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Evening administration of melatonin and Alpha Lipoic Acid combination affords better protection to rat brain against oxidative stress by induced by Trimetallic combination of Nickel, Cadmium and Chromium.

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Abstract: Differences on brain antioxidant profile together with accumulation of heavy metals have been evaluated in 30 day old rats of Wistar strain, subjected to a trimetallic (Cd- 5.12 mg /Kg BW ,Cr-7.06 mg / Kg BW ,Ni- 7.06 mg /Kg BW) mixture through drinking water for 30 days .Protective effect of melatonin (10mg /Kg BW) and alpha lipoic acid (ALA) (100 mg /Kg BW) either singly or in combination have also been tested by co- administration with metals either in the morning or evening hours. Toxicity was assessed by quantitative assay of non-enzymic (GSH, vitamin C, vitamin E) and enzymic (SOD, catalase, glutathione peroxidase) antioxidants and tissue metal content. Increased lipid peroxidation, the significant depletion in antioxidants and accumulation of metals in the brain tissue were the noticeable observations. These effects have been related with substantial generation of free radicals induced by trimetallic combination. Treatment with protectants could successfully prevent the above metal induced alterations and the potency of melatonin in this respect was better than ALA. Further evening administrations of melatonin in combination was more effective than morning dosage. Overall it is concluded that a trimetallic exposure can result in significant oxidative stress in the neural tissue and that melatonin in combination with ALA has chronotherapeutic potential against metal induced oxidative stress.

Key Words: Heavy metals, melatonin, chronotherapeutic, alpha lipoic acid, oxidative stress, brain

I. INTRODUCTION

The health benefits and harmfulness of numerous medications shift contingent upon the connection between the dosing plan and the 24-h rhythms of biochemical, physiological and neurobehavioral alterations. Additionally, a few pharmaceutical products can make modifications the 24-h rhythms prompting disease and setting up new homeostatic parameters. The alteration of biological rhythm is a new concept of adverse effects. It has been demonstrated that the latter can be minimized by optimizing the dosing schedule (Ohdo *et al*., 2001). An enormous bulk of published literature exists exhibiting the reasoning behind chronotherapy (Smolensky *et al*., 1988; Redferm *et al*., 1997; Lu *et al*., 2020; Chan and Liu,2021). Pharmaceutics is a new territory of biomedical and drug sciences that manages plan and assessment of drug dose structures (or medication conveyance frameworks) to guarantee their wellbeing, viability, quality and dependability. Chronopharmaceutics, on the other hand, is the part of pharmaceutics committed to the plan and assessment of medication conveyance frameworks that discharge a bioactive compound at a predictive rhythm, which preferably coordinates with the actual prerequisite of a given ailment. It is notable that majority of the region of the brain regions exhibits circadian rhythm (Abe *et al*., 2002). Human sleep and its pathological basis insomnia also have a neural basis (Zisapel, 2007). Even the

pathophysiologic changes accompanying the morning surge in blood pressure has a neurological basis which in turn has a lot to do with chronobiology (Cohen *et al*., 1997; Leung and Martinez, 2020). Our earlier work has showcased the deleterious effects of heavy metals (Mehrotra *et al*., 2021) such as cadmium and chromium at environmentally relevant dose (Ramachandran, 2003, Mukherjee et al., 2010; Mukherjee et al., 2021) and the combination of melatonin and alpha lipoic acid (ALA) was shown to afford greater cardio protective effect than either of them singly. In addition, the metals such as mercury, zinc, lead, copper, cadmium and chromium have been shown to exhibit circadian rhythm in terms of absorption, metabolism and excretion (Yokoyama *et al*., 2000). Hence there is a high probability of correlation between the chronotoxicity of heavy metals and possible chronotherapeutics using melatonin + ALA combination. It is the suprachiasmatic nucleus which modulates the rate of neuronal firing based on the ability of melatonin to alter circadian rhythm (Hunt et al., 2001; Armstrong and Redman, 2020). Thus, in the present study, the chronotherapeutic benefit administering melatonin + alpha Lipoic Acid in the evening is possibly due to the chronobiotic property of melatonin.

II. Materials and Methods

2.1 Selection of heavy metals and dosage:

The dosage of Nickel, Cadmium and Chromium selected in the present study is an environmentally relevant estimated average dose based on the actual concentration of chromium found in the cereals and vegetables grown across the Baroda Effluent channel as reported in our earlier publication (Mukherjee et al., 2010; Mukherjee and Ramachandran, 2021).

2.2 Experimental Animals:

Adult female rats of *Wistar* Strain used in the present study were kept in polypropylene cages were fed with pelleted rat diet. Animal experiments were conducted according to the guidelines of CPCSEA from the ministry of Social Justice and Empowerment, Government of India vide CPCSEA (827/ac/04/CPCSEA).

2.3 Experimental Groups:

Control: Rats administered with plain drinking water for 30 days.

Trimetallic (T): Rats administered with mixture of Nickel (7.06 mg / Kg Body Weight) + Cadmium (5.12 mg / Kg Body Weight) + Chromium (7.06 mg / Kg Body Weight) through drinking water. The salts used in the study were Nickel Chloride, Cadmium Chloride and hexavalent chromium trioxide, (CrO_3) for 30 days.

Trimetallic + Melatonin + Alpha Lipoic Acid in the Morning [TMA(M)]: Rats administered with mixture of Nickel (7.06 mg / Kg Body Weight) + Cadmium (5.12 mg / Kg Body Weight) + Chromium (7.06 mg / Kg Body Weight) through drinking water. The same animals were fed with melatonin (10 mg / Kg Body Weight) + Alpha Lipoic Acid (25 mg / Kg BW) through oral gavage at 7:00 Hours for 30 days.

Trimetallic + Melatonin + Alpha Lipoic Acid in the Morning [TMA(E)]: Rats administered with mixture of Nickel (7.06 mg / Kg Body Weight) + Cadmium (5.12 mg / Kg Body Weight) + Chromium (7.06 mg / Kg Body Weight) through drinking water. The same animals were fed with melatonin (10 mg / Kg Body Weight) + Alpha Lipoic Acid (25 mg / Kg BW) through oral gavage at 19:00 Hours for 30 days.

At the end of the experimental duration, animals were sacrificed using cervical dislocation and the whole brain was excised. 10 % Tissue homogenate was used for assessment of enzymic and nonenzymic antioxidants.

2.4 Assay of Lipid peroxidation (LPO) and Reduced Glutathione (GSH): Lipid peroxidation was measured by the method of (Beuge and Aust, 1978) while reduced glutathione was done by the method of Beautler et. (1969)

2.5 Estimation of Vitamin C and Vitamin E: Vitamin C was assayed by the method of Omaye et al (1979) while calorimetric assay of Vitamin E was carried out by the method of Desai (1984).

2.5 Assay of Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx): Enzymic determination of SOD was carried out spectophotometrically at 25 °C by assay protocol designed by Marklund and Marklund (1974). Neural catalase was assayed by the method of Sinha *et al* (1972) while Glutathione Peroxidase was assayed by the method of Paglia and Valentine (1967).

2.6 Brain accumulation of Ni, Cd and Cr: Metal analysis was done as per the protocol of Zmudzki (1977) as described in our earlier work (Mukherjee and Ramachandran, 2021; Mehrotra et al., 2021)

2.7 Statistical analysis

Graphing software from San Diego California USA, www.graphpad.com viz. GraphPad Prism version 6.00 for windows was used for One-way ANOVA with Bonferroni index post test. was performed using GraphPad Prism version 3.00 for Windows. For all statistical analysis, confidence limit was taken as 95 ($p < 0.05$) percent.

III. RESULTS

3.1 Lipid peroxidation and reduced glutathione:

The level of lipid peroxidation and reduced glutathione are shown in Fig.1. The level of lipid peroxidation increased significantly ($P < 0.001$) by 39 % with respect to control. Co-administration of melatonin and alpha lipoic acid both during morning as well as evening prevented this surge in lipid peroxidation. The brain of metal treated animal showed 35% decline in cellular glutathione. Administration of protectants in the evening afforded significantly ($P < 0.05$) better protection against lipid peroxidation and depletion of glutathione.

3.2 Vitamin C and Vitamin E:

The data of neural vitamin levels (Vitamin C and Vitamin E) are presented in Fig.2. The concentrations of vitamins C and E decreased significantly ($P < 0.001$) on toxic exposure to environmentally relevant trimetallic mixture. While Vitamin C decreased by 75%, Vitamin E decreased by 70%. Co-administration of Melatonin + Alpha Lipoic acid combination in the evening hours not only prevented this decline in antioxidant vitamins, but also rendered significantly ($P < 0.01$) better protection when compared to morning dosing.

3.3 Antioxidant Enzymes (SOD, CAT, GPx):

The effects of trimetallic exposure (T) on brain superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) are depicted in Fig 3. Administration of mixture of three heavy metals led to significant ($P < 0.001$) decrease in SOD (52%↓), CAT (68%↓) and GPx (51%↓). Evening administration of melatonin + alpha lipoic acid combination had non-significant effect on SOD, while CAT ($P < 0.05$) and GPx ($P < 0.01$) exhibited chronotherapeutic benefit over morning administration of protectants.

3.4 Brain levels of Ni, Cd and Cr:

Fig 4 shows the accumulation of heavy metals in the brain of experimental animals. Administration of trimetallic mixture resulted in significant neural accumulation of Ni (40%↑), Cd (42%↑) and Cr (50%↑). Although melatonin + alpha lipoic acid combination prevented the buildup of heavy metals in the brain, there was non-significant role of time of administration over neural metal accumulation.

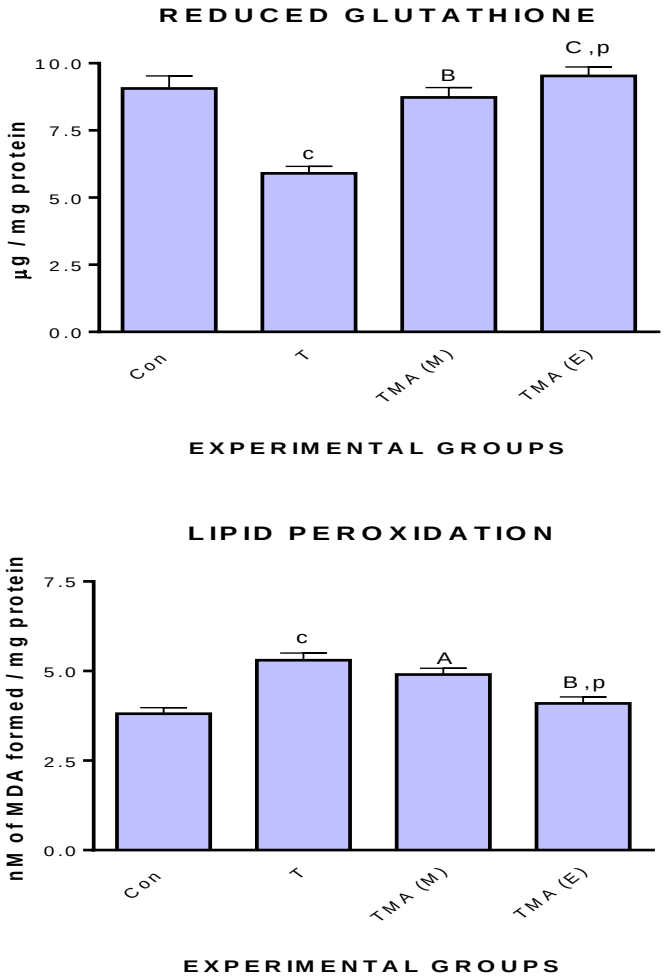

Fig. 1. Effect of Trimetallic mixture of Ni, Cr and Cd (T) and melatonin + alpha lipoic acid (M + A) combination on the levels of reduced Glutathione (GSH) and Lipid Peroxidation (LPO)

Data are expressed as mean ± SEM. n = 6 for each groups

Statistical significance of Control (C) Vs Trimetallic mixture (T): [a] P <0.05, [b] P < 0.01, [c] P<0.001 and ns nonsignificant

Statistical significance of Trimetallic mixture (T) Vs TMA (M) and TMA (E): [A] P <0.05, [B]P < 0.01, [C] P<0.001 and NS nonsignificant

Statistical significance of TMA(M) Vs TMA(E): [p] P <0.05, [q] P < 0.01, [r] P<0.001

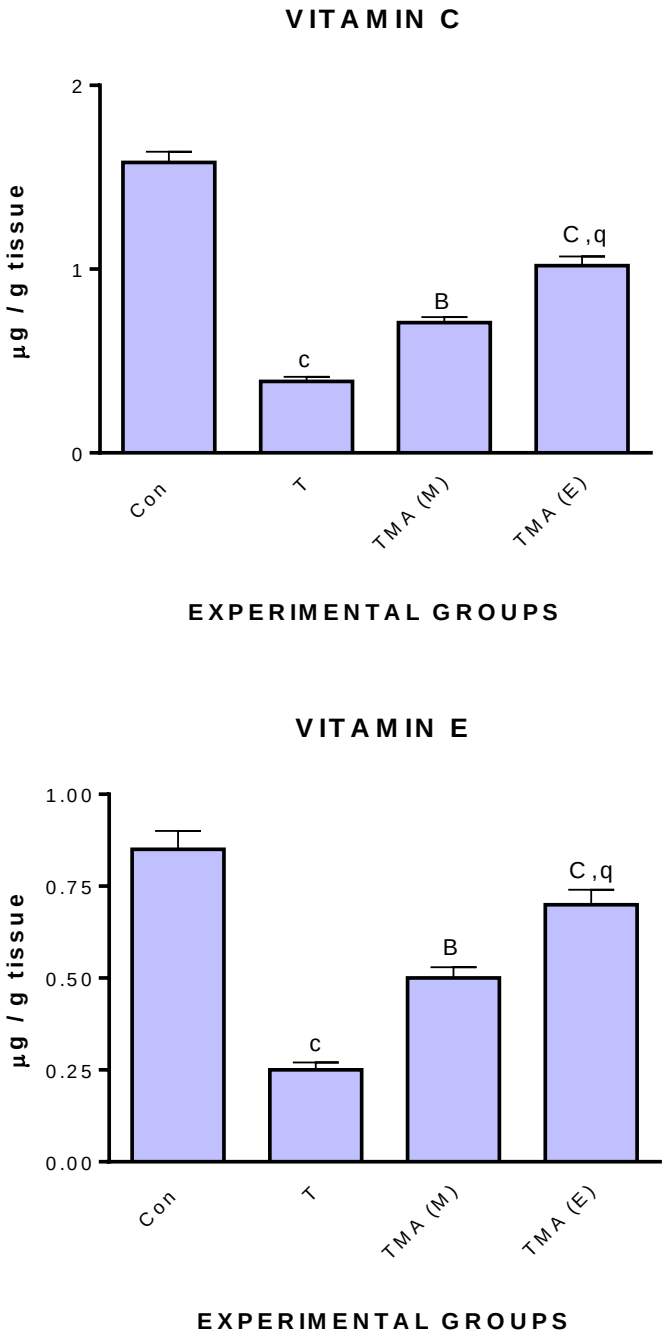

Fig. 2. Effect of Trimetallic mixture of Ni, Cr and Cd (T) and melatonin + alpha lipoic acid (M + A) combination on the level of Vitamin C and Vitamin E in rat brain.

Data are expressed as mean ± SEM. n = 6 for each groups

Statistical significance of Control (C) Vs Trimetallic mixture (T): [a] P <0.05, [b] P < 0.01, [c] P<0.001 and ns nonsignificant

Statistical significance of Trimetallic mixture (T) Vs TMA (M) and TMA (E): [A] P <0.05, [B]P < 0.01, [C] P<0.001 and NS nonsignificant

Statistical significance of TMA(M) Vs TMA(E): [p] P <0.05, [q] P < 0.01, [r] P<0.001

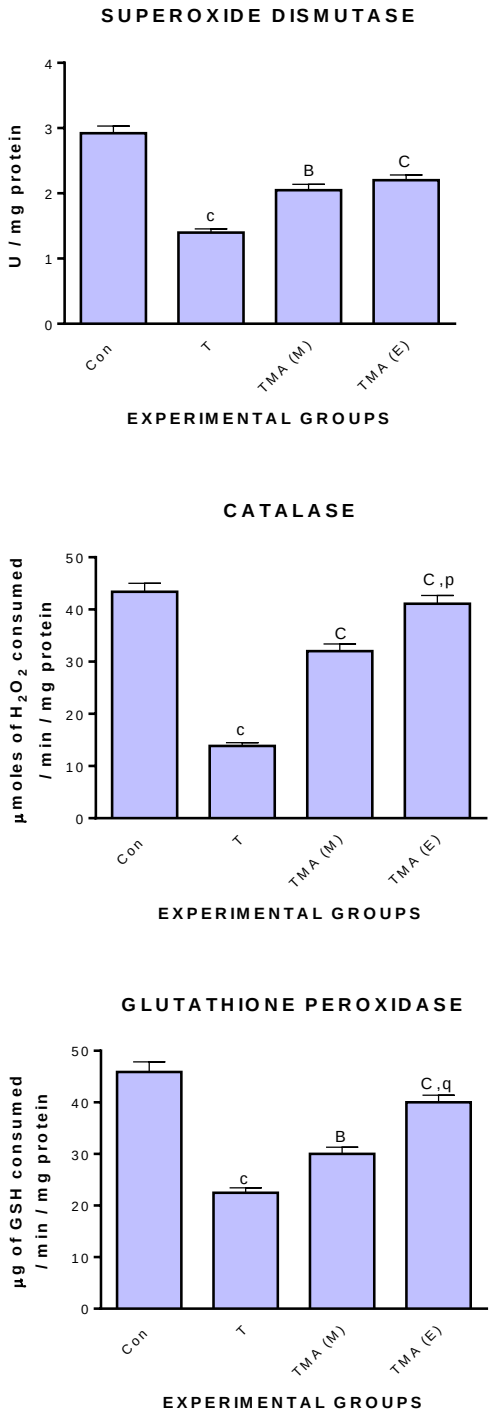

Fig. 3. Effect of Trimetallic mixture of Ni, Cr and Cd (T) and melatonin + alpha lipoic acid (M + A) combination on Superoxide Dismutase, Catalase and Glutathione Peroxidase

Data are expressed as mean ± SEM. n = 6 for each groups

Statistical significance of Control (C) Vs Trimetallic mixture (T): [a] P <0.05, [b] P < 0.01, [c] P<0.001 and ns nonsignificant

Statistical significance of Trimetallic mixture (T) Vs TMA (M) and TMA (E): [A] P <0.05, [B]P < 0.01, [C] P<0.001 and NS nonsignificant

Statistical significance of TMA(M) Vs TMA(E): [p] P <0.05, [q] P < 0.01, [r] P<0.001

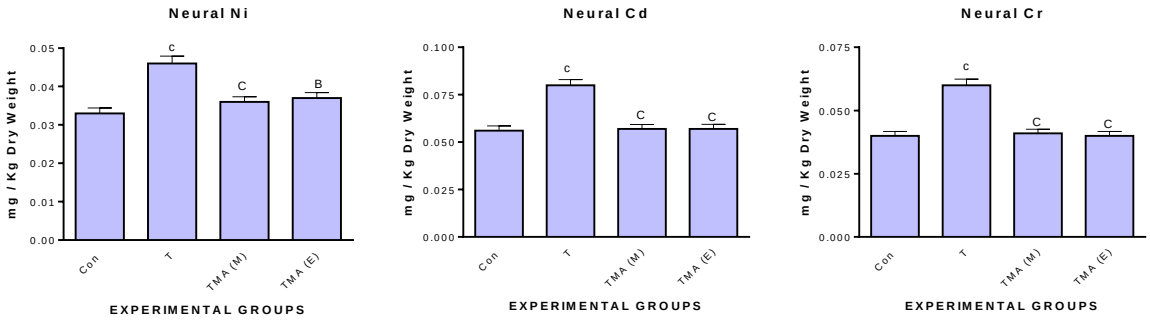

Fig. 4. Effect of Trimetallic mixture of Ni, Cr and Cd (T) and melatonin + alpha lipoic acid (M + A) combination on accumulation of Ni, Cd and Cr in rat brain.

Data are expressed as mean ± SEM. n = 6 for each groups

Statistical significance of Control (C) Vs Trimetallic mixture (T): [a] P <0.05, [b] P < 0.01, [c] P<0.001 and ns nonsignificant

Statistical significance of Trimetallic mixture (T) Vs TMA (M) and TMA (E): [A] P <0.05, [B]P < 0.01, [C] P<0.001 and NS nonsignificant

Statistical significance of TMA(M) Vs TMA(E): [p] P <0.05, [q] P < 0.01, [r] P<0.001

IV. DISCUSSION:

The major highlights of the present investigation are as follows; (1) Exposure of 30 day old wistar rats to a trimetallic mixture of nickel, cadmium and chromium for a period of 30 days results in depletion of enzymic (SOD, CAT, GPx) and nonenzymic (Vitamin C, Vitamin E, GSH) antioxidants and increased lipid peroxidation (2) Administration of the tri metallic mixture in sublethal dose results in considerable accumulation of these metals in the brain of rats. (3) Simultaneous administration of melatonin or ALA either singly or in combination prevents the depletion in antioxidants. (4) Combination of melatonin + ALA is in particular more potent in action as compared to the individual protectants. (5) Compared to morning administration of protectants, therapeutic efficacy is better when the protectants are administered in the evening.

There is a significant increase in the amount of lipid peroxidation in the brain of experimental animals. The decrease in nonenzymic antioxidants such as glutathione and enzymic antioxidants such as catalase and GPx may possibly be the contributory factor for the reported elevation in lipid peroxidation. Glutathione is the most prevalent cellular nonprotein thiol that occurs ubiquitous in eukaryotic cells (Sies *et al*., 1999). It plays a crucial role in protecting tissues against oxidizing conditions and in maintaining intracellular redox balance (Rehman *et al*., 2000). Amongst the enzymic antioxidants, it is GPx which is capable of removing lipid peroxides and hence the decline in the activity of GPx in the current study possibly favours lipid peroxidation. Evans (1993), explained the reason behind greater susceptibility of the brain to free radical damage. Higher rate of metabolic activity, readily oxidisable substrate such as membrane lipid polyunsaturated fatty acids, coupled with low level of protective antioxidant enzymes (Catalase and Glutathione Peroxidase) account for greater vulnerability of brain (Evans, 1993). Signal transduction ion like calcium leak into the cell as a result of loss in membrane integrity due to lipid peroxides. Most transition metals contribute to lipid peroxidation, a chain reaction catalyzed by hydroxyl radicals (Halliwell and Gutteridge, 2015). Thus the depletion of enzymic and nonenzymic antioxidants observed in the current study is possibly due to generation of free radicals which might have affected the transition site of the enzymic antioxidants.

On simultaneous administration of trimetallic mixture along with the protectant, a chronotherapeutic response is observed in almost all the known antioxidant parameters. Level of protection is significantly higher when melatonin or melatonin + Alpha lipoic acid (M+ALA) is administered during the evening hours. Several speculations can be made on the basis of this observed result ; upregulation of the antioxidant defense during evening hours, chronotoxicological basis of heavy metals with lesser susceptibility during the evening hours or the chronobiotic property of the protectant. It has been shown that heavy metals like Cr, Cu, Pb and Hg exhibit chronotoxicological basis with greater toxicity during the evening hours (Yokayama *et al*., 2000). This corresponds with the lower glomerular filtration during evening hours leading to higher plasma concentration of metals (Yokoyam *et al*., 2000). In the present study, cadmium is administered through drinking water and rats being nocturnal, the rate of water consumption is higher during the evening hours posing a great threat for free radical induced damage.

Even the levels of routine lipid peroxidation are significantly higher during the evening hours i.e. between 20:00 hours to 4:00 hours with a peak at 24:00 hours (Diaz-Munoz *et al*., 1985) GPx which is the principal antioxidant enzyme involved in neuroprotection has its peak at 4: 00 hours (Baydas *et al*., 2002). Melatonin peak is at 1:00 hours and the level of lipid peroxidation decreases in response to melatonin peak. Kidney as an organ exhibits tremendous alterations in circadian rhythm (Firsov and Bonny, 2018) glomerular filteration is low at night (Pons et al., 1994). Thus reduction in glomerular filteration may account for the decrease in the urinary excretion of creatine during night hours. The time of excretion of all heavy metals except zinc also tends to decrease during night hours. Therefore, the observed neural accumulation of administered metals in our study is due to reduction in GFR (glomerular filteration rate) as urinary excretion of these heavy metals decreases during night hours.

The chronotherapeutics of melatonin in preventing symptoms of trimetallic induced free radical damage may be implicated on the basis of the observed results. MT_2 receptors are localized in the CNS and the brain (Naji *et al*., 2004). The up regulation of the antioxidant enzymes by melatonin is mediated by its genomic action and hence in the backdrop of sufficient receptors, supposedly in the evening, melatonin exhibits superior protective action.

Glomerular filtration rate assumes the central theme as a major determinant involved in urinary excretion of several metals (Aono and Arki, 1988). In the present study the metals are administered through drinking water and in rats the maximum intake is during night since the creature is nocturnal and hence maximum absorption of metal is at night. Thus there is a multitude of risk factors during night with increased level of lipid peroxidation, low level of glutathione peroxidase activity and

lower rate of glomerular filteration rate contributing to increased risk of free radical mediated injury. Even the circadian rhythm in the occurrence and severity of cerebral hemorrhagic form being most common late in the evening (Schallner et al, 2014). It is apparently the chronobiotic property of melatonin that takes care of these factors during the nocturnal phase.

Conclusion: Overall, it is concluded that a trimetallic exposure led to significant oxidative stress in the neural tissue and that evening administration of melatonin in combination with ALA has a greater neuroprotective role in mitigating oxidative stress as compared to morning administration of the antioxidant combination. Thus chronotherapeutic benefit of melatonin can be fine tuned to protect cells and tissues against damage due to oxidative stress.

V. AUTHORS CONTRIBUTIONS:

Dr. Raktim Mukherjee carried out the animal experimentation, assay of enzymic and non-enzymic antioxidants and statistical analysis of results and wrote the first draft of the manuscript. Ms. Megha Dave helped in data analysis, preparation of Graphs and tables and determination of metals from preserved samples. Prof. A.V. Ramachandran conceived the study and designed the experiments.

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