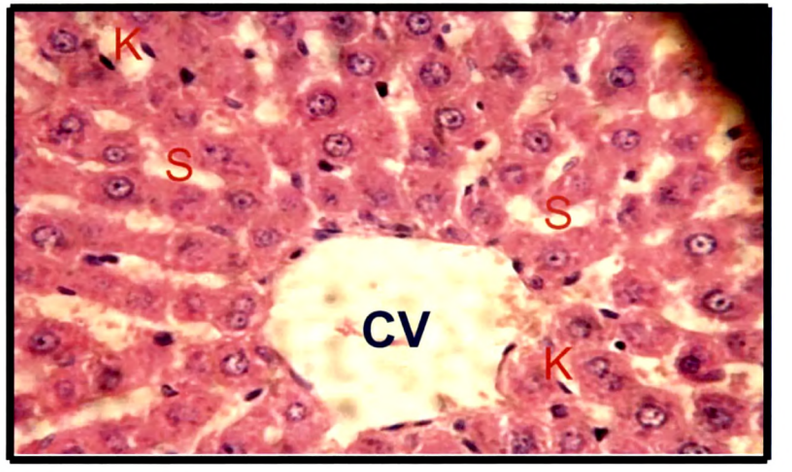


Fig 1.9 Melatonin

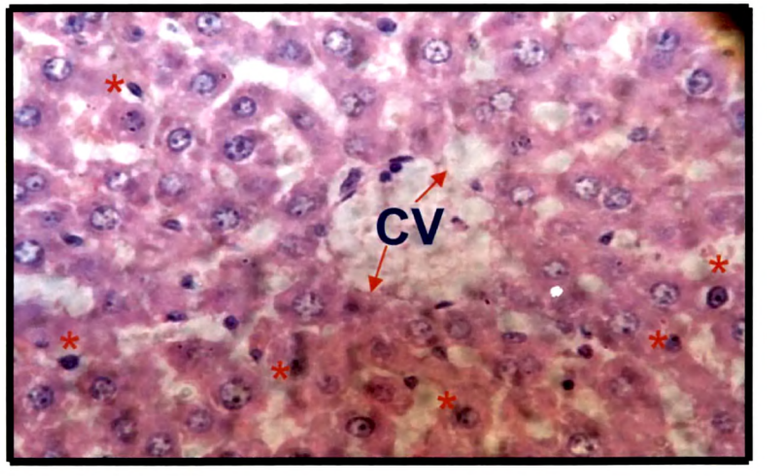


Fig 1.10 Chromium

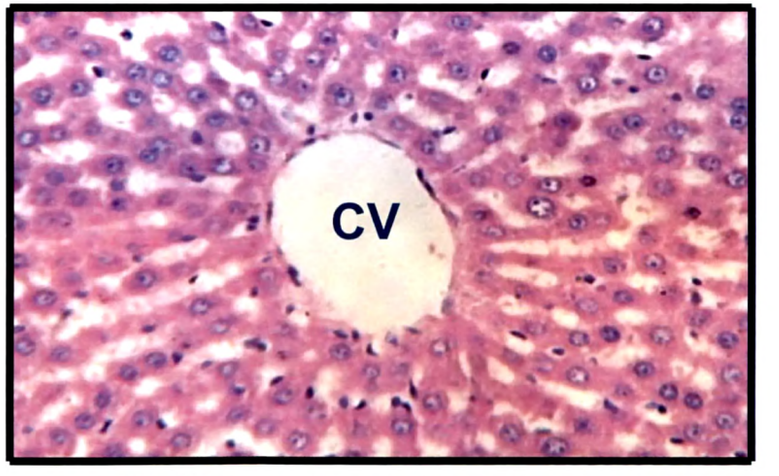


Fig 1.11 Chromium + Melatonin

## PLATE II

**Fig 1.8(a)** Photomicrograph of control liver showing periportal area (400X) PPA-Periportal area

**Fig 1.9(a)** Photomicrograph of melatonin treated liver showing periportal area (400X); PV-portal vein HA-hepatic artery.

**Fig 1.10(a)** Photomicrograph of 15 day chromium treated liver showing periportal area (400X). Note the breaches in the hepatic artery (arrow). PV-Portal vein; HA Hepatic artery

**Fig 1.11(a)** Photomicrograph of liver exposed to Cr+Mel for 15 days showing periportal area (400X). Note the near normal organization. PV- Portal Vein.

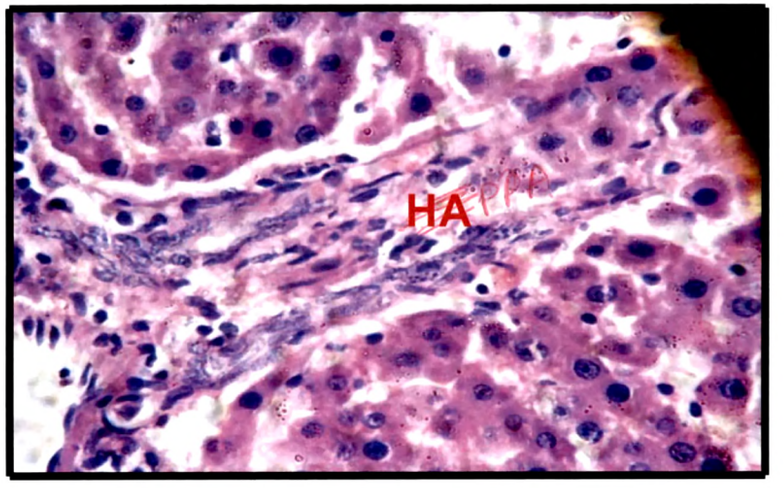


Fig 1.8(a) Control

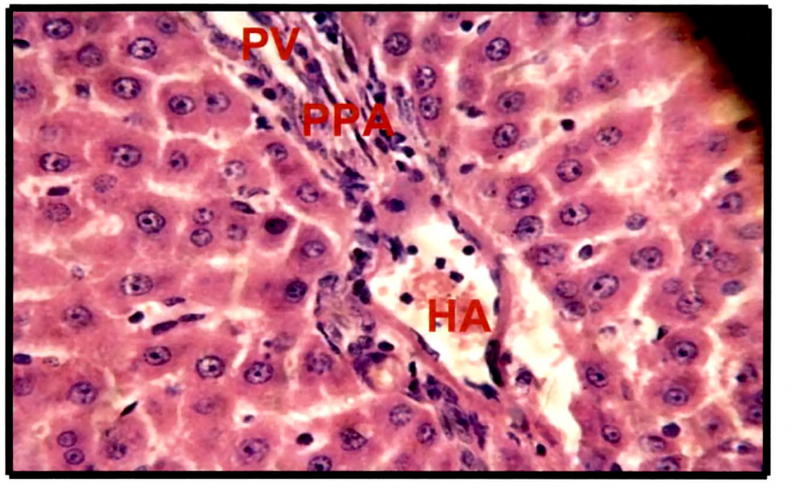


Fig 1.9(a) Melatonin

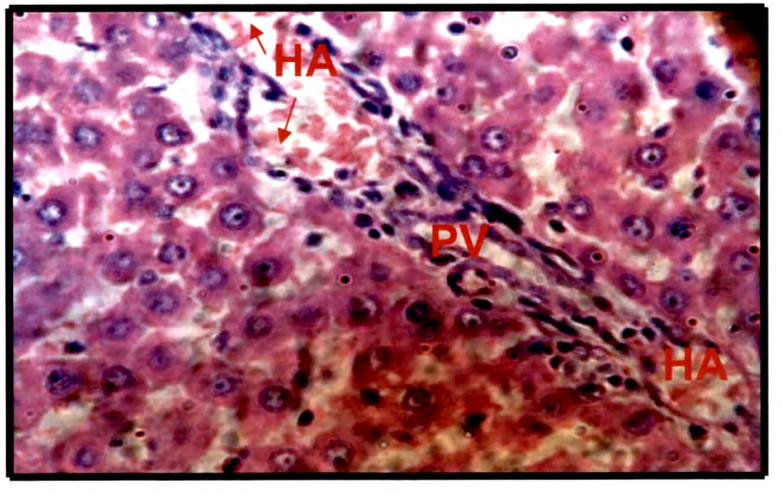


Fig 1.10(a) Chromium

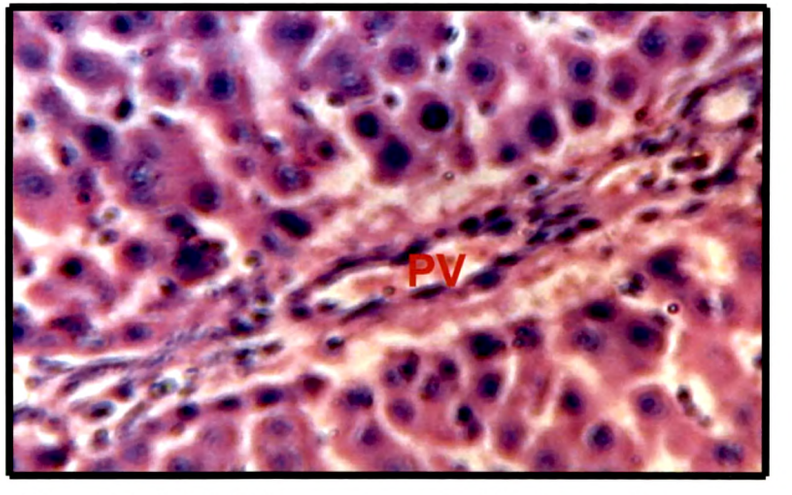


Fig 1.11(a) Chromium + Melatonin

## PLATE III

**Fig 1.12** Photomicrograph of control liver showing a hepatic lobule (400X). CV- Central Vein; S- Sinusoid.

**Fig 1.13** Photomicrograph of 30 day melatonin treated liver (400X). Showing central vein and hepatic cords.

**Fig 1.14** Photomicrograph of 30 days chromium treated liver showing a hepatic lobule (400X). Note the breached endothelium of central vein (arrows) and distended sinusoids (s).

**Fig 1.15** Photomicrograph of liver exposed to Cr+Mel for 30 days showing a hepatic lobule (400X). Note the near normal hepatic cords and central vein.

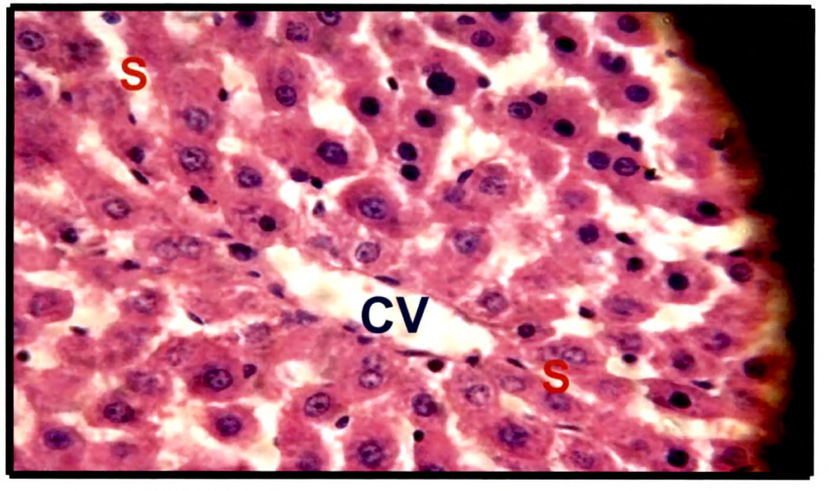


Fig 1.12 Control

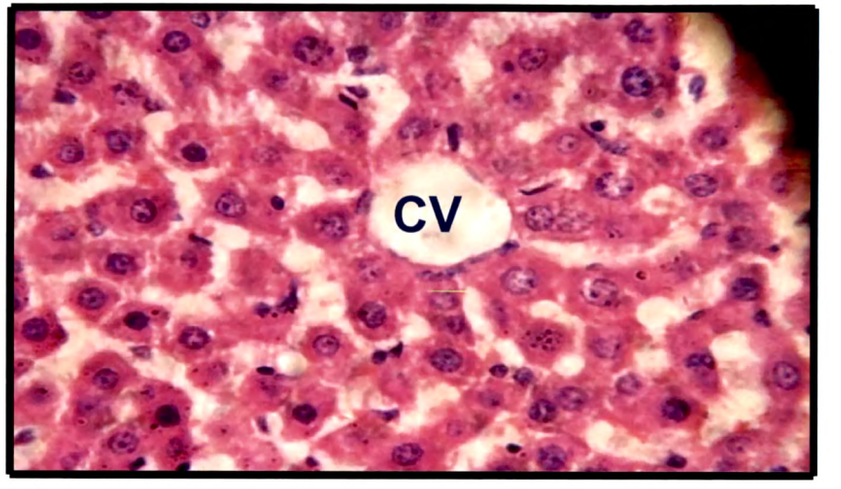


Fig 1.13 Melatonin

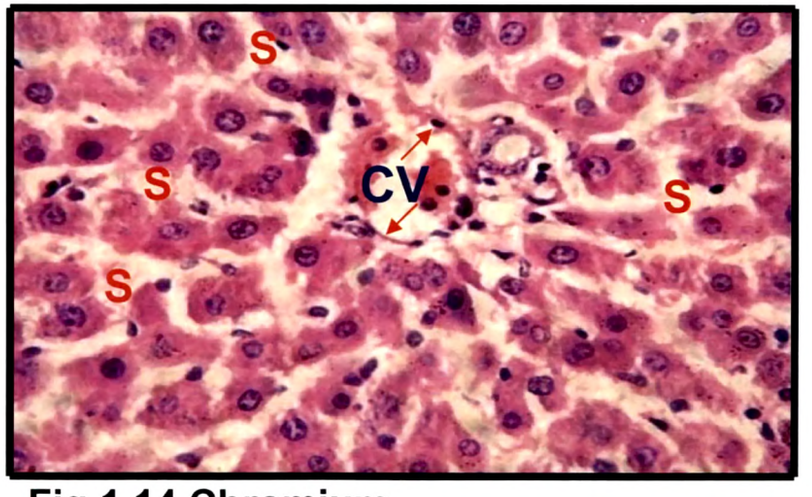


Fig 1.14 Chromium

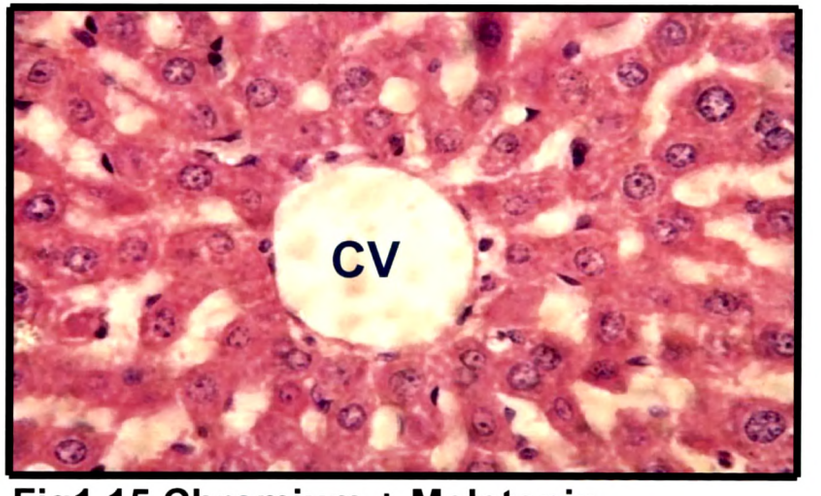


Fig1.15 Chromium + Melatonin

## PLATE IV

**Fig 1.12(a)** Photomicrograph of control liver showing periportal area (400X) PPA-Periportal Area

**Fig 1.13(a)** Photomicrograph of melatonin treated liver for 30 days showing PPA (400X)

**Fig 1.14(a)** Photomicrograph of liver exposed to Cr for 30 days showing a hepatic lobule (400X). Note the congested periportal vein (PV).

**Fig 1.15(a)** Photomicrograph of liver exposed to Cr+Mel for 30 days (400X). Note the near normal appearance of periportal area.

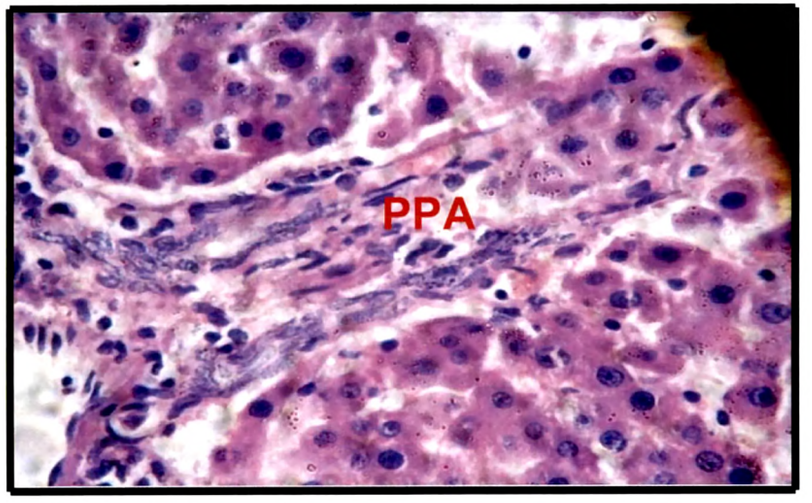


Fig 1.12(a) Control

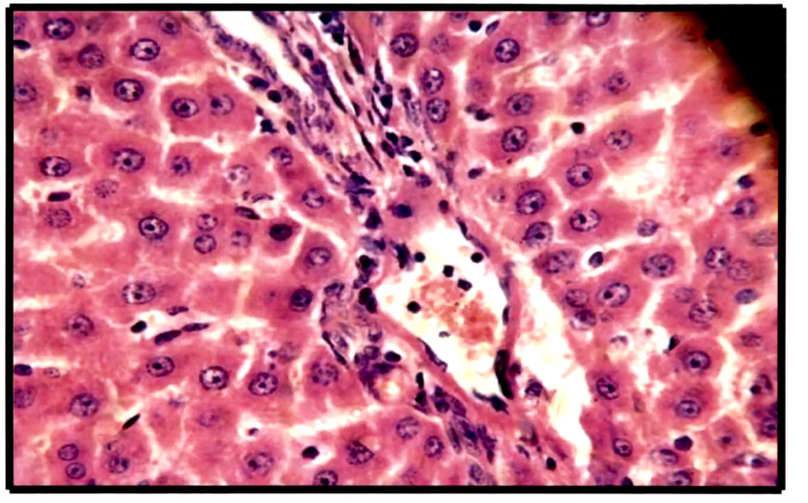

Fig 1.13(a) Melatonin

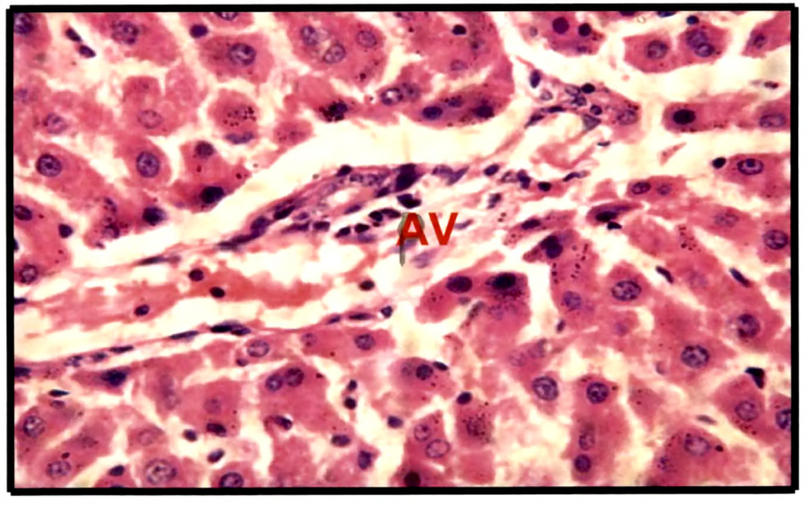


Fig 1.14(a) Chromium

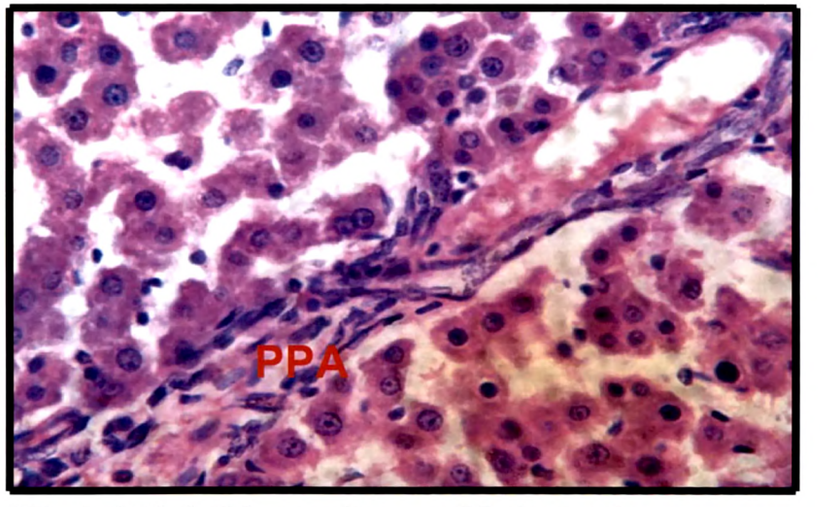


Fig1.15(a) Chromium + Melatonin

## PLATE V

**Fig 1.16** Photomicrograph of 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 1.17** Photomicrograph of 60 day melatonin treated liver (400X). Note the well organized hepatic cords and central vein and robustness of hepatocytes. S- Sinudois K- Kupfer cells

**Fig 1.18** Photomicrograph of 60 day chromium exposed liver (400X). Note the breached central vein confluencing with distended sinusoids.

**Fig 1.19** Photomicrograph of liver exposed to Cr+Mel for 60 days (400X). Note the near normal organization of hepatic cords and minor disruptions in central vein.

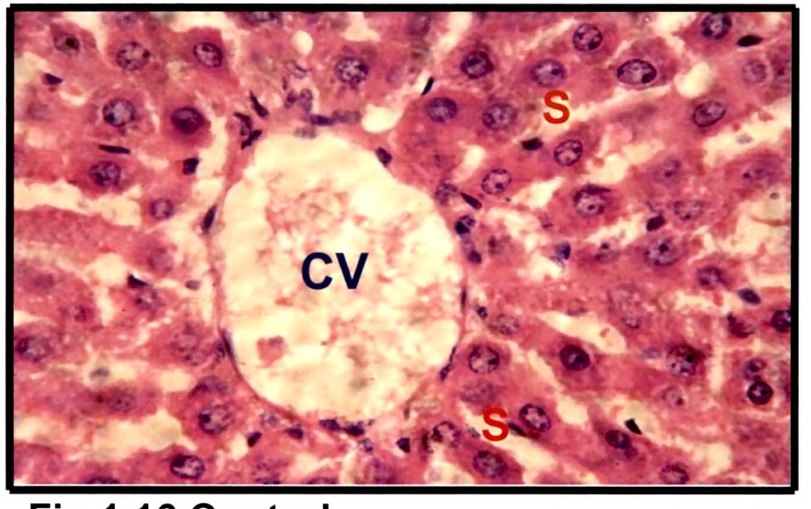


Fig 1.16 Control

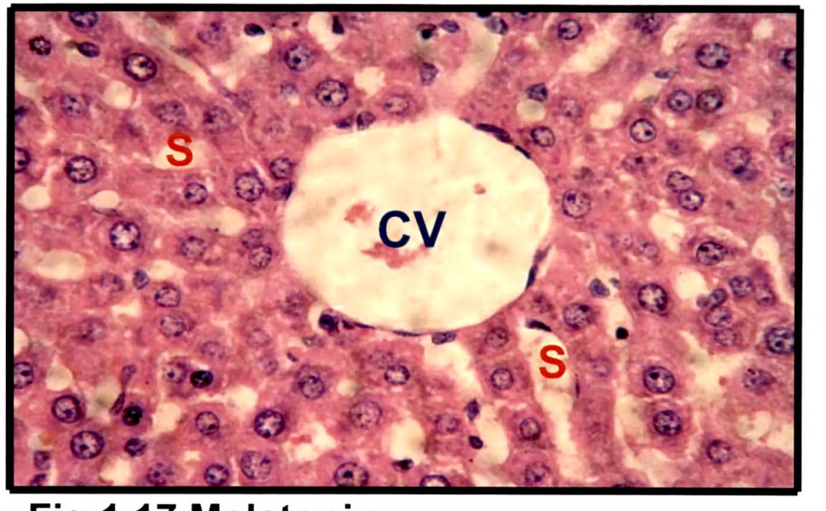


Fig 1.17 Melatonin

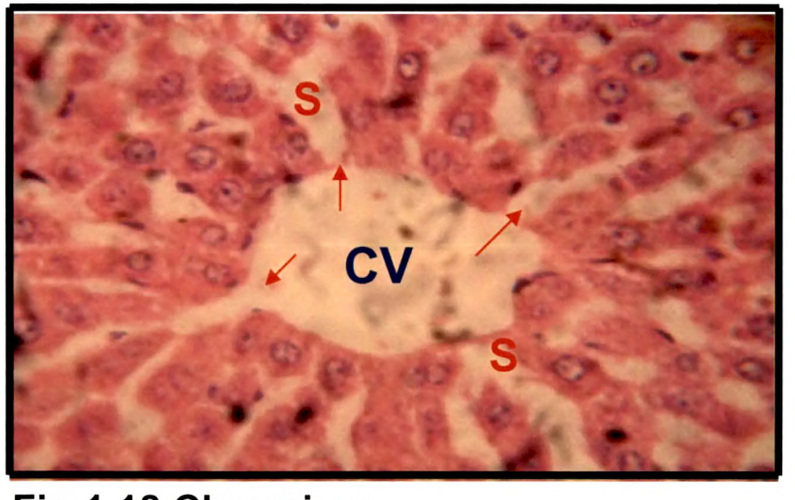


Fig 1.18 Chromium

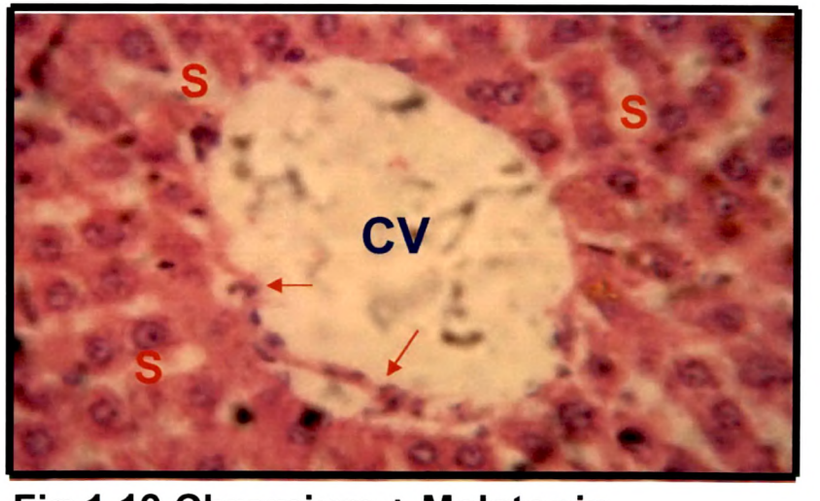


Fig 1.19 Chromium + Melatonin

## PLATE VI

**Fig 1.16(a)** Photomicrograph of Control liver showing periportal area (400X). PPA-periportal area

**Fig 1.17(a)** Photomicrograph of liver treated with melatonin for 60 days showing periportal area (400X)

**Fig 1.18(a)** Photomicrograph of liver exposed to Cr for 60 days showing disintegrated periportal area and confluencing sinusoids (400X). many apoptotic cells can be seen (arrows).

**Fig 1.19(a)** Photomicrograph of liver exposed to Cr+Mel for 60 days showing periportal area (400X). Hepatocytes appear normal but some breaches in periportal area can be seen.

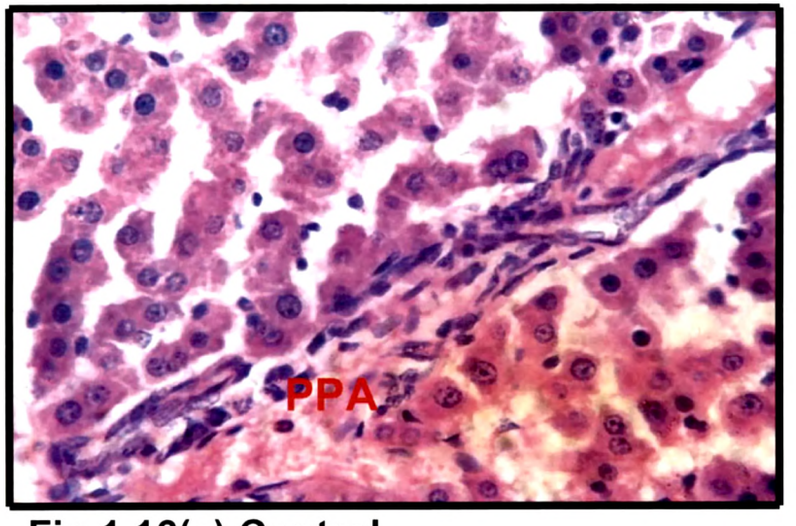


Fig 1.16(a) Control

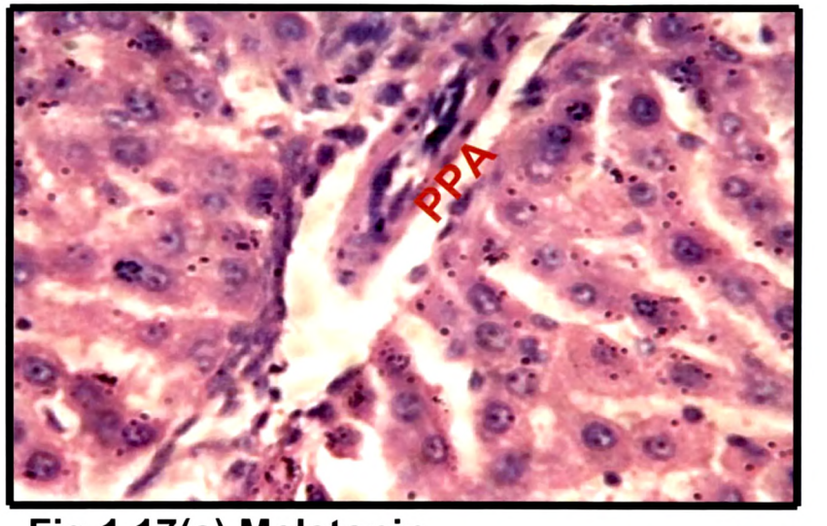


Fig 1.17(a) Melatonin

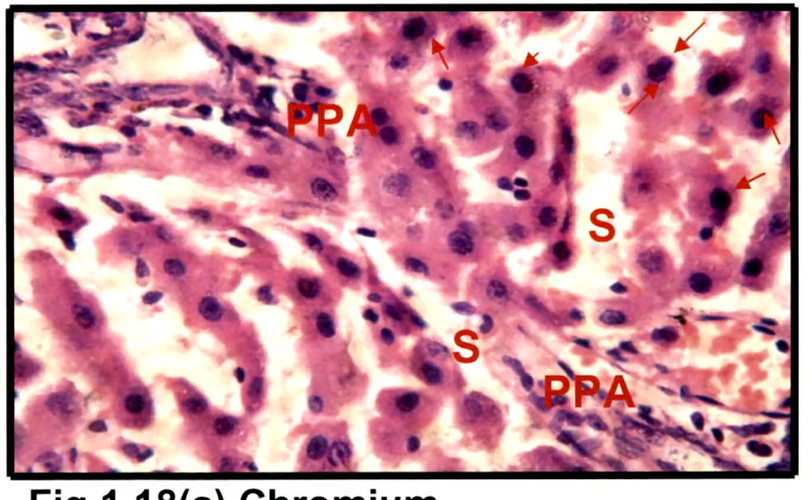


Fig 1.18(a) Chromium

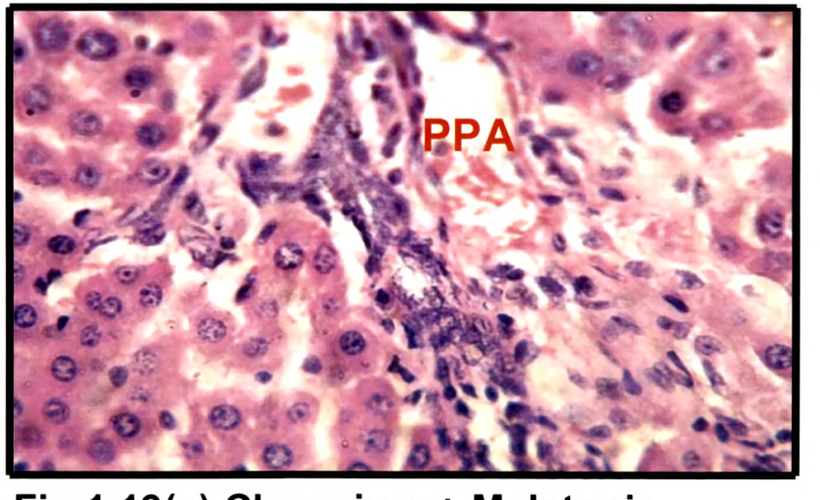


Fig 1.19(a) Chromium + Melatonin

**Discussion:**

The present study undertaken to evaluate hepatic responses to chronic Chromium (VI) exposure has shown definite oxidative stress and certain degree of toxic manifestations. It is well documented, that Cr (VI) is not a systemic toxicant as most of the Cr (VI) gets reduced to Cr (III) in body fluids and long lived non-target cells (De Flora *et al.,* 1987, 1997). Trivalent $Cr^{3+}$ is an essential trace mineral micronutrient involved in favourable regulation of metabolism by stimulating insulin action (Anderson, 1986, 1989, 1993). Its role in potentiating insulin action and in anabolism are well established. However, excessive production and/or accumulation of $Cr^{3+}$ can be paradoxically toxic to cells and organs. In this context, the present study on chronic differential duration of exposure to a realistic dosage of Cr (VI) has revealed substantial hepatic oxidative stress and toxicity together with glycemic dysregulation and tendency to lower levels of serum cholesterol and triglycerides.

Hepatic oxidative stress is marked by significantly increased LPO and decreased content of non-enzymatic antioxidants (GSH and Vit C) and activity of enzymatic antioxidants (SOD, CAT and GPx). Maximally high LPO seen at 15 days decreased gradually by 60 days of Cr (VI) exposure. The decreasing degree of LPO with increasing duration tends to suggest the induction of some adaptive/protective mechanism to stem the oxidative damage. Concomitant reduction in both enzymatic and non-enzymatic antioxidants attests to the observed increase in LPO to be due to increased oxidative stress. Cr (VI) and Cr (III) induced oxidative stress is reported in a wide variety of organs marked by increased LPO and decreased endogenous antioxidant status. However, most of these studies are on short term basis with the Cr exposure period ranging from hours to days or even a single acute administration (Bosgelmez and Girvendik, 2004; Anand, 2005; Wang *et al.,* 2006; Patlolla *et al.,*

2008). The present study involving short (15 days), medium (30 days) and long (60 days) duration of oral Cr 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 the optimal commissioning of the endogenous antioxidant machinery to resist oxidative damage to liver, the metabolic work force of the vertebrate body. A steady level of depletion of GSH and Vit C and decline in CAT and GPx activities right from short to long duration of Cr (VI) exposure highlight the effective functioning of the hepatic antioxidant system in the suggested protection against persistent oxidative stress. A report in this context suggests liver to have a more robust antioxidant system compared to kidney in terms of Cr (VI) toxicity (Anand, 2008).

Despite the fact that both CAT and GPx remain persistently inhibited (inactivated) to the same degree during all durations of Cr (VI) exposure, interestingly, SOD activity showed substantial recovery in the long duration exposure (60 days). This may have to be looked upon in the context of Reactive Oxygen Species (ROS) generated by Cr. It is already documented that Cr (VI) and Cr (III) can generate both superoxide anion and hydroxyl radicals (Bagchi *et al.,* 1995, 2001, 2002), predominantly the latter (Bagchi *et al.,* 2002). Apparently, it is presumable that during the initial periods of exposure to Cr (VI), more superoxide anion is generated which is being effectively quenched by SOD (Dong *et al.,* 2006) and, with persisting Cr (VI) stress, there is more hydroxyl radical generation which is being neutralized by the catalase-glutathione reductase and/or GPx pathways. Recovery of SOD and steady persistent inactivation of CAT and GPx seen herein attest to this notion. Since both CAT-GR or CAT-GPx mediated removal of hydroxyl and peroxide radicals are dependent on ready availability of NADPH (Kirkman *et al.,* 1987; Lei and Chang, 2005), it is

presumable that liver under increasing and persistent Cr induced stress would be generating NADPH by the operation of HMP shunt pathway. Though, G-6-PDH, the key enzyme of shunt pathway is also subjected to inactivation by metals, as are other antioxidant enzymes, its maintenance along with CAT and GPx can be attributed to the protective action of Vit C and GSH. The maintenance of activity levels of CAT and GSH at the same level even at 60 days of exposure as on 15 days suggests the robustness of the redox machinery of liver for sustained protection against Cr induced oxidative insult. Ascorbic Acid (AA) is likely to help in GSH formation for CAT-GR/GPx mediated neutralization of hydroxyl radicals while at the same time also facilitating reduction of Cr (VI) to less toxic Cr (III) (Bradberry and Vale, 1999; Dey *et al.,* 2001; Mahmond *et al.,* 2006).

Though oxidative stress seems to be managed well by the endogenous biochemical antioxidant machinery, cytotoxicity is clearly indicated by the 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. Apparently, there is also disruption of the perilobular portal area with appearance of fibrosis. These changes are very prominently manifested in the 60 day liver sections indicating the increasing cytotoxicity due to Cr (VI) exposure. In support of these observations are some reports based on both *in vitro* and *in vivo* studies indicating hepatic histological lesions, DNA damage and apoptosis with short term exposure to Cr (da Neves *et al.,* 2002; Da Silva *et al.,* 2006; Wang *et al.,* 2006). The acute single dose study of da Neves *et al.* (2002) documented vacuolation and hypertrophy of hepatocytes with disorganization of hepatic parenchyma. The study of da Silva *et al.* (2006) using a dosage of Cr similar to that of present study given through drinking water for

protracted period of 120 days has reported varying degrees of parenchymal cell vacuolization with many hepatocytes depicting ballooned appearance with karyolysis. Dilation and congestion of centrilobular vein, dilated sinusoids containing erythrocytes and with the portal area showing fibrosis and biliary duct proliferation were some of the other observations of the study. The present study of a lesser duration of 60 days has nevertheless failed to show ballooned hepatocytes with extensive vacuolation. There are indications of vacuolation in some of the hepatoctyes by 60 days. 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 with evidences of nuclear pyknosis. Mild signs of perilobular fibrosis and biliary ductal proliferation could be seen in the portal area. Chromium induced hepatic cell apoptosis has been shown to be increased in the apoptotic deficient/tumor suppersor gene p53 deficient mice and, this has led to a suggestion that Cr (VI) induced DNA fragmentation and apoptosis may be modulated through p53 (Bagchi *et al.,* 2001, 2002). It is likely in this context that, the presently observed cell loss in the hepatic parenchyma, may involve p53 suppressed apoptosis. These cytotoxic manifestations indicating Cr induced hepatotoxicity find biochemical co-relation in the observed increased serum AST and ALT, the marker enzymes of hepatic damage.

In the light of the role of melatonin as a powerful natural antioxidant, simultaneous supplementation with melatonin in Cr intoxicated animals has shown significant protection against LPO and decrease in endogenous levels of antioxidants. The critical role of melatonin in combating Cr (VI) induced oxidative stress is marked by its significant sparing effect on GSH and AA, apart from its ability in resisting the decrement in enzymatic antioxidants. Interestingly, while GSH depletion was

insignificant, the level of AA showed an increment. Though the maintenance of GSH and AA pools in Cr intoxicated melatonin supplemented animals suggests sparing effect of melatonin on AA and GSH as a primary free radical quencher, the protective effect seen on antioxidant enzymes despite 60 days of exposure to Cr could be explained in the context of reports on melatonin induced upregulation of antioxidant gene expression (Reiter *et al.*, 2001, 2003, 2006).

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 Cr, 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 levels in Cr exposed rats. The increasing degree of oxidative stress and cytotoxicty with increased duration of Cr 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, E and A against oxidative stress generated by many chemical and environmental agents including metals (Flora *et al.*, 2008), there is no report on the ability of melatonin to resist Cr toxicity. So, this study provides evidence towards melatonin to be a powerful protectant against Cr toxicity.

Since Cr (VI) gets converted to Cr (III) in tissues and Cr (III) is known to be potent stimulator of insulin action and glucose metabolism, the present study has tried to investigate serum levels of insulin, glucose and lipids. Though melatonin administration alone is found to have favorable effect on serum parameters, Cr was found to have hypocholesterolemic and hypotriglycerdemic effects. Though Cr (III) is known to be favorsable for carbohydrate metabolism, the present study highlights

the fact that, higher levels of Cr(III) (by conversion from Cr(VI) to Cr(III)) for a longer duration as in the present study has, paradoxical effects as seen by reduced insulin level and hyperglycemia. Melatonin was again purposeful in offsetting these effects of dose and duration dependent ill effects of Cr. This study therefore, sounds an alarm signal against unregulated usage of Cr (III) compounds in the diet for glucoregulation, lean body mass and better insulin action.

Overall, the present study suggests a duration dependent effect of Cr 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 chromium.

## Summary of Chapter 1

The present study was essentially undertaken to decipher the degree of oxidative stress and toxic effects induced by Chromium on hepatic tissue in male *Wistar* rats exposed to a realistic dosage of Cr(VI) (20 mg/kgBW/day) through drinking water, based on the levels of these metals found in the environment, for a duration of 15, 30 and 60 days. Protective effect of melatonin (10 mg/kg) was also assessed by simultaneous administration with the metal. Oxidative stress manifestations were assessed by the levels of lipid peroxidation (LPO), activity and content of enzymatic (SOD, CAT, GPx) and non-enzymatic (GSH, AA) antioxidants respectively together with toxicity markers of hepatic damage (ALP and ALT). Metal exposed animals showed increased LPO levels with significantly decreased activity and content of enzymatic and non-enzymatic antioxidants respectively at all the three periods. Comparatively, maximum oxidative stress was observed in the initial period of exposure (15 days), marked by maximal level of LPO. SOD activity showed maximum inhibition at 15 days while, rest of the enzymatic antioxidants showed maximum inhibition at 60 days of exposure. Metal accumulation was also maximal at the 15 days with gradual decreases till 60 days. Chromium exposure showed favourable effect on serum lipid profile while it tended to reduce serum insulin and glucose levels. Histopathological observations also prove the fact that Cr (VI) exposure leads to cytological lesions in the hepatic tissue promoting cellular necrotic/apoptotic. Melatonin was able to counteract Cr (VI) induced insults at all the treatment periods. Melatonin was successful in decreasing the hepatic oxidative stress as reflected by the decreased LPO and, increased contents of enzymatic as well as non-enzymatic antioxidants. It also prevented the alterations in insulin and glucose levels and, also protected the Cr (VI) induced structural lesions. Overall, the present

study suggests a duration dependent effect of Cr on hepatic oxidative stress and cytotoxicity and the potent activity of melatonin in preventing the negative effects of Cr (VI).