CHAPTER 8

Chapter 8

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

Many studies have reported toxic effects induced in humans and animals when exposed to certain metals. Several essential transition metals participate in control of various metabolic and signaling pathways. However, due to the rich coordination chemistry and redox properties of metals, they are capable of escaping out of the control mechanisms such as homeostasis, transport, compartmentalization and binding to the designated tissue and cell constituents. The breakdown of these mechanisms can lead to the metal binding to protein sites other than those tailored for that purpose or displacement of other metals from their natural binding sites. A growing pool of results provide evidence that toxic metals are capable of interacting with nuclear proteins and cellular proteins and even DNA leading to oxidative deterioration of biological macromolecules. 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 sulfhydryl and other effects (Valko *et al.,* 2005). Previous environmenatal impact assessment (EIA) studies along the effluent channel at Vadodara have shown high levels of cadmium (Cd), chromium (Cr) and nickel (Ni) in vegetables and cereals. It is likely that consumption of these vegetables and cereals could lead to systemic entry of these metals (Ramachandran, 2003). The toxic effects of metals involve hepatotoxicity, neurotoxicity and nephrotoxicity.

Cadmium is one of the priority pollutants and is widely present in the environment of Vadodara. The mechanism of toxicity of Cd can be multifactoral and because of its carcinogenic properties it has been classified as number I category of carcinogen by International Agency for Cancer (IARC, 1991). A number of studies in human and experimental animals have shown kidney as the critical target organ for cadmium-induced nephrotoxicty (Renugadevi and Prabu, 2009). A variety of renal toxic effects involving proximal tubules and glomerulus (Ahn *et al.,* 1999) entire kidney, glomerular swelling, atrophic and pyknotic nuclei, glomerular basement membrane swelling, vacuolization, apoptosis and necrosis (Kaur *et al.,* 2006), and kidney stone (Hu, 2000) have all been suggested in cadmium exposed rats. Oxidative stress and reactive oxygen species (ROS) formed in the presence of cadmium could be responsible for its toxic effects in many organs or cells (Wang *et al.,* 2004; Watjen and Beyermann, 2004).

Chromium is another widely used industrial chemical known to cause many systemic injuries and the major mechanism of action is due to generation of free radicals (Stohs, 1995; Valko *et al.,* 2005). Kidney being the main target tissue for Cr, accumulation is therefore more sensitive than other tissues (Dartch *et al.,* 1998). Nephrotoxicity as well as hepatotoxicity have been reported in human and experimental animals following exposure to Cr (Sugiyama, 1992). Subcutaneous administration of Cr(VI) in rats has been shown to result in progressive proteinuria, increased urea nitrogen and creatinine along with elevated serum activity level of alanine aminotransferase and renal lipid peroxidation (Na, 1991).

Nickel is another environmental contaminant which, after entry into body, targets organs. Much of the toxicity of nickel may be associated with interference with

physiological processes regulated by magnesium, zinc, calcium and manganese (Cooghan, 1989). Kidney is the principal organ for Ni toxicity in terms of toxicokinetics and accumulation (Sundermann, 1988). Acute intraperitoneal (i.p) injections of nickel chloride in rats have shown the highest accumulation of the metal in kidney (Sarkar, 1980). Ni compounds can induce LPO and modify the cellular antioxidant system as shown by many studies. Rats treated subcutaneously with $NiCl_2$ have increased LPO in liver and kidney (Sundermann *et al.,* 1984). Treatment of rats with Ni compounds caused a decrease in the GSH level as well as a decrease in glucose-6-phosphate dehydrogenase and glutathione reductase activity (Cartana *et al.,* 1992).

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 renal 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 toxicity on long term systemic entry into humans though diet and water, a realistic dosage has been worked out based on the contents of Cr, Cd, and Ni in vegetables and food grains and, an average daily food intake. Conversion factor of 6.2 (OECD, 2005) has been used for extrapolation of

dosage from human to rat. Using such a dosage, a duration dependent (15, 30 and 60 days) evaluation of renal 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 trimetallic (TM) mixtures.

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 through drinking water.

Results:

Lipid Peroxidation (LPO): Changes in renal LPO levels following exposure to the TM mixture for 15, 30 or 60 days are shown in fig 8.1; table 8.1. An age dependent increase in LPO levels was noticeable in control (Con) rats. Oral exposure to the TM mixture showed significant increment in LPO with a duration dependent increase reaching maximum level at 60 days. Melatonin afforded similar degree of protection in animals exposed to the TM mixture irrespective of duration of exposure.

Glutathione (GSH): Changes in renal GSH levels following the TM mixture exposure for 15, 30 and 60 days are shown in fig 8.2; table 8.2. There was an age dependent gradual decrease in the GSH content in Con animals. Significant duration dependent decrease in GSH content was the feature in animals treated with the TM mixture. There was significant increase in GSH level in Con animals administered with melatonin. Melatonin showed similar degree of protection in the TM mixture treated animals in all the three durations of exposure.

Ascorbic Acid (Vit C): Changes in renal Vit C content following exposure to the TM mixture treatment for 15, 30 and 60 days are shown in fig 8.3; table 8.3. Control rats showed a gradual age dependent decrease in Vit C content. There was significant progressive decrement in Vit C content in the TM mixture exposed animals. The Vit C content was significantly elevated in animals treated with melatonin alone while, melatonin administration in animals exposed to the TM mixture showed progressively increasing protective effect with increasing duration of exposure.

Superoxide Dismutase (SOD): Changes in renal superoxide dismutase activity following the TM mixture exposure for 15, 30 and 60 days are shown in fig 8.4; table

8.4. A gradual age dependent decrease in SOD activity could be seen in the Con group of animals. The activity of SOD showed significant inhibition in the TM mixture treated group of animals with almost same degree of inhibition at all the three treatment periods. There was a significant resistance against TM mixture induced decrease in enzyme activity when co-administered with melatonin. The degree of protection with melatonin was slightly lesser in the longest duration exposure group.

Catalase (CAT): Changes in renal catalase activity following exposure to the TM mixture for 15, 30 and 60 days are shown in fig. 8.5; table 8.5. A gradual age dependent decrease in enzyme activity was the feature in the Con group of animals. There was significantly decreased CAT activity in the TM mixture treated group compared to the Con group. Melatonin showed a tendency for increased enzyme activity in Con animals. Though there was progressively increasing inhibition of enzyme activity with increasing duration of TM mixture exposure, the protective effect of melatonin with co-administration was found to be most effective in the longest duration of exposure.

Glutathione Peroxidase (GPx): Changes in renal GPx activity following exposure to the TM mixture treatment for 15, 30 and 60 days are shown in fig 8.6; table 8.6. Control animals tended to show a gradual age dependent decrement in GPx activity. The TM mixture exposure resulted in duration dependent progressive decrease in enzyme activity. Melatonin co-administration tended to resist the decrement in GPx activity. The protective effect of melatonin on co-administration with TM mixture was found to be almost to the same degree irrespective of duration of exposure.

Metal Load: Changes in renal accumulation of Ni, Cd and Cr following exposure to the TM mixture treatment for 15, 30 and 60 days are shown in fig 8.7; tables 8.7.

There was significant accumulation of all the three metals on exposure to the TM mixture. The increased metal load was found to be maximal at 15 days with prominent decrement thereafter at 30 and 60 days. There was significant decrease in metal load when melatonin was administered alone or in combination with the TM mixture with the protective effect becoming progressively greater with increasing duration of exposure.

Serum Parameters: Changes in serum renal toxicity parameters like urea and creatinine are shown in tables 8.8. Both the renal toxicity parameters showed significant increase in the TM mixture exposed group. There was gradual duration dependent increase in both the parameters with TM mixture exposure. These increases in serum urea and creatinine levels were successfully prevented by melatonin when co-administered with the TM mixture.

Serum lipid profile: The changes in serum lipid profile induced by the TM mixture is represented in table 8.9 to 8.11. Whereas melatonin has a tendency to increase the triglyceride level, TM mixture tended to decrease the same. However, administration of melatonin had a negating effect on the TM mixture induced change. Total cholesterol level was found to show a decrement in TM mixture exposed animals which was essentially due to a decrease in HDL and corresponding increase in LDL and VLDL. These changes in cholesterol components were effectively prevented by concurrent melatonin administration. All the above changes were duration dependent.

Hematological profile: The changes in total leucocyte count and differential count are depicted in table 8.12 to 8.14. Apparently, exposure to the TM mixture increased the total leucocyte count contributed essentially due to a marked increase in

polymorphs and lymphocytes. Simultaneous administration of melatonin more or less prevented these hematological changes.

Histological Observations: The histomicrographs of the renal tissue exposed to the TM mixture are represented as fig.8.9 to 8.31. Exposure to the TM mixture showed significant disruption in both, the parietal and visceral layers of Bowmann's capsule more so in the latter. Glomerular disintegrity and podocyte injury in the visceral layer of Bowmann's capsule were clearly noticeable. Proximal tubular damage was marked by disruption and necrosis/apoptosis of the cells. These changes were very prominent in the 15 days treatment schedule. However by 30 and 60 days, there was progressive improvement in proximal tubular damage with minor recovery in glomerular organization. However, disruptions in the parietal layer and podocyte injury in the visceral layer of Bowmann's capsule were still discernable.

Fig 8.1 Comparison of the temporal slope of percentage change in renal LPO between TM mixture and component metals individually

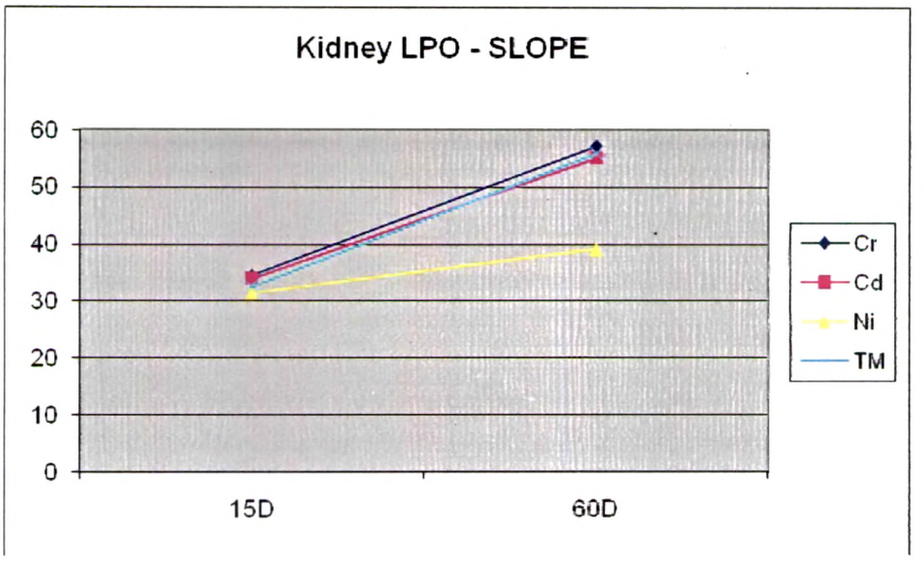

Fig. 8.2 Percentage increase in renal LPO at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

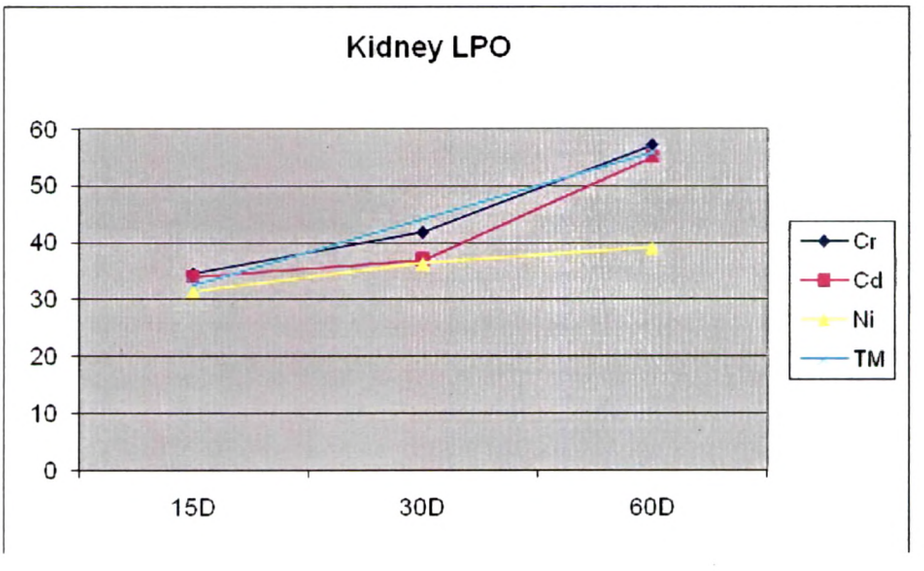

Fig 8.3 Comparison of the temporal slope of percentage change in renal GSH between TM mixture and component metals individually

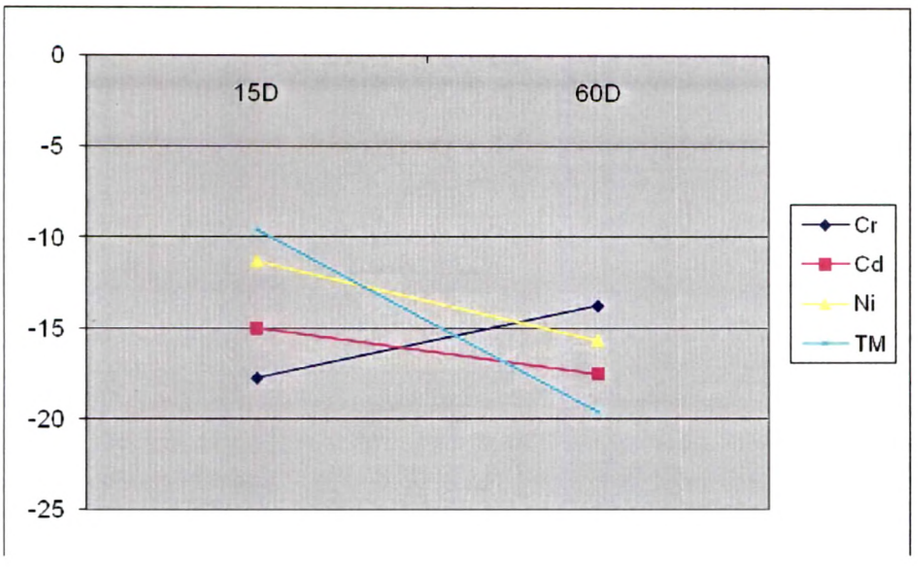

Fig. 8.4 Percentage decrease in renal GSH at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

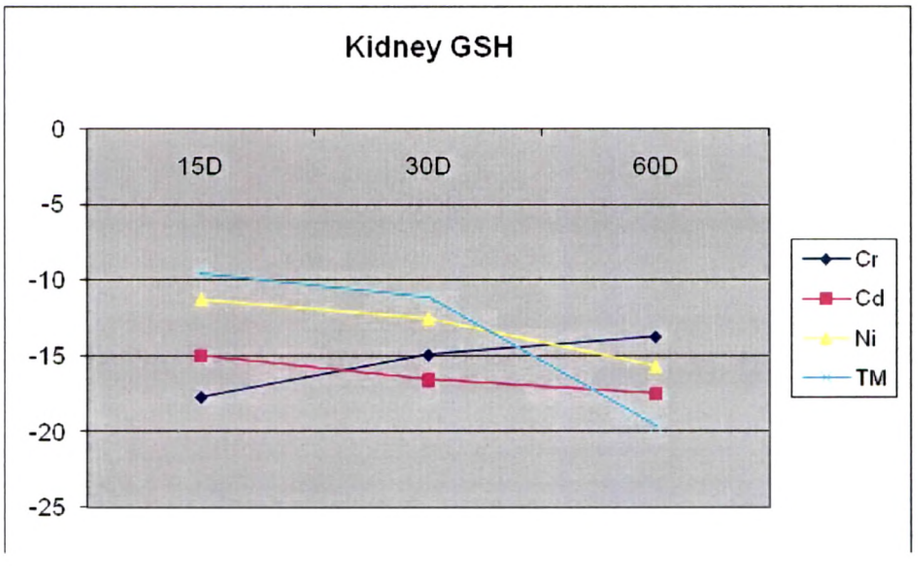

Fig 8.5 Comparison of the temporal slope of percentage change in renal AA between TM mixture and component metals individually

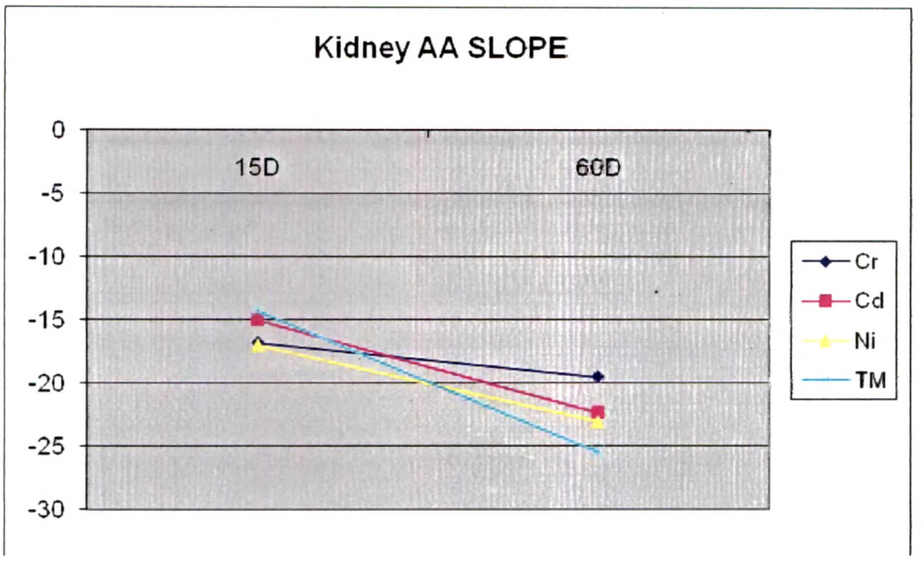

Fig. 8.6 Percentage decrease in renal AA at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

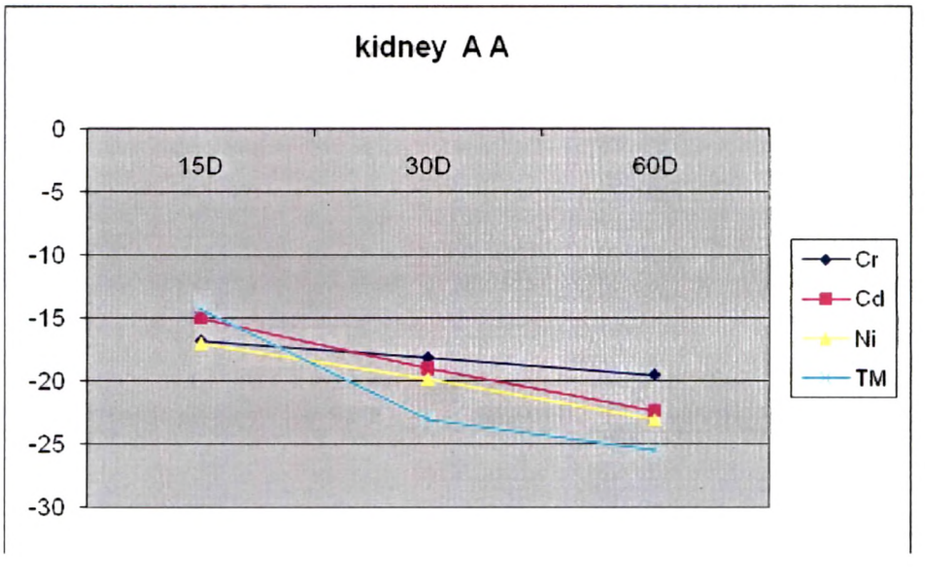

Fig 8.7 Comparison of the temporal slope of percentage change in renal SOD between TM mixture and component metals individually

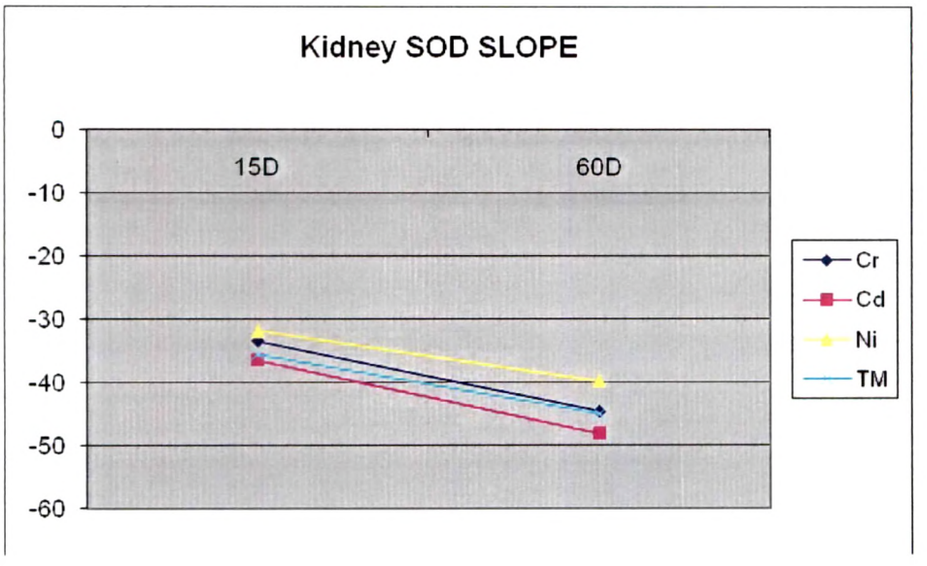

Fig. 8.8 Percentage decrease in renal SOD at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

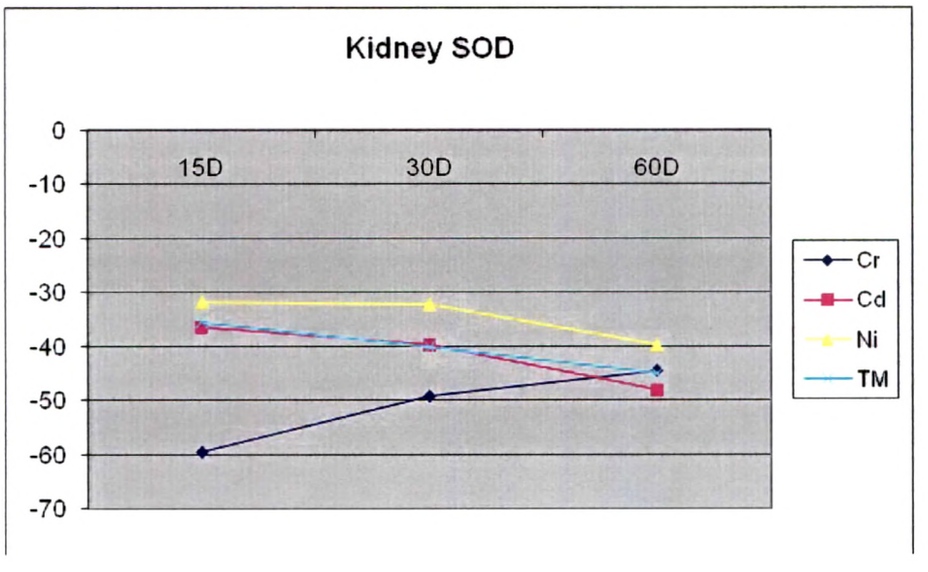

Fig 8.9 Comparison of the temporal slope of percentage change in renal CAT between TM mixture and component metals individually

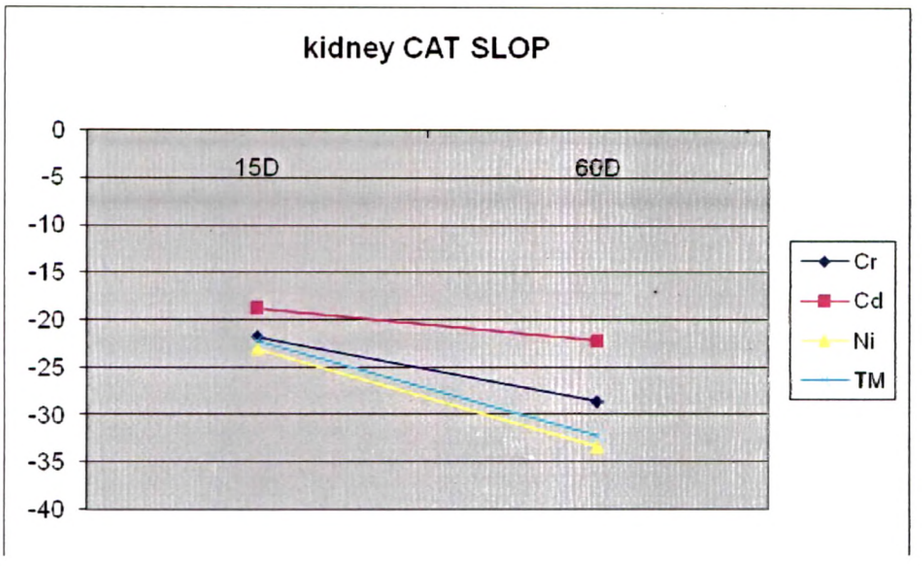

Fig. 8.10 Percentage decrease in renal CAT at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

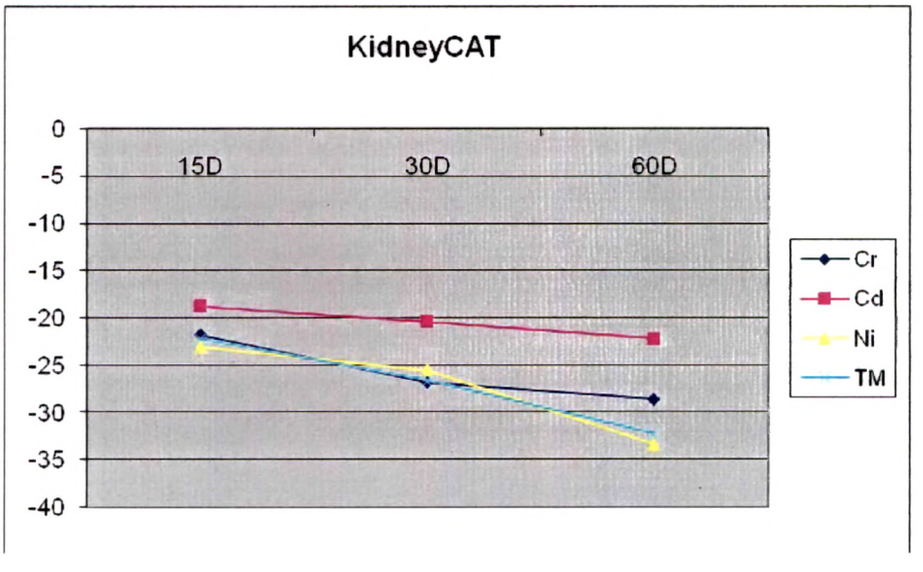

Fig 8.11 Comparison of the temporal slope of percentage change in renal GPx between TM mixture and component metals individually

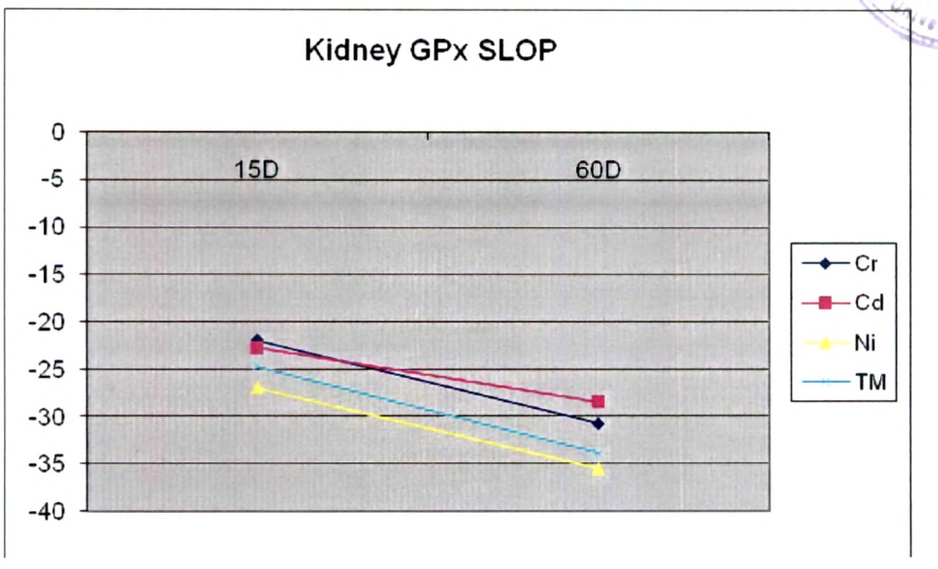

Fig. 8.12 Percentage decrease in renal GPx at 15, 30 and 60 days of exposure to the TM mixture or the component metals individually

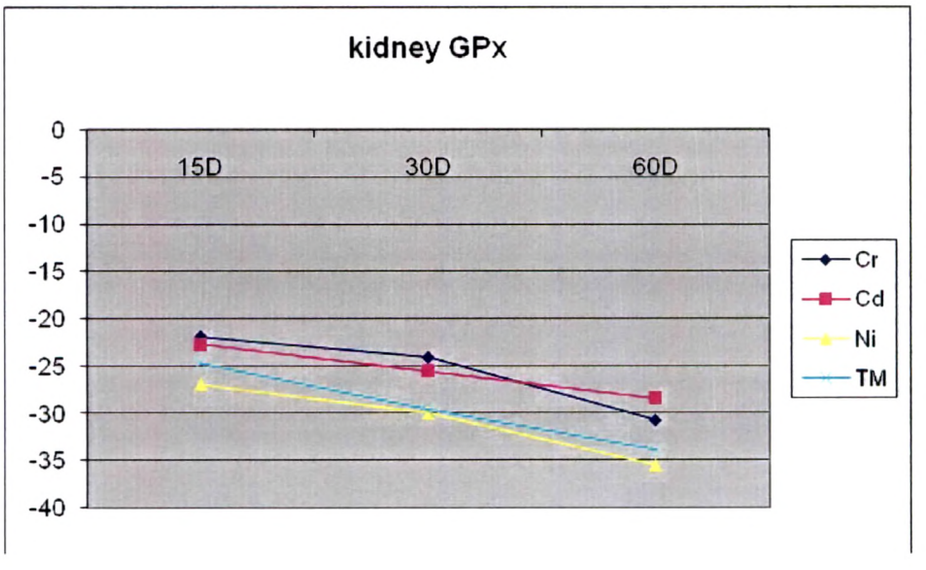

Table: 8.1 Levels of **renal lipid peroxidation (LPO)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	41.78 ± 1.26	42.49± 1.20	44.89±1.41
Mel	38.92 ± 1.32	39.20± 1.290	40.21 ±1.38
Tri	55.39±1.76@	61.23± 1.50@	69.95± 1.93@
Tri+Mel	44.64± 1.82 #	47.02±1.29#	50.59± 1.28 #

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

Table 8.2. Levels of **renal glutathione (GSH)** following 15, 30 and 60 days treatment with Nickel

	15 days	30 days	60 days
Con	0.115±0.0013	0.108±0.0014	0.102±0.0013
Mel	0.127±0.0014*	0.121±0.0018*	0.116±0.0013*
Tri	0.104±0.0013@	0.094±0.0016@	0.082±0.0019@
Tri+Mel	0.108±0.0018	0.102±0.002	0.099± 0.0013#

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

Table 8.3. Levels of **renal Ascorbic Acid (Vit C)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	0.140±0.0012	0.126±0.0011	0.106±0.0012
Mel	0.154±0.0015*	0.146±0.0016	0.129±0.0018*
Tri	0.120±0.0015@	0.097±0.0019@	0.079±0.0015@
Tri+Mel	0.145±0.0019#	0.131±0.0013#	0.113±0.0017 #

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

Table 8.4. Levels of **renal Superoxide Dismutase (SOD)** following 15, 30 and 60 days treatment with Trimetallic combinaiton

	15 days	30 days	60 days
Con	13.87±0.13	12.03±0.16	10.52±0.12
Mel	16.01±0.16*	14.27±0.11*	12.37±0.16*
Tri	8.95±0.150@	7.21±0.19@	5.79±0.189@
Tri+Mel	10.33±0.23#	9.95±0.15#	8.91±0.130#

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

Table 8.5. Levels of **renal Catalase (CAT)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	45.26±1.63	41.27±1.39	38.32±1.39
Mel	48.98±1.29	46.47±1.45	42.35±1.37
Tri	35.19±1.65@	30.26±1.75@	25.95±1.48@
Tri+Mel	43.79±1.91#	38.12±1.85#	33.29±1.66#

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

Table 8.6. Levels of **renal Glutathione Peroxidase (GPx)** following 15, 30 and 60 days treatment with TM mixture

	15 days	30 days	60 days
Con	56.87±1.28	52.38±1.82	49.92±1.43
Mel	59.98±1.09	57.04±1.39	53.76±1.52
Tri	42.85±1.95@	36.85±2.01@	32.99±1.57@
Tri+Mel	52.09±1.51#	48.52±1.98#	45.10±1.59#

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

Figure 8.13. Graph showing % accumulation of Cd, Cr and Ni in renal tissue following 15, 30 and 60 days treatment with Trimetallic Combination.

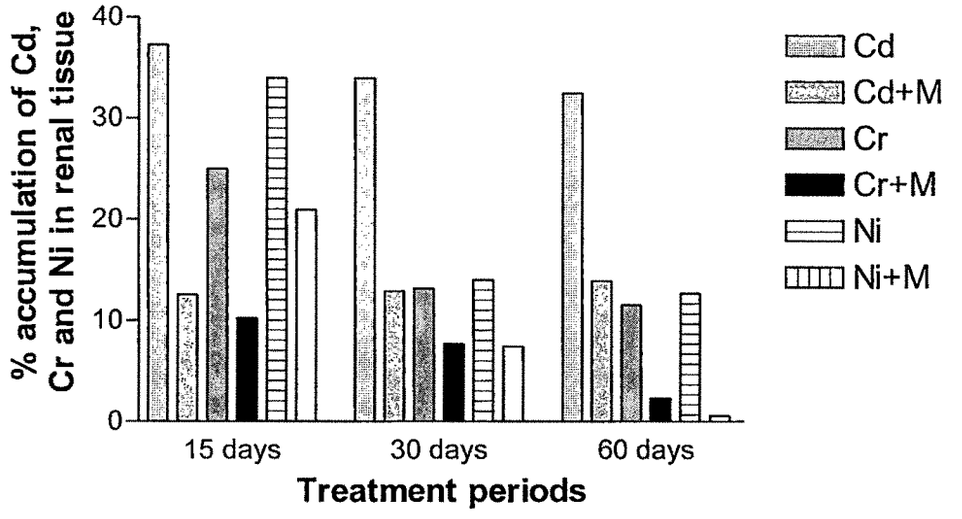

Table 8.7. % accumulation of Cd, Cr and Ni in renal tissue following 15, 30 and 60 days treatment with Trimetallic combination

	Cr	Cr+M	Cd	Cd+M	Ni	Ni+M
15 days	25	10.23	37.25	12.54	34	20.96
30 days	13.17	7.64	34.02	12.90	14	7.40
60 days	11.56	2.31	32.5	13.92	12.7	0.57

TABLE 8.8 Levels of Serum urea and creatinine following Trimetallic Mixture exposure for 15, 30 and 60 days

	15 Days		30 Days		60 Days	
	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control (Con)	38.25±0.85	0.55±0.07	38.75±1.45	0.55±0.05	31.50±0.65	0.60±0.00
Melatonin (Mel)	35.25±0.63	0.68±0.09	37.50±0.65	0.58±0.03	29.25±0.85	0.68±0.05
Trimetallic mixture (TM)	56..75±1.92@	0.75±0.09@	65.21±1.85@	0.65±0.09	74.95±1.85@	0.80±0.075@
Trimeallic + Melatonin (Cr+Mel)	35.21±2.01#	0.65±0.11	38.75±1.91#	0.60±0.10	40.75±2.15#	0.65±0.035

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

Table 8.9 Changes in serum lipid profile following 15 days of exposure with trimetallic mixture (Units expressed as mg/dl)

	Cholesterol	Triglyceride	H DL	VLDL	LDL
Control (Con)	103.50±1.04	119.25±0.85	64.25±0.85	24.25±0.85	16.25±1.11
Melatonin (Mel)	91.75±0.63*	132.00±1.08*	55.00±1.47*	26.00±1.08	11.75±1.85*
Trimetallic mixture (TM)	97.00±0.91@	101.00±1.08@	39.50±0.65@	20.75±0.48@	37.00±0.91@
Trimeallic + Melatonin (Cr+Mel)	114.00±0.82#	112.00±1.08#	58.00±0.91#	21.25±0.85	35.25±1.25

Table 8.10 Changes in serum lipid profile following 30 days of exposure with trimetallic mixture (Units expressed as mg/dl)

	Cholesterol	Triglyceride	H DL	VLDL	LDL
Control (Con)	122.75±0.85	127.75±1.11	62.50±1.04	28.50±1.19	33.75±0.85
Melatonin (Mel)	90.25±0.48*	134.50±0.65	39.50±1.04*	19.50±0.65*	33.00±1.08
Trimetallic mixture (TM)	98.50±0.65@	109.75±0.85@	38.50±1.04@	22.50±1.04@	37.00±1.08@
Trimeallic + Melatonin (Cr+Mel)	100.75±0.750	131.75±0.85	53.50±0.65#	27.00±0.91#	32.00±1.08#

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

Table 8.11 Changes in serum lipid profile following 15 days of exposure with trimetallic mixture (Units expressed as mg/dl)

	Cholesterol	Triglyceride	H DL	VLDL	LDL
Control (Con)	117.75±1.11	139.25±0.85	57.50±1.04	29.00±1.29	32.00±1.08
Melatonin (Mel)	111.75±0.85	135.75±0.25	59.00±1.08	27.25±0.85	25.25±0.85*
Trimetallic mixture (TM)	100.50±0.65@	104.00±2.04@	42.25±1.11@	22.00±1.08@	37.25±0.85@
Trimeallic + Melatonin (Cr+Mel)	116.75±0.85#	115.75±0.85#	58.75±1.55#	22.50±1.04	35.25±0.85

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

Table 8.12 Changes in hematological parameters after 15 days of Trimetallic Mixture treatment

	Leucocyte Count	Platelet Count	Polymorph	Lymphocyte	Eosin	Monocyte
Con	8275.00±85.39	1016100.00±70.83	19.00±0.91	76.00±0.41	1.25±0.25	2.25±0.25
Mel	8475.00±110.87	1025500.00±104.83	21.25±1.25	79.25±0.25	1.25±0.25	2.00±0.00
Trimetallic mixture (TM)	10000.50±34.65@	943750.00±1314.98	31.50±1.25@	79.25±0.91	1.00±0.00	1.75±0.25
Trimeallic + Melatonin (Cr+Mel)	9256.00±41.71	1193001.00±0.65#	15.25±1.04#	67.00±1.55#	1.00±0.41	1.75±0.25

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

Table 8.13 Changes in hematological parameters after 30 days of Trimetallic mixture treatment

	Leucocyte Count	Platelet Count	Polymorph	Lymphocyte	Eosin	Monocyte
Con	7175.00±125.0	1302075.00±75.08	19.25±0.63	79.00±0.91	1.50±0.29	1.75±0.25
Mel	7450.00±132.2	1423150.00±64.55	18.25±1.25	79.25±0.85	1.50±0.29	1.50±0.29
Trimetallic mixture (TM)	11800.50±1.04@	1291252±249.17@	19.75±0.85	73.75±0.86	1.00±0.00	2.00±0.00
Trimeallic + Melatonin (Cr+Mel)	10000.50±0.64	1382002.00±1.32	25.00±1.29#	80.75±1.11#	0.25±0.25	1.00±0.00

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

Table 8.14 Changes in hematological parameters after 60 days of Trimetallic mixture treatment

	Leucocyte Count	Platelet Count	Polymorph	Lymphocyte	Eosin	Monocyte
Con	8500.±91.29	1213075.00±85.39	37.75±0.85	74.75±1.25	1.00±0.00	2.00±0.00
Mel	8800±110.87	1429250.00±162.02*	52.00±0.91*	78.50±1.04	2.00±0.41	1.75±0.25
Trimetallic mixture (TM)	10200±12.36@	780000.50±0.87@	27.25±0.25@	82.25±1.11@	1.00±0.00	1.25±0.25
Trimeallic + Melatonin (Cr+Mel)	9700±21.75#	895000.80±0.48#	17.00±0.41#	80.00±0.41	1.00±0.00	1.75±0.25

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

PLATE I

Fig 8.8 Photomicrograph of control kidney showing a Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.9 Photomicrograph of 15 day melatonin treated kidney showing Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.10 Photomicrograph of 15 day Trimetallic mixture treated kidney showing Bowman's capsule with glomerulus and distal tubules (400X).Note the shrunken Bowman's capsule and glomerulus with widened distal tubular due to degenerated epithelial cells. BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.11 Photomicrograph of 15 day Trimetallic+Melatonin treated kidney(400X). Near normal appearance of the Bowmann 's capsule. BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

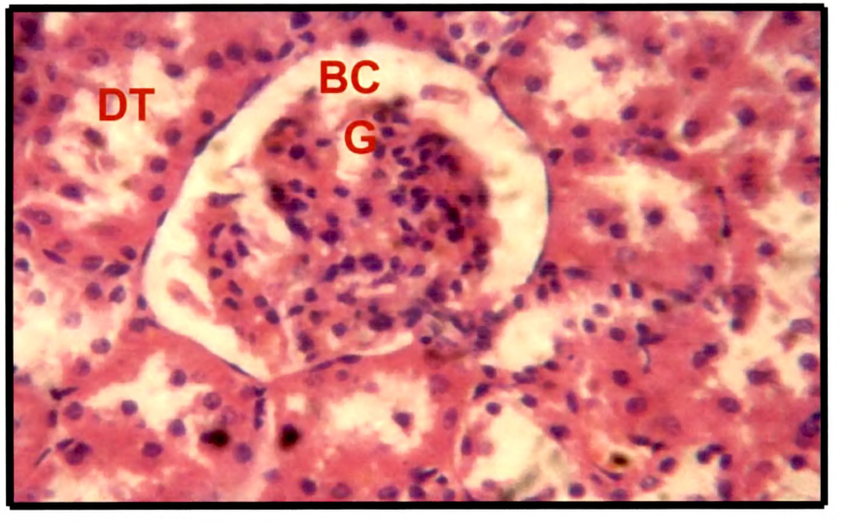

Fig 8.8 Control

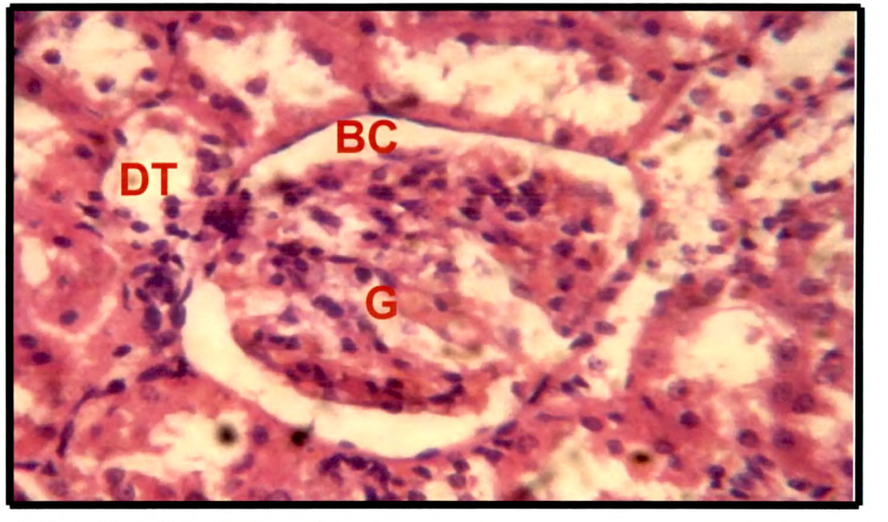

Fig 8.9 Melatonin

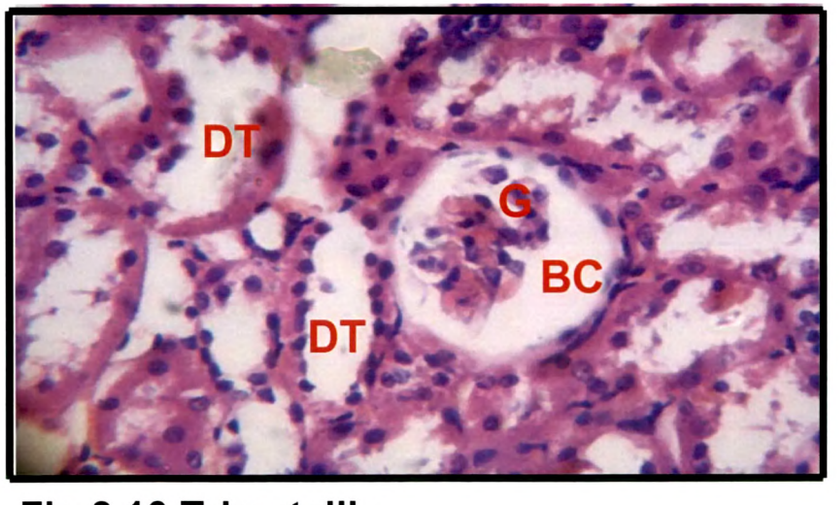

Fig 8.10 Trimetallic

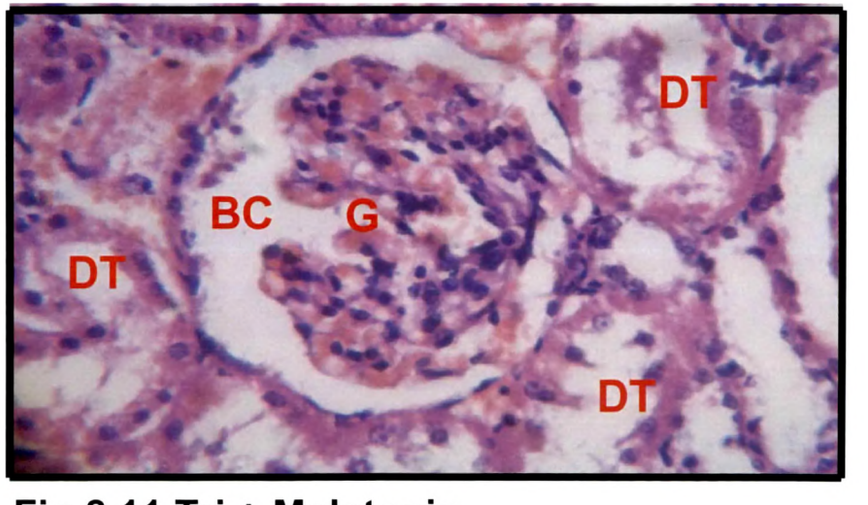

Fig 8.11 Tri + Melatonin

PLATE II

Fig 8.12 Photomicrograph of control kidney showing proximal tubules (400X), PT- Proximal tubule

Fig 8.13 Photomicrograph of kidney exposed to melatonin for 15 days showing proximal tubules (400X). PT- Proximal tubule

Fig 8.14 Photomicrograph of kidney exposed to Trimetallic mixture for 15 days showing proximal tubules (400X). Note some degeneration in proximal tubules.

Fig 8.15 Photomicrograph of kidney exposed to Trimetallic mixture+melatonin for 15 days showing near normal appearance of PT (400X).

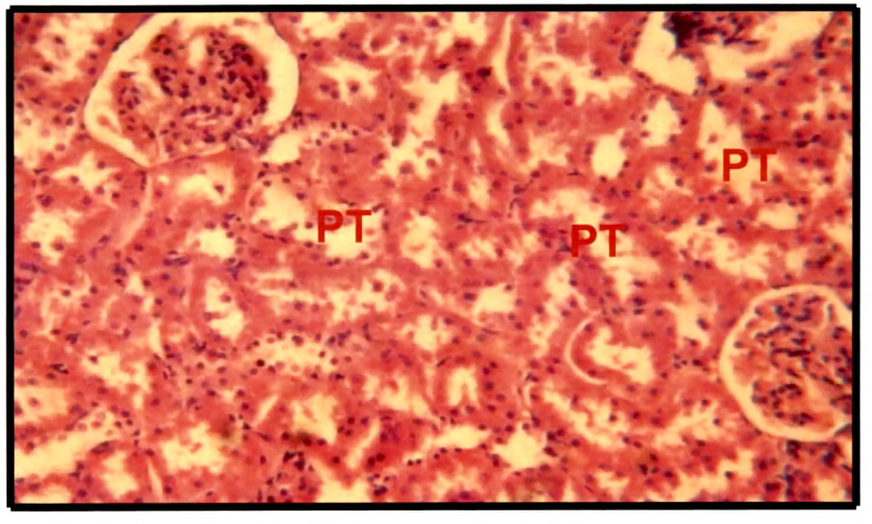

Fig 8.12 Control

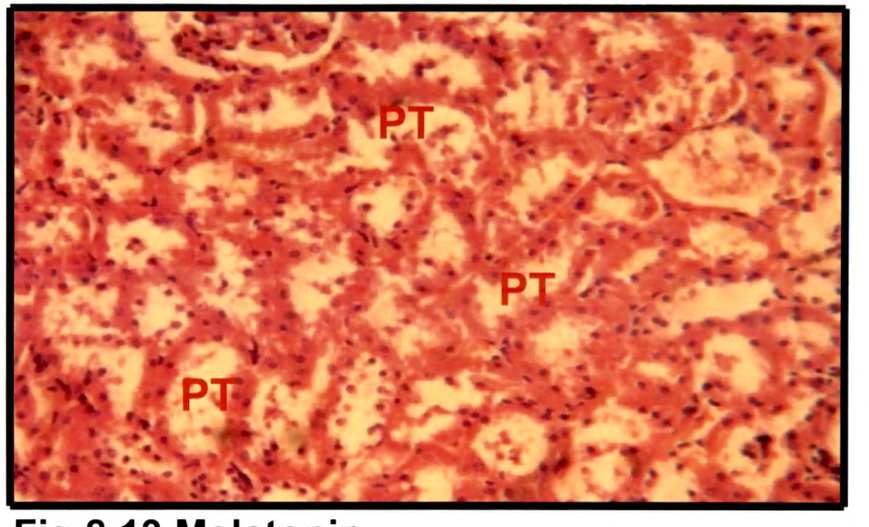

Fig 8.13 Melatonin

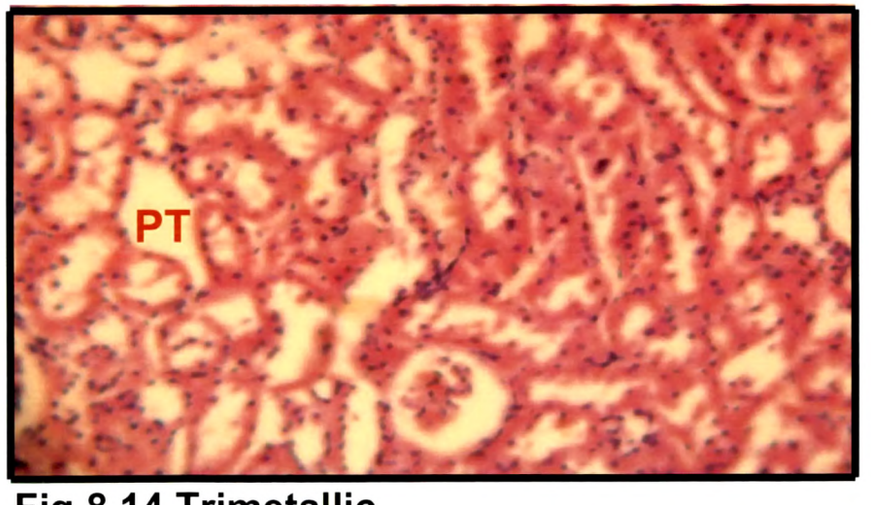

Fig 8.14 Trimetallic

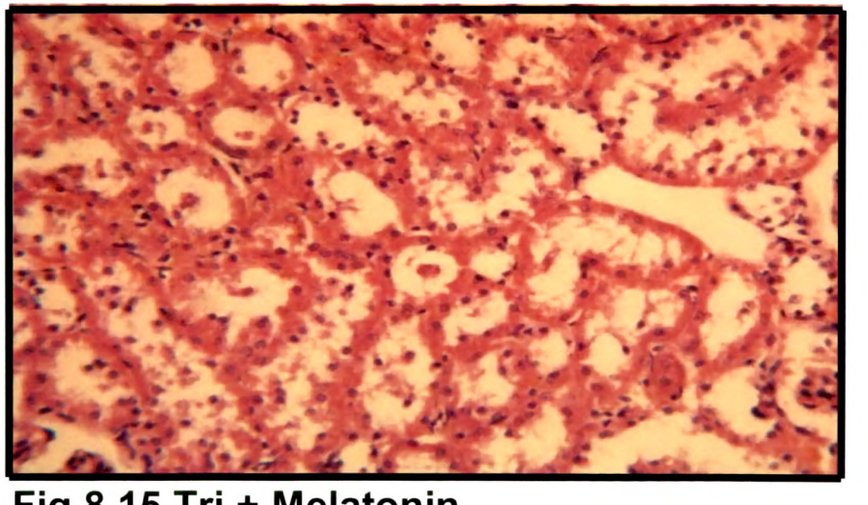

Fig 8.15 Tri + Melatonin

PLATE III

Fig 8.16 Photomicrograph of control kidney showing a Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.17 Photomicrograph of kidney exposed to melatonin for 30 days showing Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.18 Photomicrograph of kidney exposed to nickel for 30 days showing Bowman's capsule with glomerulus and distal tubules (400X). Note disrupted Bowman's capsular epithelium (arrow) and some degree of glomerular degeneration. Distal tubular disorganization is also evident. DT- Distal tubule; G- Glomerulus

Fig 8.19 Photomicrograph of kidney exposed to Trimetallic mixture+melatonin for 30 days showing normal looking Bowman's Capsule (400X).

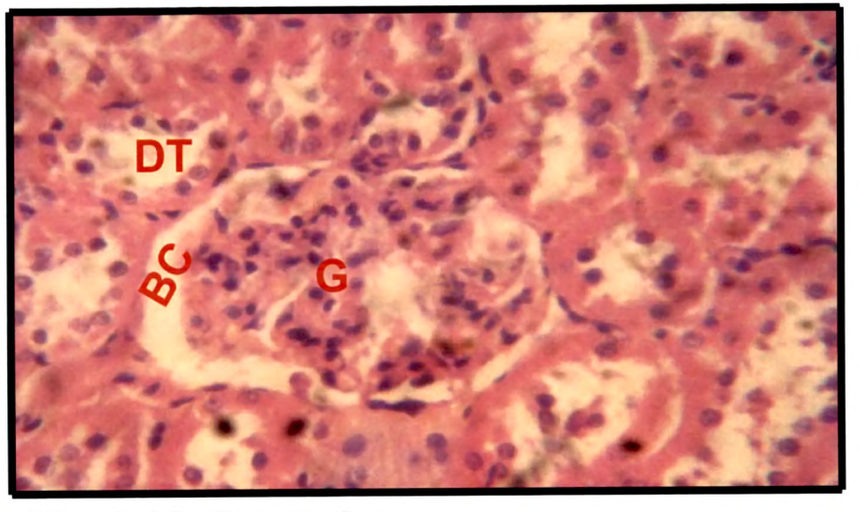

Fig 8.16 Control

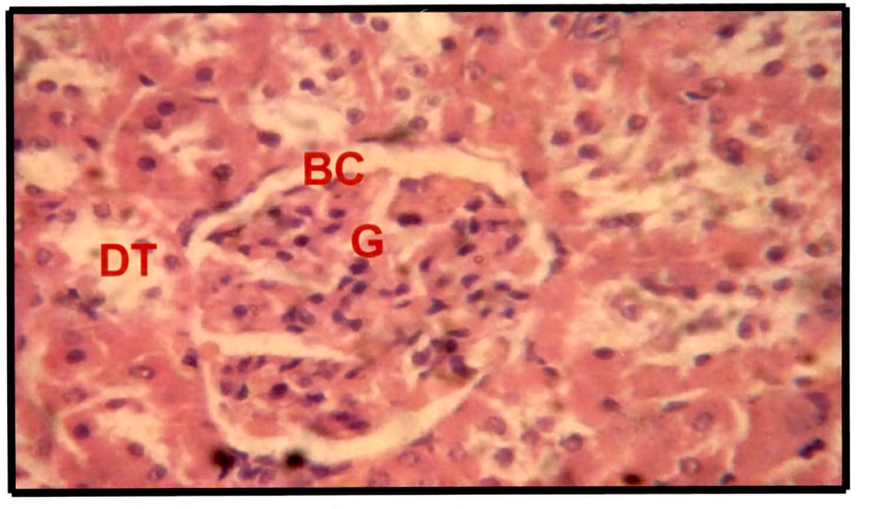

Fig 8.17 Melatonin

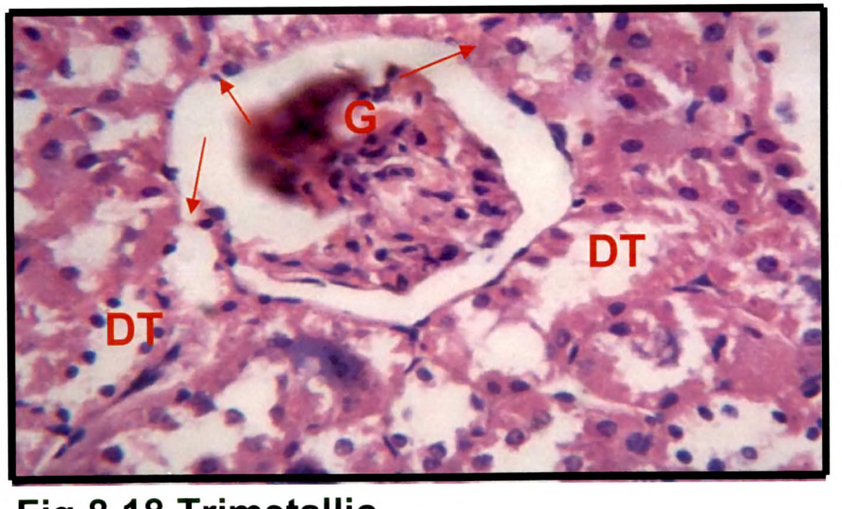

Fig 8.18 Trimetallic

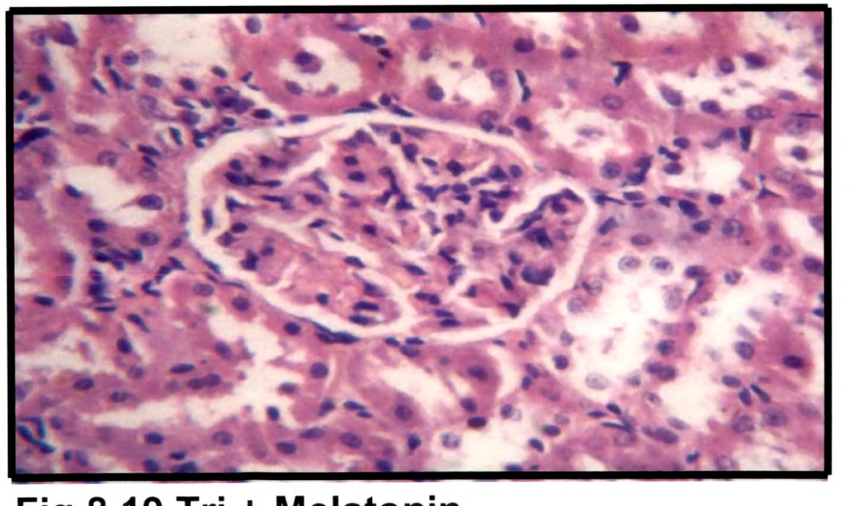

Fig 8.19 Tri + Melatonin

PLATE IV

Fig 8.20 Photomicrograph of control kidney showing proximal tubules (400X), PT- Proximal tubule.

Fig 8.21 Photomicrograph of kidney exposed to melatonin for 30 days showing proximal tubules (400X). PT- Proximal tubule

Fig 8.22 Photomicrograph of kidney exposed to trimetallic mixture for 30 days showing proximal tubules (400X).Note the greater degree of proximal tubular cell degeneration resulting in distended proximal tubules. PT- Proximal tubule

Fig 8.23 Photomicrograph of kidney exposed to Trimetallic mixture+melatonin for 30 days showing near normal proximal tubules (400X). PT- Proximal tubule

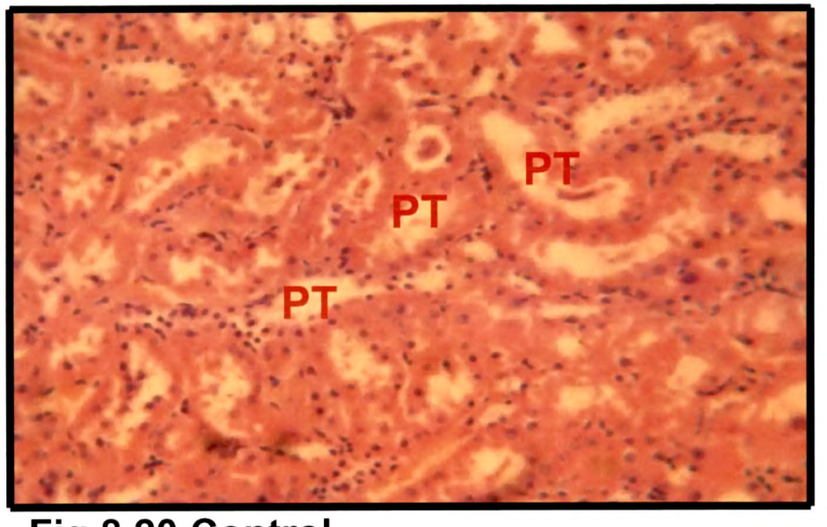

Fig 8.20 Control

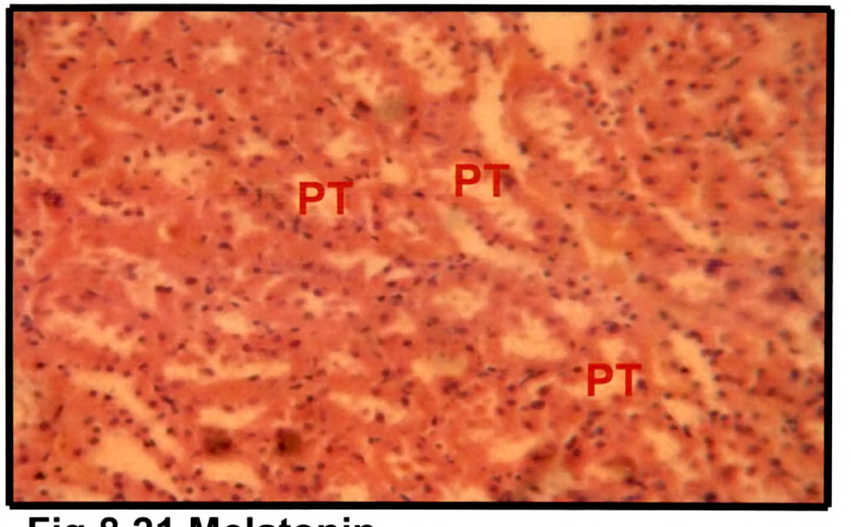

Fig 8.21 Melatonin

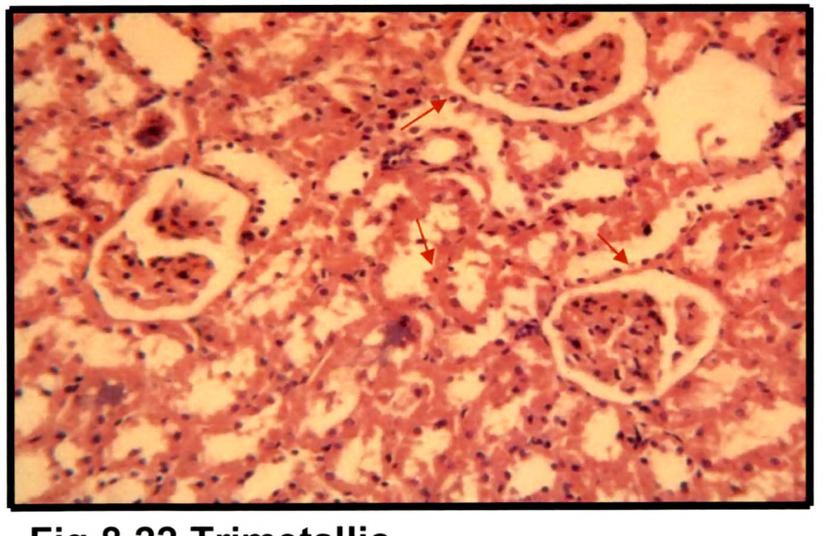

Fig 8.22 Trimetallic

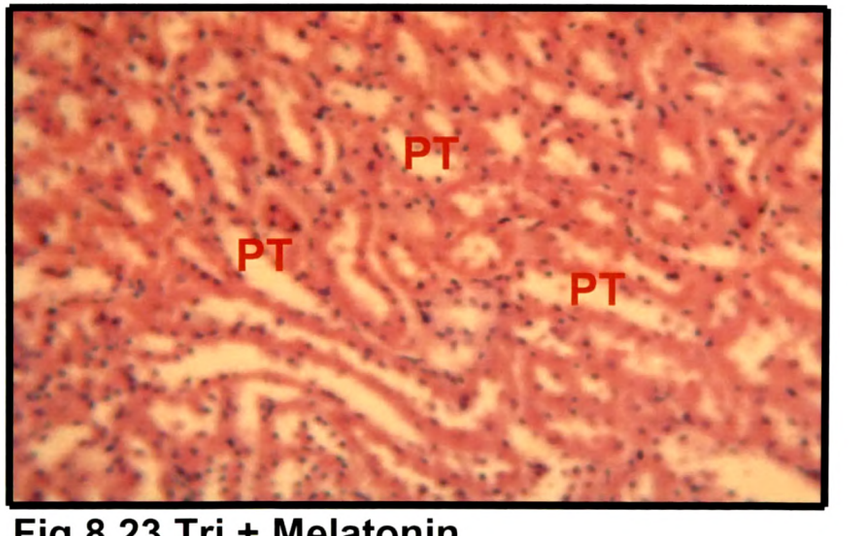

Fig 8.23 Tri + Melatonin

PLATE V

Fig 8.24 Photomicrograph of control kidney showing a Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.25 Photomicrograph of kidney exposed to melatonin for 60 days showing Bowman's capsule with glomerulus and distal tubules (400X). BC-Bowman's Capsule; DT- Distal tubule; G- Glomerulus

Fig 8.26 Photomicrograph of kidney exposed to trimetallic mixture for 60 day showing Bowman's capsule with glomerulus and distal tubules (400X). Note the disruption of Bowmann;s capsular epithelium (*) and shrunken and degenerated glomerulus. Distal tubular degeneration can be seen. DT- Distal tubule; G- Glomerulus

Fig 8.27 Photomicrograph of kidney exposed to TM+melatonin for 60 days showing normal looking Bowman's capsule. Some degree of glomerular shrinkage with visible tubular degeneration are visible (400X). DT- Distal tubule; G- Glomerulus

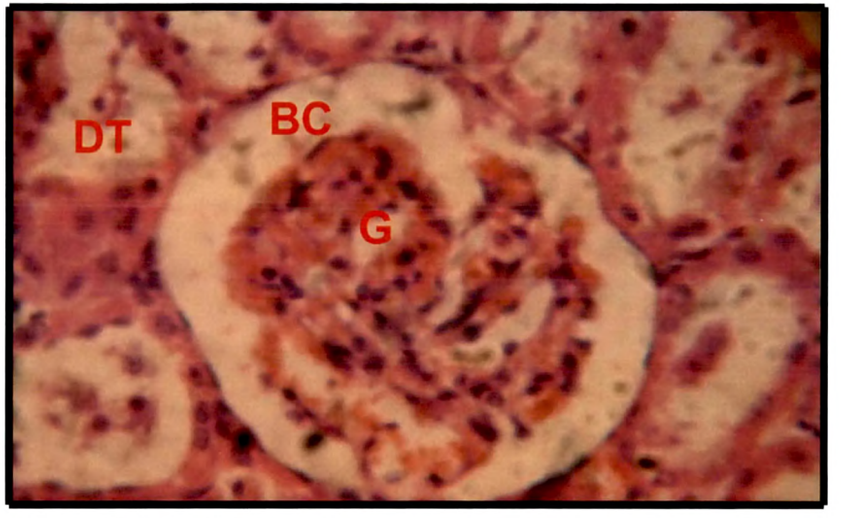

Fig 8.24 Control

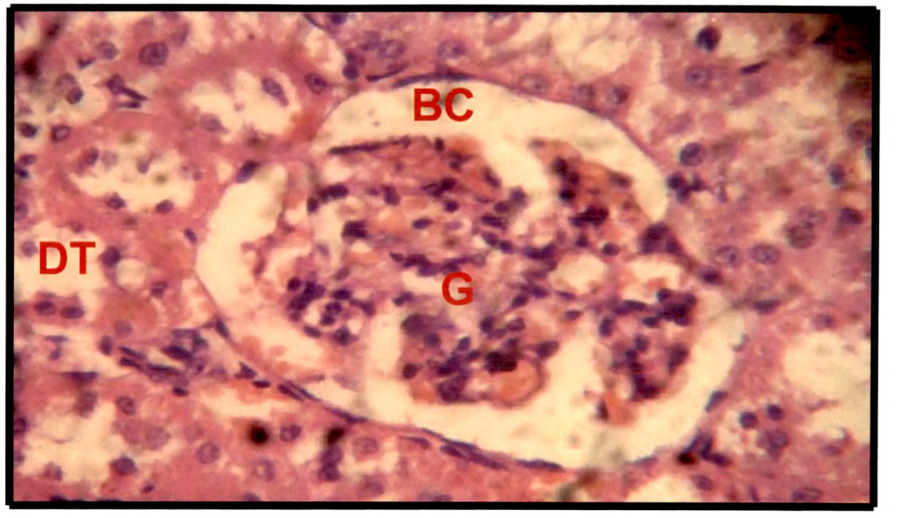

Fig 8.25 Melatonin

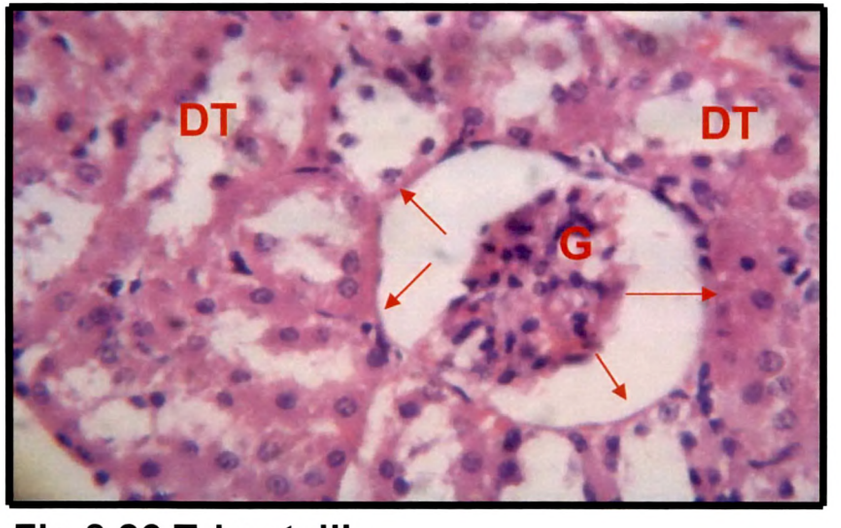

Fig 8.26 Trimetallic

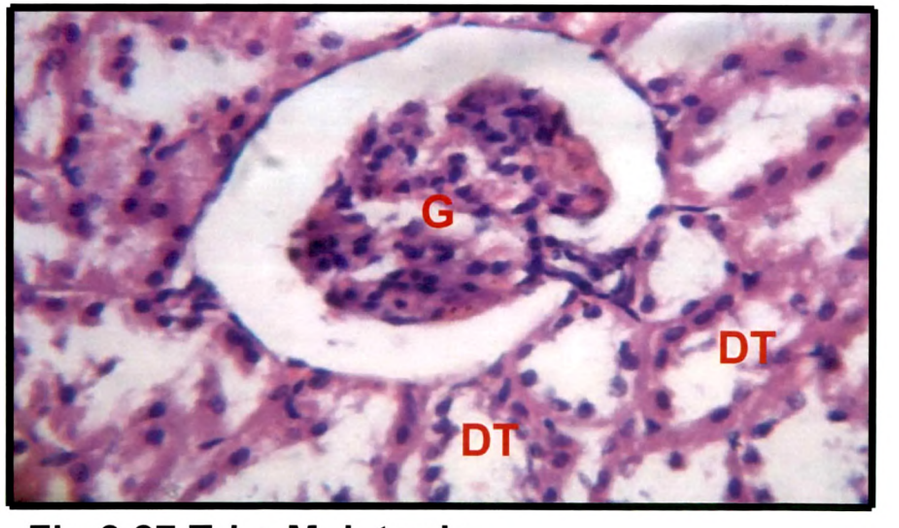

Fig 8.27 Tri + Melatonin

PLATE VI

Fig 8.28 Photomicrograph of control kidney showing proximal tubules (400X), PT- Proximal tubule.

Fig 8.29 Photomicrograph of kidney exposed to melatonin for 60 days showing proximal tubules (400X). PT- Proximal tubule

Fig 8.30 Photomicrograph of kidney exposed to Trimetallic mixture for 60 days showing proximal tubules (400X). Note the evident proximal tubular degeneration clearly evident. PT- Proximal tubule

Fig 8.31 Photomicrograph of kidney exposed to Trimetallic +melatonin for 60 days showing normal proximal tubules (400X). Minor degree of proximal tubular degeneration evident. sPT- Proximal tubule

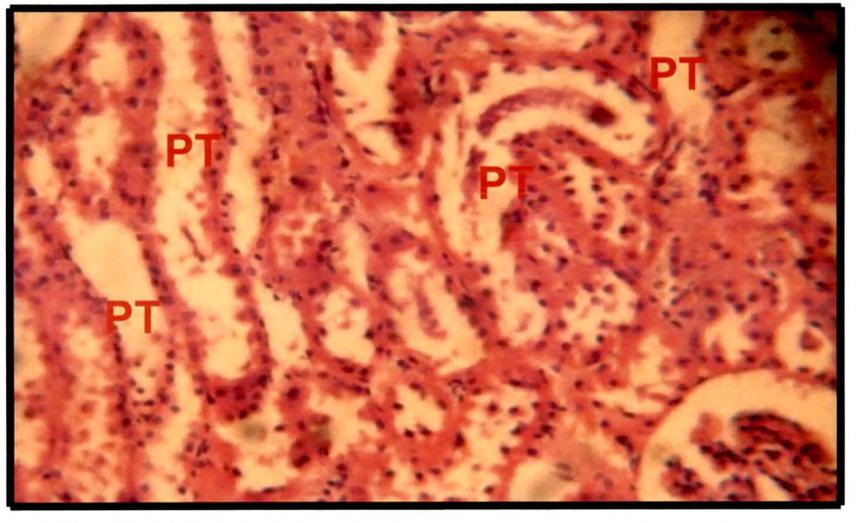

Fig 8.28 Control

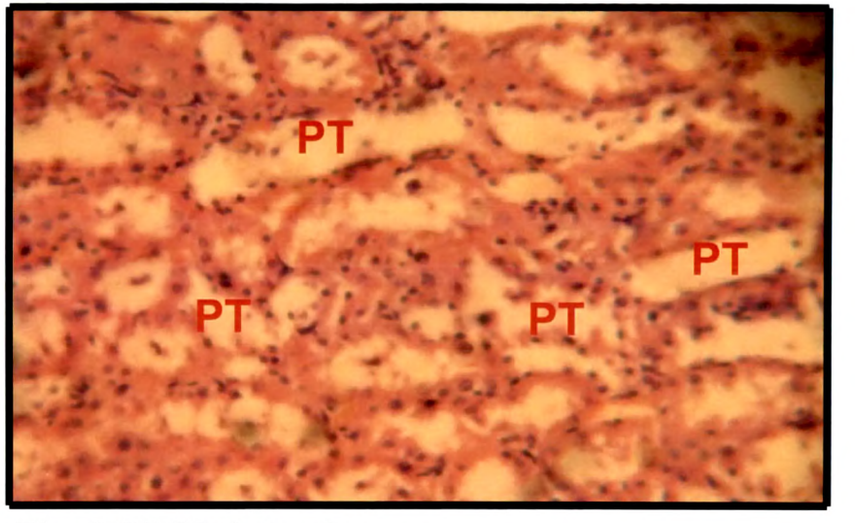

Fig 8.29 Melatonin

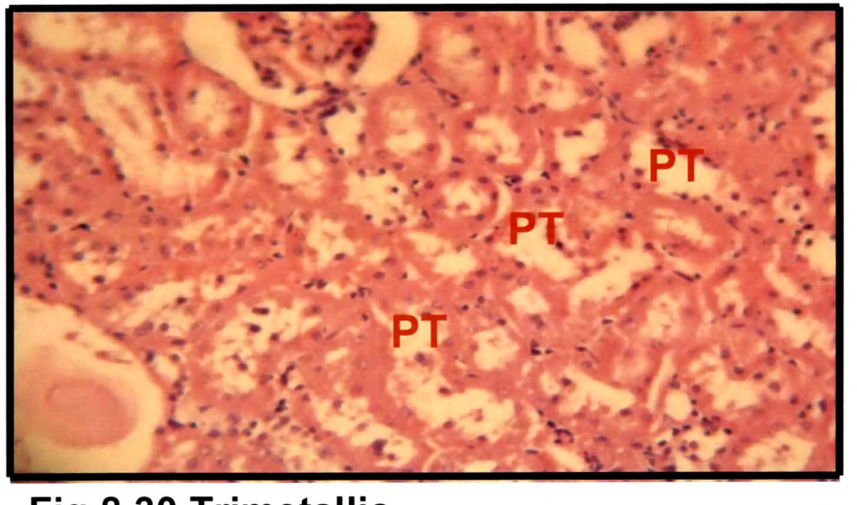

Fig 8.30 Trimetallic

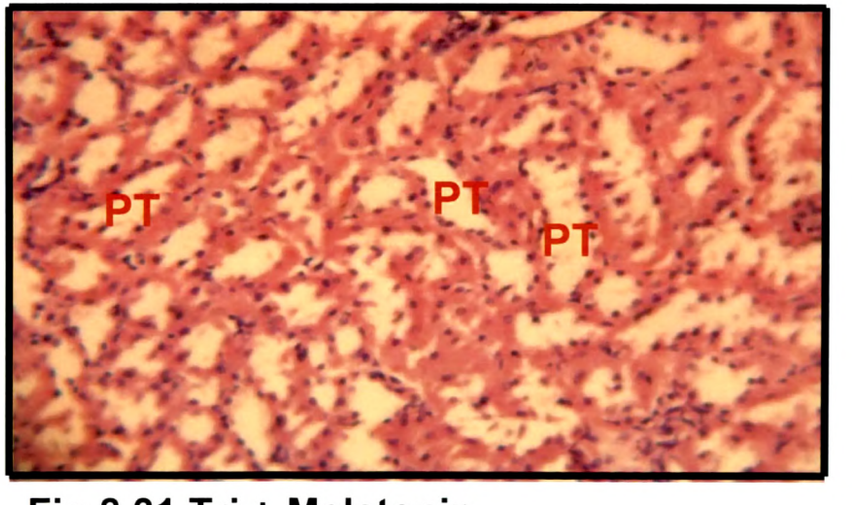

Fig 8.31 Tri + Melatonin

Discussion:

The human populace world over is steadily and recurrently being exposed to an admixture of toxic chemicals through food and drink. Such toxic chemicals include metals which find their way into the environment due to anthropogenic activities especially industrialization, and in turn find systemic entry into humans. One of the organs targeted by metals is the kidney. Acute exposure to many of the metals in higher doses is known to cause renal tubular necrosis while lower exposures lead to renal tubule cellular apoptosis (Erfurt *et al.,* 2003). Cadmium, mercury and arsenite are among the most environmentally prominent metals capable of causing renal toxicity (Fowler, 1993). Most studies todate have been with single metals though, it is becoming increasingly clear that, in a human context, the exposure is to a multiple metal *milieu.* It is relevant to note that, renal toxicological responses are by far dependent on the dosage, duration and type of metal exposure (Madden and Fowler, 2000), almost all data available todate are based on single dosage or acute studies lasting for a few days, unsuitable to draw inferences for humans from a practical point of view. The potential importance of altered toxicity due to interactions between metals and their cellular mechanisms of action are totally missed and such poorly characterized interactions such as synergistic, additive or antagonistic may determine the over all toxicity much different from that resulting due to individual metals.

The present duration dependent study on Cd, Cr and Ni, environmentally relevant in the local context, using a realistic dosage, has revealed certain subtle interactive effects in terms of oxidative stress, histoarchitectural lesions and hematological changes. 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. Though the absolute values tend to suggest similarity with those of individual metals (Chapters 2,4,6), a closer observation of temporal pattern of changes indicates subtle interactive modulations. Relatively higher and increasing inhibition of SOD activity suggests the significant participation of the dismutation reaction needed for controlling the deleterious effects of superoxide anion as well as its increasing generation. This is quite similar to the observations made for Cd and Ni but unlike that of Cr. The temporal pattern of change seen in the case of SOD clearly underlines predominant Cd effect throughout.

The disumation reaction catalyzed by SOD is likely to lead to increasing production of H_2O_2 which by participation through Fenton reaction can lead to production of more deleterious hydroxyl radical. The prominent inhibition of both CAT and GPx is not only indicative of the efficient neutralization of H_2O_2 but also the participation of both CAT and GPx mediated reactions with equal vigor. Apparently, this is a common trend for the renal tissue as similar participation of both CAT and GPx has been noted for all the three metals individually as well (Chapters 2,4,6), but quite distinct from liver where GPx mediation was noted to be prominent (Chpater 7). Even other investigators have observed the inhibition of renal CAT and GPx under metal intoxication. The overall pattern of temporal changes noted for CAT and GPx compared with those of individual metals suggest a predominant Ni effect though with a slightly antagonistic effect of Cr in the case of GPx.

The non-enzymatic antioxidants, GSH and ascorbate are not only important in scavenging of free radicals but also in regenerating each other and further, GSH aides in GPx mediated H_2O_2 detoxification. The relatively greater percentage depletion of

ascorbate than GSH with the TM mixture compared with individual metals, suggests a greater role for ascorbate in renal detoxification mechanism. However, 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. Differential interactive effects of the metals are clearly inferable from the temporal pattern of changes shown by the two non-enzymatic antioxidants. Whereas ascorbate shows more of Cd effect on short duration exposure and, additive effect of all the three metals are disernable at medium and long durations of exposure, GSH depicts more of Ni effect with a cumulative lesser depletion at short and medium durations of exposure and, an additive effect of Cd on longer duration. These recorded interactive changes can be taken to imply altered toxicokinetics of the metals and tissue response when present together.

The altered toxicokinetics and toxic effect of the metals are clearly reflected in the observed histoarchitectural alterations of the renal tissue. The deleterious effects manifested are principally disruption in the continutity of both parietal and visceral epithelial lining of Bowmann's capsule, glomerular shrinkage and disintegrity with loss of podocytes and proximal tubular damge marked by necrosis/apoptosis of the absorptive epithelium. In a recent study by Eicler *et al.* (2006), Arsenite, Cadmium and Mercury were found to be equally potent in inducing dose dependent increase in podocyte apoptosis through extrinsic pathway. They further showed that a combination of the three metals induced lesser degree of podocyte apoptosis suggesting a probable antagonistic rather than additive or synergistic effect. The present study in contrast shows that, a TM mixture of Cd, Cr and Ni has greater potential to inflict renal damage compared with the potential of each metal

individually studied earlier (Chapters 2,4,6). All the three metals had shown varying degrees of the above cited lesions in lesser intensity. However, a combination of the three metals as used in the present study exerted a significant compounding effect as early as 15 days of exposure itself. This is in contrast to the observed effects of the metals individually wherein a progressive deterioration was a feature from the shortest to the longest duration of exposure. The greater degree of renal damage marked by structural lesions of the filtration apparatus as well as proximal tubular disorganization as early as 15 days of exposure itself with the TM mixture apparently suggests a synergistic additive effect of the three metals. This becomes self explanatory when viewed in the context of maximal accumulation of all the three metals at 15 days. The discrepancy between the present results and those of Eichler *et al.* (2006) may lie in the different combination of metals used or in the type of study, *in vivo* versus *in vitro*. The current *in vivo* studies are more composite involving the entire renal tissue while that of Eichler *et al.* (2006) is an *in vitro* study involving isolated podocytes. In support of the present observation of additive toxic effect of the metals in combination as against that caused by the metals individually, is a study of Liu *et al.* (2000) involving arsenite, and cadmium showing greater renal toxicity in combination of these two metals than with either metal alone.

Differential accumulation of metals when in combination as against individually has been reported (Zalups and Barfuss, 2002; Aduayom *et al.,* 2003; Peixota *et al.*, 2003 ;Eichler *et al.,* 2005;). The present study also shows differential degree and pattern of accumulation of metals when given in combination or individually. The key differences observed are : 1) while there was a gradual increase of both Cr and Cd but a gradual decrease of Ni with increasing duration of exposure of the metals

individually, the exposure to the TM mixture showed an identical pattern of progressive decrease with increasing durations of exposure, 2) maximal accumulation with individual exposure was seen for Ni while with the TM mixture maximum accumulation was seen for Cd followed by Ni and Cr in that order. Moreover, the decrease seen with progressively increasing duration of exposure was significant for Ni and Cr but not for Cd. The potentiated effect recorded for glomerular damage seen persistently throughout the duration of exposure with the TM mixture can be related with increased apoptotic loss of podocytes as shown by Eichler *et al.* (2006). Their suggestions of extrinsic pathway mediated apoptosis of podocyte based on observed accumulation of FAS and FADD (proteins of extrinsic pathway) and increased Caspase 8 activity seems tenable in the present case too. The persistent podocyte loss and glomerular disruption noted herein may have to be accounted for as an interactive effect of high persistent Cd with Cr and Ni though declining. Paradoxically, the present observations also suggest noticeable recovery of proximal tubular lesions on prolonged exposure to the TM mixture. The initial greater deleterious effects seen at 15 days is probably due to the synergistic effect of all the three metals at their highest concentration and may be related in terms of mechanism of action to the possible activation of the intrinsic pathway by long term exposure to Cd accompanied by mitochondrial membrane oxidation and DNA deletion in rat kidney tubule cells (Takaki *et al.*, 2004). A link between the increased formation of ROS and oxidative stress (seen in the present study) and consequent effect on mitochondrial membrane triggering the intrinsic pathway of apoptosis by way of activation of Caspase 9 and 3 can also be considered feasible as has been shown by Chen *et al.* (2001). Interestingly, ROS are also implicated in FAS receptor mediated activation of extrinsic apoptotic pathway via JNK mediated induction of FAS L or FAS expression

(Baner *et al.,* 1998; Chen *et al.,* 2001; Provinciali *et al.,* 2002). Since Caspase 3 is the common terminal link for both pathways of apoptosis and as during the course of present studies, increased caspase 3 activity has been recorded both at 15 and 60 days of the TM mixture exposure (Chapter 9), a plausible hypothesis regarding renal induction of apoptosis is that, short duration exposure to the TM mixture, by cumulative/additive effect of high levels of all the three metals and the general moderate oxidative stress together activated both the extrinsic and intrinsic pathways through activation of Caspase 8, 9 and 3. Later, at the long duration exposure, Cd principally, in the wake of declining levels of Cr and Ni in conjunction with heightened oxidative stress (as discussed earlier), the extrinsic pathway of apoptosis via caspase 8 and 3 persists while the intrinsic pathway of activation becomes suppressed. Viewed in the this context, the apoptotic loss of tubular cells in the initial period is accreditable to the activation of the intrinsic pathway and, with is suppression during the later period, recovery of the proximal tubular epithelium becomes the order of the day as observed presently. However, the persistent activation of extrinsic pathway through Caspase 8, 3 activation accounts for the observed persistent glomerular lesions. The remarkable effect of the TM mixture on the histoarchitecture of the renal tissue by way of the cytotoxic interaction of the metals can be excepted to reflect on the functional status of the renal tubules. The currently recorded significant increment in serum urea and creatinine levels (markers of renal damage) was testimony to the structural and functional alterations induced by the TM mixture. The recovery of the proximal tubules seen on longer exposure to the TM mixture suggests the possible induction of protective proteins like metallothionein, iron binding proteins as well as stress proteins as a cumulative effect

of the metals. Reports are available to show induction of stress proteins on prolonged exposure to metals (Chapters 4 and 6).

The present study also throws light on the hematological and metabolic alterations brought about by the TM mixture. Subtle alteration in lipid metabolism is indicated by the recorded decrease in serum triglyceride and cholesterol levels. Since the decrease in total serum cholesterol level is induced by a differential alteration of the various cholesterol fractions marked by decrease in HDL (good cholesterol) and significant increase in LDL and VLDL (bad cholesterol), the changes in lipid metabolism induced by the TM mixture could be considered unfavorable. The observed hypotriglycerdemia and hypocholesterolemia are features akin to Cr toxicity (Chapter 1) while, the changes in HDL, LDL and VLDL components are more of effects common to the three metals. These observations warrant the need to study the modulatory effects of toxic metals in metabolic features. Hematological changes marked by increase in total leucocyte count are essentially due to a significant increase in polymorphs and a minor increase in the lymphocyte number. Distinctly different interactive effect of the three metals is revealed by the herein recorded increased leucocyte number as against the leucocytopenic effects of individual metals (Chapters 2, 4, 6). The decreased platelet count suggesting a hypercoagulatory state seems to be more of a Cd-Ni effect as both these metals individually had similar effect. Pertinently, Kosaz and Aksl (2006) have also observed similar effect due to Cd toxicity.

Supplementation with melatonin had significant protective effect in preventing and/or minimizing the various alterations induced by the TM mixture. Whereas the changes in leucocyte count and lipid profile could be completely prevented, changes in all the

other parameters could be minimized by the co-administration of melatonin. The mechanisms of combating oxidative stress by melatonin have already been discussed in detail in previous studies (Chapter 2, 4, 6). Apparently, melatonin is potent in preventing the adverse effects of metal toxicity even in a situation of exposure of multiple metals as in the case of exposure to individual metals. Since the efficacy of melatonin in combating the pro-oxidant changes induced by the TM mixture is found to be slightly lesser in degree, considering its more protective efficacy against its individual metals, there is need to look for a combination therapy of melatonin with other antioxidants like vitamin C and E, in combating the more robust changes induced by the TM mixture. Different combinations of natural antioxidants with melatonin may be needed depending on the combination of the insulting metals.

In conclusion, the present study clearly highlights differential interactive effects of Cr, Cd and Ni on renal oxidative stress, cytotoxicity and hematological and metabolic aspects. The findings clearly warrant the need to study the toxicological effects of different metal combinations mimicking environmental contaminations and human relevance so as to bring out the differential interactive effects of various metals and to explore the possible means of combating the same from the perspective of human health.

Summary of Chapter 8

The present study was essentially undertaken to decipher the oxidative stress and toxic effects induced by a Trimetallic (TM) mixture of Cd, Cr, and Ni on renal tissue in male *Wistar* rats exposed to a realiatic dosage of the TM mixture given through drinking water in keeping with the levels of these metals found in the environment, for a duration of 15, 30 and 60 days. 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 continutity of both parietal and visceral epithelial lining the Bowmann's capsule, glomerular shrinkage and disintegrity with loss of podocytes and proximal tubular damge 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 suggest 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.