CHAPTER 7

Chapter 7

Hepatic oxidative stress and toxicity due to Trimetallic exposure: Duration dependent study using realistic dosage and protective effect of melatonin.

By acting as catalysts in the oxidative reactions of biological macromolecules, transition metals contribute to oxidative tissue damage. Multiple mechanisms have been implicated in metal induced toxicity and one of the well known mechanisms is the formation of reactive oxygen species (ROS) (Nuran *et al.*, 2001). Detailed studies in the past two decades have shown that metals like iron, copper, cadmium, chromium, mercury, nickel, and vanadium have the ability to produce reactive radicals, resulting in DNA damage, lipid peroxidation, depletion of protein sulfhydryls and other effects (Valko *et al.*, 2005). Presence of metals in foods and drinks constitutes a serious threat and it has been shown by a previous environmental impact assessment study that Cadmium (Cd), Chromium (Cr) and Nickel (Ni) are found in appreciable amounts in cereals and food grains grown in the heavily industrialized north-west part of Vadodara (Ramachandran, 2003).

Cadmium is one of the priority pollutants and is widely present in the environment of Vadodara. Soluble cadmium salts can accumulate and result in toxicity to kidney, liver, lungs, brain, testis, heart and central nervous system (Stohs and Bagchi, 1995; Valko *et al.*, 2005). The mechanism of toxicity of cadmium can be multifactorial and because of its carcinogenic properties it has been classified as number I category of carcinogen by International Agency for Cancer (IARC, 1991).

Chromium another widely used industrial chemical is known to cause many systemic injuries including DNA damage, lipid peroxidation, enzyme inhibition, cytotoxicity and mutagenesis and the major mechanism of action is due to generation of free radicals (Valko *et al.*, 2005; Stohs, 1995).

Nickel is another environmental contaminant which after entry into the body, targets organs like kidney, lungs, spleen, liver, heart and testis. Much of the toxicity of nickel may be associated with interference with physiological processes regulated by magnesium, zinc, calcium and manganese (Cooghan, 1989). Formation of reactive oxygen species and oxidative stress are also related with the ability to form DNA adducts, cause DNA strand break and chromosomal aberrations, induce lipid peroxidation and promote carcinogenesis (Valko *et al.*, 2005; Stohs, 1995).

The major route of entry of toxicants in humans is the oral route through food and water. Since any environment may not be contaminated by a single metal due to industrialization and anthropogenic activities, studies involving combination of metals are the need of the day. Since chromium, cadmium and nickel have been identified as major environmental pollutants 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), the present study was planned to evaluate hepatic oxidative stress and toxicity generated by a combination of these metals in male *Wistar* rats. In this context, since the study is aimed at understanding the possible trimetallic mixture (TM mixture) toxicity on long term systemic entry into humans through diet and water, a realistic dosage has been worked out based on the Cr, Cd, and Ni contents in vegetables and food grains and also based

on 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) evaluation of hepatic oxidative stress and toxicity have been carried out.

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 the TM mixture.

Material and Methods: Same as in Chapter 1 but the metals given are Nickel (Ni), Cadmium (Cd) and Chromium (Cr) in the dose of 200 mg/kg/BW/day, 9 mg/kg/BW/day and 20mg/kg BW/day respectively in drinking water.

Results:

Lipid Peroxidation (LPO): Changes in the hepatic LPO levels following trimetallic treatment for 15, 30 and 60 days are shown in figure 7.1; table 7.1. Control animals showed an age dependent gradual increase in the LPO levels. There was significant increase in LPO levels of animals treated with the TM mixture. Treatment with melatonin significantly decreased the LPO levels. Melatonin did show duration dependent protective effect for all the exposure durations.

Glutathione (GSH): Changes in hepatic glutathione levels following trimetallic treatment for 15, 30 and 60 days are shown in figure 7.2; table 7.2. An age dependent gradual decrease in GSH level was the feature in control (Con) rats. There was significant progressive decrease in GSH level in animals treated with the TM mixture. There was significant increase in the GSH level when animals were administered melatonin alone or in combination with the TM mixture, with increasing duration tending to show slightly lesser efficacy.

Ascorbic Acid (Vit C): Changes in hepatic Vit C levels following the TM mixture treatment for 15, 30 and 60 days are shown in figure 7.3; table 7.3. A gradual age dependent decrease in Vit C content was seen in control (Con) rats. In animals treated with the TM mixture, there was significant decrease in Vit C content while, there was significant increase in the contents of Vit C when animals were administered with melatonin. Melatonin did not show any duration dependent effect but mostly showed a similar degree of protective effect in all the treatment periods.

Superoxide Dismutase (SOD): Changes in hepatic SOD activity following exposure to the TM mixture for 15, 30 and 60 days are shown in figure 7.4; table 7.4. An age dependent gradual decrease in SOD activity could be seen in the Con group of animals. The enzyme activity showed significant inhibition in animals exposed to the TM mixture and there was an increasing degree of inhibition paralleling duration of exposure. In Mel and Mel+Tri group of animals, the activity of SOD was significantly increased as compared to Con and Tri group of animals respectively. The degree of protection with melatonin was duration dependent.

Catalase (CAT): Changes in hepatic CAT activity following exposure to the TM mixture for 15, 30 and 60 days are shown in figure 7.5; table 7.5. Hepatic CAT activity tended to show an age dependent decrement. There was significantly decreased CAT activity in the TM mixture exposed group as compared to the control group of animals. Melatonin administration significantly increased the enzyme activity in the Mel+Tri and Mel groups of animals.

Glutathione Peroxidase (GPx): The changes in hepatic GPx activity following exposure to the TM mixture for 15, 30 and 60 days are shown in figure 7.6; table 7.6. Control animals tended to show a gradual age dependent decrement in the activity of GPx.. There was progressive duration dependent decrease in the activity of GPx in the TM mixture exposed animals. Melatonin administration tended to increase GPx activity in control rats. The protective effect of melatonin administration was found to be most effective at 15 days.

Metal Load: The changes in hepatic metal load following exposure to the TM mixture for 15, 30 and 60 days are shown from figure 7.7; table 7.7. There was a gradual decrease in the hepatic load of Cr and Ni in the TM mixture group with a concomitant increase in hepatic Cd metal load. There was significant decrease in the hepatic metal load of all the metals with co-administration of melatonin. Melatonin showed a decrease in metal load and the degree of decrement was almost the same in all the three durations of treatment.

Histology: Changes in hepatic histology following TM mixture treatment for 15, 30 and 60 days are shown in fig 7.8 to fig 7.32. TM induced histological changes like hypertrophy and degeneration of hepatocytes, dilation of sinusoids, disruption of hepatic cords and central vein and necrotic cells with fibrous periportal area could be seen clearly in all the exposure periods. The changes were prevented and near normal histoarchitecture was observed by co-administration with melatonin.

Serum and Hematological Parameters: The changes in serum glucose, melatonin and insulin levels and marker enzymes of hepatic marker damage enzyme are shown in the table 7.8 and 7.9 respectively. Hematological changes are shown in table 7.10. There was increase in marker enzymes of hepatic damage (ALP and ALT) while, melatonin protected against this increase. Animals showed a hypoglycemic effect after treatment with the TM mixture. There was significant decrease in the serum titre of melatonin in the TM mixture exposed animals. Hematological parameters did not show any significant change.

Fig 7.1Comparison of the temporal slope of percentage change in hepatic LPO between TM mixture and component metals individually

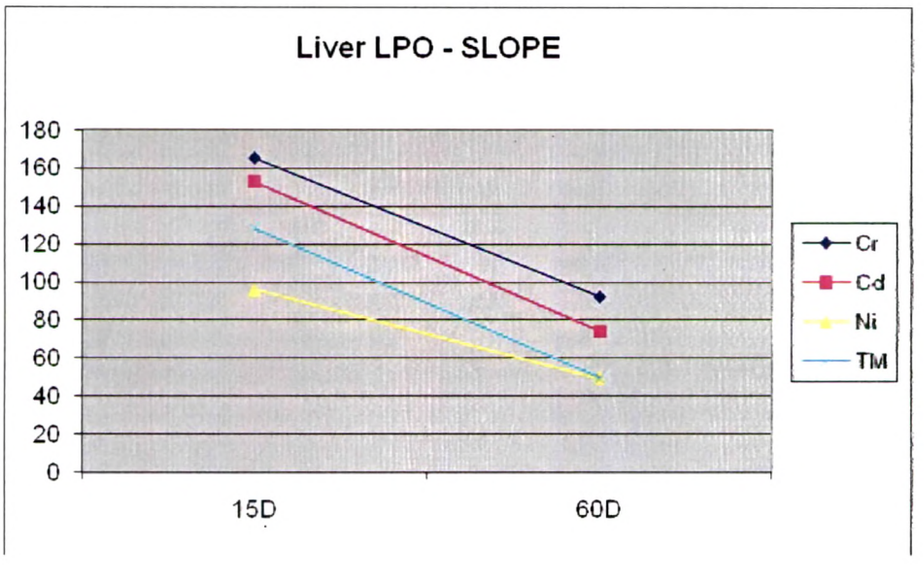

Fig. 7.2 Percentage increase in hepatic LPO at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

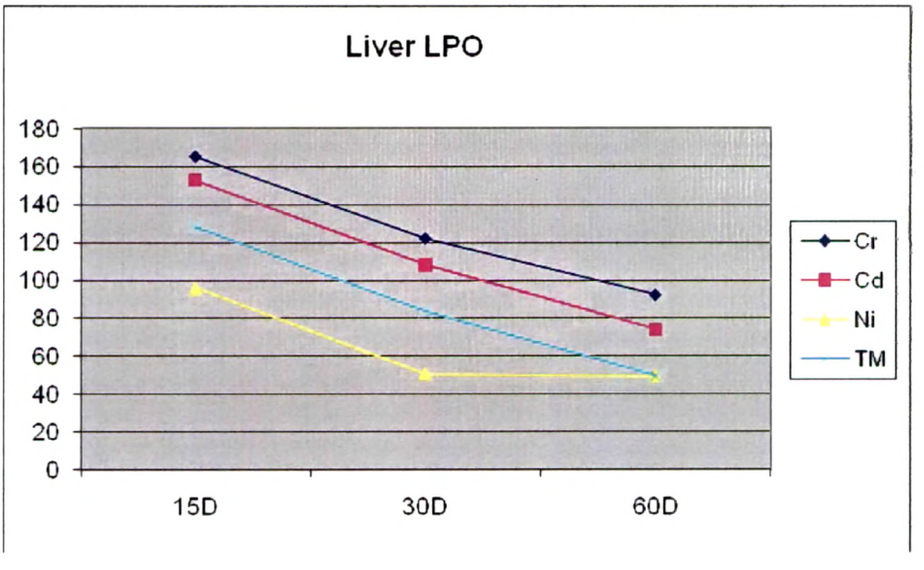

Fig 7.3 Comparison of the temporal slope of percentage change in hepatic GSH between TM mixture and component metals individually

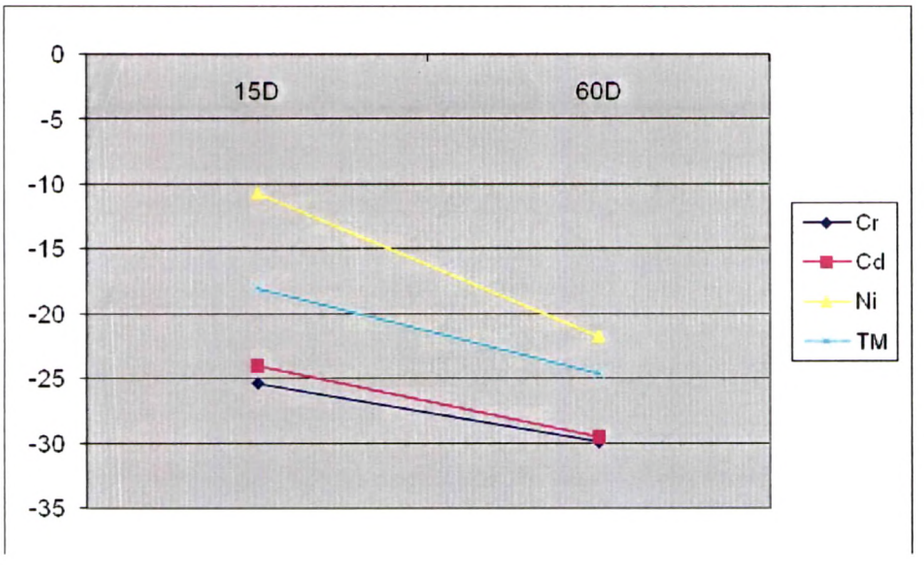

Fig. 7.4 Percentage decrease in hepatic GSH at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

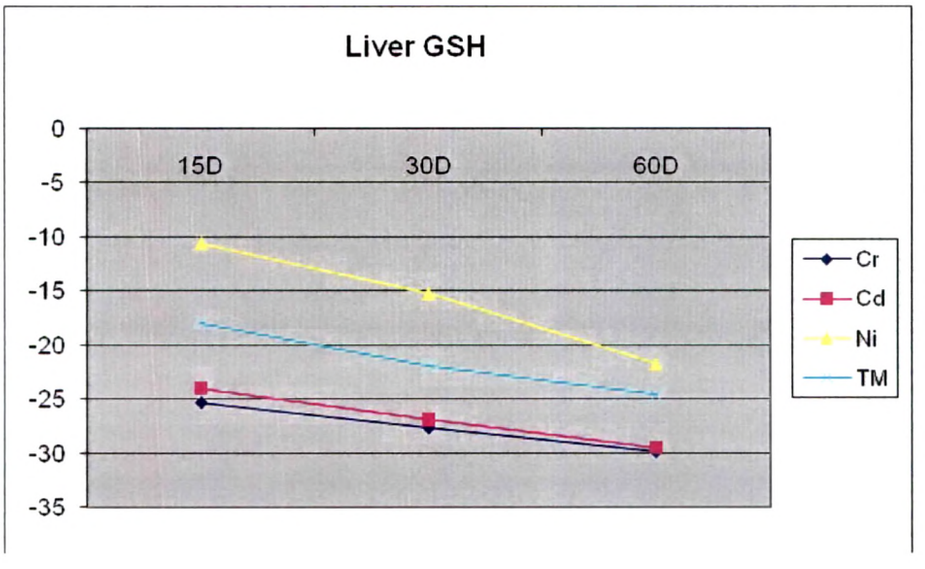

Fig 7.5 Comparison of the temporal slope of percentage change in hepatic AA between TM mixture and component metals individually

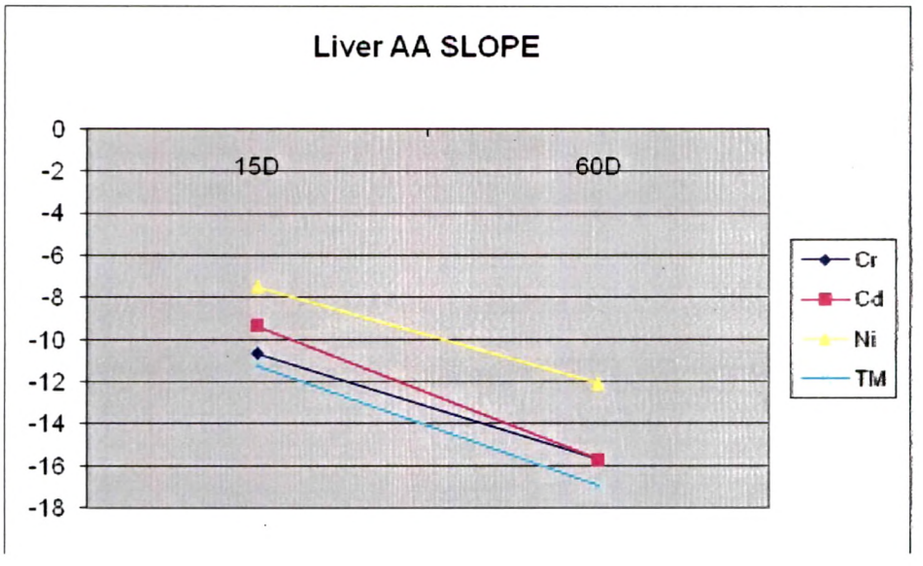

Fig. 7.6 Percentage decrease in hepatic AA at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

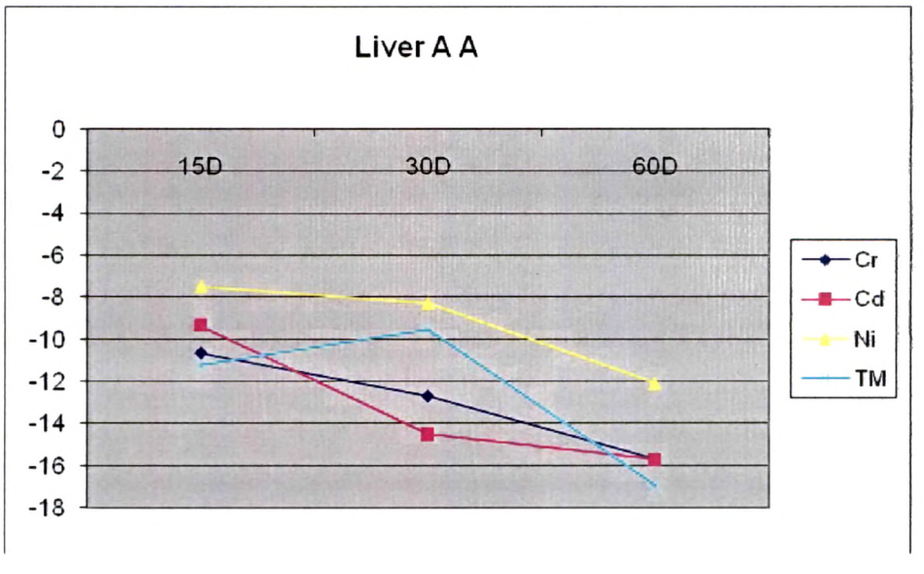

Fig 7.7 Comparison of the temporal slope of percentage change in hepatic SOD between TM mixture and component metals individually

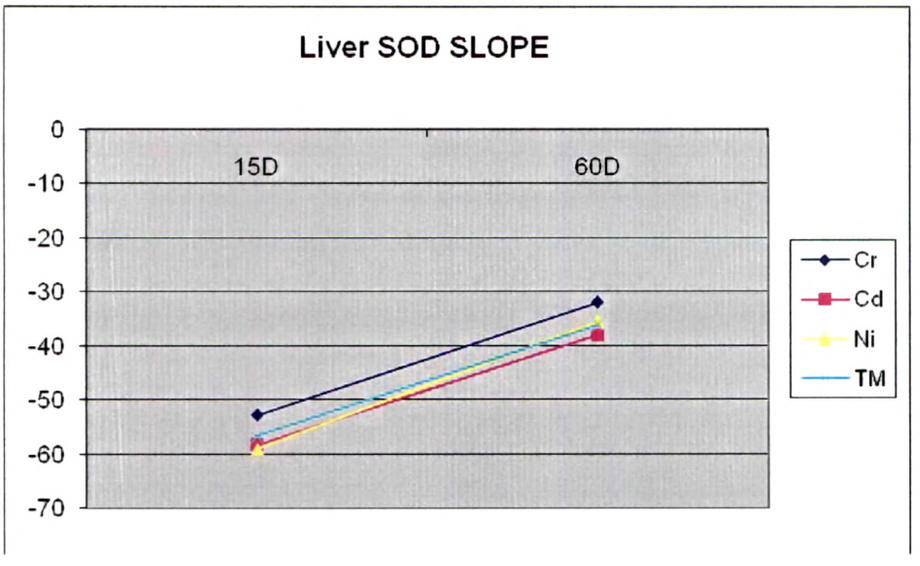

Fig. 7.8 Percentage decrease in hepatic SOD at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

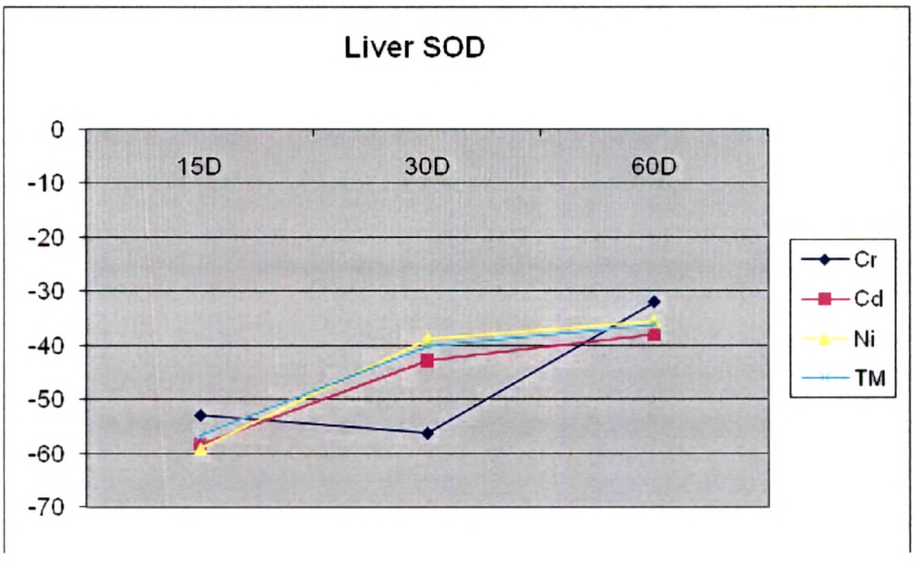

Fig 7.9 Comparison of the temporal slope of percentage change in hepatic CAT between TM mixture and component metals individually

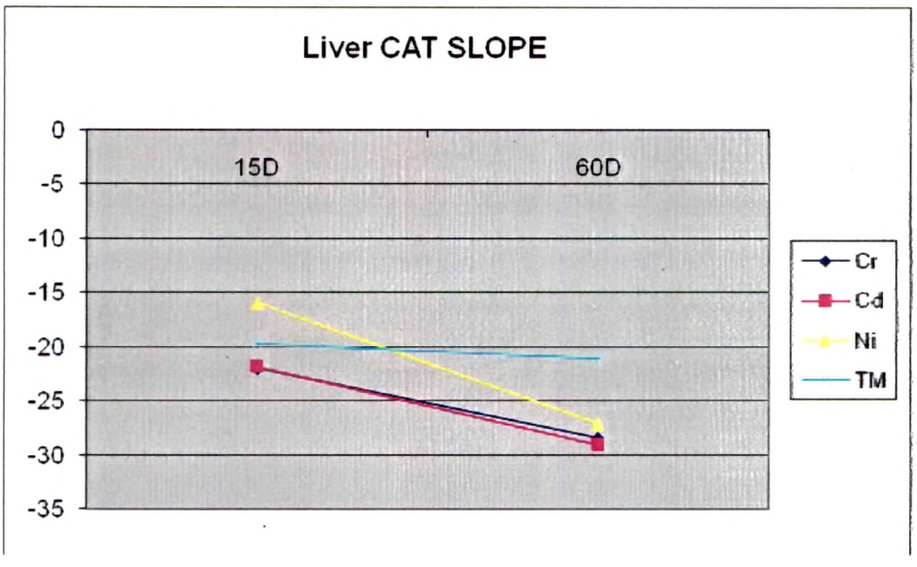

Fig. 7.10 Percentage decrease in hepatic CAT at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

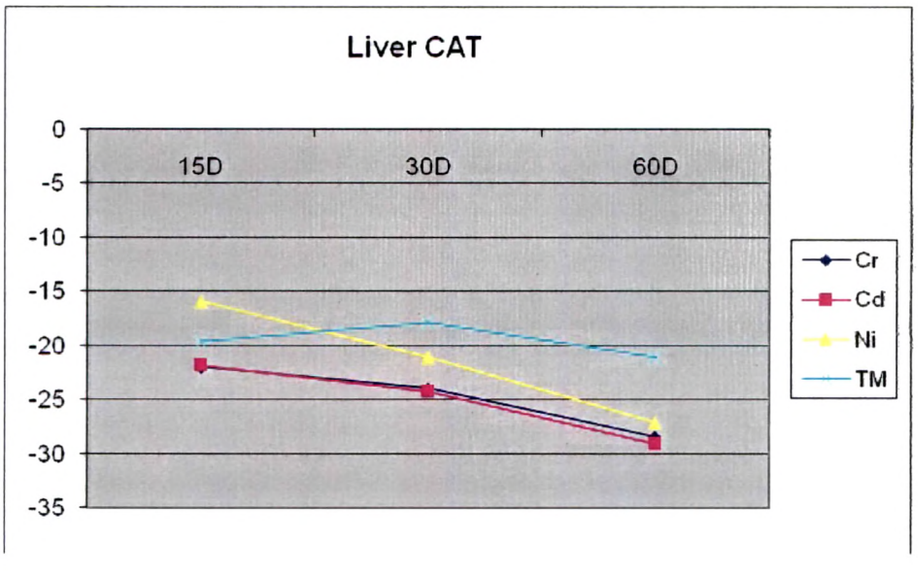

Fig 7.11 Comparison of the temporal slope of percentage change in hepatic GPx between TM mixture and component metals individually

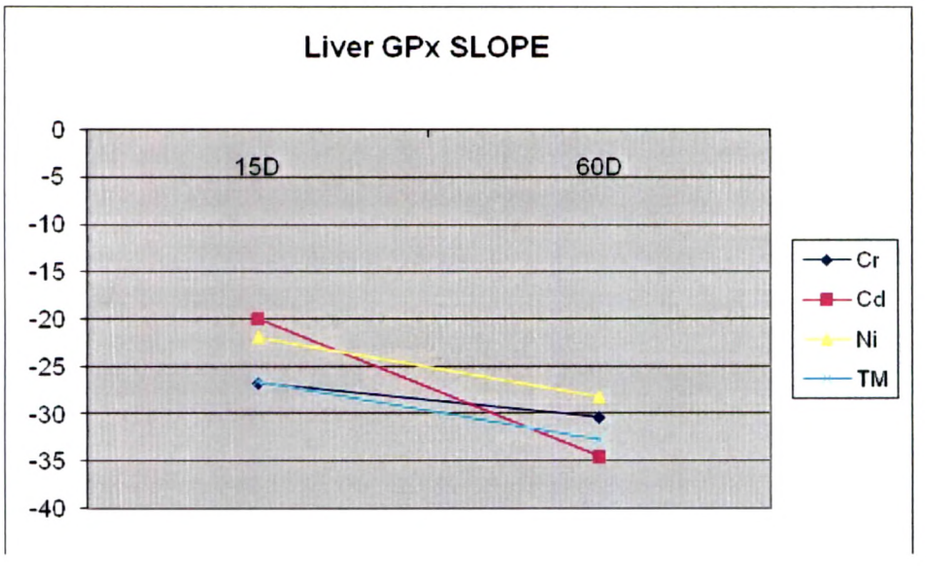

Fig. 7.12 Percentage decrease in hepatic GPx at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

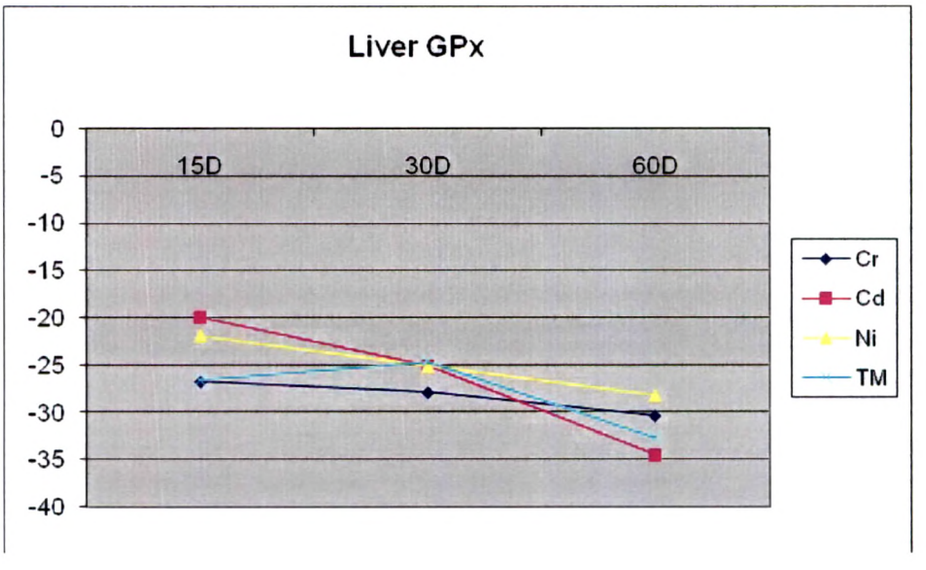

Table: 7.1 Levels of **hepatic lipid peroxidation (LPO)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	19.02 ± 1.26	20.79 ± 1.080	23.30 ± 1.02
Mel	16.490 ± 1.39	18.280 ± 1.290	21.76 ± 1.17
Tri	43.40±1.79@	38.16±1.90@	34.92±1.26@
Tri+Mel	31.50±1.58#	29.51±1.27#	27.48±2.01#

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

Table 7.2. Levels of **hepatic glutathione (GSH)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	0.150 ± 0.0012	0.130± 0.0017	0.114±0.0015
Mel	0.160±0.0017*	0.144±0.0012*	0.128±0.0014*
Tri	0.123±0.0017@	0.102±0.0014 @	0.087±0.0021@
Tri+Mel	0.145 ±0.0021#	0.120±0.0014 #	0.101±0.0012#

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

Table 7.3. Levels of **hepatic Ascorbic Acid (Vit C)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	0.160±0.0014	0.131± 0.0013	0.124±0.0019
Mel	0.179±0.0018*	0.157± 0.0018*	0.148±0.0013*
Tri	0.146±0.0016@	0.117±0.0013@	0.103±0.0019@
Tri+Mel	0.165±0.0019#	0.136±0.0016#	0.130±0.0015#

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

Table 7.4. Levels of **hepatic Superoxide Dismutase (SOD)** following 15, 30 and 60 days treatment with TM mixture

	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 *
Tri	6.01 ± 0.145@	7.16 ±0.128@	6.72 ± 0.155@
Tri+Mel	8.81 ± 0.156#	9.13± 0.109#	8.65 ± 0.128#

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

Table 7.5. Levels of **hepatic catalase (CAT)** following 15, 30 and 60 days treatment with TM mixture

	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*
Tri	41.55±1.91@	36.29±2.01@	32.85±1.61@
Tri+Mel	45.12±1.20 #	41.28 ±1.09#	39.07±1.97#

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

Table 7.6. Levels of **hepatic Glutathione Peroxidase (GPx)** following 15, 30 and 60 days treatment with Trimetallic Combication

	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
Tri	45.78±1.10 @	40.19±1.09@	32.79±1.61@
Tri+Mel	58.61±1.02 #	49.12 ±1.39#	43.38±1.90#

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

Figure 7.7. Graph showing % accumulation of Cd, Cr and Ni in hepatic tissue following 15, 30 and 60 days treatment with Trimetallic Combination.

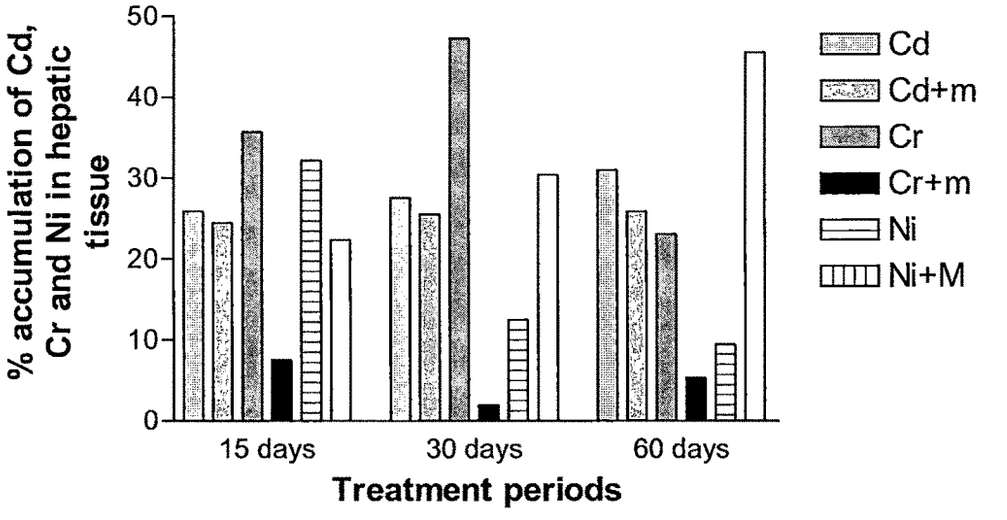

Table 7.7. % accumulation of Cd, Cr and Ni in hepatic tissue following 15, 30 and 60 days treatment with Trimetallic combination

	Cr	Cr+M	Cd	Cd+M	Ni	Ni+M
15 days	35.74	7.58	25.89	24.46	32.24	22.38
30 days	47.30	1.92	27.59	25.53	12.48	30.43
60 days	23.15	5.25	31.08	25.90	9.44	45.66

Table 7.8. Changes in serum glucose, insulin and melatonin levels following 15, 30 or 60 days treatment with Trimetallic Mixture

	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)	109.5±0.64	0.52±0.01@	75±8.65@	81.75±1.49@	0.88±0.09@	60±5.49@	106.48±1.85@	1.20±0.27@	20±6.50@
Cr+Melatonin (Cr+Mel)	110.38±0.85	0.64±0.01#	102±4.85#	105.52±1.09	1.08±0.09#	109±6.47	127.25±1.11#	0.57±0.09#	87±4.98#

@ p<0.05 between Con vs Tri

# p<0.05 between Tri vs Tri+mel

*p<0.05 between Con vs Mel

Table7.9 Changes in serum ALP and ALT following 15, 30 and 60 days of treatment with Trimetallic mixture

	15 days		30 days		60days	
	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±3.4	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
Trimetallic Mixture (TM)	205.7±1.95@	60.19±2.51@	230.65±2.79@	68.8±3.10@	250.15±1.9@	75.4±3.15@
Trimetallic mixture+Melatonin (TM+Mel)	179.59±2.95#	49.95±3.05#	185.89±1.98#	55.2±2.9#	175.15±3.7#	61.6±1.59#

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

Table 7.10 Changes in hematological parameters following treatment with Trimetallic mixture following 15, 30 and 60 days

	15 Days			30 Days			60 Days		
	Hemoglobin	**Erythrocyte count**	**Packed Cell Volume (PCV)**	**Hemoglobin**	**Erythrocyte count**	**Packed Cell Volume (PCV)**	**Hemoglobin**	**Erythrocyte count**	**Packed Cell Volume (PCV)**
Control (Con)	15.05±0.06	9.66±0.48	51.75±0.13	16.75±0.01	10.27±0.01	51.50±0.09	15.75±0.85	10.68±1.32	60.25±1.25
Melatonin (Mel)	15.60±0.15	10.1325±1.08	56.00±0.85	16.00±0.01	9.92±0.01	56.25±0.01	15.75±0.91	10.72±1.11	54.00±1.83
Trimetallic Mixture (TM)	14.25±1.31	9.3825±0.09	48.50±0.09@	13.58±0.02@	9.28±0.01@	42.00±0.01@	14.73±0.65	10.78±0.91	57.00±0.91
Trimetallic mixture+Melatonin (TM+Mel)	14.68±0.13	14.675±0.09#	51.75±0.06#	13.70±0.05	10.42±0.01	52.50±0.01#	14.85±0.85	10.86±0.29	56.25±0.63

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

PLATE I

Fig 7.8 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-Kuffer cells

Fig 7.9 Photomicrograph of 15 day melatonin treated liver (400X). Note the robust appearance of hepatic cords. S- Sinudois

Fig 7.10 Photomicrograph of 15 day Trimetallic mixture exposed liver (400X). Note the seriously disrupted endothelium of central vein (arrow) and disturbed hepatic cords with apoptotic cells (*).

Fig 7.11 Photomicrograph of liver exposed to Tri+Mel for 15 days (400X). Note the near normal organization of hepatic lobule and intact central vein. CV-Central Vein

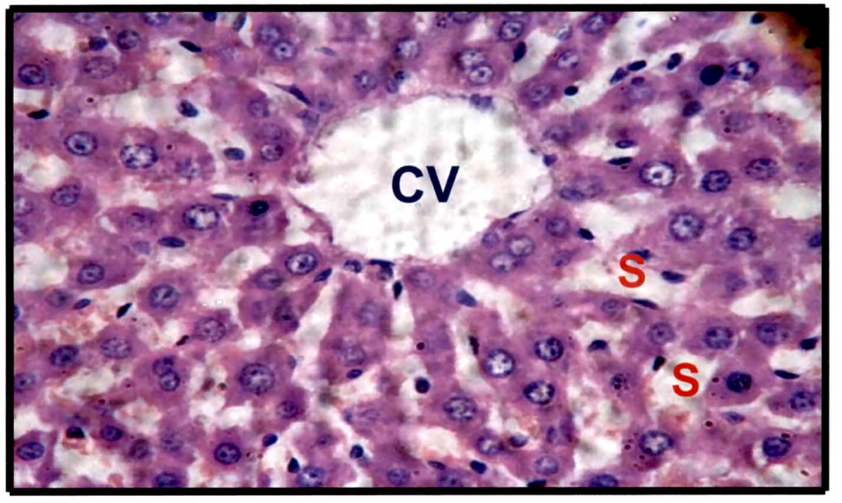

Fig 7.8 Control

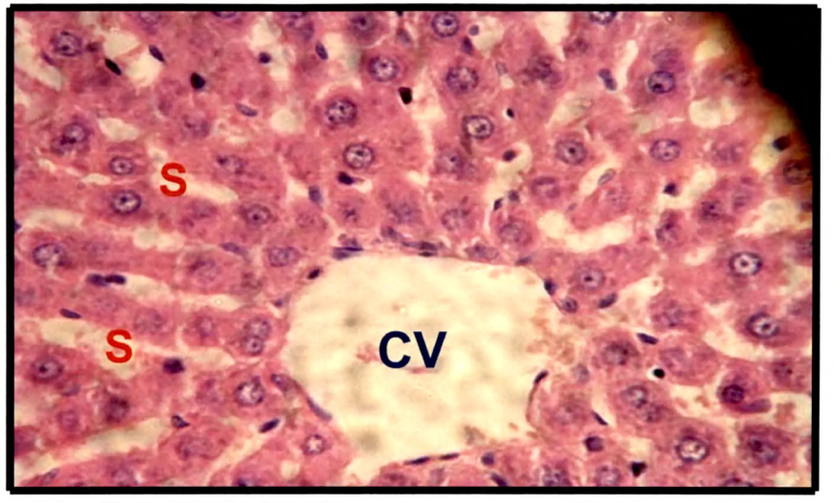

Fig 7.9 Melatonin

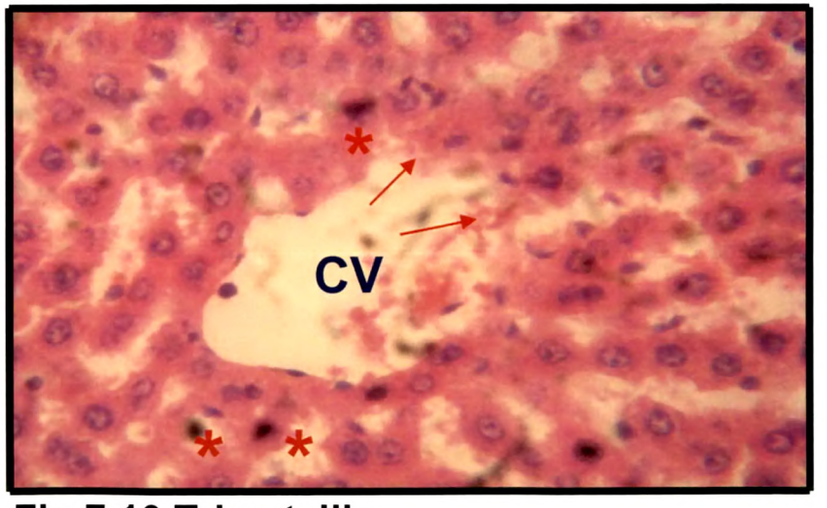

Fig 7.10 Trimetallic

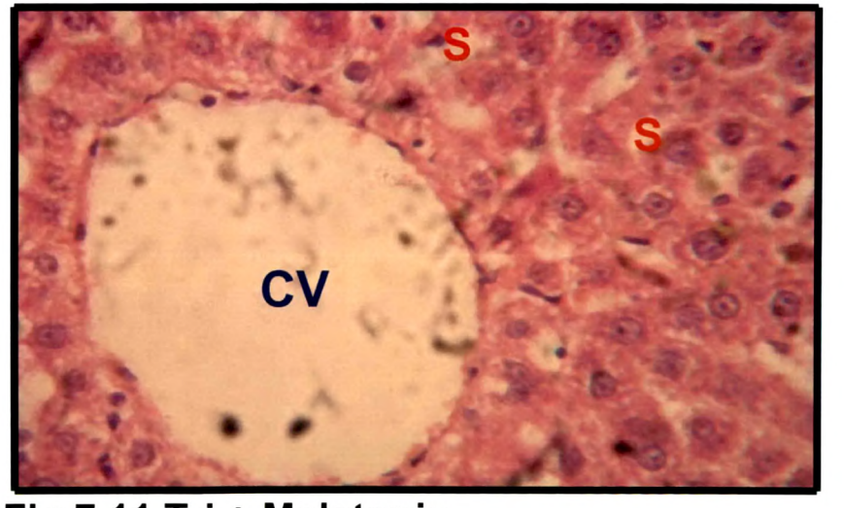

Fig 7.11 Tri + Melatonin

PLATE II

Fig 7.8(a) Photomicrograph of control liver showing periportal area (400X). PPA-Periportal Area

Fig 7.9(a) Photomicrograph of melatonin treated liver showing PPA area (400X); PPA-Periportal Area

Fig 7.10(a) Photomicrograph of 15 day trimetallic exposed liver showing periportal area (400X). Note the dilated portal vein and its disruption and many apoptotic cells (*). PV-Portal vein;HA Hepatic artery

Fig 7.11(a) Photomicrograph of liver exposed to Tri+Mel for 15 days showing periportal area (400X). Note the near normal appearance of the periportal area. Some apoptotic cells can be seen (*) PV - Portal Vein.

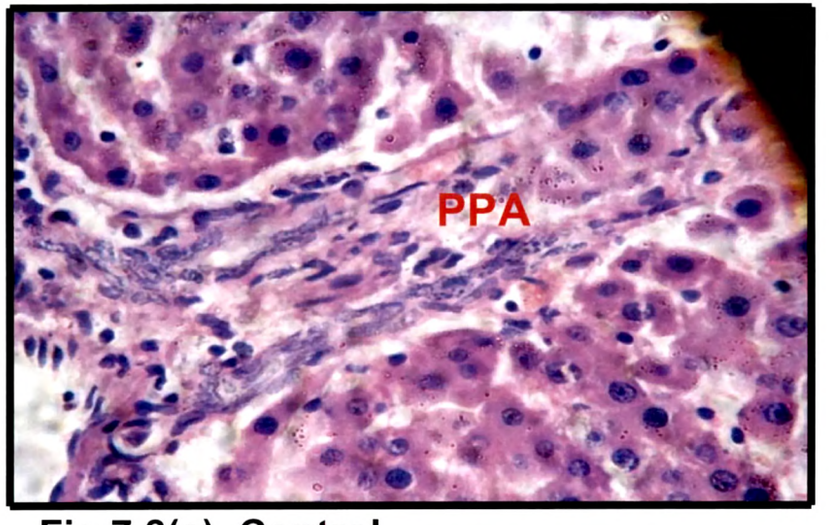

Fig 7.8(a) Control

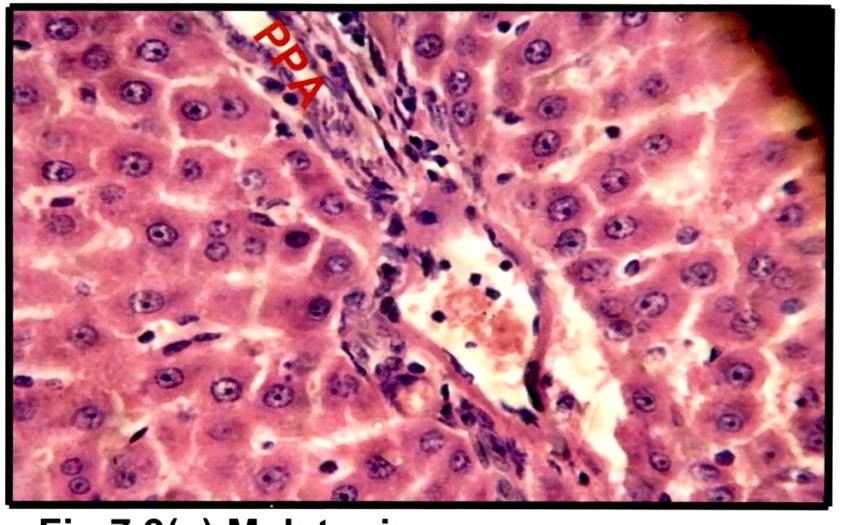

Fig 7.9(a) Melatonin

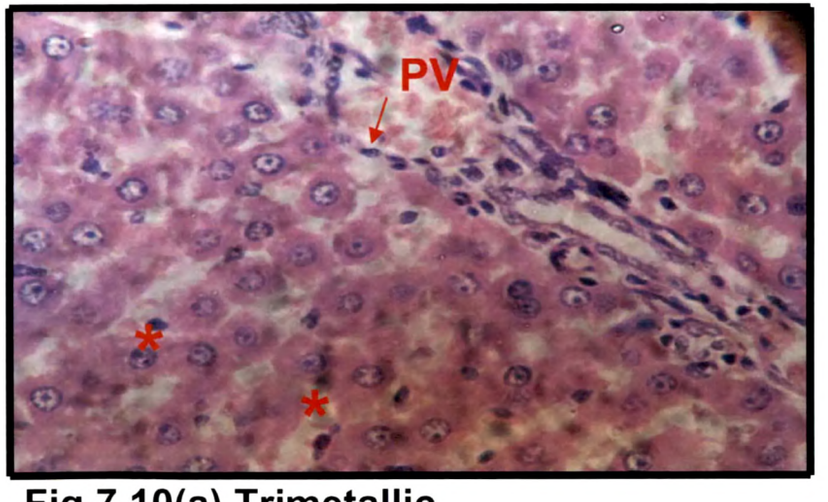

Fig 7.10(a) Trimetallic

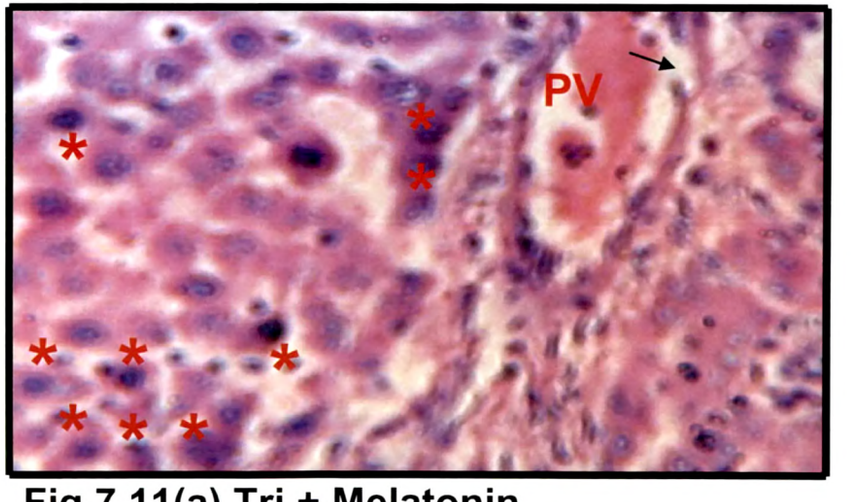

Fig 7.11(a) Tri + Melatonin

PLATE III

Fig 7.12 Photomicrograph of control liver showing a hepatic lobule (400X). CV Central Vein; S Sinusoids.

Fig 7.13 Photomicrograph of 30 day melatonin treated liver (400X). Showing central vein and hepatic cords. CV Central Vein; S Sinusoids.

Fig 7.14 Photomicrograph of 30 day trimetallic treated liver showing a hepatic lobule (400X). Note the near complete breakdown of endothelium of central vein (arrow), disturbed hepatic cords with degenerating and apoptotic hepatocytes(*).CV-Central Vein

Fig 7.15 Photomicrograph of liver exposed to Tri+Mel for 30 days showing a hepatic lobule (400X). Note the better organization with near normal central vein with few apoptotic and degenerative hepatocytes(*).

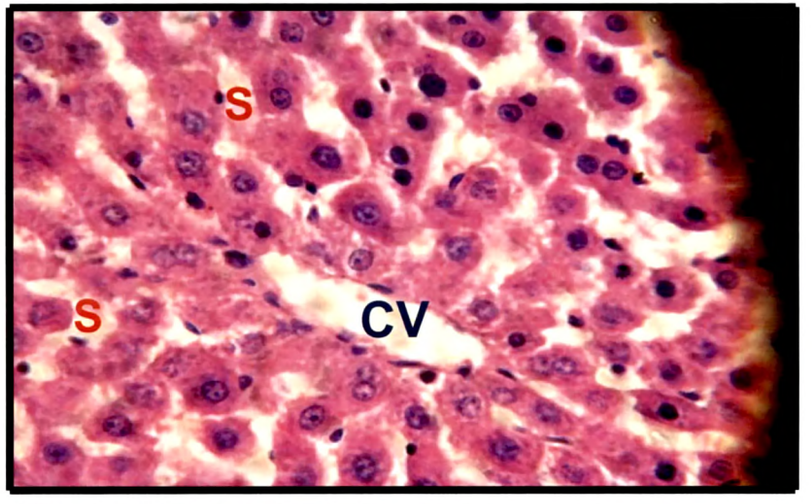

Fig 7.12 Control

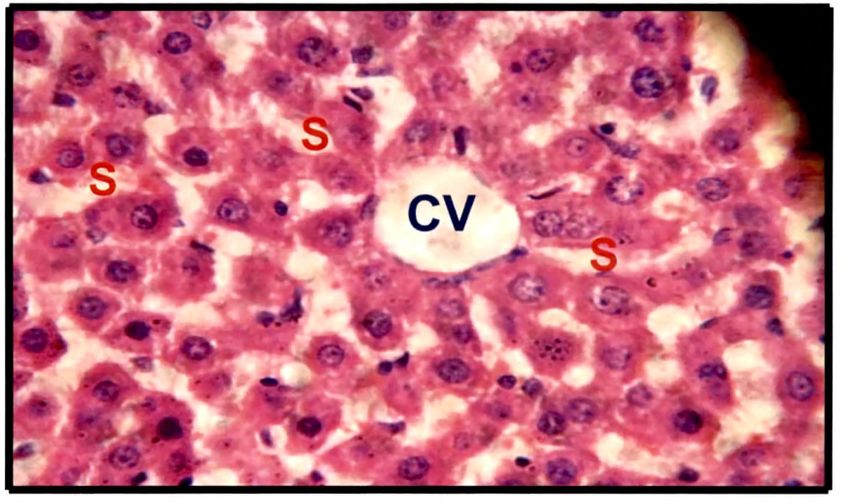

Fig 7.13 Melatonin

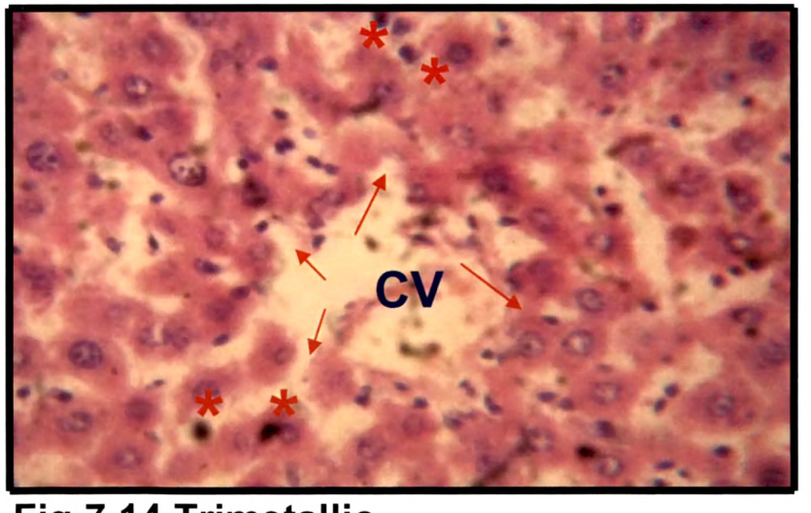

Fig 7.14 Trimetallic

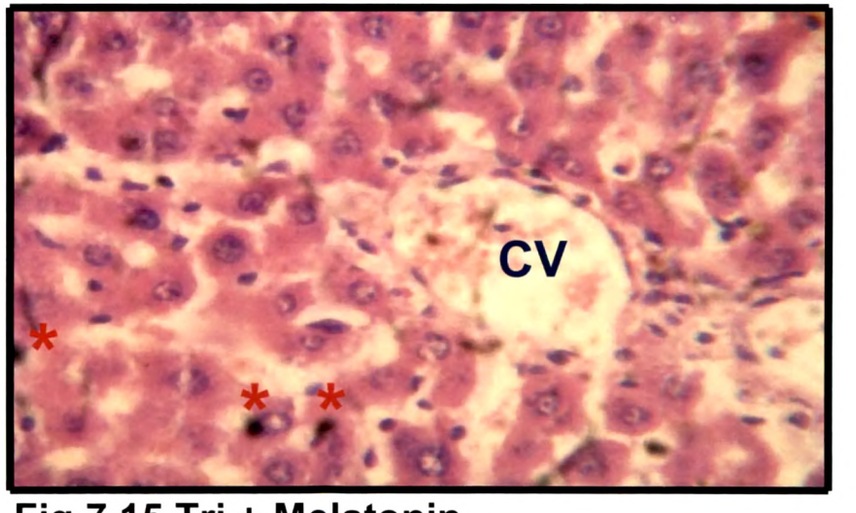

Fig 7.15 Tri + Melatonin

PLATE IV

Fig 7.12(a) Photomicrograph of control liver showing periportal area (400X) PPA-Periportal Area

Fig 7.13(a) Photomicrograph of melatonin treated liver for 30 days showing PPA area (400X)

Fig 7.14(a) Photomicrograph of liver exposed to Trimetallic mixture for 30 days showing PPA area (400X). Note the apoptotic cells and hepatocytes (*).

Fig 7.15(a) Photomicrograph of liver exposed to Tri+Mel for 30 days showing PPA area(400X). Note the near normal appearance of the periportal area can be seen.

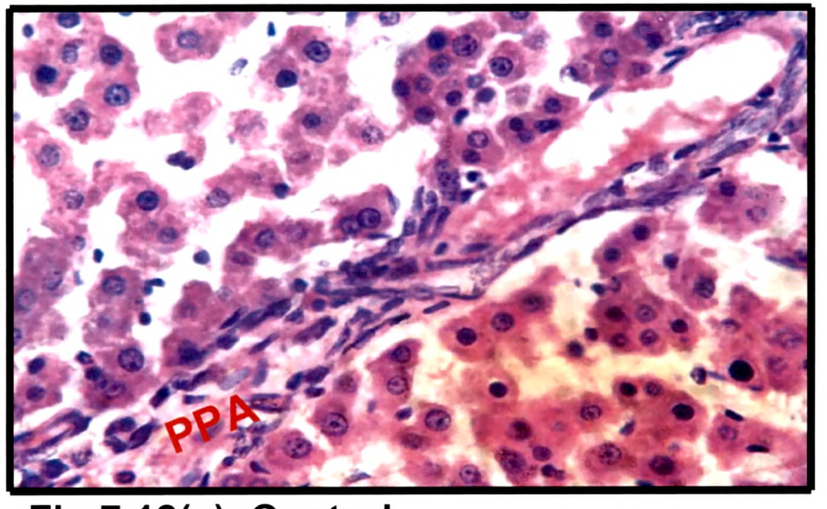

Fig 7.12(a) Control

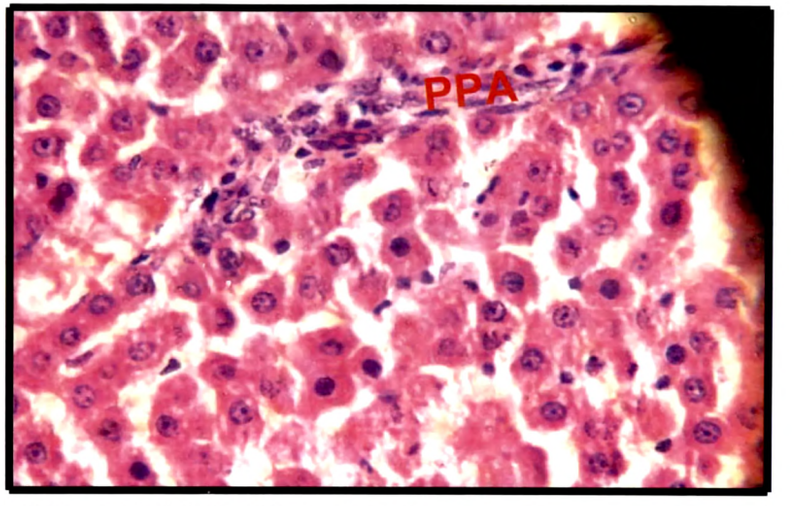

Fig 7.13(a) Melatonin

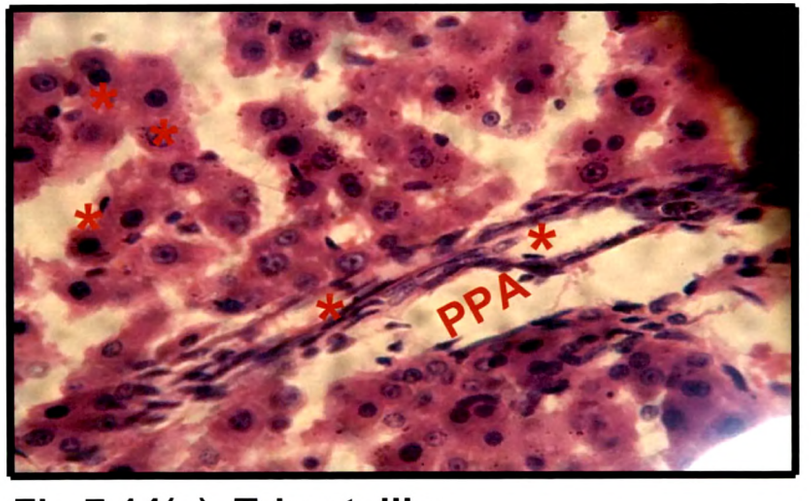

Fig 7.14(a) Trimetallic

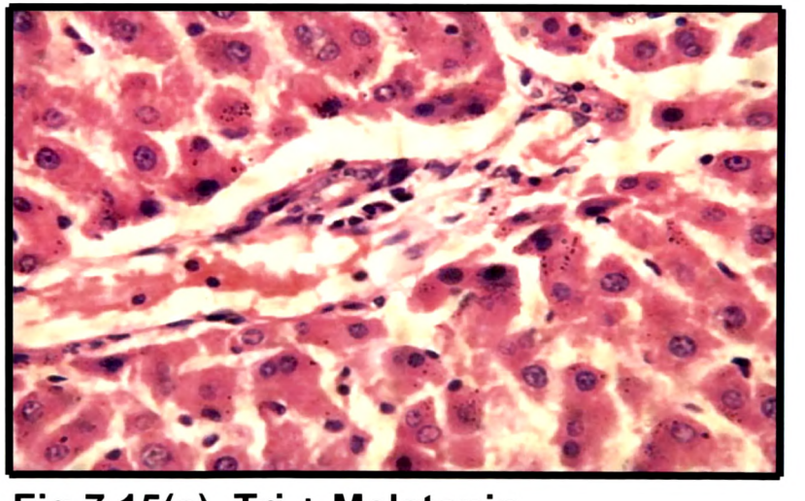

Fig 7.15(a) Tri + Melatonin

PLATE V

Fig 7.16 Photomicrograph of 60 day control liver(400X) showing a hepatic lobule. Note the cord like organization of hepatocytes and a central vein. CV- Central Vein, S- Sinusoid

Fig 7.17 Photomicrograph of 60 days melatonin treated liver (400X). Note the robustness of hepatocytes.

Fig 7.18 Photomicrograph of 60 day Trimetallic mixture exposed liver (400X). Note the severe endothelial breakdown of central vein and its confluencing with distended sinusoids. The hepatocytes appear to be degenerative. BC-Bowman's Capsule, DT-Distal Tubule, G-Glomerulus

Fig 7.19 Photomicrograph of liver exposed to Tri+Mel for 60 days (400X). Near normal appearance of periportal area.

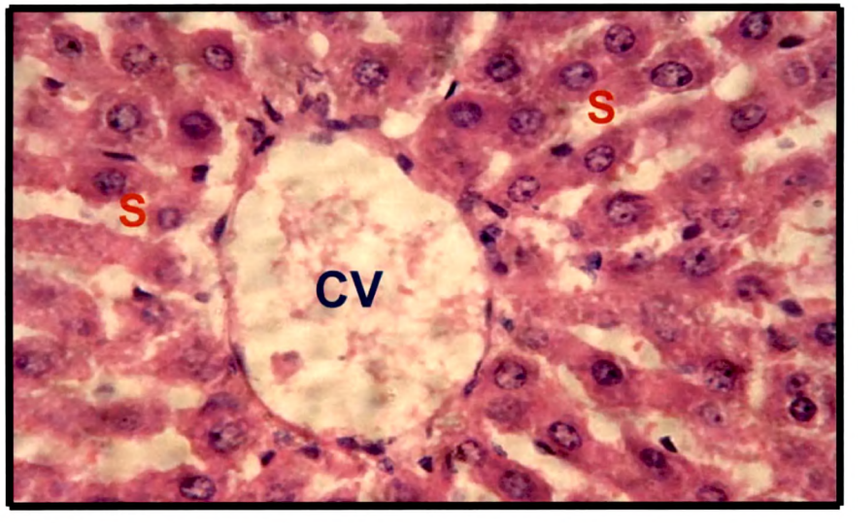

Fig 7.16 Control

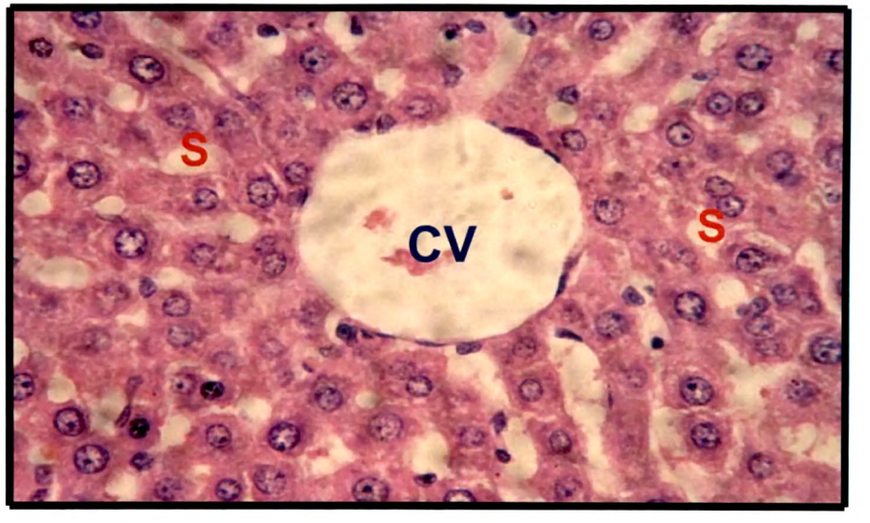

Fig 7.17 Melatonin

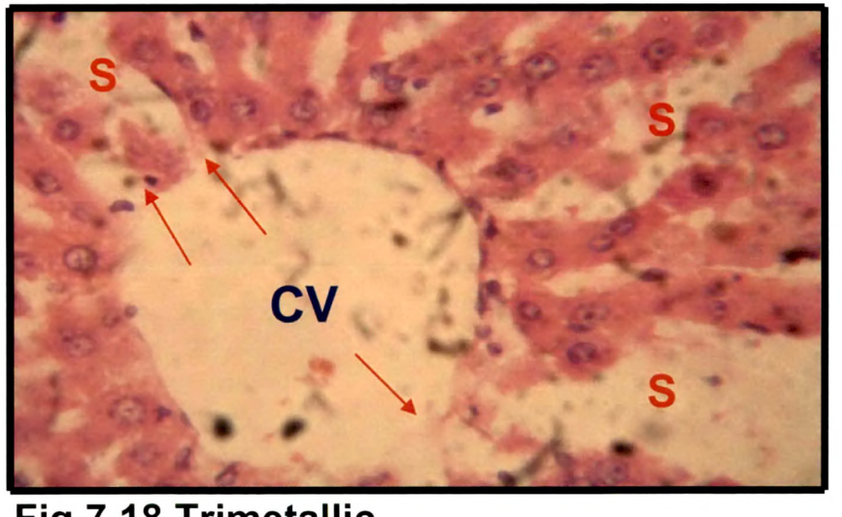

Fig 7.18 Trimetallic

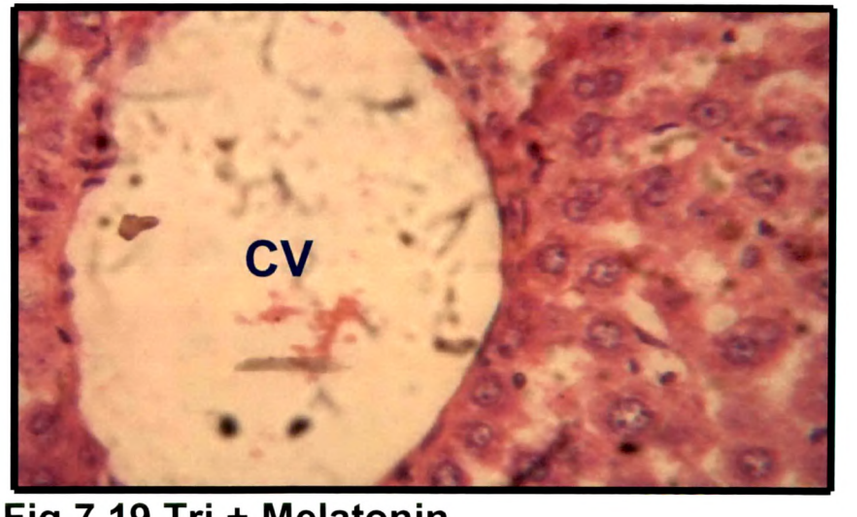

Fig 7.19 Tri + Melatonin

PLATE VI

Fig 7.16(a) Photomicrograph of control liver showing periportal area (400X). PPA-periportal area

Fig 7.17(a) Photomicrograph of liver treated with melatonin for 60 days showing periportal area (400X)

Fig 7.18(a) Photomicrograph of liver exposed to Trimetallic mixture for 60 days showing disintegrated periportal area (400X). Note the degenerated periportal area leading to meeting of adjoining hepatic lobules. Also note the rampant apoptotic hepatocytes (*). PPA- Periportal Area

Fig 7.19(a) Photomicrograph of liver exposed to Tri+Mel for 60 days showing periportal area (400X). Better looking periportal area but apoptotic and degenerating hepatocytes can be seen.

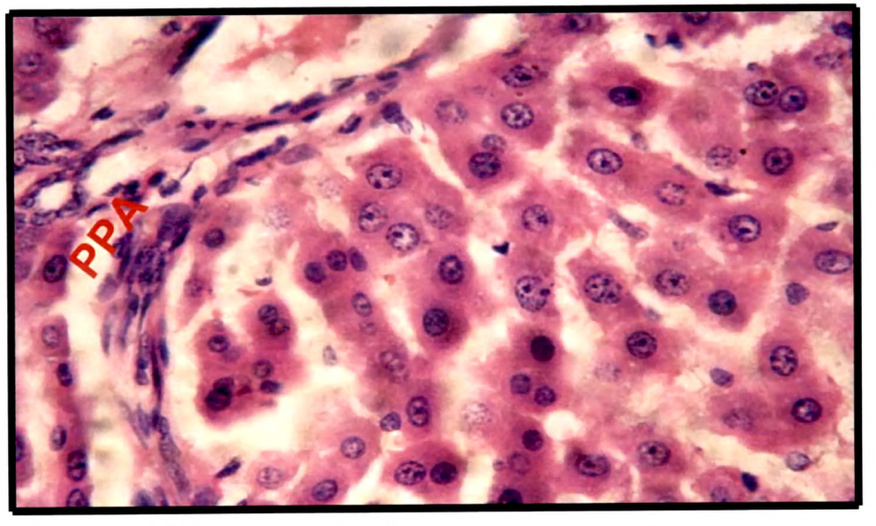

Fig 7.16(a) Control

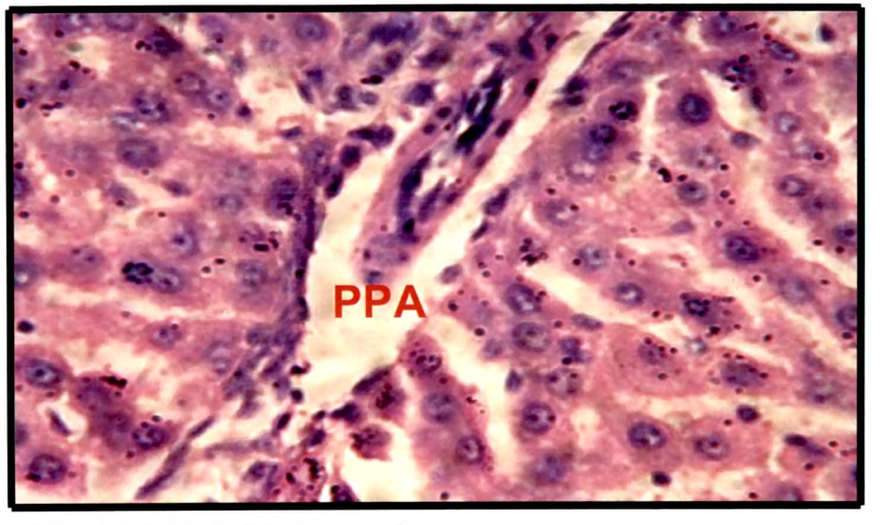

Fig 7.17(a) Melatonin

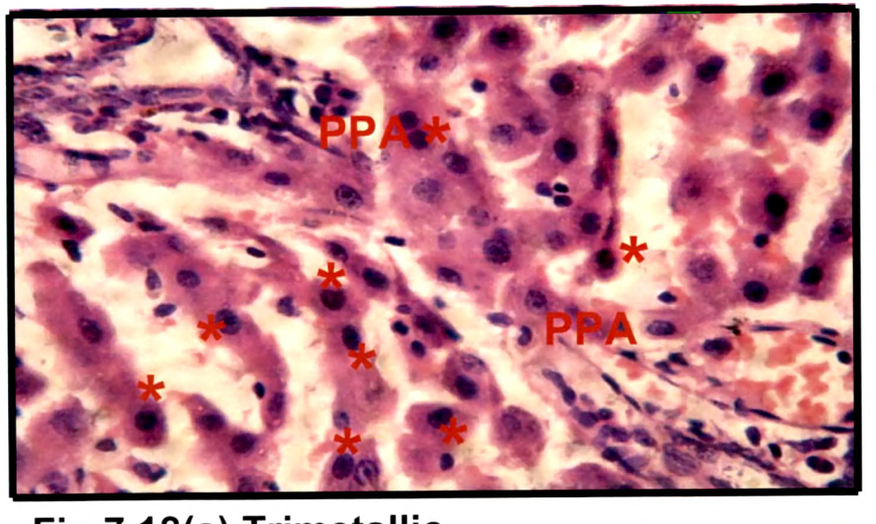

Fig 7.18(a) Trimetallic

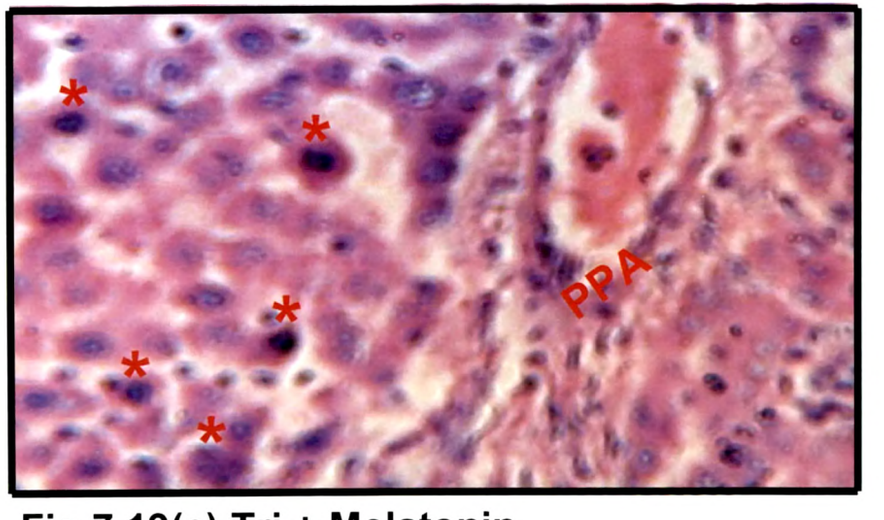

Fig 7.19(a) Tri + Melatonin

Discussion:

Classical toxicology has in recent times given way to genetic and molecular toxicology and even a new developing field called toxicogenomics and these reductionist fields of toxicology are better placed to characterize the effect of a chemical at molecular and genetic levels (Feren *et al.*, 2002; Thomas *et al.*, 2002). However, chemical toxicants including metals may affect very many different physiological systems and further, even different systems interact with each other (Carpenter *et al.*, 1998) so much so that, the biologic relevance or health impacts of these changes may be indeed difficult to interpret. Added to this is the complication of chemical mixtures. Based on relevant studies, evidence is coming forth to link biochemical responses and the interactive effect of chemical mixtures, more so, at the chronic level (Ishaque, 1998; Kluste *et al.*, 2002). Some studies have based the composition of the mixture on the toxicity of each individual component (Saleva *et al.*, 1999; Bae *et al.*, 2001) while, others have based it on quality criteria, composition at superfund sites and average composition in the environment (Spehar and Fiandt, 1986; Enserink *et al.*, 1991; Buhl, 1998). In the present study, exposure of experimental animals to the TM mixture (Cr, Cd and Ni) has been on a realistic dosage based on environmental relevance and systemic entry.

This study has revealed no compounding effect of the metals when present together in terms of hepatic oxidative stress. As has been the case with individual metal exposure (Chapters 1,3,5), the TM mixture shows greatest degree of LPO at the short exposure period of 15 days with gradually but significantly decreasing degree of LPO at the medium and long durations of exposure. The observed degree of LPO and pattern of

temporal alteration on comparison with those of individual metals (Chapters 1,3,5), tends to indicate an additive interaction of Cd and Cr. However, persistence of oxidative stress during continued exposure to the TM mixture is clearly indicated by the decreased contents of non-enzymatic (GSH and Vit C) and enzymatic (SOD,CAT, GPx) antioxidants. Apparently, the hepatic tissue is overwhelmed by a sudden induction of oxidative stress in the initial period but resistance and recovery seem to be the order on continued exposure as marked by the decreasing degree of LPO, 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 (Chapters 1, 3, 5). The observed persistent depletion in GSH and Vit C and inhibition of CAT and GPx despite decrease in LPO as in the case of observations made with single metals (Chapters 1, 3, 5) again attest to the notion of substantial recovery of LPO and continued generation of free radicals with effective scavenging of the same on continued exposure to the TM mixture. The recovery in LPO seen on longer duration exposures could be accredited to both prevention of propagation of membrane peroxidation and reversal of peroxylated lipids to normal lipids as well as effective scavenging of free radicals involved in LPO. The candidate molecules in this context are GSH and ascorbic acid (Vit C) and α-tocopherol (Vit E) (Buettner, 1993) of which, the former two have shown significant and persistent depletion in the present study. It is likely that all the three function together in not only preventing further LPO but also in removing the peroxyl group from lipids by chain breaking reactions. Glutathiolation of peroxylated membrane lipids is an effective mode of detoxification and reversal of lipid peroxidation and moreover, it is

also protective as, lipid peroxidation products in excess can prevent apoptosis and favor mitogenesis by activating appropriate cell signaling pathways (Ramana *et al.*, 2006). These workers have also shown that depletion in GSH is adaptive in preventing such cell signaling by moderating the formation of glutathiolated conjugates of peroxylated lipids. In this context, the presently observed decrease in GSH in TM mixture exposed hepatic tissue of rats can find justification in not only aiding in detoxification and removal of hydrophobic metabolites like lipid peroxidation products by glutathione but also in minimizing the chances of stimulated mitogenesis and cancer induction. The fact that GSH decrease was only by 25% at 60 days of TM mixture exposure suggests that, despite its continued and active involvement in minimizing oxidative stress, there is certain degree of rebound GSH synthesis as has been shown by Alptebin *et al.* (1996) in combating lipid peroxidation in tissues of water-immersion stressed rats. So the decrease in GSH content can be essentially correlated with modulation of lipid peroxidation and not with depressed GSH synthesis and/or GSH breakdown. Concurrently, increasing depletion of ascorbic acid seen herein as well as the purported involvement of α-tocopherol are all relevant in the recorded decrement in hepatic LPO on prolonged periods of exposure to the TM mixture. It is pertinent to note that, the changes in GSH and ascorbate seen in the present study on TM mixture apparently seem to be accreditable to an interactive antagonistic effect of Cd and Cr on one side and Ni on other side in the case of GSH and more of a Cr induced effect with slight additive effect of Cd and Ni in the case of ascorbate as compared to the changes recorded for individual metals (Chapters 1,3,5).

The present TM mixture seems to generate higher level of superoxide radical in the initial period as marked by the greater degree of inhibition of SOD. The marked inhibition of SOD at 15 days exposure to the three metals individually has been attributed to active quenching of superoxide radical in the case of Cr and Ni (Chapters 1, 5) and, Cd substitution of Mn or Zn in the enzyme in the case of Cd (Chapter 3). Since the present study on TM mixture has shown exactly the same degree of inhibition of SOD at 15 days, it is difficult to infer whether it is a Cr or Ni mediated mechanism or Cd mediated mechanism. However, a comparison of the temporal alterations in SOD activity seen presently with those recorded for individual metals (Chapters 1,3,5) seem to confirm a non-additive combined Cd-Ni effect. However, with increasing duration there is a shift from preponderance of superoxide radicals to peroxide and hydroxyl radicals which are being managed by CAT and GPx mediated pathways as seen by the increasing inhibition of these enzymes. The relatively greater inhibition of GPx tends to suggest the GPx mediated mode of neutralization of peroxide and hydroxyl radicals to be the dominant mechanism

The recorded data on oxidative stress parameters do suggest some interactive effects of Cd, Cr and Ni. However, potentiating effects of a mixture of 25 groundwater contaminating metals on oxidative stress on liver, kidney and brain, when given through drinking water, has been reported for male rats (Jadhav *et al.*, 2007). Similarly, a synergistic affect of Pb, Mn and Hg on central and peripheral nervous activity in rats have also been reported (Papp *et al.*, 2006). At the same time, Kluste *et al.* (2006) have demonstrated a concentration dependent differential interaction of As, Cd, Hg and Pb with a synergistic effect at lower concentration and antagonistic

effect at higher concentration. These reports and the present observations taken together suggest the need to evaluate the interactive toxic effects of metals in experimental animals on the basis of environmental relevance and in the context of human health. The combination of Cr, Cd and Ni taken in the present study in the above context has shown at least an antagonistic effect as, degree and pattern of LPO and changes in antioxidants indicate an effect which is lesser to the one observed with Cr or Cd individually but more than that recorded for Ni (Chapters 1,3,5). On a comparative basis from the previous studies, it is inferred that the generation of hepatic oxidative stress is in the hierarchical order of Cr< Cd < Ni. A combination of these however shows oxidative stress greater than Ni but lesser than Cr and Cd. The exact mechanism of antagonism between the three metals is not clear. Interestingly, Cd-Ni interaction has been reported to stimulate detoxification mechanism by way of hepatic monoxygenases and certain cytochrome P450 isoenzymes (Iscan *et al.*, 1992). Again, Iscan at al. (1992) had shown increased GST activity on Ni exposure and no augmentation or inhibition by Cd when given in combination. On the other hand, a combination of Cr and Cd has shown a protective effect of Cr on Cd induced toxicological effects in rats (Stacey *et al.*, 1983) and a protective effect of Cr on Cd induced negative effect on the quality of Quail eggs (Shalicka *et al.*, 2008). In this context, it is likely that, even in the present study, Cr might be exerting some protective effect on Cd toxicity and even vice versa but at the same time with a slightly potentiating effect of Ni. There also seems to be an effect on metal uptake by hepatic tissue as the hepatic metal load of all the three metals when given in combination is found to be significantly lower than that shown by individual metals.

Presumably, there is some interactive interference in the hepatic entry of these metals or even possibly there is a better evacuation of metals. The decreased degree of oxidative stress compared to Cd or Cr alone may also suggest induction of protective mechanism involving metallothionein and iron binding proteins as shown for Cd and Ni (Chapters 3, 5). These however need to be ascertained to provide validity.

Though the TM mixture seems to have an antagonistic interaction with the evaluated oxidative stress parameters showing an intermediatery level between Cr, Cd and Ni. Though this suggests a reduced cellular oxidative stress, the TM mixture seems to have greater cytotoxic effects. This is clearly discernable from the histological observations of disruption of hepatic cords, cellular necrosis or apoptosis, distended sinusoids and disintegration of endothelial lining of capillaries and central vein. These cytoarchitectural distortions are much severe than those observed on exposure to metals individually (Chapters 1, 3,5). Such severe impact on the hepatic histoarchitectural organization, in the midst of no additive oxidative stress and decreased tissue metal load, are perplexing and warrant more detailed investigations on the mechanism of cytotoxic manifestations of a combination of Cr, Cd and Ni. The additive or synergistic cytotoxic effect of the three metals is clearly confirmed by 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. Apparently, there is cumulative additive or synergistic effect of Cr, Cd and Ni on the structural and functional damages inflicted by the three metals. Such effects may involve cell adhesion molecules, cytoskeletal proteins and vascular endothelial integrity. Only future studies can throw light on these aspects.

Another interesting revelation from the present study is the paradoxical effect on glycemic status which tended to be hypoglycemic as against hyperglycemic effect of the metals individually (Chapters 1,3,5). The serum insulin titres are also slightly lower in animals exposed metals individually. This seems to project another compounding effect of the metallic mixture on carbohydrate metabolism and glycemic status. Since metals may function as endocrine disruptors, the combination of Cr, Cd and Ni may have an endocrine effect leading to subtle alteration in insulin:glucagon molar ratio which could account for the observed glycemic effect. However, this is another aspect which needs to be ascertained and, in the context of carbohydrate metabolism, the interactive effects of the metals seem to be much different from the effects of the metals individually. Trimetallic mixture induced hematological changes suggest a drop in hemoglobin content and platelet count. The drop in hemoglobin content is an indication of disturbed iron metabolism. This is likely to be a Cd effect as it can displace iron from membrane and other bound proteins creating iron deficiency and consequently affect the bone marrow and hemoglobin synthesis. This has been discussed earlier (Chapter 3).

As in the case of individual metals as reported in previous chapters (1,3,5), even in the case of TM mixture, melatonin seems to have a potent ability in combating and protecting the hepatic tissue against oxidative stress as, the degree of LPO and reduction in antioxidants are much lesser when melatonin was given as supplement along with the TM mixture. Though the degree of protective effect with respect to certain parameters appeared to be lesser as compared to the effects against individual metals, as a whole, melatonin seems adept in protecting the hepatic tissue against the

adverse effects of the three metals in combination. However, a combination of melatonin along with Vit C or Vit E may be much more potent. The involvement of melatonin in combating oxidative stress is also clearly indicated by its declining serum level in animals exposed to the TM mixture. Obviously, melatonin might be exerting protective effect by acting as a direct scavenger of free radicals and, by inducing antioxidant enzymes and even by negating the effects of metal by complexing with them and acting as a chelator. The protective effect of melatonin even on glycemic status and hepatic damage is visible in the form of normalization of serum glucose levels and hepatic damage marker enzymes (ALP and ALT). The protective effect of melatonin against the cytotoxic effects of metals is very remarkable as seen by near normal histoarchitecture of hepatic tissue. Apparently, the role of melatonin in the cytological and histological organization of tissues needs to be studied at some depth and, the present observation suggests a possible role of melatonin in maintaining tissue organization. The mechanism and site of action need to be understood in this context.

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 endocrine status and 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. This study highlights the need to evaluate the toxicological manifestations of relevant metal

combinations for understanding the possible complications to human health.

Summary of Chapter 7

The present study was undertaken to evaluate hepatic oxidative stress induced by a realistic dosage of Trimetallic (TM) mixture (Cr, Cd and Ni) based on environmental relevance and provided through drinking water for 15, 30 and 60 days. 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 overhelmed 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.