Chapter 4: Trimetallic induced oxidative stress in Testes and Epididymis: Realistic dosage and duration dependent studies and, role of Melatonin

Introduction:

Race for industrialization without any foresight has resulted in polluted human environs and, among the various pollutants, metal ions are important causes of environmental pollution as they are waste products of many industrial processes. Vadodara city is also a victim of such an industrial race which has led to increased accumulation of metals in the food grains and vegetables grown in and around it due to the use of effluent water for the purpose of irrigation. Vadodara population is knowingly or unknowingly ingesting such metal contaminated diet everyday and as a matter of fact, concentration of some of the metals is many folds higher than permitted by WHO. Previous study from our laboratory had suggested concentration of chromium, cadmium and nickel in the food grains and vegetables, grown in and around Vadodara to be predominantly high, suggesting their wide applications in the industries located around the city. Absorption of Cr (VI), Cd and Ni varies depending upon their concentration but systemically they getting distributed into various organs including male reproductive organs.

'Under normal homeostasis, cells maintain critical balance between free radical generated due to routine metabolic activity and antioxidants; however, presence of

xenobiotic compounds like metals can result in disturbed prooxidant – antioxidant homeostasis leading to increased oxidative stress. It is now well established that all the three metals namely chromium(VI), cadmium and nickel directly or indirectly generate free radicals; these notorious free radicals in turn cause increased lipid peroxidation, altered antioxidant enzyme activity and disturbed calcium and sulfhydryl homeostasis resulting in enhanced oxidative stress.

The toxic effects of metals on various organisms have received great scientific interest in the last couple of decades. However, most of the animal studies focus on hepatic, renal or neurotoxicity, and information related to male reproductive toxicity of metals is scarce. Even then, the studies on above lines are based on various routes of administration like subcutaneous, intramuscular, dermal or intraperitoneal all of which are of least relevance in terms of actual human exposure to toxic metals. Further, most of the research papers discuss metal induced toxicity based on acute treatment or single high dose exposures which are far by predominant to long term low level environmental exposure to humans.

Literature survey suggests that toxic manifestations of single metal are well documented, but unfortunately there is no record available regarding metal combination induced insult of the organism. To the best of our knowledge, a point of much greater concern which none of the toxicologists have taken into the consideration is that, no individual is ever environmentally exposed to a single metal alone; instead, humans are exposed to a combination of metals. Considering the above shortcomings of the available

literature, the present study has designed an experimental setup of orally exposing rats to a realistic and physiologically simulatable concentration of metal combination (Cr (VI), Cd and Ni), as prevailing in the dietary components of the Vadodara population, for three different duration (i.e. 15, 30 and 60 days). Male reproductive toxicity has been assessed by evaluating LPO and levels of antioxidants in testes and epididymis.

Human exposure to xenobiotic compounds including heavy metals is unavoidable however, the use of a powerful antioxidant can help us to maintain the proper redox state of the body. One such natural, efficacious antioxidant identified is Melatonin. Potency of this indole is tested in the present investigation as a possible protectant to counter the oxidative stress mediated ill effects caused due to metal intoxication on male reproductive organs (testis and epididymis).

Material and Method:

For treatment, methodology employed and protocols refer material and methods section (Page no. 17).

Results:

Lipid peroxidation (LPO):

Trimetallic exposure resulted in duration dependent increment in testicular (Figure 4.1a, Figure 4.1b and Table 4.1) and epididymal (Figure 4.2a, Figure 4.2b and Table 4.2) lipid peroxidation compared to control animals. Co-administration of melatonin along with trimetallic mixture significantly reduced lipid peroxidation levels of both testis and epididymis.

Reduced Glutathione (GSH):

Testis (Figure 4.3a, Figure 4.3b and Table 4.3) and epididymis (Figure 4.4a, Figure 4.4b and Table 4.4) of rats treated with the TM mixture showed significant reduction in GSH content in a duration dependent manner. Simultaneous supplementation with melatonin exerted a protective effect on the depletion of GSH content in both testis and epididymis with maximum protection being seen the longest duration exposure group.

Ascorbic acid (AA):

The testis ascorbic acid content showed progressive depletion in animals exposed to trimetallic mixture (Figure 4.5a, Figure 4.5b and Table 4.5). Same trend was observable even for epididymal ascorbic acid content (Figure 4.6a, Figure 4.6b and Table 4.6) . In general, melatonin increased ascorbic acid content in control rats. The protective effect of melatonin on ascorbic acid content when co-administered with trimetallic mixture was found to be more pronounced with increasing duration in the case of testis compared to epididymis.

Catalase (CAT) activity:

Trimetallic exposure recorded significant decrement in testis catalase activity which tended to decrease with increasing duration (Figure 4.7a, Figure 4.7b and Table 4.7). Progressively linear decrease in catalase activity was recorded in epididymis of the trimetallic treated animals (Figure 4.8a, Figure 4.8b and Table 4.8). Concurrent melatonin administration showed significant protective effect in both testis and epididymis of the treated animals in a duration dependent manner.

Superoxide dismutase (SOD) activity:

Testis (Figure 4.9a, Figure 4.9b and Table 4.9) and epididymis (Figure 4.10a, Figure 4.10b and Table 4.10) of trimetallic mixture exposed rats showed a duration dependent linear increase in SOD inhibition. Though the short term trimetallic exposure showed a slightly lesser SOD inhibition in testis, the long term exposures showed similar SOD inhibition in both the organs. Melatonin administration showed significant protective effect against SOD inhibition due to Trimetallic treatment in both testis and epididymis.

Glutathione peroxidase (GPx):

Trimetallic mixture induced GPx inhibition was duration dependent in both testis (Figure 4.11a, Figure 4.11b and Table 4.11) and epididymis (Figure 4.12a, Figure 4.12b and Table 4.12). Conversely, the protective effect on co-administration with melatonin was found to be better in short duration exposure compared to long duration in both testis and epididymis.

Glutathione reductase (GR) activity:

GR activity of both testis (Figure 4.13a, Figure 4.13b and Table 4.13) and epididymis (Figure 4.14a, Figure 4.14b and Table 4.14) depicted a duration dependent linear decrement with trimetallic exposure, more pronounced in testis. Concurrent protective effect with simultaneous administration of melatonin also showed a duration dependent effect with protective effect at the short duration exposure being better than at the longer durations of metal exposure.

Figure 4.1a: Percentage changes (increase) in Testicular LPO at 15,30 and 60s of exposure to the TM mixture or the component metals individually.

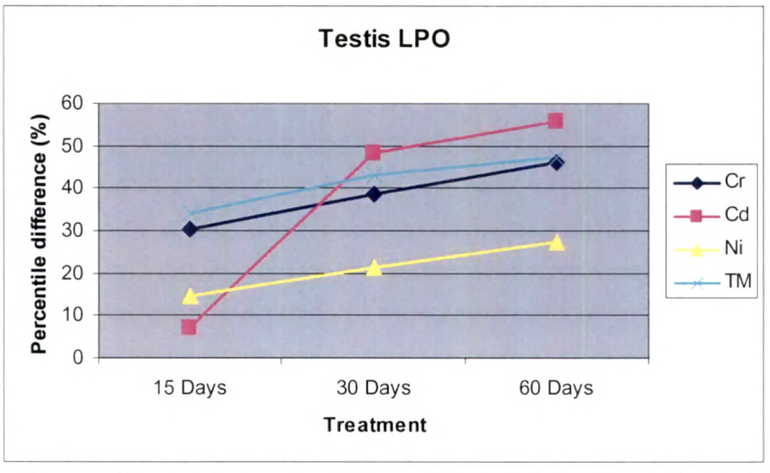

Figure 4.1b: Comparison of the temporal slope of percentage change in testicular LPO between TM mixture and component metals individually.

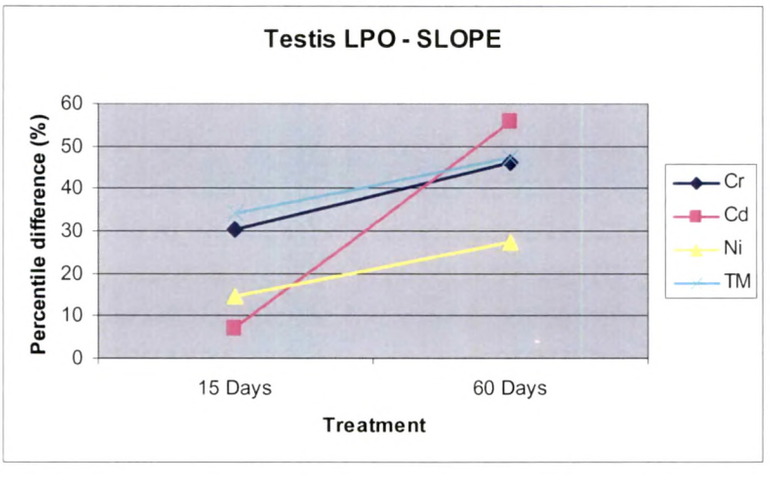

Table 4.1: Trimetallic induced Lipid peroxidation (LPO) levels (nmoles of MDA formed/ 100 mg tissue) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	1.44 ± 0.069	1.33 ± 0.032	1.93 ± 0.15^{b}	1.61 ± 1.09
30 Days	2.09 ± 0.16	1.84 ± 0.047	2.99 ± 0.15 ^{b}	2.49 ± 0.14^{c}
60 Days	2.67 ± 0.061	2.37 ± 0.116	3.94 ± 0.20 ^{b}	3.04 ±0.16^{c}

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.2a: Percentage changes (increase) in Epididymal LPO at 15,30 and 60s of exposure to the TM mixture or the component metals individually.

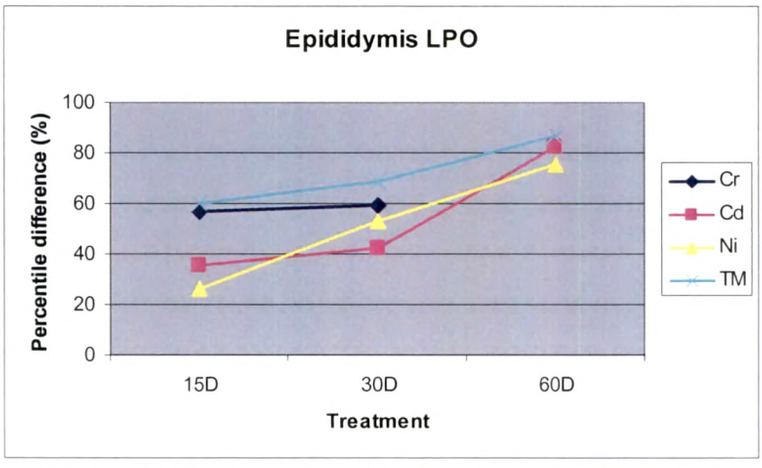

Figure 4.2b: Comparison of the temporal slope of percentage change in Epididymal LPO between TM mixture and component metals individually.

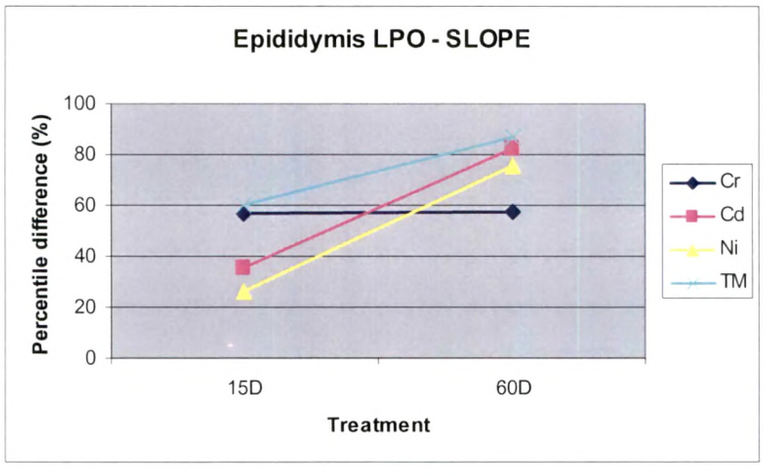

Table 4.2: Trimetallic induced Lipid peroxidation (LPO) levels (nmoles of MDA formed/ 100 mg tissue) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	1.83 ± 0.24	1.37 ± 0.08	2.93 ± 0.20^{b}	2.11 ± 0.18^{c}
30 Days	2.18 ± 0.17	2.05 ± 0.10	3.68 ± 0.11^{b}	2.41 ± 0.13^{c}
60 Days	2.66 ± 0.21	2.37 ± 0.10	4.98 ± 0.21^{b}	3.16 ± 0.22^{c}

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.3a: Percentage changes (decrease) in testicular GSH at 15,30 and 60s of exposure to the TM mixture or the component metals individually.

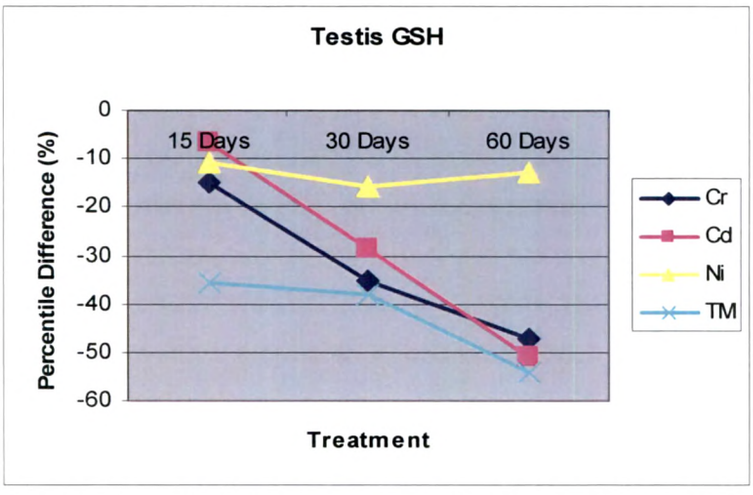

Figure 4.3b: Comparison of the temporal slope of percentage change in testicular GSH between TM mixture and component metals individually.

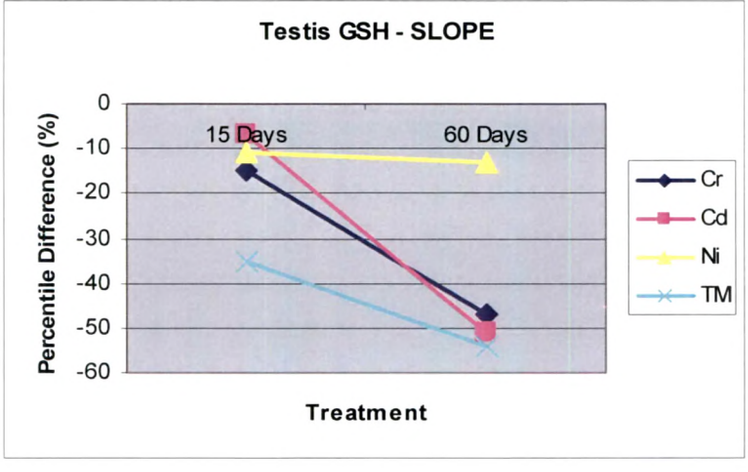

Table 4.3: Trimetallic induced alterations in Glutathione (GSH) levels (µg GSH/ 100 mg tissue) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	22.89 ± 0.51	26.45 ± 0.80[a]	14.79 ± 0.32[b]	21.98 ± 0.43[c]
30 Days	20.66 ± 0.31	24.71 ± 0.81[a]	12.76 ± 0.31[b]	19.98 ± 0.16[c]
60 Days	21.80 ± 0.28	27.39 ± 0.22[a]	9.99 ± 0.25[b]	17.75 ±0.31[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.4a: Percentage changes (decrease) in epididymal GSH at 15,30 and 60s of exposure to the TM mixture or the component metals individually.

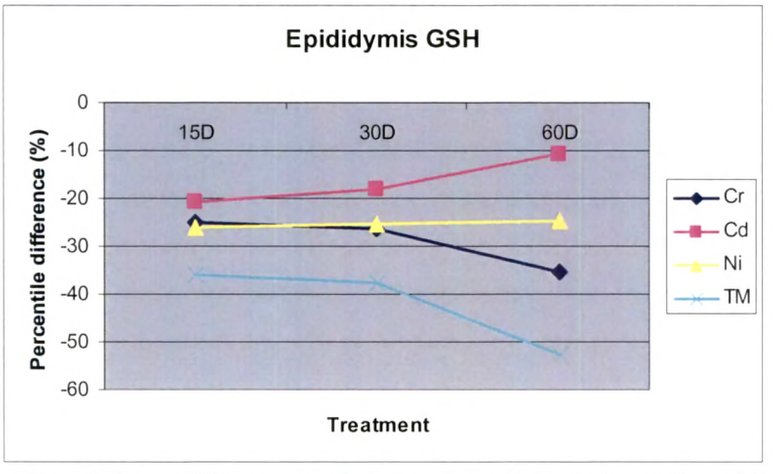

Figure 4.4b: Comparison of the temporal slope of percentage change in epididymal GSH between TM mixture and component metals individually.

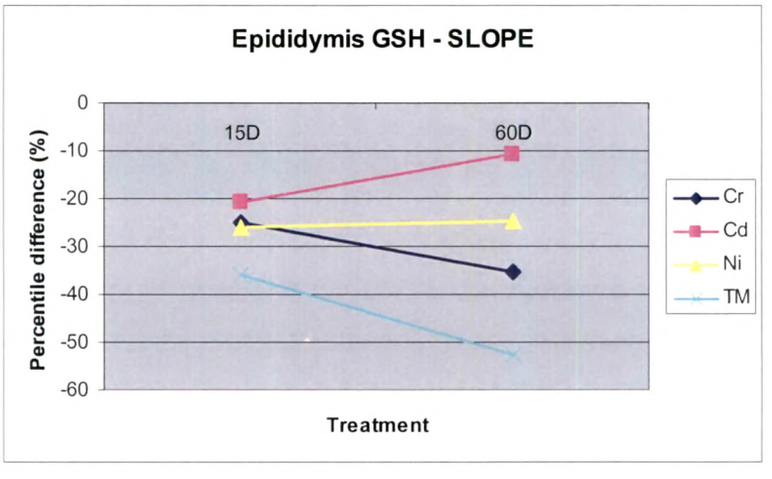

Table 4.4: Trimetallic induced alterations in Glutathione (GSH) levels (µg GSH/ 100 mg tissue) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	14.83 ± 0.14	19.27 ± 0.33[a]	9.48 ± 0.17[b]	13.25 ± 0.14[c]
30 Days	14.56 ± 0.46	17.87 ± 0.26[a]	9.08 ± 0.15[b]	12.03 ± 0.15[c]
60 Days	13.86 ± 0.20	15.04 ± 0.37[a]	6.58 ± 0.13[b]	11.39 ± 0.08[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.5a: Percentage changes (decrease) in testicular Ascorbic acid at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

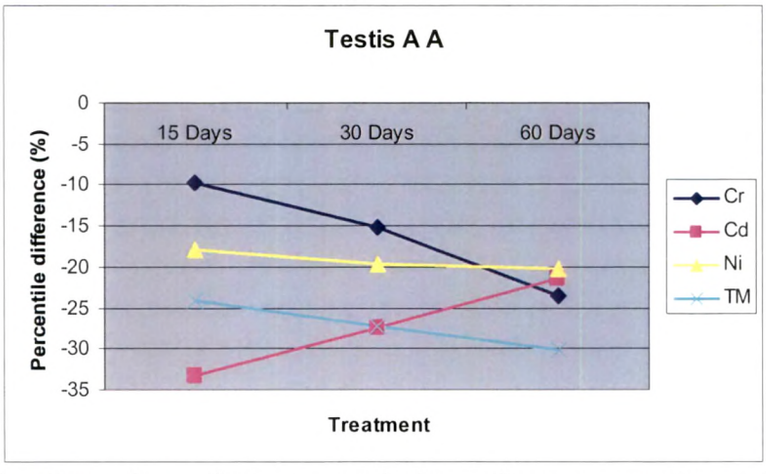

Figure 4.5b: Comparison of the temporal slope of percentage change in testicular Ascorbic acid between TM mixture and component metals individually.

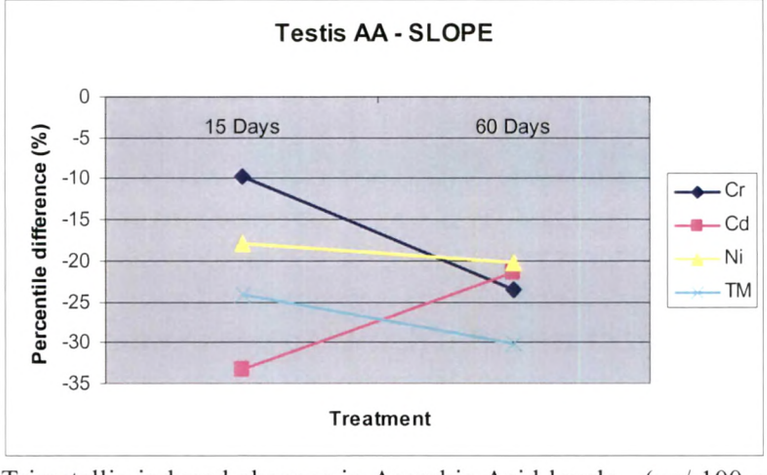

Table 4.5: Trimetallic induced changes in Ascorbic Acid levels (µg/ 100 mg tissue) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	44.87 ± 0.97	54.99 ± 0.26[a]	34.03 ± 1.57[b]	42.08 ± 2.17[c]
30 Days	43.08 ± 0.51	45.73 ± 0.35[a]	31.34 ± 0.12[b]	26.16 ± 0.27[c]
60 Days	37.41 ± 0.20	42.54 ± 0.14[a]	26.16 ± 4.2[b]	35.08 ± 2.59

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.6a: Percentage changes (decrease) in epididymal Ascorbic acid at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

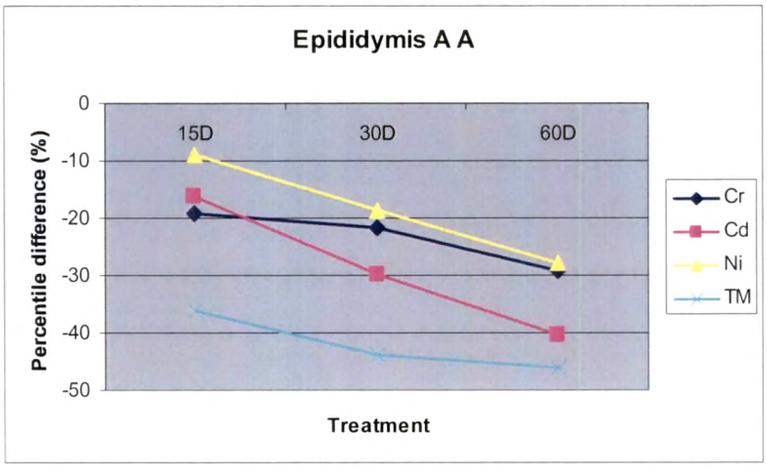

Figure 4.6b: Comparison of the temporal slope of percentage change in epididymal Ascorbic acid between TM mixture and component metals individually.

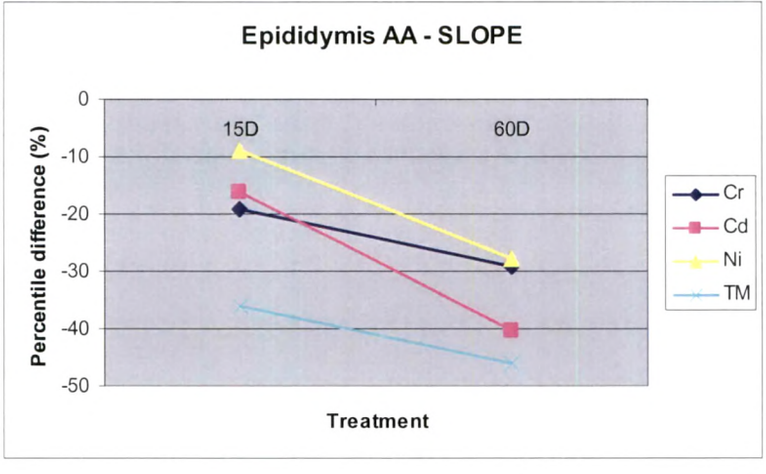

Table 4.6: Trimetallic induced changes in Ascorbic Acid levels (µg/ 100 mg tissue) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	32.38 ± 0.37	33.76 ± 0.44[a]	20.68 ± 0.22[b]	24.79 ± 0.41[c]
30 Days	33.42 ± 0.10	35.47 ± 0.66[a]	18.74 ± 0.27[b]	25.97 ± 0.23[c]
60 Days	31.76 ± 0.42	34.67 ± 0.34[a]	17.13 ± 0.17[b]	24.63 ± 0.31[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.7a: Percentage changes (decrease) in testicular Catalase at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

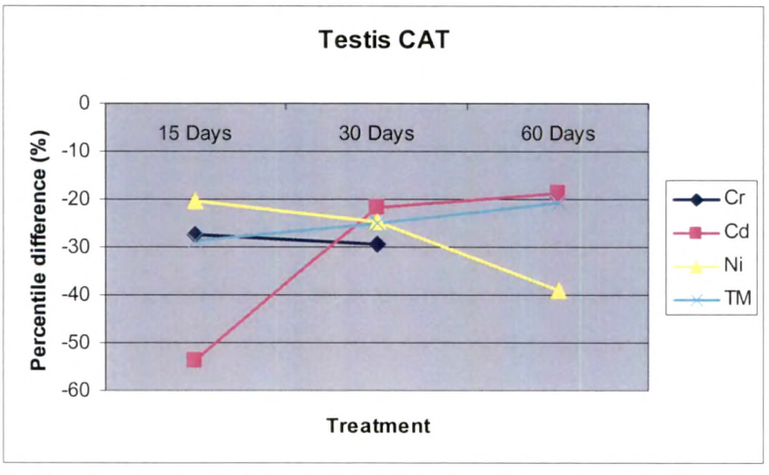

Figure 4.7b: Comparison of the temporal slope of percentage change in testicular Catalase between TM mixture and component metals individually.

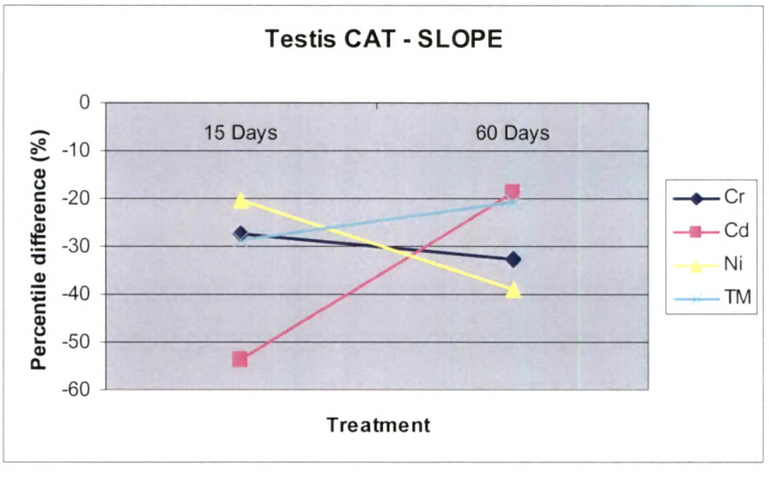

Table 4.7: Trimetallic induced changes in Catalase (CAT) activity (µmoles of H_2O_2 consumed/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	18.05 ± 0.07	18.70 ± 0.25	12.89 ± 0.24[b]	15.67 ± 0.16[c]
30 Days	16.33 ± 0.23	17.93 ± 0.54[a]	12.26 ± 0.21[b]	14.48 ± 0.28[c]
60 Days	16.68 ± 0.13	16.74 ± 0.16	13.22 ± 0.30[b]	15.08 ± 0.27[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.8a: Percentage changes (decrease) in epididymal Catalase at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

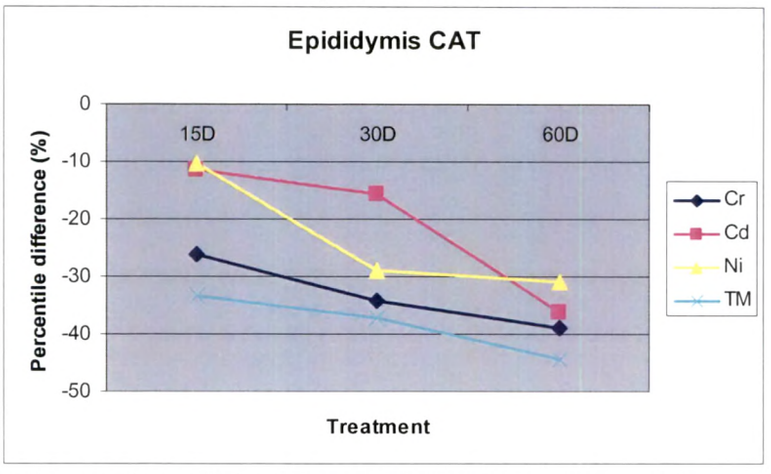

Figure 4.8b: Comparison of the temporal slope of percentage change in epididymal Catalase between TM mixture and component metals individually.

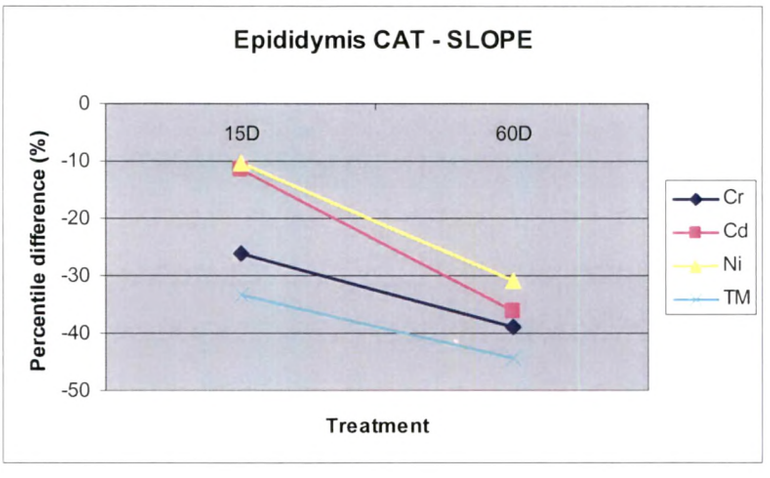

Table 4.8: Trimetallic induced changes in Catalase (CAT) activity (µmoles of H_2O_2 consumed/ min/ mg protein) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	19.43 ± 0.08	25.02 ± 0.44[a]	12.95 ± 1.23[b]	17.81 ± 0.41[c]
30 Days	22.42 ± 0.07	23.46 ± 0.16	14.07 ± 0.17[b]	20.41 ± 0.57[c]
60 Days	24.57 ± 0.15	26.89 ± 0.27[a]	13.64 ± 0.11[b]	19.23 ± 0.49[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.9a: Percentage changes (decrease) in testicular Superoxide dismutase (SOD) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

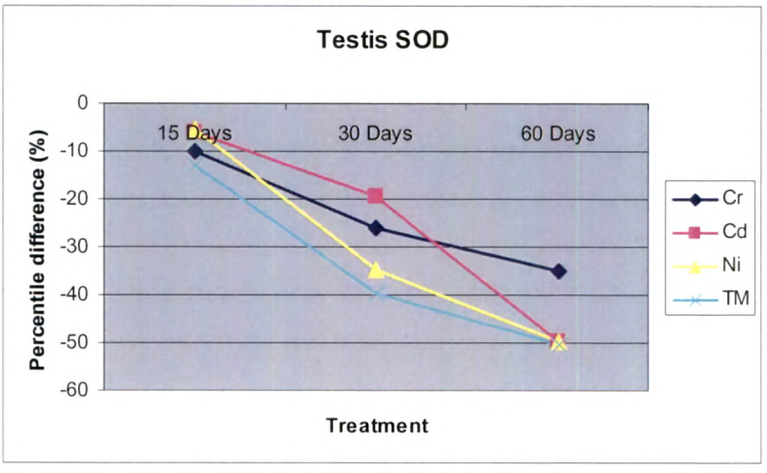

Figure 4.9b: Comparison of the temporal slope of percentage change in testicular Superoxide dismutase (SOD) between TM mixture and component metals individually.

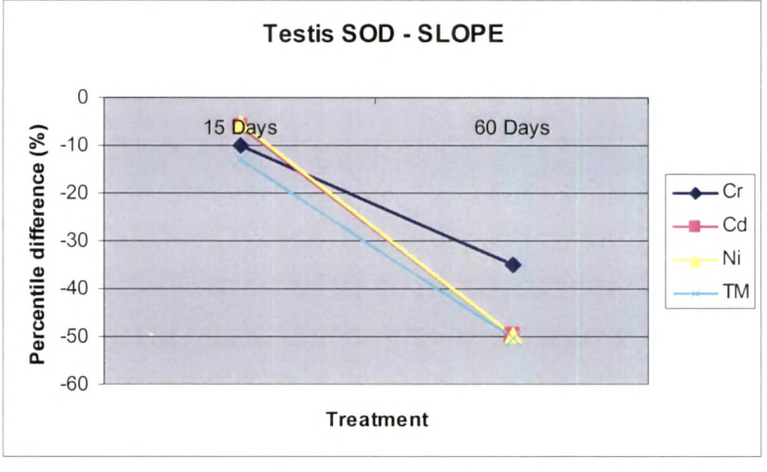

Table 4.9: Trimetallic induced changes in Superoxide dismutase (SOD) activity (units/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	3.01 ± 0.15	4.59 ± 0.14[a]	2.62 ± 0.19	2.89 ± 0.14
30 Days	3.59 ± 0.14	4.79 ± 0.07[a]	2.16 ± 0.26[b]	3.08 ± 0.02[c]
60 Days	3.64 ± 0.11	4.87 ± 0.11[a]	1.81 ± 0.16[b]	3.19 ± 0.16[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.10a: Percentage changes (decrease) in epididymal Superoxide dismutase (SOD) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

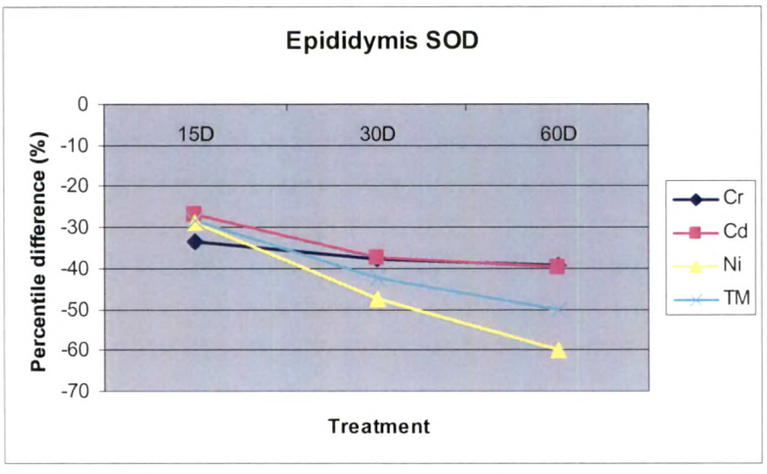

Figure 4.10b: Comparison of the temporal slope of percentage change in epididymal Superoxide dismutase (SOD) between TM mixture and component metals individually.

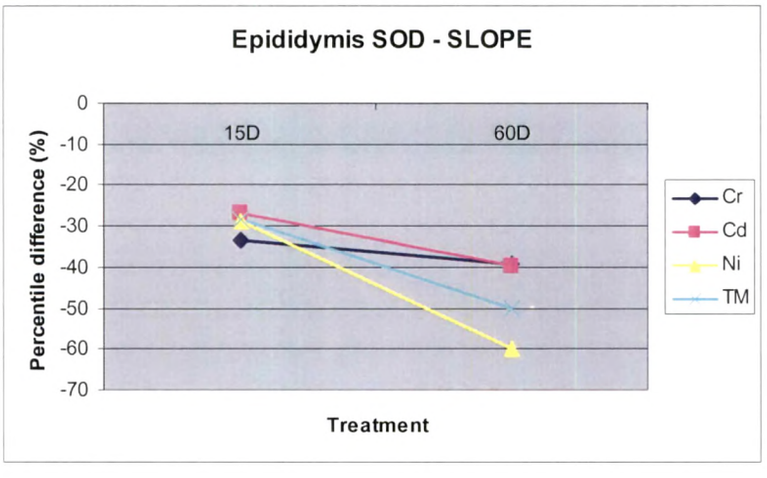

Table 4.10: Trimetallic induced changes in Superoxide dismutase (SOD) activity (units/ mg protein) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	12.87 ± 0.25	14.23 ± 0.13[a]	9.21 ± 0.24[b]	11.70 ± 0.26[c]
30 Days	16.42 ± 0.17	17.39 ± 0.16[a]	14.55 ± 0.05[b]	13.40 ± 0.15[c]
60 Days	16.56 ± 0.17	18.94 ± 0.41[a]	13.51 ± 0.24[b]	14.68 ± 0.34[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.11a: Percentage changes (decrease) in testicular Glutathione peroxidase (GPx) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

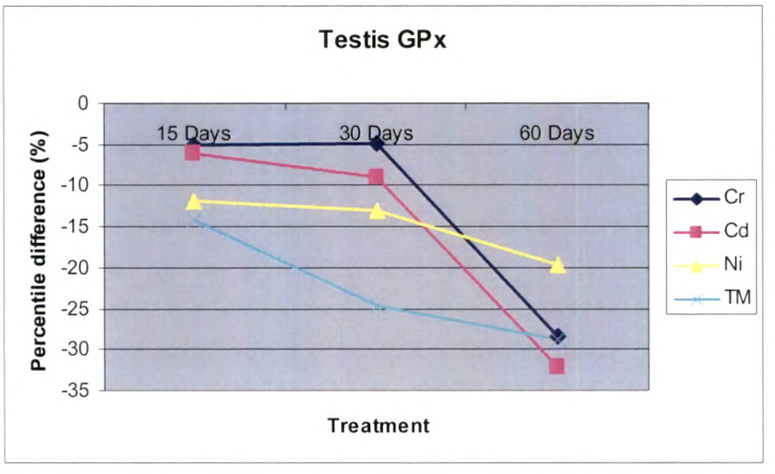

Figure 4.11b: Comparison of the temporal slope of percentage change in testicular Glutathione peroxidase (GPx) between TM mixture and component metals individually.

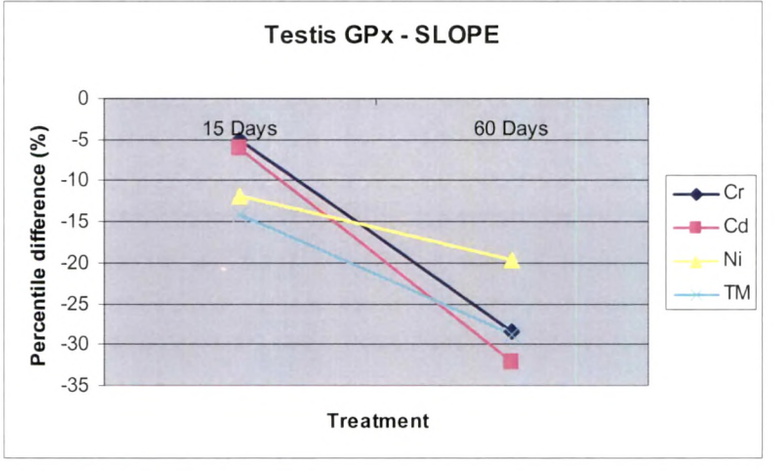

Table 4.11: Trimetallic induced changes in Glutathione peroxidase (GPx) activity (µmoles of GSH consumed/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	173.02 ± 1.10	188.09 ± 2.28[a]	148.61 ± 0.45[b]	159.76 ± 0.34[c]
30 Days	142.64 ± 0.93	144.35 ± 0.40	107.43 ± 0.43[b]	131.1 ± 0.30[c]
60 Days	144.68 ± 1.21	150.38 ± 1.1[a]	102.47 ± 0.35[b]	129.33 ± 0.38[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic

C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.12a: Percentage changes (decrease) in epididymal Glutathione peroxidase (GPx) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

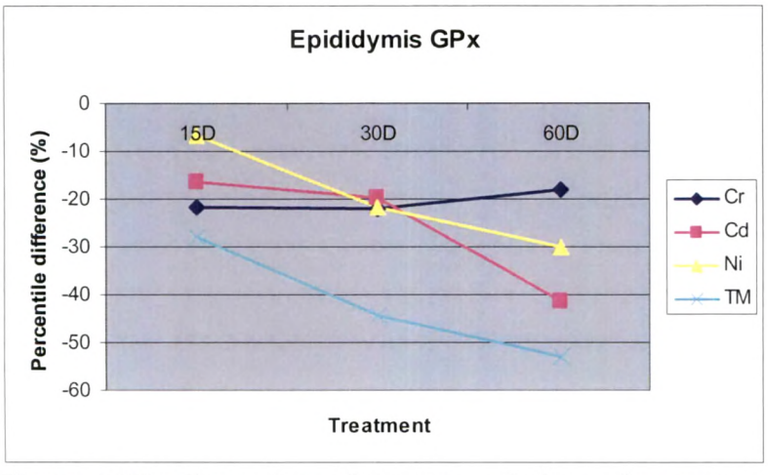

Figure 4.12b: Comparison of the temporal slope of percentage change in epididymal Glutathione peroxidase (GPx) between TM mixture and component metals individually.

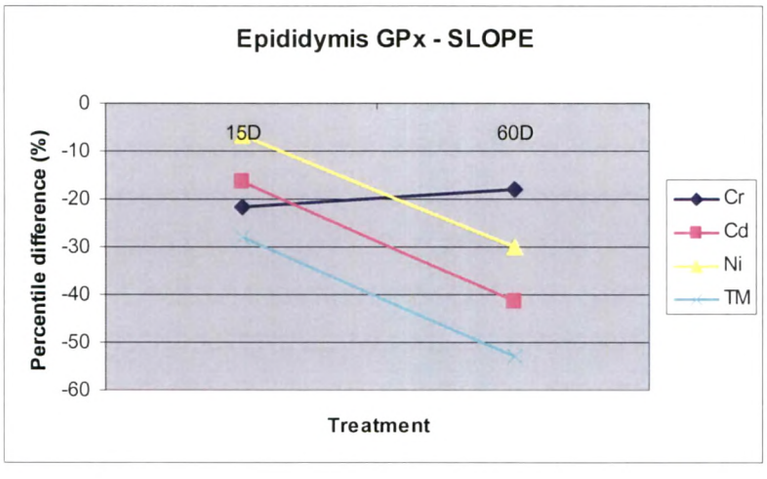

Table 4.12: Trimetallic induced changes in Glutathione peroxidase (GPx) activity (μmoles of GSH consumed/ min/ mg protein) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	89.17 ± 0.24	100.77 ± 0.18[a]	64.11 ± 0.76[b]	86.62 ± 3.63[c]
30 Days	82.26 ± 0.37	87.32 ± 0.18[a]	45.83 ± 0.46[b]	60.22 ± 0.22[c]
60 Days	83.43 ± 0.19	87.12 ± 0.32[a]	39.17 ± 0.17[b]	54.23 ± 0.26[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic

C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Figure 4.13a: Percentage changes (decrease) in testicular Glutathione reductase (GR) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

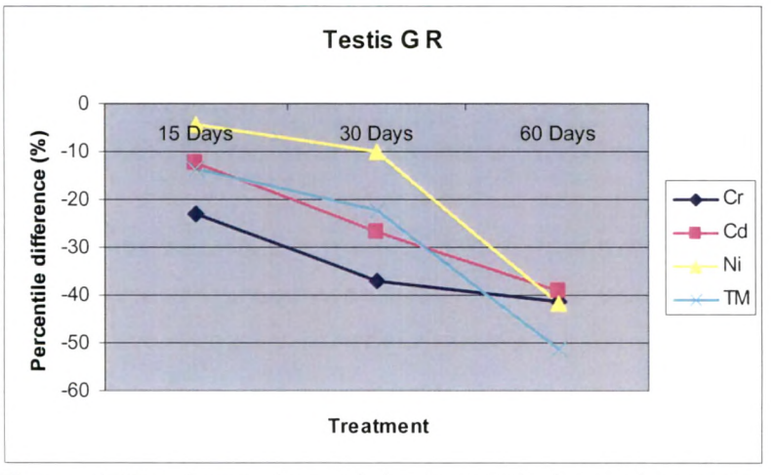

Figure 4.13b: Comparison of the temporal slope of percentage change in testicular Glutathione reductase (GR) between TM mixture and component metals individually.

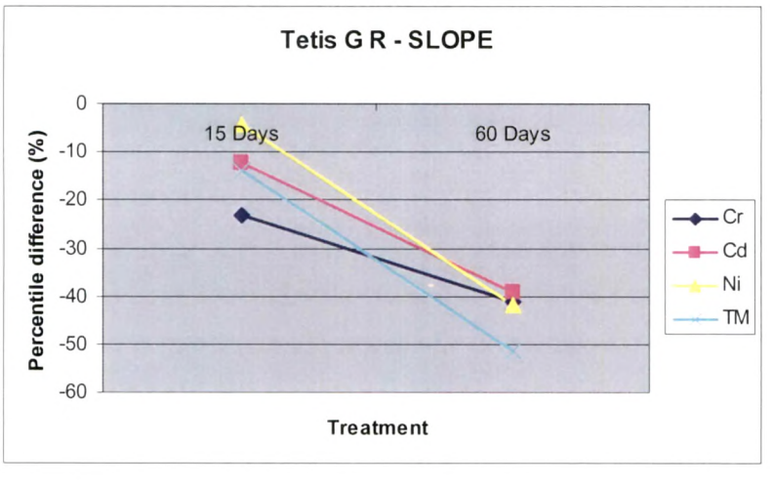

Table 4.13: Trimetallic induced changes in Glutathione reductase (GR) activity (nmoles of NADPH oxidized/ min/ mg protein) in testis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	112.35 ± 0.63	119.90 ± 0.21[a]	96.87 ± 0.26[b]	107.31 ± 0.60[c]
30 Days	114.87 ± 0.58	118.47 ± 0.09[a]	89.12 ± 0.11[b]	101.56 ± 0.27[c]
60 Days	108.34 ± 0.13	111.72 ± 0.71[a]	52.66 ± 0.36[b]	94.15 ± 0.11[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;

Tri + Mel – Tri + Melatonin

Figure 4.14a: Percentage changes (decrease) in epididymal Glutathione reductase (GR) at 15, 30 and 60s of exposure to the TM mixture or the component metals individually.

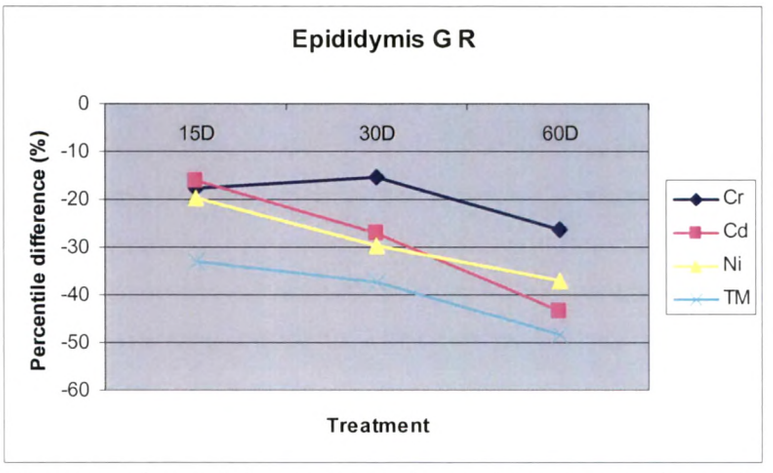

Figure 4.13b: Comparison of the temporal slope of percentage change in epididymal Glutathione reductase (GR) between TM mixture and component metals individually.

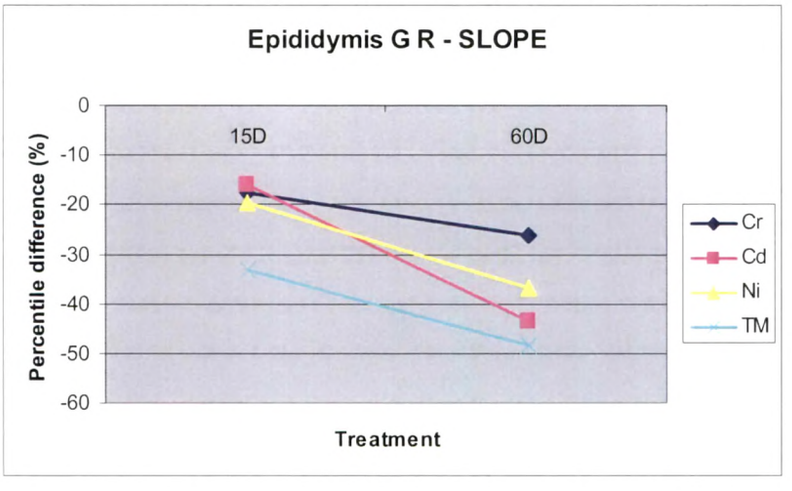

Table 4.14: Trimetallic induced changes in Glutathione reductase (GR) activity (nmoles of NADPH oxidized/ min/ mg protein) in epididymis with or without Melatonin.

Treatment	C	C + Mel	Tri	Tri + Mel
15 Days	129.21 ± 0.36	133.45 ± 0.37[a]	86.42 ± 0.21[b]	112.67 ± 0.20[c]
30 Days	128.45 ± 0.39	129.53 ± 1.39	60.37 ± 0.06[b]	116.40 ± 0.03[c]
60 Days	130.43 ± 0.13	134.09 ± 0.53[a]	82.23 ± 0.24[b]	106.54 ± 0.15[c]

Values expressed as Mean ± SEM of 6 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;

c $p<0.05$, compared with Trimetallic
C – Control; C + Mel – Control + Melatonin; Tri –Trimetallic;
Tri + Mel – Tri + Melatonin

Table 4.15a: Trimetallic Mixture induced percentage changes in chromium, cadmium and nickel content of testis with or without melatonin.

Treatment	Cadmium			Chromium (VI)			Nickel		
	15 Days	30 Days	60 Days	15 Days	30 Days	60 Days	15 Days	15 Days	30 Days
TM	28.95	31.11	55.74	35.00	15.38	23.81	10.88	10.83	24.38
TM + Mel	-11.90	4.26	15.13	23.08	2.38	23.21	6.34	6.58	11.41

C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

Table 4.15b: Trimetallic Mixture induced percentage changes in chromium, cadmium and nickel content of epididymis with or without melatonin.

Treatment	Cadmium			Chromium (VI)			Nickel		
	15 Days	30 Days	60 Days	15 Days	30 Days	60 Days	15 Days	15 Days	30 Days
TM	31.12	12.39	28.76	18.49	22.00	23.72	3.58	11.99	21.28
TM + Mel	34.52	10.14	27.49	1.59	-0.76	9.60	-0.43	11.20	6.45

C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

Table 4.16: Trimetallic mixture induced changes in the serum titre of melatonin (pg/ ml) with or with out melatonin.

Treatment	C	C + Mel	TM	TM + Mel
15 Days	121.00	140.00	75.00	102.00
30 Days	112.00	143.00	60.00	109.00
60 Days	93.00	126.00	20.00	87.00

Values expressed as Mean ± SEM of 4 animals per group.
a $p<0.05$, compared with the control; **b** $p<0.05$, compared with the Control;
c $p<0.05$, compared with Chromium (VI)
C – Control; C + Mel – Control + Melatonin; TM – Trimetallic Mixture;
TM + Mel – Trimetallic Mixture + Melatonin

Discussion:

Exposure to the TM mixture has recorded significant oxidative stress marked by increasing LPO and decrease in both non-enzymatic and enzymatic antioxidants. Compared to testis, epididymis has shown significantly greater degree of LPO and decrement in antioxidants. This pattern of changes is similar to what have been observed with each of the metals individually (Chapter 1-3). However, the absolute values and the degree of increase in LPO or decrease in levels and activity of antioxidants are quite different and suggestive of complex interactions between the metals and tissues in a parameter and duration dependent manner. Humans are no doubt exposed to multiple metals and other toxicants especially if residing in industrialized areas with the composition of the chemical mixture depending on the local features and anthropogeneic activities. It is therefore relevant to understand the physiological responses and/ or the systemic deleterious effects to such a situation. To this end, animal experimentations involving multiple metal exposure, more so to a specific combination depending on the local environmental status, are needed. Unfortunately, there are hardly any studies with chemical mixtures, much less, based on a realistic exposure with reference to composition and dosage. Though a certain degree of normal endogenously generated oxidative stress is unavoidable or is even favorable with reference to the functioning of reproductive organs, heightened oxidative stress generated by xenobiotic exogenous agents could be deleterious and contribute to reproductive features or infertility (Agrawal and Prabhakaran, 2005; Cenzza *et al.*, 2007; Tremellen, 2008).

The present study using a realistic concentration of a TM mixture selected based on environmental relevance has shown significant induction of oxidative stress in testis and epididymis with increasing degrees of LPO and depletion of non-enzymatic antioxidants together with marked inhibition/ inactivation of enzymatic antioxidants. Though this is a general trend of changes brought about by chronic exposure to even single pro-oxidant metals (Chapter 1-3), the antioxidant systems that are affected, the degree to which they are affected and even the induction of protective mechanisms at the molecular level can all be widely different in a situation of exposure to multiple metallic mixture. These differences can depend upon the site and mode of action consequent to synergistic, additive or antagonistic interactions (Carpenter *et al.*, 2002).

A comparative scrutiny delving deeper into the changes brought about by the three metals individually as well as in combination brings out the above paradigm quite clearly. Lipid paroxidation which is a reflection of the degree of oxidative stress shows that, the TM mixture has mixed effect of the component metals. Relatively higher induction of LPO at the shortest exposure itself is a feature of chromium compared to cadmium and nickel. The testicular induction of LPO on exposure to the TM mixture seems to exhibit more or less a Cr effect with an antagonistic and synergistic actions of Cd at 15 and 30 days respectively over that of Cr. The interaction of cadmium and chromium seem therefore to have a bearing on testicular LPO when present as a mixture with nickel. Interestingly, the decrease in the content of non enzymatic antioxidants and in the activity of enzymatic antioxidants seems to have two distinct interactive effects.

Whereas the pattern of depletion of both GSH and ascorbate (non enzymatic antioxidant) tends to show a Cr effect with a significant synergistic action of Ni on ascorbic acid and an additive effect on GSH, the pattern of decrease in activities of SOD, GPx and GR tends to be a Ni effect with synergistic action of Cd. Distinctly, CAT shows a pattern of decreasing inhibition mimicking Cd with, pronounced antagonistic actions of Cr and Ni over it at 15 days. The maximal induction of LPO on exposure to TM mixture is a reflection of the cumulative effect of all the three metals, especially Cd and Cr in generating ROS like superoxide anion, hydrogen peroxide and hydroxyl radicals. The individual mechanism of ROS generation for all these three metals is already explained previously (Chapters 2 - 5). Higher level of $O^{\bullet}_2^-$ generation is predictable looking at the significant decrement in SOD activity. The greater inhibition of SOD seen with the TM mixture can not only be related with the persistent dismutation of $O^{\bullet}_2^-$ but also by the inactivatig effect of Cd by displacement of Zn from Cu-Zn-SOD (Cytosolic or SOD1) (Amara *et al.*, 2008)The hydrogen peroxide generated by dismutation of $O^{\bullet}_2^-$ by SOD can lead to generation of more deleterious hydroxyl radical via Fenton reaction facilitated by Cr, Ni, Fe (released by Cd from membrane bound protein) and Zn (released from Cu-Zn-SOD). In this scenario, testis is subjected to an increasing exigency of decomposing H_2O_2. Of the two enzyme systems, CAT or GPx-GR system, the latter seems to play a predominant role in testis as both GPx and GR show progressively greater decreasing activity. As this system is dependent on GSH for the reductive power, the currently observed significant decrease in GSH content is self explanatory. Involvement of CAT in the reduction of H_2O_2 to H_2O and molecular oxygen seems to be of lesser significant in testis as there is progressive recovery in CAT activity. The decrease in ascorbate is

explainable in term of its role in regeneration of GSH and Vit. E as well as its direct ability of scavenging free radicals. Overall, exposure to TM mixture seems to generate greater oxidative stress marked by greater degree of lipid peroxidation and decrease in antioxidants. The effects seen are clearly interactive additive effect of Cr and Ni for non enzymatic antioxidants and of Ni and Cd for enzymatic antioxidants.

The interactive effects of metals are also likely to be tissue specific as is depicted by the herein documented changes in epididymal LPO and endogenous antioxidants in contradistinction to that of testis. The epididymis seems to be vulnerable to oxidative stress as noted by significantly higher LPO as well as marked decrease in both non enzymatic and enzymatic antioxidants. Increased epididymal lipid peroxidation is more of an additive effect of Cd and Ni unlike the Cr effect seen in testis. The TM mixture exposure seems to generate significant amounts of $O^{\bullet}_2{}^-$ and H_2O_2 as can be made out by the marked inhibition of SOD, CAT and GPx. Whereas the inhibition of CAT and GPx is an additive effect of all the three metals, the inhibition of SOD is more of a nickel effect. Apparently, the inhibition of SOD activity can be correlated with both increased dismutation of $O^{\bullet}_2{}^-$ as well as the earlier proposed inhibitory effect of nickel by way of inhibition of copper chaporonin SOD (CCS) (Chapter: 3). Equally significant decrement in the activities of both CAT and GPx is indicate of the probable greater H_2O_2 centered oxidative stress in epididymis and the involvement of both CAT and GPx mediated decomposition of H_2O_2. Marked decrease in GR activity and depletion of GSH content support the role of GPx in handling H_2O_2 based oxidative stress. Parallel depletion of both GSH and ascorbic acid seems to suggest not only their complementary role in the

actions of antioxidants enzymes but also on their competence to quench the free radicals. Whereas the depletion in epididymal GSH content is more of a synergistic effect of Cr and Ni, that of ascorbate is more of additive effect of all the three metals. In totality, the recorded changes in epididymis are not only indicative of greater oxidative stress compared to testis but also more of additive effects of Cd and Ni or even an additive effect of all the three metals in certain cases.

The indoleamine, melatonin, now gaining recognition as a natural antioxidant had been used as a supplement in the present study to evaluate its potential to protect testis and epididymis against the TM mixture induced pro-oxidant state. It is very encouraging to note form the observations that melatonin has proved competent to control the pro-oxidant changes induced by the TM mixture even at the longest duration exposure. It has been found to be very effective in controlling LPO, in preventing depletion of GSH and in resisting inhibition/ inactivation of SOD and GR. Even in the case of ascorbate depletion and decrease in activities of CAT and GPx, melatonin seems to exert reasonably good protective effect. Compared with the degree of protection afforded by melatonin against intoxication by individual metals of the TM mixture (Chapter: 1-3), quite interestingly, its protective effect against the TM mixture recorded herein seems to be much better. It is well neigh impossible to offer an explanation for the better protective effect shown by melatonin against the metallic mixture as against individual metals. The comparison has also revealed that though it has an overall redeeming effect against the TM mixture in both testis and epididymis, its competence against individual metals seems to be of the order Cr < Cd < Ni in the case of testis and Cd < Ni < Cr in the case of

epididymis. This once again attests to the earlier inferred predominant Cr effect in testis and Cd-Ni effect in epididymis. In the light of the known role of melatonin as an effective antioxidant, it is presumable that the protective effect of this indoleamine could be considered to be by different mechanisms like, direct quenching of free radicals, helping to restore GSH, ascorbic acid and Vit. E as well as, induce antioxidant enzymes at the genomic level. The importance of melatonin in this context is discernible not only by its activity to protect effectively against the TM mixture induced prooxidant changes when given exogenously but, also by the recorded significant lowering of the serum titre of melatonin which becomes greater in a duration dependent manner. Since this is probably the only report of change in serum melatonin level and, that too in a situation of exposure to metallic mixture, it is apparent that melatonin is an important and effective *In vivo* agent in combating metal toxicity. There are hardly few report of usage of melatonin to combat metal toxicity as either an ameliorative or a protective agent.

In conclusion, it is inferable from the present study on exposure to a TM mixture that, the responses are not only tissue specific but also a consequence of complex interactions between the constituent metals. The study also brings out the involvement of subtle duration dependent differential interactions such as synergistic, additive or antagonistic in terms of reproductive organ responses on exposure to a combination of metals. It is also inferable from the present study that melatonin could be considered as a potent therapeutic agent against metal intoxication either used alone or in combination with vitamins.