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Melatonin protects against chromium (VI) induced hepatic oxidative stress and toxicity: Duration dependent study with realistic dosage

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ABSTRACT

The present study was undertaken to assess 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/kg/b.w./day) through drinking water, based on the levels of these metals found in the environment, for a duration of 15, 30 and 60 days. The protective effect of melatonin (10 mg/kg) was also studied by simultaneous administration with the metal. Levels of enzymatic and non-enzymatic antioxidants as well as lipid peroxidation were assessed. There was a significant decrease in enzymatic as well as non-enzymatic antioxidants and an increase in the lipid peroxidation level, which were prevented and maintained at near-normal levels by the administration of melatonin in all treatment periods. Metal accumulation was maximal at 15 days, with gradual decreases till 60 days. Histopathological observations also demonstrated the fact that Cr (VI) exposure leads to cytological lesions in the hepatic tissue promoting cellular necrotic/apoptotic changes, while melatonin was able to counteract insults induced by Cr (VI) at all treatment periods. It also prevented alterations in insulin and glucose levels. Overall, the present study suggests a duration-dependent effect of Cr on hepatic oxidative stress and cytotoxicity and shows the potent activity of melatonin in preventing the negative effects of Cr (VI).

KEY WORDS: melatonin; oxidative stress; toxicity, chromium (VI)

Introduction

Hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)] are the principal forms of chromium of common occurrence in workplaces. Hexavalent chromium compounds are used extensively in various 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 & Liu, 1993; Barceloux, 1999). Adverse renal and hepatic effects on exposure to chromium were reported (Love, 1983; Verschoor *et al.,* 1988). Functional disruption of several organs on accumulation of Cr due to long term exposure has been suggested (Nieboer &

Jusys, 1988). The exact mechanism of action of chromium compounds on tissues has not been extensively studied, but chromium was observed to generate massive amounts 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 & Dalai, 1990; Suden *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 proteins, lipids and DNA (Nordberg & Arner, 2001). Though Cr (III) is considered essentially as a trace element in glucoregulation and supplements have been used for combating diabetes, its ability to form cross-links with DNA and protein makes it a potentially dangerous agent, particularly when present in excessive amounts.

Studies in mammals have suggested harmful effects of Cr (VI). Subcutaneous administration of Cr (VI) in rats was shown to result in progressive proteinuria, increased urea nitrogen and creatinine, along with elevated serum activity level of alanine aminotransferase and hepatic

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lipid peroxidation (Kim and Na, 1991). Increased hepatic lipid peroxidation was shown in mice administered 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 were 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. Most studies evaluated Cr toxicity by intraperitoneal or subcutaneous administration. Further, there are but few 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 (Ramachandran 2003). This has necessitated the present study on Cr induced hepatic and 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. 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 therapeutics. 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.

Materials and methods

Animals
Adult male albino rats of Wistar strain were used as animal model for the experiments. All the selected rats were of 180 days old and in the weight range of 275–300 g. The animals were kept in the animal house under controlled conditions of ambient temperature (25±2 °C) and a 12:12 hours light and dark photoperiod throughout the experimental study. Food pellets (Pranav Agro Industried Ltd, Sangli, MHR, India) and water was provided *ad libitum.* The rats were acclimatized for a period of 10 days prior to commencement of the experiment. After acclimatization, the intake of water was observed for 5–7 days and an average was taken for preparation of the dose. Animal experiments were conducted according to the guidelines of CPCSEA from the Ministry of Social Justice and Empowerment, Government of India, vide CPCSEA (827/ac/04/CPCSEA).

Experimental protocol
The rats were randomly divided into four groups of six animals each. The control group was given normal drinking water while the chromium alone and the chromium + melatonin group of animals received chromium (20 mg/kg/b.w./daily) dissolved in drinking water during the acclimatization period. The melatonin group of animals received intraperitoneal melatonin (10 mg/kg/b.w.) at 6:00 pm daily, while the control group received saline at the same time. Following the treatment schedule of 15, 30 or 60 days, animals were sacrificed on the 16th, 31st and 61st day. The time of sacrifice was 07:30 hrs (7:30 am). On completion of the treatment period, the animals were weighed and sacrificed by cervical dislocation. Blood sample was collected by jugular vein puncture. The organs were quickly excised, cleared of the adhering fat, blotted and weighed, and then processed for biochemical studies.

Chemicals
All chemicals used in the study were of highest purity and of analytical grade. The dosage of chromium selected in the present study is an environmentally relevant realistic dose based on the actual concentration of chromium found in cereals and vegetables grown across the Baroda effluent channel as reported in our earlier publication (Ramachandran, 2003). The actual chromium content administered to animals was calculated on the basis of average food consumption in rats empirically based on field values of routinely consumed cereals and vegetables grown along the Baroda effluent channel.

Hepatic lipid peroxidation (LPO)
Lipid peroxidation was assayed colorimetrically in the liver by the method of Buege and Aust (1978) and expressed as nmol of MDA formed/g tissue.

Assay of non-enzymatic and enzymatic antioxidants
Hepatic total reduced glutathione (GSH) (Beutler *et al*., 1963), vitamin C (Omaye *et al.,* 1979), superoxide dismutase (SOD) (Marklund & Marklund *et al.,*1974), catalase (CAT) (Sinha, 1972) and glutathione peroxidase (GPx) (Rotruck *et al.,* 1973)were assessed in tissue homogenate.

Hepatic chromium content
Hepatic load of chromium was assessed by the method of Rubio *et al.* (2008) 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/g of tissue.

Clinical chemistry parameters
About 3 ml of whole blood was collected in a test tube and centrifuged for 10 mins at 10,000 rpm at 4 °C. The serum collected was then stored at −20 °C for further use. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, insulin and melatonin were measured. Except insulin and melatonin, all the other parameters were evaluated using Merck Diagnostic kit method using Merck Microlab 300 semi-automatic autoanalyser.

Insulin

Insulin was measured using Mercodina Rat Insulin ELISA kit (Mercodina 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 (NIHFW, New Delhi).

Histology

Liver tissue was blotted free of blood and other body fluid and fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (3 μM) were cut on glass slides and stained with hematoxilin and eosin (H&E). The sections were observed under Leica Image Analyser.

Statistical analysis

One way ANOVA with Bonferroni index posttest 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)

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 Con animals. Significant decrease in LPO was seen in rats treated with melatonin while there was significant resistance against increase in LPO in Con, 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 showed the same degree of protective effect (Figure 1A).

Superoxide dismutase (SOD)

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 to 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 a relatively and significantly lesser decrement in the
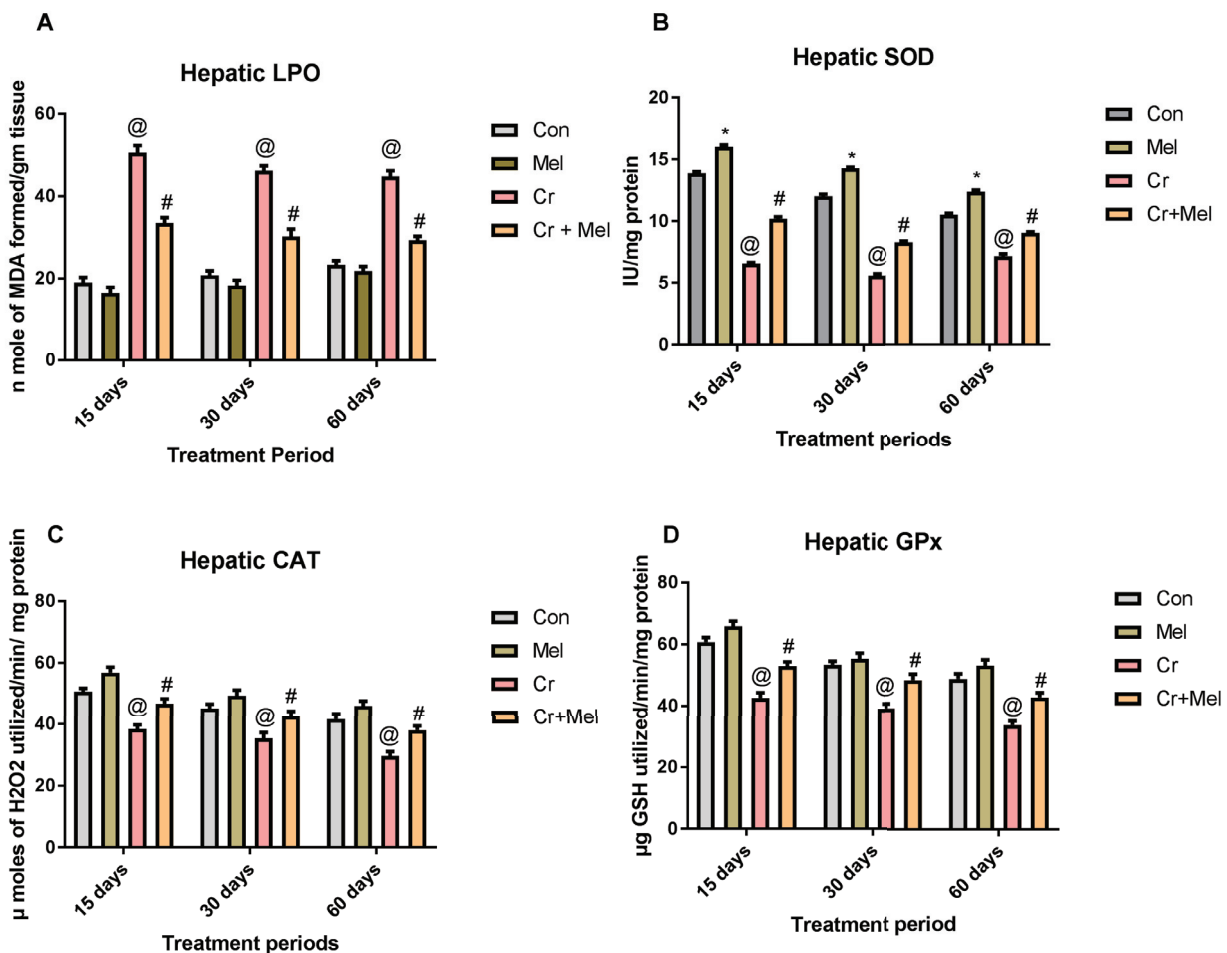

Figure 1. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 15, 30 and 60 days treatment on A: hepatic lipid peroxidation, B: superoxide dismutase (SOD), C: catalase (CAT) and D: glutathione peroxidase (GPx). Values are expressed as mean ± SEM for n = 6, where @$p<0.05$ between Con vs Cr, #$p<0.05$ between Cr vs Cr+Mel, *$p<0.05$ between Con vs Mel.

long duration Cr exposure groups. Similarly, the corresponding degree of protective effect with melatonin was also less in the 60 day Cr exposure group (Figure 1B).

Catalase (CAT)

Hepatic CAT activity tended to show an age dependent decrement. There was significant decrease in CAT activity in the Cr group of animals compared to the Con group of animals. Catalase activity was significantly decreased to the same degree in Cr exposed rats, irrespective of duration of exposure. The degree of protective effect of melatonin was also found to be duration independent (Figure 1C).

Glutathione peroxidase (GPx)

An age dependent decrement in GPx 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 found to be duration independent (Figure 1D).

Glutathione (GSH)

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. A similar degree of decrement in GSH by Cr exposure and the same degree of protective effect by melatonin on co-administration with Cr, irrespective of duration, was found. A gradual age dependent decrease in GSH level was the feature in Con rats (Figure 2A).

Ascorbic acid (Vitamin C)

Liver, as 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 level in the Cr group of animals when compared to the Con group. The degree of depletion of hepatic vitamin content was progressively less with increasing duration of Cr exposure and was also relatively loewer than that of GSH. There was a duration independent similar degree of protection by melatonin at all three time periods of Cr exposure (Figure 2B).

Metal load

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 (Figure 3).

Serum parameters

There is an age dependent decrease in melatonin levels and there was a significant decrease in melatonin level when the animals were treated with Cr. Melatonin administration increased the circulating levels of melatonin more than in control animals (Figure 4). The enzyme markers of hepatic damage ALT and ALP (Table 1) showed an increase in Cr treated rats while they tended to remain within the normal range in rats co-administered with melatonin. Cr induced hypoinsulinemia and hyperglycemia with decrease in melatonin levels. Administration of
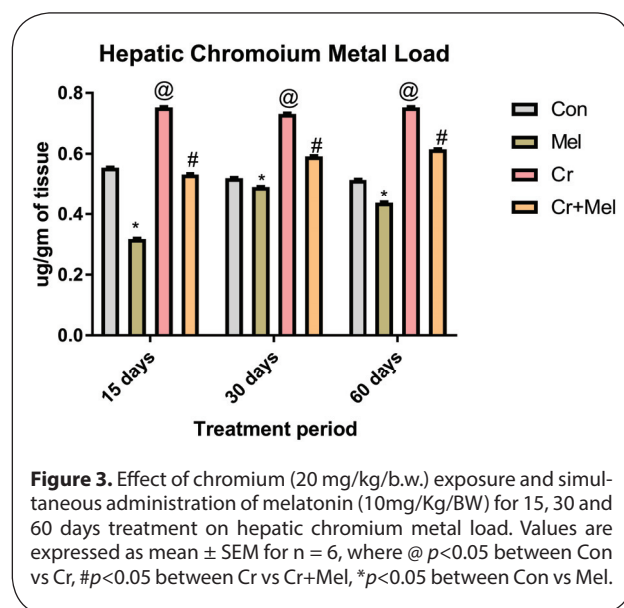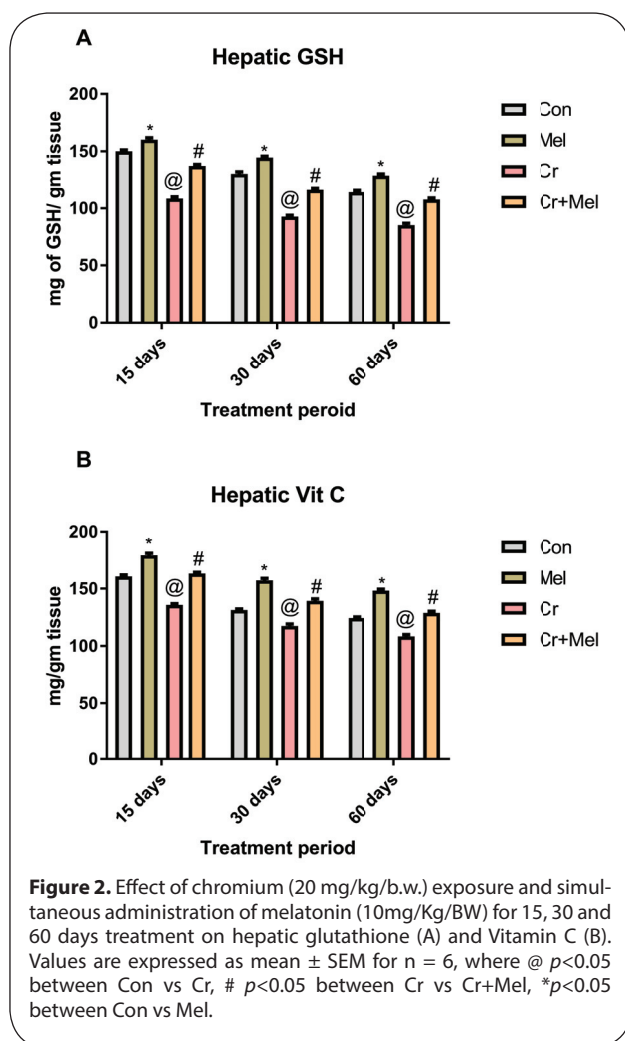

Figure 2. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 15, 30 and 60 days treatment on hepatic glutathione (A) and Vitamin C (B). Values are expressed as mean ± SEM for n = 6, where @ *p*<0.05 between Con vs Cr, # *p*<0.05 between Cr vs Cr+Mel, **p<0.05 between Con vs Mel.

Figure 3. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 15, 30 and 60 days treatment on hepatic chromium metal load. Values are expressed as mean ± SEM for n = 6, where @ *p*<0.05 between Con vs Cr, #*p*<0.05 between Cr vs Cr+Mel, **p<0.05 between Con vs Mel.

Table 1. Changes in serum ALP and ALT activities following 15, 30 or 60 day exposure to Cr(VI).

	15 days		30 days		60 days	
	ALP(U/l)	ALT(U/ml)	ALP(U/l)	ALT(U/ml)	ALP(U/l)	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) (10 mg/kg/b.w.)	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) (20 mg/kg/b.w.)	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#

Table 2. Changes in serum glucose and insulin levels following 15, 30 or 60 day exposure to Cr(VI).

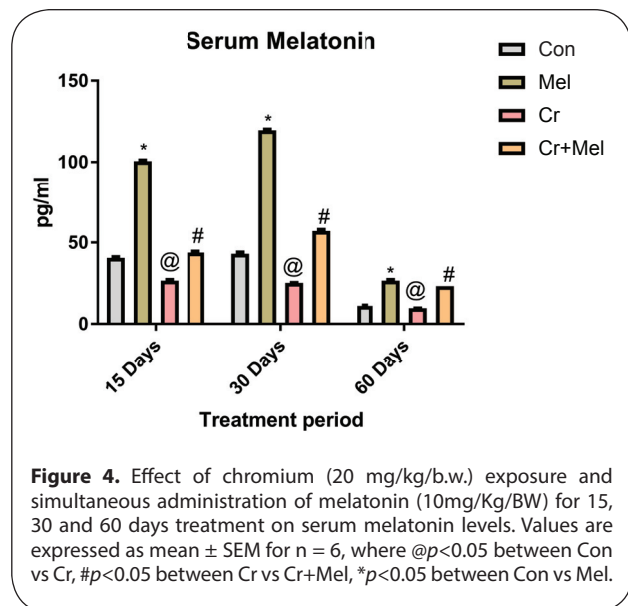|	15 Days		30 Days		60 Days	
	Blood Glucose (mg/dl)	Insulin (mg/dl)	Blood Glucose (mg/dl)	Insulin (mg/dl)	Blood Glucose (mg/dl)	Insulin (mg/dl)
Control (Con)	112.5±2.51	1.7±0.06	104.25±2.85	1.58±0.09	115.75±3.85	1.79±0.01
Melatonin (Mel) (10 mg/kg/b.w.)	125±3.11*	0.6±0.09*	115.50±1.65*	0.95±0.01*	120.50±2.65*	0.81±0.01*
Chromium (Cr) (20 mg/kg/b.w.)	132.50±1.04@	0.43±0.01@	159.62±4.13@	0.60±0.09@	165.75±2.85@	0.46±0.01@
Cr+Melatonin (Cr+Mel)	128.58±2.15	0.94±0.01#	129.52±3.09#	1.50±0.01#	121±2.06#	1.61±0.01#

melatonin along with Cr prevented the changes induced by chromium most effectively (Table 2).

Histology

Changes in the hepatic histology following Cr treatment for 15, 30 and 60 days are shown in figures 5 to 7. Cr-induced histological changes are seen clearly in all duration periods. These changes were brought to a near normal histoarchitecture by simultaneous administration of melatonin. Thus melatonin was able to protect the hepatic tissue in all the three treatment periods.

Discussion

The present study was undertaken to evaluate hepatic responses to chronic Chromium (VI) exposure has shown definitive oxidative stress and a 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 (Bagchi *et al.,* 2001; De Flora *et al.,* 1987, 1997; Hininger *et al.,* 2007; Patlolla *et al.,* 2009). Trivalent Cr^{3+} is an essential trace mineral micronutrient involved in favorable regulation of metabolism by stimulating insulin action (Anderson, 1986, 1989, 1993; Hininger *et al.,* 2007). Its role in potentiating insulin action and in anabolism is well established (Vincent *et al.,* 2015; Hua *et al.,* 2012). However, excessive production and/or accumulation of Cr^{3+} can be paradoxically toxic to cells and organs (Dillon *et al.,* 2009; Shrivastava *et al.,* 2005). In this context, the present study on chronic differential duration is exposure to a realistic dosage of Cr (VI) revealed substantial

Figure 4. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 15, 30 and 60 days treatment on serum melatonin levels. Values are expressed as mean ± SEM for n = 6, where @*p*<0.05 between Con vs Cr, #*p*<0.05 between Cr vs Cr+Mel, **p<0.05 between Con vs Mel.

hepatic oxidative stress and toxicity along with glycemic dysregulation.

Hepatic oxidative stress is marked by significantly increased LPO and decreased content of non-enzymatic antioxidants (GSH and Vit C) and by the activity of enzymatic antioxidants (SOD, CAT and GPx). Maximally high LPO seen on day 15 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
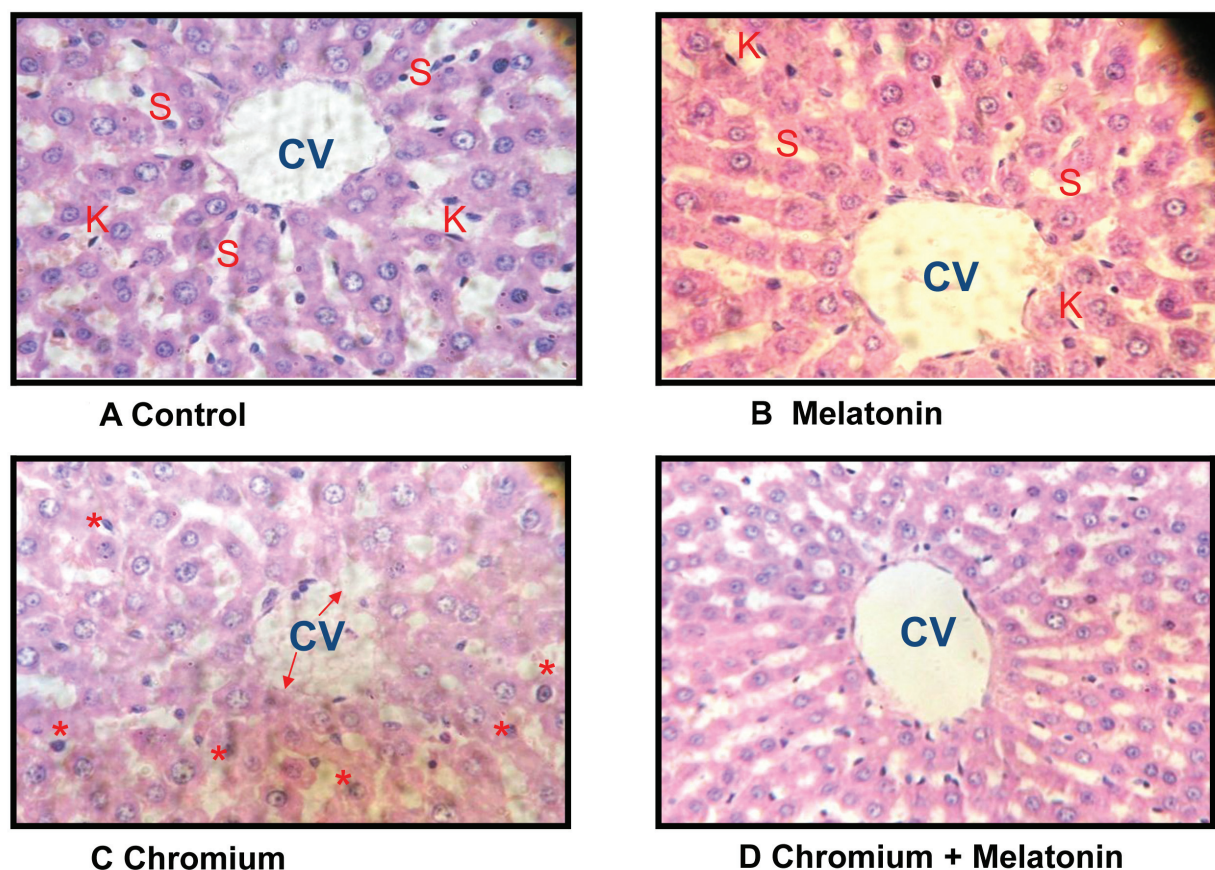
Figure 5. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 15 days of treatment. (A)Photomicrograph of control liver showing a hepatic lobule. (B) Photomicrograph of melatonin treated liver. (C) Photomicrograph of chromium exposed liver (D) Photomicrograph of liver exposed to Cr+Mel. Note the cord like organization of hepatocytes and a central vein(CV). CV – Central Vein; S – Sinusoid; endothelial rupture of central vein (arrows) and apoptotic cells (* mark)

increase in LPO to be due to increased oxidative stress. Cr (VI) and Cr (III) induced oxidative stress has been 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 (Bagchi *et al.,* 2001,2002; Bosgelmez & Girvendik, 2004; Anand, 2005; Wang *et al.,* 2006; Dong *et al.,* 2006; Patlolla *et al.,* 2009). 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 the 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 the liver to have a more robust antioxidant system compared to the kidney in terms of Cr (VI) toxicity (Anand, 2005).

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 assessed in the context of 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), concerning predominantly the latter (Marouani *et al.,* 2017). 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 (Garcia-Nino *et al.,* 2013, 2015) 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 and CAT-GPx mediated removal of hydroxyl and peroxide radicals are dependent on ready availability of NADPH (Kirkman *et al.,* 1987; Lei & Chang, 2005), it is presumable that the
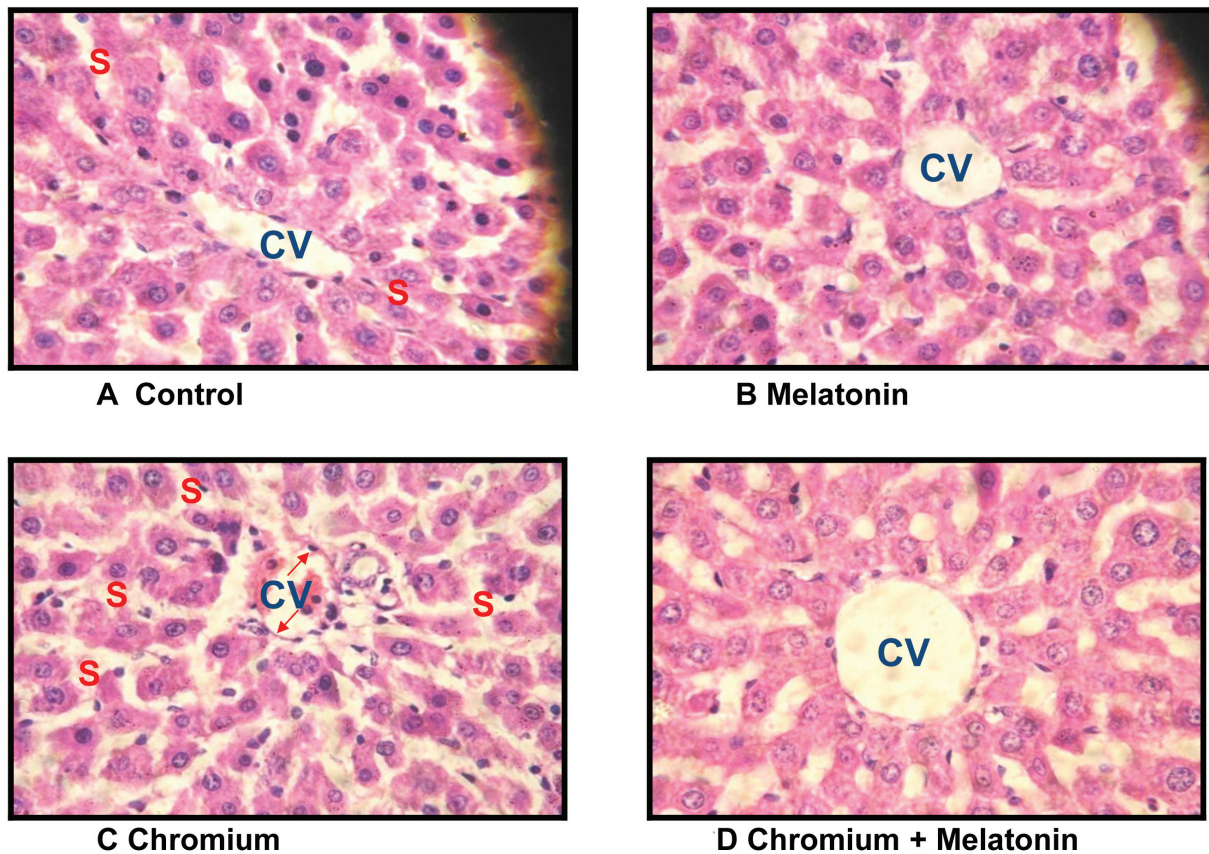
Figure 6. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10mg/Kg/BW) for 30 days of treatment. (A)Photomicrograph of control liver showing a hepatic lobule. (B) Photomicrograph of melatonin treated liver. (C) Photomicrograph of chromium exposed liver (D) Photomicrograph of liver exposed to Cr+Mel. Note the cord like organization of hepatocytes and a central vein(CV). CV – Central Vein; S – Sinusoid; endothelial rupture of central vein (arrows).

liver under increasing and persistent Cr induced stress would be generating NADPH by operation of the HMP shunt pathway. A similarly increased mRNA level of NADPH was seen in cisplatin induced oxidative stress in hepatic tissue (Palipoch *et al.,* 2014; Arivarasa *et al.,* 2008). 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 at 15 days suggests the robustness of the redox machinery of the 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 facilitating reduction of Cr (VI) to less toxic Cr (III) (Bradberry & 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 organization of the hepatic cords with disruption of

the endothelial lining of sinusoids and central vein and the 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 60-day liver sections indicating 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 the present study given through drinking water for a protracted period of 120 days, reported varying degrees of parenchymal cell vacuolization with many hepatocytes depicting ballooned appearance with karyolysis. Dilation and congestion of the centrilobular vein, dilated sinusoids containing erythrocytes and the portal area showing fibrosis and biliary duct proliferation were some of the other observations of the study. The present study of a
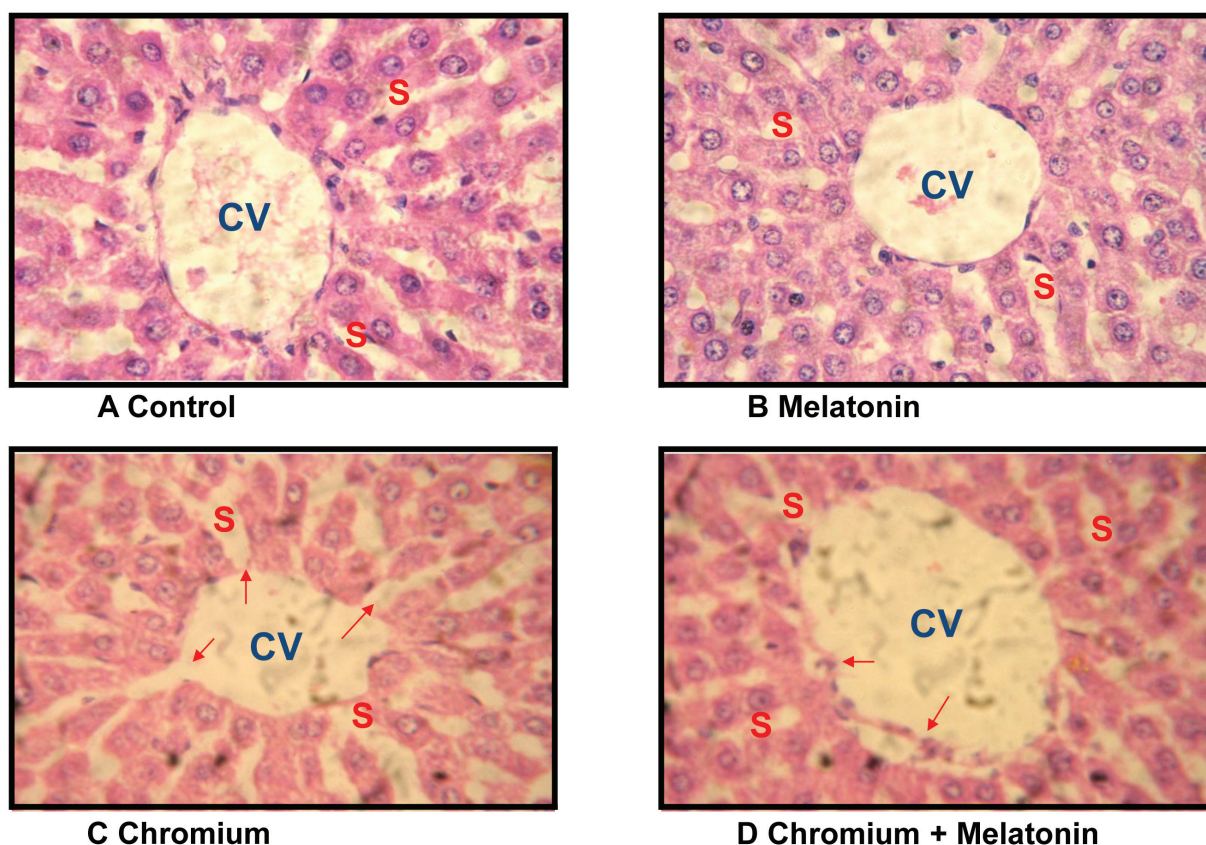
Figure 7. Effect of chromium (20 mg/kg/b.w.) exposure and simultaneous administration of melatonin (10 mg/kg/b.w.) for 60 days of treatment. (A)Photomicrograph of control liver showing a hepatic lobule. (B) Photomicrograph of melatonin treated liver. (C) Photomicrograph of chromium exposed liver (D) Photomicrograph of liver exposed to Cr+Mel. Note the cord like organization of hepatocytes and a central vein(CV). CV – Central Vein; S – Sinusoid; endothelial rupture of central vein (arrows)

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 evidence 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 apoptotic deficient/tumor suppressor gene p53 deficient mice and this has led to the suggestion that Cr (VI) induced DNA fragmentation and apoptosis may be modulated through p53 (Bagchi *et al.,* 2001, 2002). 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 showed 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.,* 2000, 2001, Rodriguez *et al.* 2004).

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 the duration dependent significant decline in serum melatonin titer. 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. 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 a potent stimulator of insulin action and glucose metabolism, the present study has tried to investigate serum levels of insulin, glucose and lipids. While melatonin administration alone was found to have a favorable effect on serum parameters, Cr was found to have hypocholesterolemic and hypotriglycerdemic effects. Though Cr (III) is known to be favorable 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 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.

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