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"An *In vivo* and *In vitro* Study on Multiple Metal Toxicity and the Protective Effect of Melatonin on Organ Specific Effects"

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An *In vivo* and *In vitro* Study on Multiple Metal Toxicity and the Protective Effect of Melatonin on Organ Specific Effects

Recently great attention has been paid to not only to the effects of toxic xenobiotics but also to their interactions with one another or with dietary factors (Brzoska *et al.*, 2000). It has been known that uptake accumulation and toxicity of xenobiotics can be modified (potentiated or reduced) by dietary factors (Grosicki and Domanska, 1997; Brzoska and Moniuszko-Jakoniuk, 1998). Amongst various xenobiotics, a growing body of evidence indicates that transition metals act as catalysts in the oxidative deterioration of biological macromolecules, and therefore, the toxicities associated with these metals may be due at least in part to oxidative tissue damage. Recent studies have shown that heavy metals exhibit the ability to produce ROS (reactive oxygen species), resulting in lipid peroxidation, DNA damage, depletion of enzymic and non enzymic antioxidants (Stohs and Bagchi, 1995). Hepatotoxicity and nephrotoxicity are common toxic manifestations of heavy metals. Hence interactive studies involving heavy metals are of special relevance and in this context. To this end, based on the industrial profile of Vadodara, three metals viz. nickel, cadmium and chromium were chosen for the study.

STUDY BACKGROUND:

Vadodara (22°17''59') North Latitude, (73°15''18') East Longitude is the chemical hub of Gujarat with diverse nature of industries. A revelation of the diverse nature of chemicals used in these industries stems from the fact that the industrial effluent contains about 50 different hazardous chemicals. Interestingly, because of the diversity these industries produce, even the effluents are of diverge chemical nature and composition. Industrial effluents from all these

factories are collected into a common effluent channel and are ultimately discharged into the Gulf of Cambay. This Baroda effluent channel, 86 KM long is also Asia's second longest one. A sizable density of populace from the vadodara city consumes vegetables irrigated from the water of Baroda effluent channel. Earlier study conducted in the same laboratory has quantified the heavy metal content in these cereals and vegetables which exceeded the WHO recommended maximum permissible limit of metals (especially nickel, cadmium and chromium) by 3 to 20 times (Ramachandran, 2003). This can be potentially dangerous for subjects having dietary dependence on these food sources. In accordance to the healthcare scenario of the geographic zone, a realistic dosage schedule was employed in the *in vivo* studies whereas *in vitro* studies were designed to have mechanistic insight into the toxicity of metals.

There are number of reports regarding the usage of melatonin as a protectant under experimental conditions of metal toxicity essentially due to its potent antioxidant property. In contrast to many other antioxidants, melatonin crosses all morpho physiological barriers, i.e. the blood brain barrier and the placenta, and is distributed throughout all cells (Rodriguez *et al.*, 2004). It is both water as well as lipid soluble. The metabolites of melatonin are by themselves potent antioxidants. Upon oxidation, melatonin converts to a number of antioxidant compounds such as cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine and N1-acetyl-5-methoxykynuramine. Therefore, melatonin is considered to be a versatile antioxidant that is more powerful than glutathione in neutralizing free radicals and that can protect cell membranes from oxidative damage more effectively than other antioxidants (Reiter *et al.*, 1997). Melatonin has the capability of scavenging both oxygen- and nitrogen-based reactants, including those formed from peroxynitrite, and

blocking transcriptional factors, which induce proinflammatory cytokines. In addition melatonin is reported to protect against pro –oxidant enzymes and reduces lipid peroxidation in distinct membranes induced by the hydroxyl and ascorbyl radicals and by peroxynitrite (Teixeira *et al.*, 2003). In view of these background information, melatonin was selected as a protectant against heavy metal induced hepatotoxicity and nephrotoxicity. Protective effect of melatonin (10 mg/kg) was also assessed by simultaneous administration with respective metals.

The present study addresses the following issues in male *Wistar* rats subjected to chromium (20 mg / Kg BW), cadmium (9 mg / Kg BW) and nickel (200 mg / Kg BW) exposure, either singly or in combination in male *Wistar* rats:

- Temporal changes in tissue accumulation of metals, degree of lipid peroxidation, level of enzymic and non enzymic antioxidants.
- Serum marker enzymes of hepatic and renal dysfunction.
- Marker(s) of apoptosis.
- Tissue burden of metals.
- Histopathological alterations.
- *In vitro* studies using normal and immortalized hepatocytes.

The major findings in Liver and Kidney are summarized in the subsequent sections:

LIVER (*in vivo* studies)

Chromium:

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 (15, 30 and 60 days). 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).

Cadmium:

In cadmium treated animals, hepatic oxidative stress was 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.

Nickel:

Toxic exposure to nickel for 15, 30 and 60 days have revealed significantly elevated LPO at the short duration period of exposure, which tended to decrease on prolonged exposure. There was steady progressive depletion in GSH and Vit C contents with increased inhibition of CAT and GPx. Maximal inhibition was seen at 15 days followed by decreased inhibition by 30 and 60 days. Maximal hepatic Ni load was seen at 15 days which tended to decrease by 30 and 60 days of exposure. Nickel exposure lead to hypoinsulinemia and an overall increase in blood glucose level with increased RBC count, hemoglobin content and packed cell volume. Cytotoxicity is clearly indicated by the histopathological manifestations seen in the hepatic tissue during all the treatment periods. The cytotoxic manifestations indicating Ni induced hepatotoxicity find biochemical co-relation in the recorded increase in serum ALP and ALT, marker enzymes of hepatic damage. Melatonin showed

significant protection against LPO and endogenous levels of antioxidants in Ni intoxicated animals when co-administered. Overall, the present study suggests a duration dependent Ni induced oxidative stress and the ability of hepatic tissue to activate counteractive measures to minimize the same on prolonged exposure to the metal. Melatonin was very effective in combating Ni induced oxidative stress ad hence worthy of consideration as a therapeutic/protective agent against nickel toxicity.

Trimetallic exposure:

The study did not reveal any compounding effect of the metals when present together in terms of hepatic oxidative stress. The TM mixture shows greatest degree of LPO at the short exposure period of 15 days with gradual but significantly decreasing degree of LPO at the medium and long durations of exposure. Persistence of oxidative stress during continued exposure to the TM mixture is clearly indicated by the decreased contents of non-enzymatic antioxidants (GSH and Vit C) and activity levels of enzymatic antioxidants (SOD, CAT, GPx). The hepatic tissue is overwhelmed by a sudden induction of oxidative stress in the initial period and is indicative of activation of the detoxification mechanism and/or effective commissioning of adaptive/protective mechanisms. This feature was seen even with exposure to individual metals in the TM mixture. Oxidative stress parameters do suggest some interactive effects of Cd, Cr and Ni. Though the TM mixture seems to have an antagonistic interaction with the evaluated oxidative stress parameters showing an intermediate level between Cd, Cr and Ni or Cd, Ni and Cr. **The TM mixture seems to exert greater cytotoxic effects.** The additive or synergistic cytotoxic effect of the three metals is clearly confirmed by the serum levels of ALT and ALP, both of which are significantly elevated and more in comparison to serum levels seen when exposed to individual metals. The TM mixture had a paradoxical effect on glycemic status

which tended to be hypoglycemic as against hyperglycemic effect of the metals individually. Melatonin seems to have a potent ability in combating and protecting hepatic tissue against oxidative stress and also protect against cytotoxic effects of metals to near normal histoarchitecture of hepatic tissue. Overall, the present study throws up certain intriguing interactive effects of Cr, Cd and Ni, such as antagonistic and/or additive or even neutralizing effects on hepatic oxidative stress, altered glycemic dysregulation as well as synergistic cumulative potentiating effect on histoarchitectural organization. Melatonin has proved to be an effective agent in maintaining the structural integrity of hepatic tissue, in minimizing the oxidative stress and in normalizing glycemic dysregulation brought about by the interactive effects of Cr, Cd and Ni.

***In vitro* studies:**

A *in vitro* study using two human liver cell lines namely HepG2 (cancerous) and Chang (non-cancerous) was conducted to assess the cytolethality, ROS generation and, Caspase 3 activity to monitor the apoptotic pathway in presence of Cd, Cr, and Ni individually or in combination with or without melatonin. Cytolethality was essessed in both the cell lines following incubation for 3, 6, 12 and 24 hrs with Cd, Cr, or Ni individually as well as combination with or without melatonin. Cadmium and Chromium showed maximum cytolethality followed by the TM mixture and Ni at all the time periods. Maximum cytolethality was seen in the 100μM concentration of metals individually or in combination. ROS generation was seen at 12 and 24 hrs of incubation with the maximum concentration of metals. At 12 hr of incubation, Cd showed maximum ROS generation while at 24 hrs, Cr showed the maximum. Caspase 3 activity was seen to be maximally induced by Cd in both the cell lines followed by Cr, TM mixture and Ni in that order. Simultaneous administration of

melatonin significantly reduced the cytolethality induced by the metals individually as well as in combination. Melatonin is a known scavenger of free radicals and hence in its presence, the ROS generation was found to be reduced to a significant level. Melatonin had a positive effect on caspase 3 in lowering its activity in presence of the metals individually as well as a mixture. The present study has shown differential mechanism of cytolethality (apoptosis/necrosis) by Cd, Cr and Ni alone or in combination and the protective role of melatonin as an antioxidant and anti-apoptotic agent stands clearly established.

Overall trend in hepatotoxicity: Cr > Cd > TM > Ni

***in vitro* studies:** HepG2 (cancerous) > Chang (non-cancerous)

KIDNEY (*in vivo* studies)

Chromium:

Development of oxidative stress, marked by significantly high levels of lipid peroxidation (LPO) and decreased contents of non-enzymatic (GSH and AA) as well as enzymatic antioxidants (SOD, CAT, GPx) was the feature in Cr (VI) exposed animals. The degree of LPO progressively increased from 15 to 60 days. The progressive increase in LPO was well paralleled by the concurrent increase in renal metal load. A steady decrease in the levels of GSH and AA together with decreased activity levels of enzymatic antioxidants has been noted. The duration dependent increase in LPO suggests the lack of any adaptive/protective mechanism to control the oxidative stress. The histopathological alterations affecting the integrity and functioning of malphigian tubules and proximal tubules are indicative of cytological lesions due to Cr (VI) exposure. Dysfunctional renal tubules and other renal damages are well reflected in the significantly elevated serum levels of urea and creatinine.

Hematological alterations were marked by decreased leucocyte count mainly due to change in polymorph/neutrophil number. It is interesting to note that all alterations induced by chronic Cr exposure including oxidative stress, lipid peroxidation, structural lesions in relation to renal response and even the reduced neutrophil count in the blood were all checked substantially on co-administration with melatonin.

Cadmium:

Decreased content of non-enzymatic antioxidants (GSH and Vit C) and activity of enzymatic antioxidants (SOD, CAT, GPx) with markedly increased LPO levels are indicative of Cd induced renal oxidative stress. The duration dependent increasing level of LPO suggests absence of any adaptive/protective mechanism. The gradual increase in LPO can be co-related with the parallel increase in the renal metal load through 30 to 60 days. For, the all three periods of Cd exposure, there is a gradual depletion of GSH and Vit C along with decreased activities of enzymatic antioxidants. Clearly, the renal tissue shows greater Cd toxicity on longer duration of exposure. Progressive degeneration/shrinkage of the glomerulus, vacuolization, hemorrhage and swelling of tubules and, proximal tubule cellular damage were the cytotoxicity features induced by Cd treatment. The histopathological alterations were maximally seen at 60 days of Cd exposure. Cadmium induced fall in leucocyte count, significantly reduced platelet count and hypertriglyceridemia ware also evident. All these changes were brought to a near normal state with co-administration of melatonin. Overall, the present study suggests a duration dependent effect of Cd on increased renal oxidative stress and cytotoxicity with melatonin being very effective in counteracting the negative effects of cadmium.

Nickel:

Renal oxidative stress and lipid peroxidation due to metal toxicity have shown progressive increase with prolonging duration of Ni exposure, well paralleled by temporally increasing renal Ni load. Elevation in LPO and heightened oxidative stress were marked by equally potent depletion of GSH and Vit C and inhibition of SOD, CAT and GPx. Nickel induced renal cytotoxic manifestations involving renal tubular damage are marked by structural alterations. The overall structural disorganization marked by these changes is accreditable to increasing oxidative stress generated by Ni. Nickel intoxication has shown a tendency for hypertriglyceridemia and hypercholesterolemia with decreased HDL and increased LDL and VLDL cholesterol. Exposure to Ni resulted in subtle hematological alterations marked by decreased leukocyte count, essentially corelatable with lymphocyte number, and a transient increase in platelet count at 15 days followed by decrease during longer periods. Melatonin was able to counter the effects of Ni on renal LPO, oxidative stress as well as histopathological alterations when used as a protectant. It was able to significantly minimize the Ni induced effects on many fronts and even normalize certain parameters. Overall, the present study has revealed significant Ni induced renal LPO and oxidative stress along with structural changes and hematological modulations on exposure to Ni. Melatonin was successful in negating most of the major Ni induced toxic manifestions.

Trimetallic Exposure:

Increasing oxidative stress marked by progressively increasing LPO and decreasing concentrations and activities of non-enzymatic and enzymatic antioxidants respectively with interactive alterations are the principal observations made in this study. The progressively decreasing level of both ascorbate and GSH together with LPO tends to project a picture of increasing renal oxidative stress due to exposure to

the TM mixture. The deleterious effects manifested principally are, disruption in the continuity of both parietal and visceral epithelial lining the Bowman's capsule, glomerular shrinkage and disintegrity with loss of podocytes and proximal tubular damage marked by necrosis/apoptosis of the absorptive epithelium when exposed to the TM mixture. The present study showed differential degree and pattern of accumulation of metals when given in combination or individually. Subtle alteration in lipid metabolism is indicated by the recorded decrease in serum triglyceride and cholesterol levels. Hematological change marked by increase in total leucocyte count is essentially due to a significant increase in polymorphs and a minor increase in lymphocyte number. The observed decrease in platelet count suggests a hypercoagulatory state induced by the TM mixture. Melatonin was potent in preventing the adverse effects of metal toxicity even in a situation of exposure to multiple metals as in the case of exposure to individual metals. Overall, the present study clearly highlights differential interactive effects of Cr, Cd and Ni on renal oxidative stress, cytotoxicity and hematological and metabolic aspects and, indicates that melatonin can be used as an effective therapeutic agent against metal induced oxidative stress and cytotoxicity.

Overall trend in nephrotoxicity: Cd > Cr > TM > Ni

OVERALL CONCLUSIONS:

1. Chromium seems to the potent inducer of hepatoxicity. Overall the observed trend in liver is of the following order:

   Cr > Cd > TM > Ni

2. Down regulation of antioxidant defense is the common mechanistic basis behind the detrimental changes and this is well correlated with the corresponding histopathological alterations in both liver and kidney.

3. There is pronounced adaptive response observed at the early stage of metal exposure (i.e. at 15 day treatment schedule) for all the metals administered either singly or in combination.
4. On the other hand, the maximum detrimental effect in the kidney is due to cadmium with the following trend:

   Cd > Cr > TM > Ni

5. The duration dependent increasing level of LPO in the kidney of cadmium treated animals suggest absence of any adaptive / protective mechanism.

6. Results from *in vitro* studies indicate greater sensitivity of HepG2 (cancerous) cell line when compared with Chang (non-cancerous) cell line. Cadmium induced apoptosis was evident in both cell lines.
7. Melatonin was able to alleviate the symptoms of oxyradical changes in both tissues under all conditions of metal exposure.
8. Unlike reported prooxidant effects of conventional antioxidants (vitamin C and Vitamin E) which when administered in large amount, there is any available report on melatonin toxicity even at extra supraphysiological dose. This makes it a potent antioxidant and it is worthy to try combination of melatonin with other antioxidants as a therapeutic alternative in cases repeated of low dose metal exposure.

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