CHAPTER 9

Chapter 9

Induction of cytolethality and generation of ROS by Cadmium, Chromium and Nickel alone or in combination: *In vitro* studies on HepG2 and Chang liver cells and protective effect of melatonin.

Cadmium (Cd), Chromium (Cr) and Nickel (Ni) have been identified as the three most abundant heavy metals in the environment in soil and, cereals and vegetables grown in and around Vadodara (Ramachandran, 2003). Due to their abundant presence in cereals and vegetables, systemic entry into humans through food and water is unavoidable. All the three metals shown to have toxic effects in different tissues of experimental animals, primarily by the generation of oxidative stress and/or alterations in endogenous antioxidants (Chapters 1-8). Besides the above, the hepatic and renal toxicities of these metals can result in apoptosis, cancer and even other functional disorders. Since any experimental toxicological evaluations have to be of human relevance in terms of dose and route of administration, realistic dosages of all these three metals have been employed in the previous studies. Such studies involving realistic dosages of Cd, Cr and Ni administration through drinking water singly or in combination (as human consumption of these metal containing food ingredients lead to exposure to all three), have recorded extensive induction of oxidative stress and histopathological lesions in both liver and kidney and even subtle but differential interactive effects of the metals (Chapters 1-8).

All the three metals (Cr, Cd and Ni) have been considered potent hepatotoxicants causing hepatic dysfunctioning and histopathological lesions. Though the cause or

effect relation between hepatic and oxidative stress is not clear, damages caused by generation of oxidative stress leading to hepatic dysfunctioning is considered more feasible. However, even Cr, Cd and Ni are also reportedly weakly carcinogenic in that order. Toxic manifestations of all the three metals also involve modulation of apoptosis, very much associated with the process of carcinogenesis, to varying degrees with Cr and Cd being more apoptotic (in that order) than Ni.

Previous *in vivo* studies have releaved both hepatic and renal cytotoxicity of all the three metals with characteristic histopathological alterations including cell death (apoptotic/necrotic) (chapters 1-8). Since these studies have shown significant generation of oxidative stress, the possibility of oxidative stress mediated DNA damage/apoptosis cannot be overruled as a possible mechanism of metal induced cytotoxicity. It is in this context, the present *in vitro* evaluation has been undertaken on two different cell lines (HepG2-cancinogenic and Chang-normal cell line). The response of the cells towards Cd, Cr or Ni individually or in combination has been in terms of cell viability, total ROS generation and Caspase 3 activity. The *in vitro* data is being complemented with *in vivo* assay of caspase 3 and metallothionein (MT).

Material and Methods:

Chemicals: Metal salts, Chromium trioxide (CrO_3), Cadmium chloride ($CdCl_2$) and Nickel chloride ($NiCl_2$) were purchased from Qualigens (India). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was obtained from SIGMA Chemicals (St.Louis, MO), 6-Carboxy-2',7'-chlorofluorescin diacetate (DCFH-DA) was obtained from FLUKA, Minimal Essential Medium (MEM), trypsin-EDTA and Streptomycin and Penicillin were obtained from Sigma (St. Louis, MO), Fetal Bovine Serum (FBS) was obtained from HiMedia (Mumbai, India).

Cell lines and culture conditions: A human hepatoma cell line (HepG2) and a normal Chang cell line were used in the study. Both the cell lines were obtained from NCCS (National Centre for Cell Sciences, Pune, India). The cultures were grown in tissue culture flasks with 75 cm^2 surface area in MEM supplemented with 10% FBS and 1% streptomycin and penicillin. The cells were incubated for 24 hrs at 37° C in a 5% CO_2 atmosphere in a CO_2 incubator and the cells were allowed to grow, and form a monolayer in the flask. Cells grown to 80-90% confluency were washed with phosphate buffer saline, trypsinized with 3ml of trypsin-EDTA, diluted and counted and seeded (5x 10^5 cells/well) in 96 well plates.

Treatment protocol: Cells were seeded in 96 well microtiter plates (Nunc) at a cell density of 5x 10^5 cells/well in unaltered media. After 24 hrs, the media were replaced with media containing various concentrations of metals alone or in combination with or without melatonin (0,1,10 and 100 μM). Cells were exposed to the metals for

designated periods of time (3, 6, 12 and 24 hrs). At the end of the designated time period, cells were used for assaying cell viability and ROS generation.

Measurement of Cytolethality: The effect of the metals individually or in combination with or without melatonin, on cell viability was assessed by an MTT assay, which measures viable cells by assessing metabolic integrity. Mitochondrial function was evaluated spectrophotometrically by measuring the degree of mitochondrial reduction of tetrazolium bromide to formazan by succinic dehydrogenase (Carmihael *et al.,* 1987). The absorbance was read at a wavelength of 550nm using microtiter plate reader (Bio-Tek instruments Inc).

Reactive Oxygen Species (ROS) Detection: ROS generation was determined using dichlorodihydrofluorescein diacetate (Wang and Joseph, 1999). The fluorescence of the samples was monitored at an excitation wavelength of 485 nm and an emission wavelength of 538 nm in a flurospectrophotometer (Jasco, Japan).

Caspace 3 Activity: Caspase 3 activity was measured using Caspase 3 colorimetric kit by R&D systems, Inc.

Results:

Effect of Melatonin on viability Test: Viability test for two different concentration of melatonin (10μM and 100μM) was tested on HepG2 and Chang liver cell lines. The lower concentration of melatonin showed a better viability test in both the cell lines as shown in fig 9.1.

Effect of Cd on viability test: The effect of different concentrations of Cd with different incubation time on HepG2 and Chang cell lines are shown in fig 9.2(A) and 9.2(B) respectively. In both the cell lines the highest concentration of Cd (100μM) showed least viability. Minimum viability was seen in the 24 hrs of exposure while the Chang cell line showed more resistance to Cd than the HepG2 cell line. Melatonin did protect both the cell lines at all the time intervals.

Effect of Ni on viability test: The effect of different concentrations of Ni with different incubation times on HepG2 and Chang cell lines are shown in fig 9.3(A) and 9.3(B) respectively. Maximum viability was seen at 1 μM concentration and incubation time of 3 hours in both the cell lines while, minimum viability was seen with 100 μM of concentration and 24 hrs of incubation. Nickel showed the least cytotoxicity in both the cell lines. Chang cell line showed more resistance to Ni toxicity as compared to HepG2 cell line. Melatonin was able to protect both the cells at all concentrations and in at time intervals. Both the cell lines showed dose and duration dependent effect with Ni.

Effect of Cr on viability test: The effect of different concentrations of Cr with different time periods of exposure to HepG2 and Chang cell lines are shown in fig

9.4(A) and 9.4(B) respectively. Chromium showed minimum viability at the highest concentration. The incubation period of 24 hrs of Cr was the most cytotoxic with minimum viability of both the cell lines. Chromium showed both dose and duration dependent effect on both the cell lines with the Chang cells being more resistant than HepG 2 cell line. The cytotoxicity of Cr was reduced in both the cell lines when co-incubated with melatonin. Melatonin showed remarkable protection against cytotoxicity induced by Cr in both the cell lines.

Effect of Trimetallic (TM) mixture on viability test: The effect of different concentrations of TM mixture with different time periods of exposure to HepG2 and Chang cell lines are shown in fig 9.5(A) and 9.5(B) respectively. At the highest concentration, minimum viability was seen in both the cell lines. Maximum cytotoxicity was seen at 24 hrs of incubation, with Chang cell line showing less cytotoxicity than HepG2 cell line. There was no additive effect of the individual metals and the viability of the cell lines was more than Cr and Cd individually at all the time intervals. Melatonin showed marked protection against cytotoxicity of the cells when incubated with TM mixture. Both the cell lines showed dose and duration dependent effect of TM mixture.

Reactive Oxygen Species (ROS) generation in HepG2 cell line: The generation of ROS in HepG2 cell line following 12 and 24 hrs of incubation with different concentrations of metals individually as well as in combination is shown in fig 9.7(A) and 9.7(B) respectively. At 12 hrs of incubation, Cd induced maximum ROS but at 24 hrs of incubation Cr showed maximum generation of ROS. The TM mixture showed less generation of ROS than Cd and Cr but more than that of Ni at both the

time intervals. Melatonin a known antioxidant is able to reduce the production of ROS at both the time intervals when incubated together with the metals.

Reactive Oxygen Species (ROS) generation in Chang cell line: The generation of ROS in Chang cells following 12 and 24 hrs of incubation with different concentrations of metals individually as well as in combination are shown in fig 9.8(A) and 9.8(B) respectively. After 12 hrs, TM mixture showed maximum generation ROS while at the end of 24 hrs, Cd showed maximum generation of ROS. Chang cell line showed relatively less production of ROS as compared to HepG 2 cell line. The ROS generation was decreased at the cells incubated with melatonin at both the time intervals.

Caspase 3 activity: The changes in Caspase 3 activity in HepG2 and Chang cell lines are shown in fig 9.10(A) and 9.10(B) respectively. In both the cell lines, Cd induced maximum Caspase 3 activity. The least induction of Caspase 3 as shown by Ni while, Cr showed less induction of the enzyme than Cd but more than that of Ni. TM mixture showed less induction than Cd and Cr but more than Ni. There was a marked decrease in Caspase 3 activity when the cells were treated with melatonin in presence of metals.

Fig 9.1. % viability of HepG2 and Chang treated with different Con. Of Melatonin

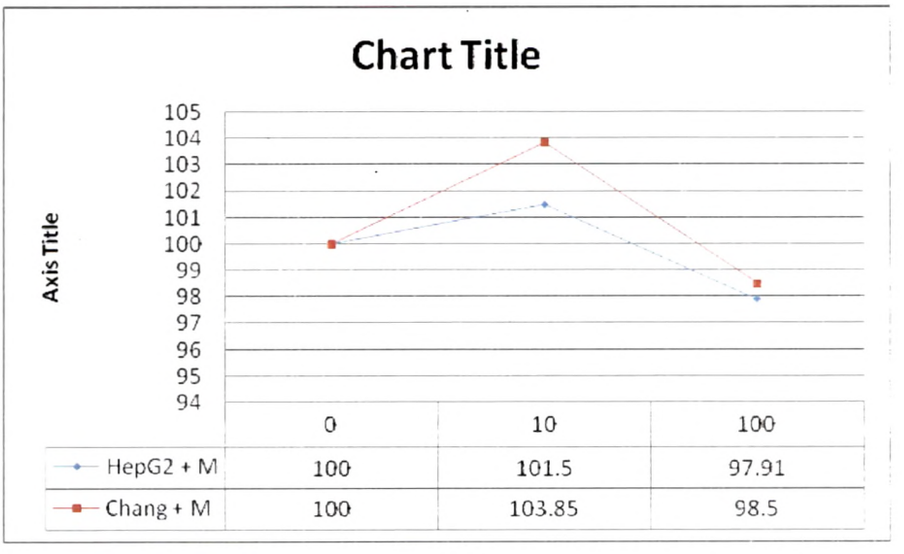


Fig 9.2(A). % viability of HepG2 cell lines following different incubation time and concentration of Cd

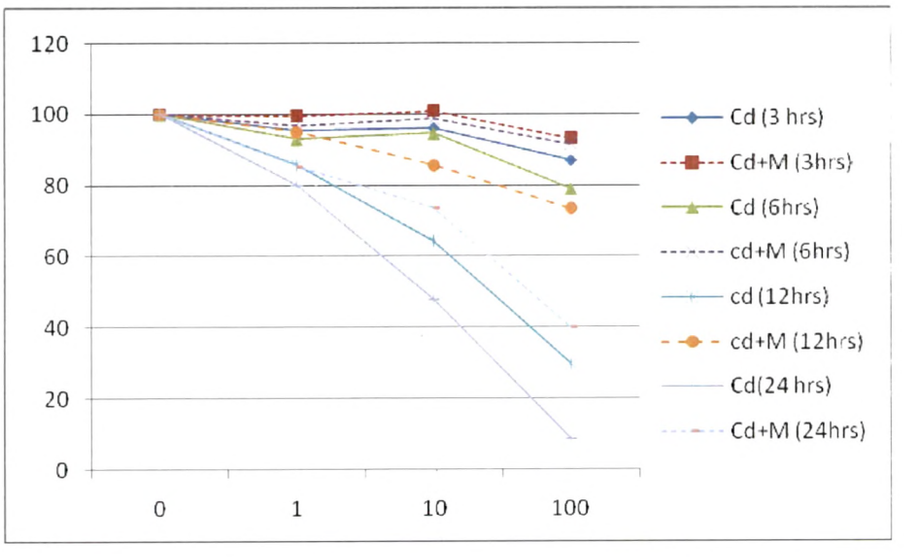


Fig. 9.2 (B) % viability of Chang cell lines following different incubation time and concentration of Cd

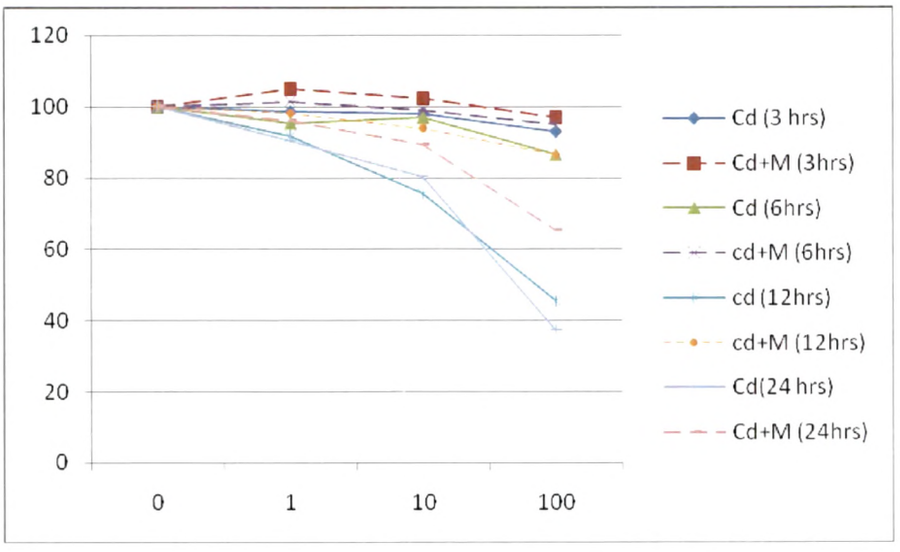


Fig 9.3(A). % viability of HepG2 cell lines following different incubation time and concentration of Ni

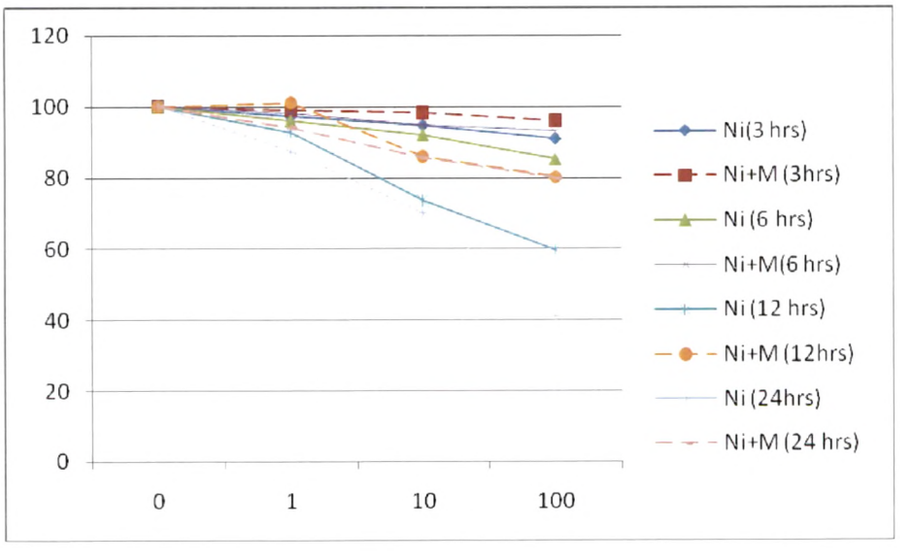


Fig 9.3(B). % viability of Chang cell lines following different incubation time and concentration of Ni

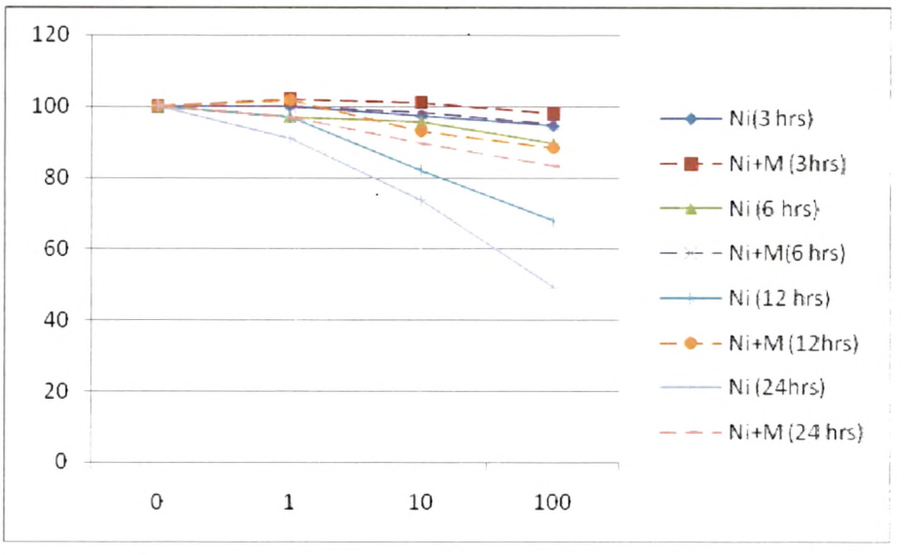


Fig 9.4(A). % viability of HepG2 cell lines following different incubation time and concentration of Cr

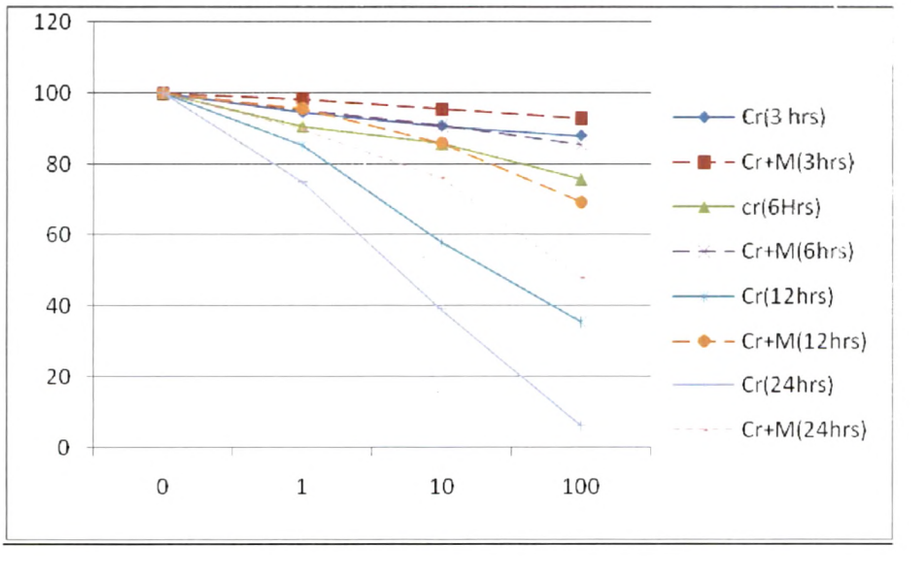


Fig 9.4(B). % viability of Chang cell lines following different incubation time and concentration of Cr

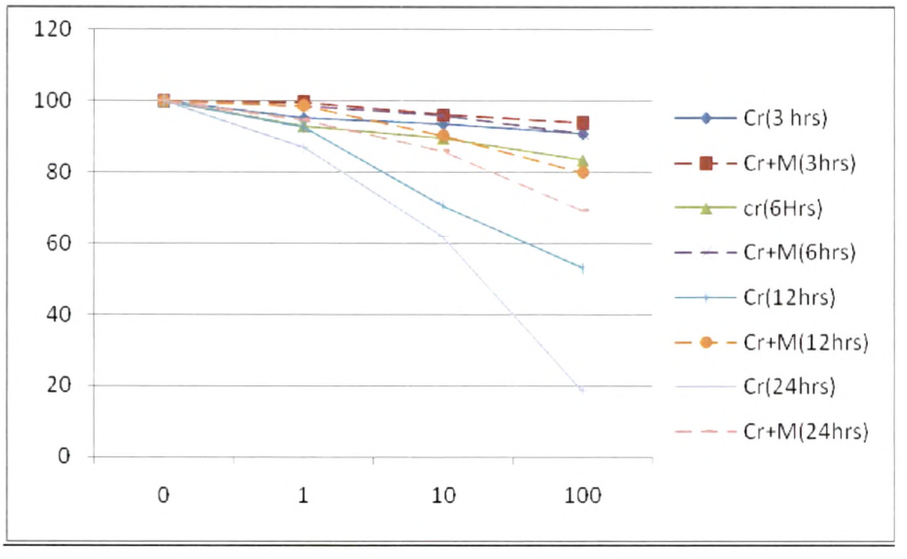


Fig 9.5(A). % viability of HepG2 cell lines following different incubation time and concentration of Trimetallic combination

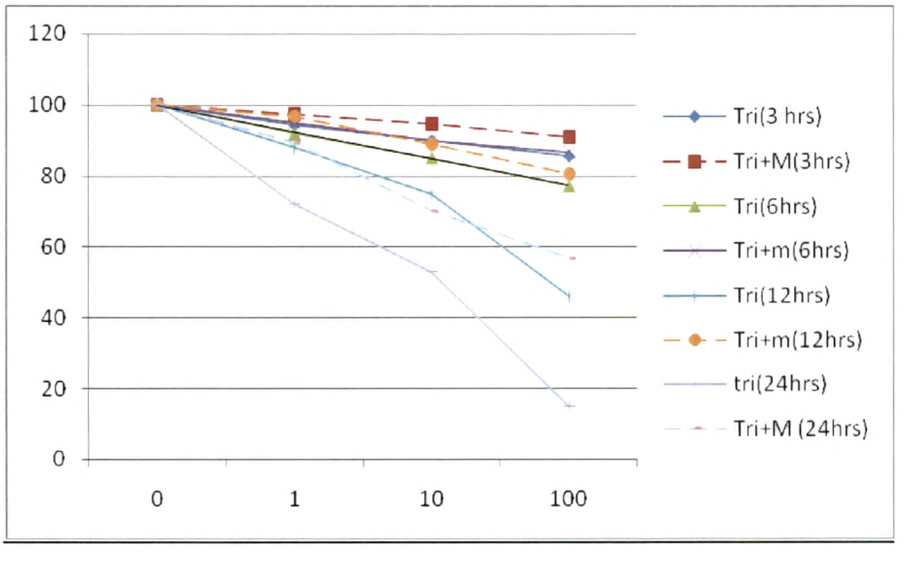


Fig 9.5(B). % viability of Chang cell lines following different incubation time and concentration of Trimetallic combination

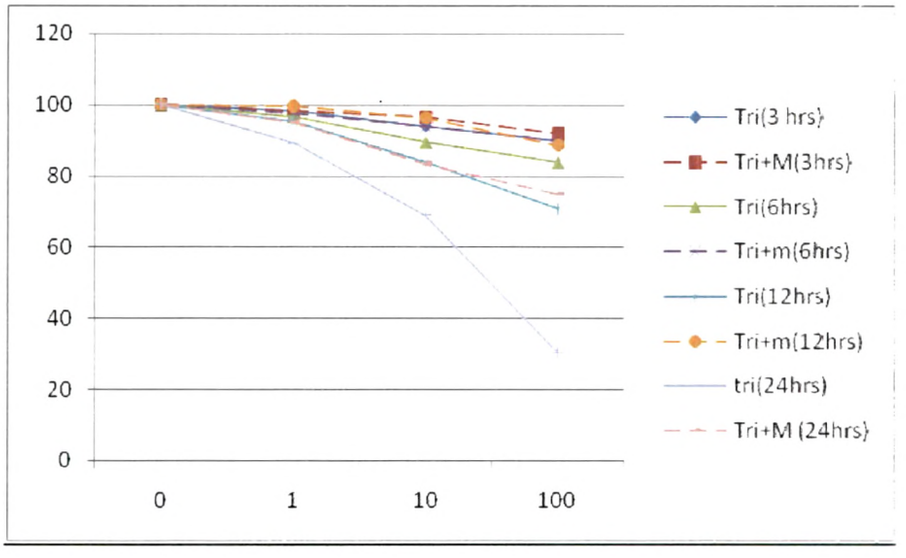


Fig9.6 (A) % cell viability after 24 hrs incubation with different metals and combination in HepG2 cell line

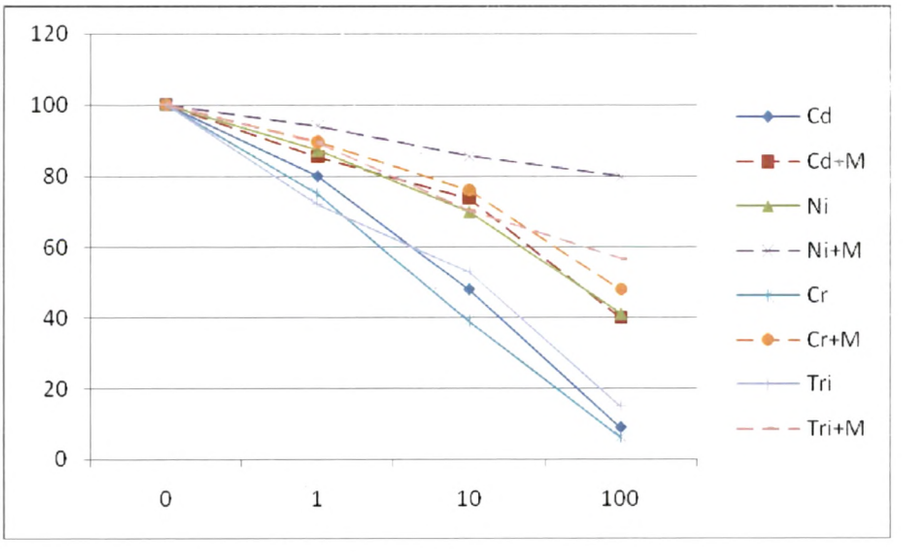


Fig9.6 (B) % cell viability after 24 hrs incubation with different metals and combination in Chang cell line

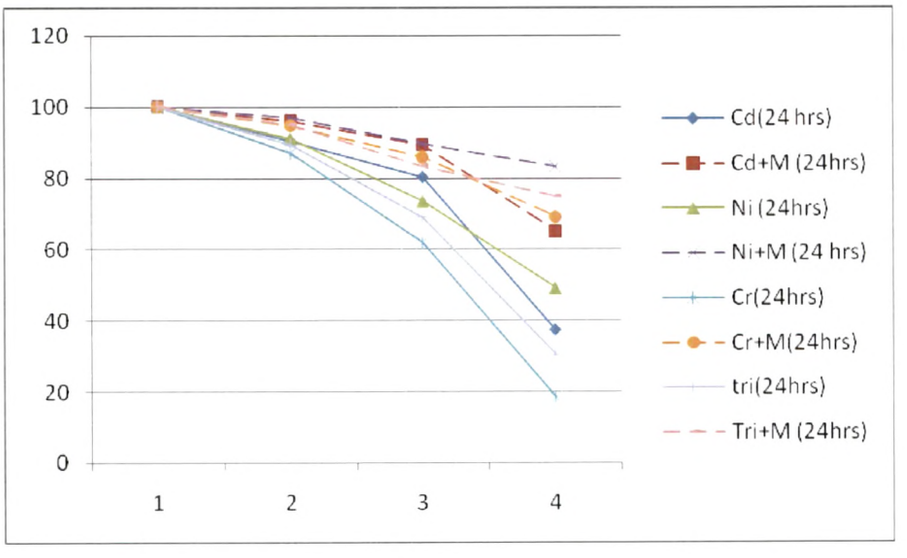


Fig 9.7 (A) ROS generation in HepG2 after 12 hrs of exposure to different metals

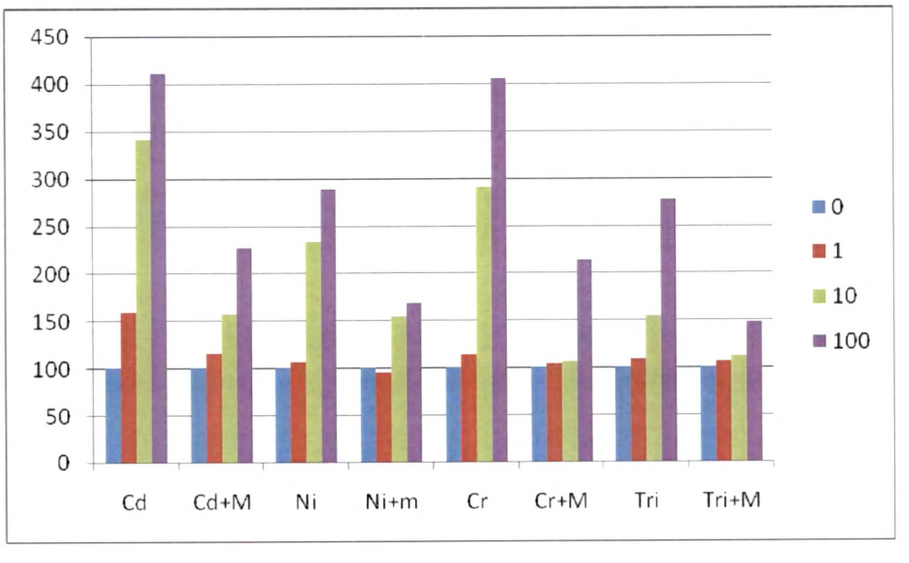


Fig 9.7 (B) ROS generation in HepG2 after 24 hrs of exposure to different metals

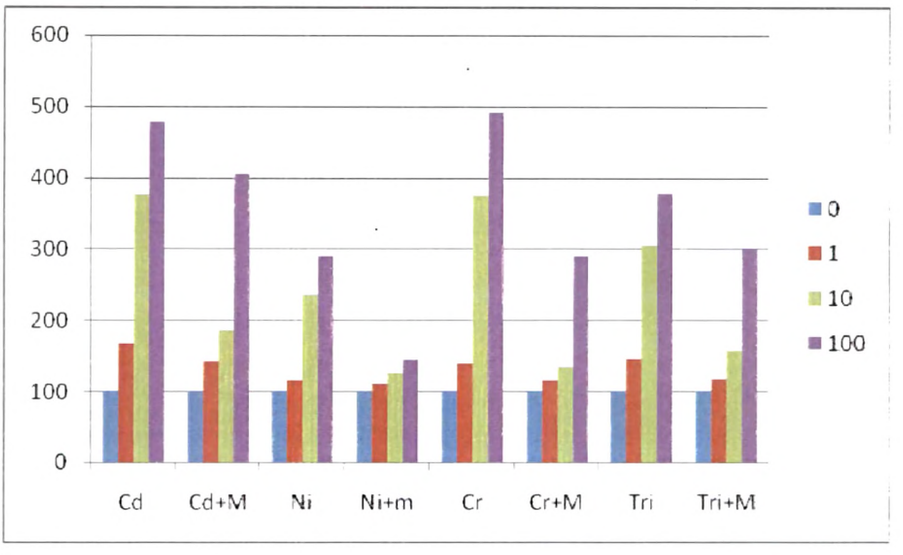


Fig 9.8 (A) ROS generation in Chang after 12 hrs of exposure to different metals

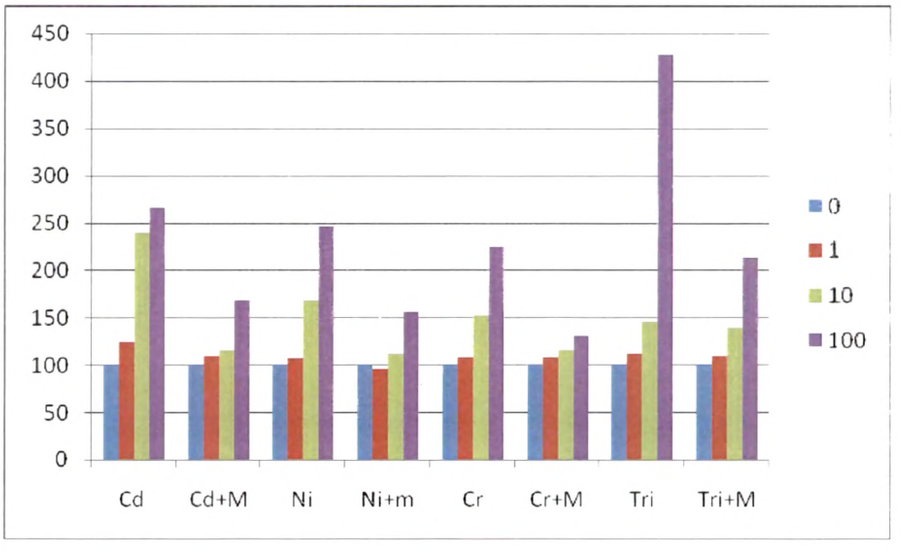


Fig 9.8 (B) ROS generation in Chang after 24 hrs of exposure to different metals

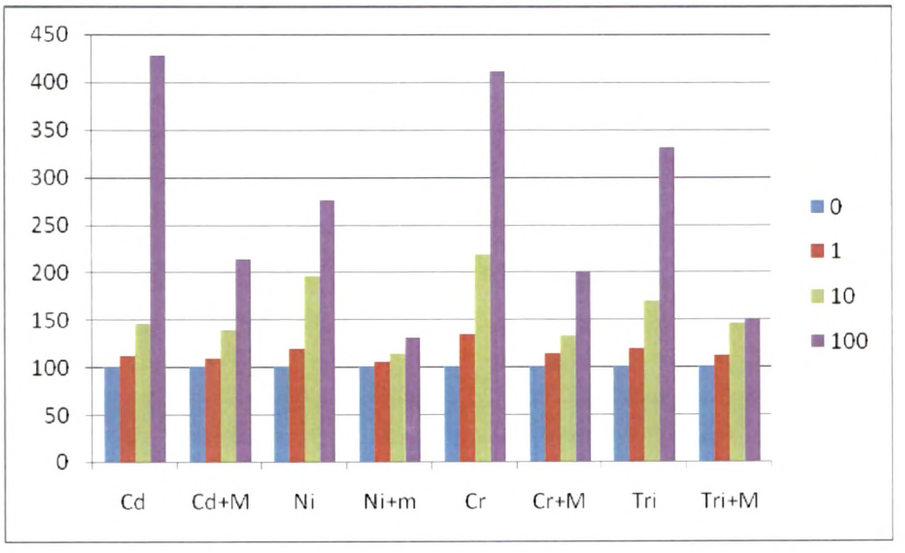


Fig 9.9 (A) ROS generation after 12 and 24 hrs with maximum concentration of metals in HepG2 cell line

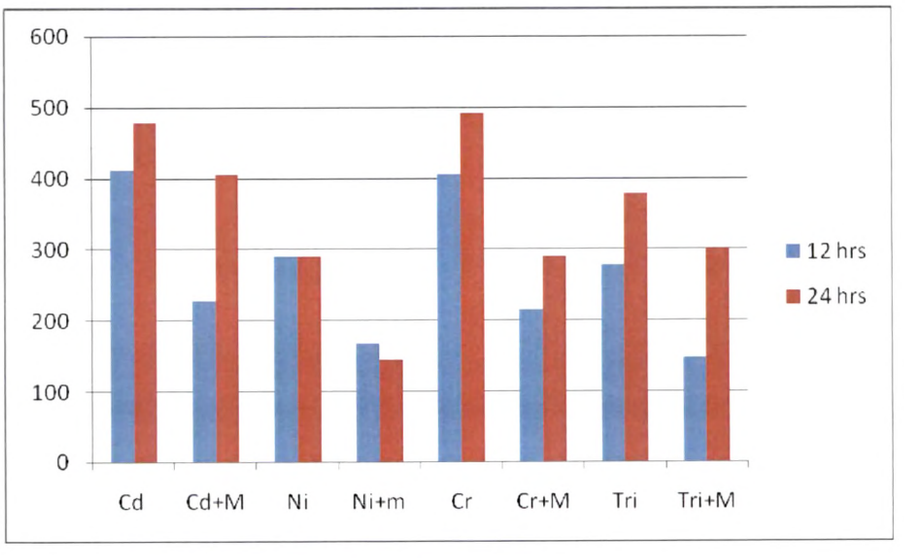


Fig 9.9 (B) ROS generation after 12 and 24 hrs with maximum concentration of metals in Chang cell line

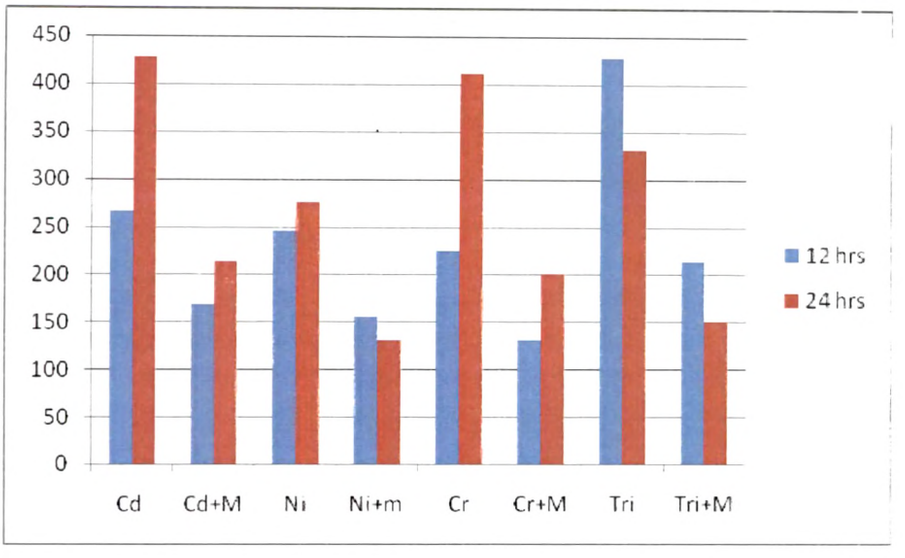


Fig 9.10 (A) % increase in the Caspase 3 activity after 24 hrs of incubation with 100µM of metals with or without melatonin in HepG2 cell line

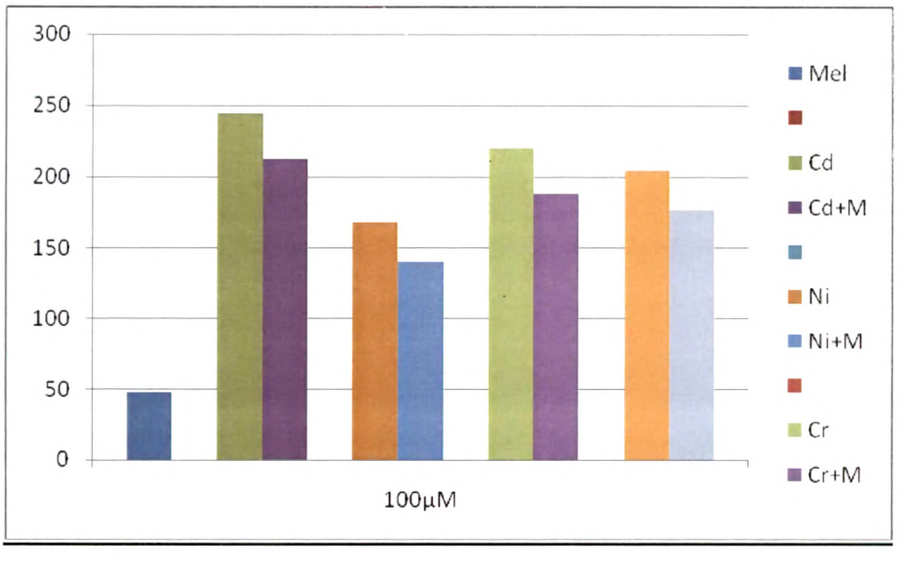
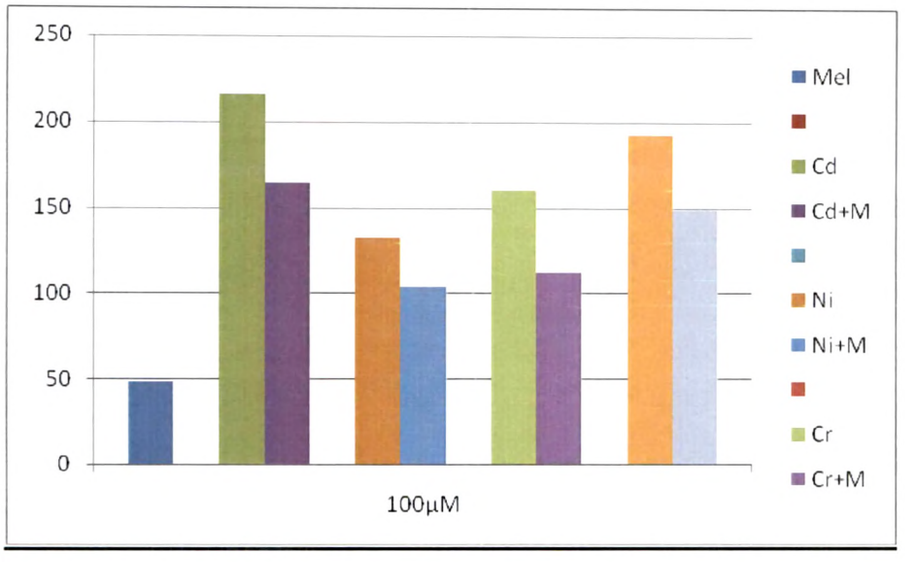


Fig 9.10 (B) % increase in the Caspase 3 activity after 24 hrs of incubation with 100µM of metals with or without melatonin in Chang cell line



Discussion:

The present *in vitro* studies using HepG2 and Chang cell lines have revealed more robust generation of ROS and greater cytolethality in HepG2 relative to Chang cells on exposure to Cd, Cr, Ni or even a combination of all the three metals Though there are dosage and duration dependent increase in ROS and cytolethality in general, nevertheless, metal specific differential degrees of responses are shown by both the cell lines. Comparatively, Cr and Cd have shown maximum cytolethality followed by the TM combination. Nickel tended to show the least cytolethality. Though both Cr and Cd showed an almost similar degree of effect on cell viability at the highest concentration, a closer look at the data reveals that Cd is maximally toxic at 12 hrs of exposure while Cr becomes more lethal than Cd at 24 hrs of exposure. Apparently, Cd is likely to be more cytotoxic during short duration of exposure while Cr might manifest greater toxicity at longer durations. Previous *in vivo* studies on duration dependent induction of oxidative stress and histopathological lesions by Cd and Cr are in ready agreement with the present inference. The greater effect of Cr on a longer duration surpassing that of Cd is very clearly indicated from the data on Chang cells compared to HepG2 cells. It is of relevance to note the fact that, while HepG2 is a cancer cell line, the Chang cell is a normal hepatic cell line. Apparently, non cancerous normal cells seem to be more sensitive to the long term toxic effect of Cr. Other *in vitro* studies involving these metals using either same or different cell lines have also obtained results quite similar to the present study (Shimoda *et al.,* 2001; Eichler *et al.,* 2006; Curtis *et al.,* 2007; Patlolla *et al.,* 2009). Interestingly, the TM combination has instead of a cumulative additive effect shown reduced degree of

cytolethality compared to Cr and Cd. This might bespeak of nullifying interactive effect of Cd and Cr or even an antagonistic effect of Ni on both Cd and Cr.

The observed reduced degree of cell viability on long duration exposure to the metals could be accredited to cell death caused by mitochondrial dysfunctioning as the MTT assay used in the present study is essentially a mitochondrial function test. The least effect of cell viability in presence of Ni could be due to its minimal cytotoxicity. Cell death due to metals indicated by the viability test could be accredited to apoptotic loss as metals have been reported to induce apoptosis. The minimal cell loss recorded for Ni in the present study in both HepG2 and Chang cells is probably due to low potency of Ni to induce apoptosis as has been reported by Holmes and Reinsford (2001) in HepG2 and JTC15 hepatic cell lines and Curtis *et al.* (2007) in isolated keratinocytes. The relatively greater cell death seen in HepG2 cells relative to Chang cells could find justification in the reported higher content of MT in the latter as compared to former (Shimoda *et al.,* 2001). The near 90% HepG2 cell loss by 24 hrs of exposure to 100μM of Cd clearly indicates activation of apoptotic pathway by Cd. The endogenous concentration of MT seem to be related with modulation of Cd induced apoptosis in a variety of circumstances; for example, Waalkes *et al.* (1991, 1993) and Shimoda *et al.* (2001) have shown Cd sensitivity to apoptosis in hepatocellular tumors and cell lines derived from them as, tumor cells are known to be down regulated for MT expression. This down regulation of MT has been clearly demonstrated in human and murine liver tumors (Onosaka *et al.,* 1996; Waalkes *et al.,* 1996; Ghoshal *et al.,* 2000). Similar poor expression of MT are seen in murine lung cancer (Waalkes *et al.,* 1991, 1993, 1996). The inherent conclusion that can be drawn from these reports is

under expression of MT and the sensitivity of such cells to Cd induced apoptosis as can be related to the present observations on HepG2 cells. As against these, the concurrently observed greater Chang cell survival under similar duration of exposure and concentration of Cd corroborate this contention as, the absolute basal and Cd induced levels of MT are both higher in the Chang cells (Shimoda *et al.,* 2001). Further correlation to Cd induced apoptosis and the protective effect of MT comes from our unpublished observations of more than 8 fold induction of MT in the hepatic tissue of rats exposed to Cd for 60 days (Mukherjee *et al.,* unpublished). Though Shimoda *et al.* (2001) had indicated Cd induced hepatotoxicity and apoptosis, the mechanism of induction of apoptosis was not elucidated. However, Eichler *et al.* (2006) have clearly elucidated the mechanism of apoptosis in renal podocytes as due to an activation of the extrinsic apopototic pathway. Their study has clearly documented a marked induction of Caspase 8 but not Caspase 9 and also accumulation of proteins involved in the extrinsic apoptotic pathway. These observations of Eichler *et al.* (2006) taken together with the presently observed maximal induction of Caspase 3 by Cd in HepG2 provide compelling evidence for the induction of extrinsic pathway of apoptosis in hepatic cell lines with relatively greater induction in HepG2 cells relative to Chang cells. Taken together, Cd can be considered to induce hepatic cell apoptosis by the activation of extrinsic pathway in the absence of an optimal critical level of MT. The induction of MT by Cd is apparently, an adaptive response to protect against Cd induced apoptosis. While excessive free Cd induced cellular apoptosis can be accredited to an activation of extrinsic pathway, Cr induced cell loss may not be related with the MT content. This

is understandable in the light that Cr is a poor inducer of MT and our *in vivo* studies on Cr exposure had hardly shown 3 fold increase in MT content from the basal level in the hepatic tissue (Mukherjee *et al.,* unpublished). Since Cr itself is not a cytotoxic agent but rather an oxygen free radical generator (Bagchi *et al.,* 2001), the observed higher cell loss on long term exposure to Cr may have to find an alternate explanation other than that suggested for Cd. Chromium is a potent generator of ROS (superoxide, hydrogen peroxide, hydroxyl radicals) and the generated ROS are capable of attacking proteins, DNA and membrane lipids thereby disrupting cellular functioning and integrity (Bagchi *et al.,* 1997; Patolla *et al.,* 2009). A consequent effect is lipid peroxidaiton (LPO) and as been shown by Bagchi *et al.* (2001) in K562 and J774 cells as well as our previous *in vivo* studies on Cr induced hepatic and renal oxidative stress (Chapters 1 and 2). The *in vivo* studies have clearly indicated the observed higher degree of hepatic and renal LPO to be due to the hydroxyl radical through Fenton/Haber-Weiss reactions catalyzed by Cr. This radical is capable of removing a hydrogen atom from methylene group of polyunsaturated fatty acids augmenting LPO. Apart form LPO, Cr induced ROS can also generate DNA damages (Blasiak and Kowalik, 2000; Devi *et al.,* 2001; Wang *et al.,* 2006; Wise *et al.,* 2008; Patolla *et al.,* 2009). Since the peroxidation of mitochondrial membrane can lead to its rupture and lead to release of its contents, an activation of an intrinsic pathway cannot be overruled. Mitochondrial damage coupled with different DNA lesions induced by Cr can contribute to a switching on, of intrinsic pathway (Takaki *et al.,* 2001). Current observation of higher induction of Caspase 3 by Cr exposure, relatively more in HepG2 cells than in Chang cells, attest to this fact. In this context,

it is worth mentioning that, toxic metals have been previously shown to activate Caspase 9 and 3, markers of intrinsic apoptotic pathway in neuroblastoma (Humphrey *et al.,* 2005) and glioma cells (Watjen *et al.,* 2002). The observations of Curtis *et al.* (2001) on the effects of Cr and Ni on human keratinocytes have tended to suggest activation of necrosis as a possible cause of cell death at high concentrations of Cr and Ni. This leads to a conclusion that Cr induced cell death may be due to both the intrinsic pathway of apoptosis on exposure to low concentration as well as activation of necrotic pathway under exposure to high concentration. More focussed studies on concentration and duration dependent effect of Cr under both *in vivo* and *in vitro* conditions are needed to validate these assumptions. In the same context, the presently observed Ni induced low level of cytotoxicity in both HepG2 and Chang cell lines suggest the relative resistance of induction of apoptosis by Ni and possible cell death by way of necrosis at higher concentration and longer durations of exposure. Resistance to Ni mediated apoptosis can also be attributed to synthesis of specific metal binding proteins as well as stress proteins (Chapter 5).

Interestingly, the TM mixture of Cd, Cr and Ni has shown relatively less cytolethality compared to Cd and Cr. From the observations on cell loss, ROS generation and Caspase 3 activity, it is clear that, the mixture of Cd, Cr and Ni induces the same extrinsic pathway of apoptosis as deduced for Cd. Similar inductions of extrinsic pathway of apoptosis by Cd, Hg and As individually as well as combination have been shown in cultured podocytes (Eichler *et al.,* 2006). Nevertheless, the observed reduced intensity of responses on exposure to the TM mixture suggests interactive effects of the metals by way of Cd and Cr inhibiting each other's responses as well as

antagonistic action of Ni on Cd and Cr mediated effects. The lesser cytotoxic effect of TM mixture can also be related to the ability of metallic mixture to induce many protective proteins including stress protein as was also inferred in the earlier studies (Eichler *et al.,* 2006; Chapter 8). Another possible explanation could be, differential accumulation of the metals when present together (Zalups and Barfuss, 2002; Aduayom *et al.,* 2003). Further, even desperate results have been recorded in studies involving metallic mixtures (Hochdal and Waalkes, 1997; Liu *et al.*, 2000; Peixto *et al.,* 2003)

Due to the known properties of melatonin as a direct free radical scavenger (Mutusszak *et al.,* 1997; Tan *et al.,* 1998; Xan *et al.,* 1999; El-Sokkarky *et al.,* 2002) and as a inducer of antioxidant enzyme (Reiter *et al.,* 2003; Rodrique *et al.,* 2004) and also based on the efficacy of melatonin in our *in vivo* studies on metal induced hepatic and renal toxicity (chapter 1-8), melatonin has been used in the present *in vitro* study to assess its competence to prevent cytolethality, induction of ROS and inhibition of apoptosis. The obtained results clearly show that melatonin can check to a significant extent metal induced cytolethality, ROS generation as well as Caspase 3 activity in both the cell lines. However, the degree of protective effect seems to be differential as, maximal effect for cytolethality was seen with Cd and Cr followed by TM mixture and Ni while protection against ROS generation was found to be maximum with Cr followed by TM mixture and least with Cd. Caspase 3 activity was reduced by melatonin to almost same degree against all exposures. Number of studies have shown protective effect against liver injury caused by various agents, in all of which, melatonin's antioxidant property had a principal role (Ohta *et al.,* 2000; Calvo *et al.,*

2001; Noyam *et al.,* 2006). Cytoprotective effect of melatonin under conditions of hepatic insult by way of prevention of hepatic malfunction, inhibition of generation of free radicals and accumulation of neutrophils in damaged areas has also been reported (Okatani *et al.,* 2003). Its protective effects against symptoms of severe sepsis/shock in both humans and animals have been documented (Escames *et al.,* 2006). A direct protective effect of melatonin in cultured rat hepatocytes against LPO and cytotoxocity induced by Cr has also been recorded (Susa *et al.,* 1997). In the present study, melatonin seems to afford protection against apoptosis and/or necrosis induced by mitochondrial damage as well as ROS mediated mechanisms. It also seems to afford protection against generation of ROS generation and endogenous antioxidants as well as in prevention of cytolethality in inducing synthesis of antioxidants and protective proteins like MT and stress proteins.

In conclusion, the present study has shown differential mechanisms 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. Further investigations are required to predict the usage of melatonin as a safe therapy for acute and chronic liver damage by toxic metals.

Summary of chapter 9

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. Melatonin was used as an antioxidant along with the metals and it 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.