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Protective effects of succimer against lead induced neurotoxicity in developing brain of rats

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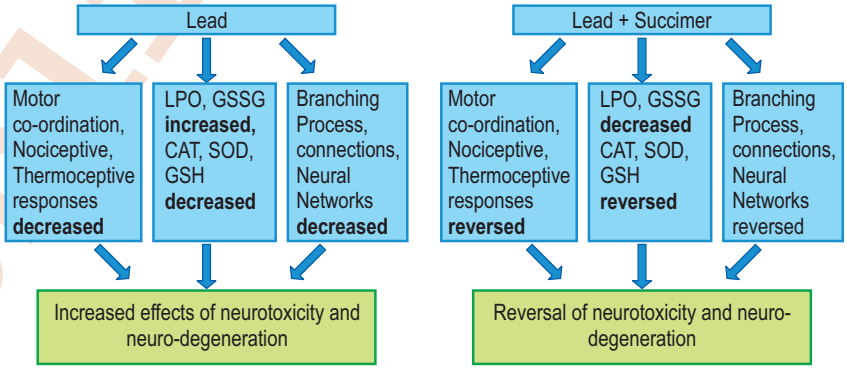
Abstract

Aim: The aim of this study was to evaluate the protective effects of succimer against lead induced neurotoxicity in developing brain of rats.

Methodology: Healthy albino Wistar rats were segregated into four groups, Control (receives normal water), Lead (100 ppm through drinking water), Lead+Succimer (100 ppm + 50 mg kg^{-1} b.wt. day^{-1}) and succimer alone (50 mg kg^{-1} b.wt. day^{-1}). Doses were started from the first day of pregnancy confirmed and continued till day 30 post-natal pups. The 1st, 15th, 30th day post-natal pups were used for oxidative stress markers assessment, histological study, whereas 15th, 30th day pups were used for behavioral assessment.

Results: Lead treated rats showed lowered motor coordination, thermal and mechanical pain sensitivity when compared to control group and these responses reversed on treatment with succimer ($p< 0.01$). Lead treated rats showed a significant ($p< 0.01$) decrease in CAT, SOD activity and GSH levels, while LPO and GSSG levels were increased as compared to control group, and succimer treatment reversed the altered oxidative metabolism. Lead treated rats showed a decrease in number of branches in neurons and branching of neuronal networks. The number of branches and branching of neuronal networks were reverted on treatment with succimer.



Interpretation: This study concludes that succimer has considerable therapeutic value against lead induced neurotoxicity along with neurodegeneration with its chelation as well as anti-oxidant properties reverse neuro-behavioral alterations, oxidative stress and histological impairments caused with lead during pre- and post-natal exposure to rats.

Key words: Anti-oxidants, Lead, Neurodegeneration, Neurotoxicity, Succimer.

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Introduction

Lead plays a significant role in modern industry and a large population are at a risk of lead toxicity due to occupational exposure (Fracasso *et al.,* 2002). Lead is one of the toxic heavy metals, posing threat to humans if exposed to exceeded levels. Lead toxicity depends on its chemical form administrated to the animal, the route of administration and the frequency and duration administered to the animals (Ibrahim *et al.,* 2012). Lead accumulation produces damaging effects on the haematological and renal system, causes various forms of cancer, nephrotoxicity, affects nervous, cardiovascular and respiratory systems, retards growth and leads to hepatic, reproductive dysfunction (ATSDR, 2005; Shilpa *et al.,* 2014). The poorly developed blood-brain barrier in infants and young children facilitates the easy entry of lead into the brain. Their bodies absorb a larger percentage of lead, thereby exhibiting lead toxicity at lower exposure level than adults (Ibrahim *et al.,* 2012). The reactive oxygen species (ROS) play a major role in lead induced toxicity (Flora *et al.,* 2008). Oxidative stress has been implicated for its contribution to lead-associated tissue injury in the liver, kidneys, brain and other organs. Prenatal exposure of lead at any concentration results in a significant neurochemical alterations in the rats' brain (Sarath *et al.,* 2007). It is also evident that disruption of neurotransmitter systems may be involved in Pb related neuro-behavioral dysfunction (Anderson *et al.,* 2016). Epidemiological investigations have established the relationship between chronic developmental lead exposure and cognitive impairments in young children (Kim *et al.,* 2012). Inhalation of lead can permanently lower intelligence quotient (IQ), damage emotional stability and cause hyperactivity, poor school performance and hearing loss. Lead toxicity remains a significant public health problem because of its global pervasiveness and its adverse effects on the nervous system (Azzaoui *et al.,* 2009).

Elimination of lead from body is possible through chelation. Heavy metal chelating compounds not only eliminate metal toxicity but also reduces the production of free radicals. Apart from other forms of chelating methods 2, 3-dimercapto-propane-1-sulfonate and calcium disodium ethylene-di-amine-tetra-acetic acid ($CaNa_2EDTA$) has been the main stay of chelation therapy for lead poisoning for last 40 years. A combination of EDTA along with British Anti Lewisite (BAL), also known as dimercaprol, and a penicillin derivative called oral D-penicillamine are also in use in chelation until the availability of succimer (Flora and Pachauri, 2010). Succimer is a chelating agent commonly used for the treatment of lead in blood. Moreover, succimer, a dithiol compound, has a greater therapeutic index and more recent studies have proven that succimer is safer than Ca-EDTA (Aaseth *et al.*, 2016). Succimer has advantages over dimercaprol and $CaNa_2EDTA$. Succimer is a potent chelator for moderate lead toxicity and is a standard preferable antidote for lead in many countries, including USA (Bradberry and Vale, 2009). Succimer has been effectively lower lead accumulation in tissues and decrease lead content in brain (Stangle *et al.,* 2004). Reported to succimer along with lipoic acid showed excellent anti-oxidant properties against lead oxidative stress (Flora *et al.,* 2007). Succimer eliminates free radicals and also reduce the production of free radicals, and thus has therapeutic effect against heavy metal toxicity (Mikirova *et al.,* 2011). The present study reports the protective effects of succimer against lead induced neurotoxicity in developing brain through oxidative markers, neurodegeneration in brain and behavioral studies.

Materials and Methods

Healthy albino Wistar rats (*Rattus norvegicus*) weighing between 180 to 200 g were obtained from National Institution of Nutrition, Hyderabad. They were assigned randomly to experimental groups and housed 2 females and 1 male for cage (polypropylene cages) to allow breed. Light cycles were maintained as 12Light : 12Dark hours (6.00 AM to 6.00 PM), and temperature between 22 ± 2º C. The laboratory conditions of animals are maintained as per IAEC of Zoology, Osmania University as per the guidelines of CPCSEA (CPCSEA No: 383/01/a/CPCSEA). The study protocol on experiment in animals (to evaluate the protective effects of succimer against lead induced neurotoxicity in developing brain of rats) was approved by IAEC of Osmania University, Hyderabad.

After confirmation of day one pregnancy, rats were randomly grouped into four. Rats of group I were given drinking water and treated as control. Rats of groups II, III and IV were given lead acetate (100 ppm through drinking water), lead acetate (100 ppm) + succimer (50 mg kg^{-1} body weight orally with plastic gavage) and succimer alone (50 mg kg^{-1} body weight; orally with plastic gavage), respectively. Doses were continued for 51 days (gestational 21 days and post natal 30 days).

Behavioural tests: Rats were trained on the rota-rod at 30 rpm for 2 min each for three days from the day prior to experiment. On the day of analysis, animals were placed on the rotating rod at 30 rpm, and duration each rat remained on the rotating rod was recorded (Dunham and Miya, 1957). Hot plate (thermal, 52.0±0.5° C) latency time was recorded on Remi hot plate following the method of Eddy and Leimbach (1953). The response time was recorded in seconds. The nociceptive pain was assessed using the Randall Selitto electronic algesimeter. The maximum force applied was limited to 50 g (calibrated force) to avoid skin damage. The withdrawal of paw was noted as response and expressed in pounds (Randall and Selitto, 1957).

Oxidative stress markers: LPO was estimated by the method of Bhuyan *et al.* (1981). The results were expressed in nano-mol of MDA g^{-1} tissue. Catalase activity in the brain tissue was assessed on the basis of disappearance of H_2O_2 in the presence of enzyme

source at 26 ºC. CAT activity was expressed in terms of µ mol min^{-1} mg^{-1} tissue (Brannan *et al.,* 1981). SOD activity in the brain tissue was measured on the basis of auto-oxidation of pyrogallol in the presence of EDTA. The enzyme activity was expressed as Unit mg^{-1} protein (Murkland and Murkland, 1974). Glutathione content in the brain tissue was estimated by the method of Hissin and Hilf (1976). Glutathione disulfide (GSSG) in the brain tissue was estimated by the method of Hissin and Hilf (1976). GSSG concentration was measured by spectrophotometry by GSH recycling method, based on the principle of conversion of GSSG to GSH by glutathione reductase and NADPH and reaction with DTNB.

Histological study : The isolated brains from experimental rats were immediately preserved in Golgi cox solution (equal volumes of potassium chromate, potassium dichromate, mercuric chloride and distilled water [5:5:5:5] and filtered) for 10 days in dark room. The brain tissue was washed with water and transferred to 30% sucrose solution for 3 to 4 hrs. The brain was fixed on vibrotome stage with cyanoacrelate glue in vibrotome tank filled with 6% sucrose solution. A 10 µ thick sections of brain were sliced on vibrotome. The brain sections were transferred in the following serve to hypo, water and ammonium solution and dehydrated by passing through increasing concentration of alcohol (30%, 50%, 70%, 90% and 100%) and finally in iso-propanol. The slides were prepared with care and observed at 4x magnification under Lawrence digital microscope (Robbin and Bryan, 1998).

Statistical analysis: The data obtained were subjected to one-way analysis of variance (One–way ANOVA) and t-test for comparison of significance between the groups. Data were presented as mean±SE.

Results and Discussion

Lead is a naturally occurring heavy metal, having profound neurotoxicity (Sarath *et al.,* 2007). Developing pups are more susceptible to heavy metal intoxication, particularly lead, due to the fact that they have comparatively less established anti-oxidant mechanisms and regulatory pathways. Lead exposure alters biochemical and neurotransmitter system of the brain during development (*Reddy et al.,* 2003). In view of this, in the present study, succimer chelation therapy towards lead intoxication was investigated. The results revealed that lead exposure (100 ppm, intra-gastrically through drinking water) for a duration of fifty one days (21 pre-natal and 30 post-natal) resulted in behavioral alteration, oxidative stress and disturbance of neuronal structure of brain in terms of neural connections and networks.

The motor coordination in lead administered rats was comparatively lowered ($p<0.001$) than control, increased ($p<0.05$) in succimer protective group against lead as compared to lead alone treated group (Fig. 1a). The effect of lead on motor activity was more on day 15 than day 30. The results of succimer group were found similar to that of control group. Lead acetate obstructs the cholinergic pathway causing depletion of acetylcholinesterase in the brain (Richetti *et al.,* 2011), and

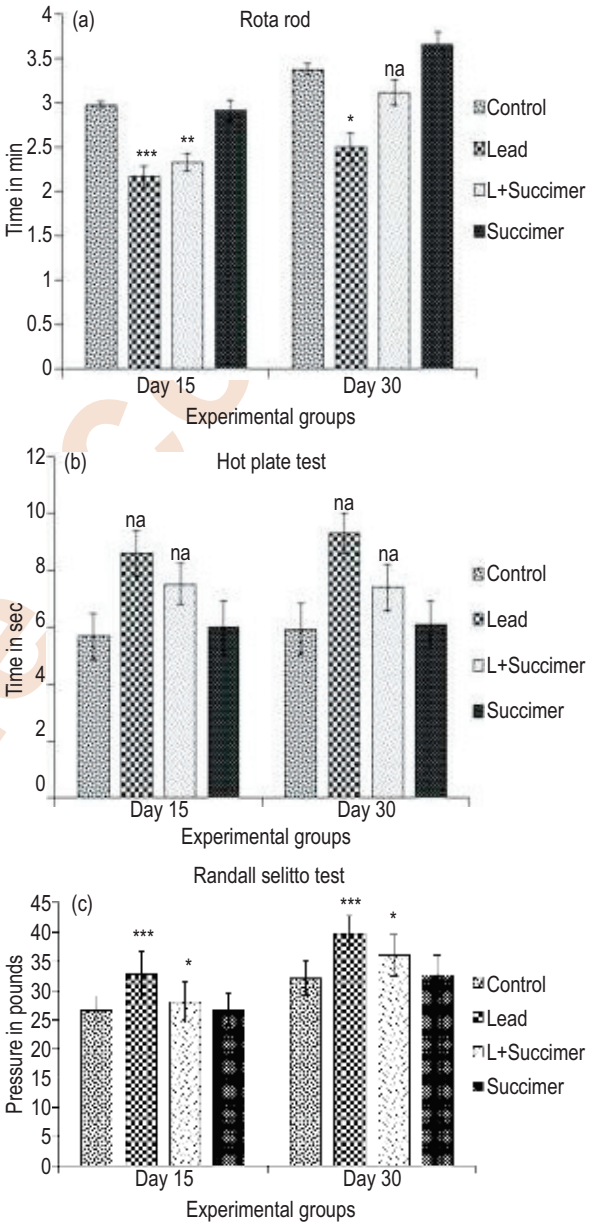

Fig. 1: Succimer protective effect on motor co-ordination (a); temperature induced nociceptive pain response (b); and mechanically induced nociceptive pain response (c) of rats exposed to lead. The motor co-ordination time is in min, the hot plate latency time in sec and the mechanical pain response in pressure in pounds is shown in respective figures. Each bar graph representing the mean±SE (n= 5 animals). Significance of the data is shown as 0.001 (***), 0.05 (**), 0.01 (*) and non- significant (na).

Table 1: Effect of succimer on oxidative stress markers (LPO, GSH, GSSG levels and SOD and CAT activity) in brain of rats exposed to lead during gestational and lactating periods

Groups → Oxidative stress marker & Day variation ↓	Control	Lead	% of change from control	Lead+Succimer	% of change from control	Succimer	% of change from control
LPO							
Day 1	0.49±0.019	0.59±0.036*	20.40%	0.53±0.038**	8.16%	0.50±0.020	2.04%
Day 15	0.46±0.018	0.61±0.029*	32.60%	0.54±0.023**	17.39%	0.46±0.016	0%
Day 30	0.53±0.033	0.65±0.032*	22.64%	0.56±0.028*	5.66%	0.52±0.035	-1.88%
GSH							
Day 1	0.67±0.021	0.62±0.030*	-7.46%	0.65±0.024*	-2.98%	0.67±0.029	0%
Day 15	0.74±0.024	0.64±0.036*	-10.00%	0.71±0.031*	-4.05%	0.74±0.035	0%
Day 30	0.77±0.033	0.61±0.024*	-20.77%	0.73±0.033*	-5.47%	0.77±0.036	0%
GSSG							
Day 1	0.37±0.041	0.47±0.022*	27.02%	0.41±0.026*	10.81%	0.37±0.029	0%
Day 15	0.37±0.027	0.50±0.034*	35.13%	0.41±0.023*	10.81%	0.37±0.026	0%
Day 30	0.45±0.043	0.57±0.031*	26.66%	0.50±0.046*	11.11%	0.41±0.023	-8.88%
SOD							
Day 1	1.10±0.036	0.96±0.058*	-12.72%	1.01±0.060*	-8.18%	1.09±0.036	-0.90%
Day 15	1.00±0.039	0.90±0.039*	-10.00%	0.96±0.044**	-4.00%	0.99±0.040	-1.00%
Day 30	0.97±0.029	0.88±0.027*	-9.27%	0.93±0.026*	-9.27%	0.98±0.024	1.03%
CAT							
Day 1	0.25±0.029	0.17±0.016*	-32.00%	0.22±0.028**	-12.00%	0.25±0.033	0%
Day 15	0.26±0.025	0.20±0.025*	-23.07%	0.24±0.033*	-7.69%	0.27±0.030	3.84%
Day 30	0.30±0.021	0.23±0.030*	-23.33%	0.27±0.025*	-10.00%	0.31±0.030	3.33%

LPO, GSH, GSSG levels and SOD and CAT activity in brain tissue of rats exposed to lead and suucimer. Data expressed as mean±SE (n=5 animals). Significance of data is $p<0.01$ (p*), $p<0.05$ (p**). Units: LPO levels were expressed as nano-mol of TBARS g^{-1} tissue; GSH content was expressed in terms of µg mg^{-1} protein; GSSG levels were presented as µg mg^{-1} protein; SOD activity was presented as unit mg^{-1} protein; CAT activity was expressed as µ mol min^{-1} mg^{-1} tissue

ultimately resulting in the crippling of motor coordination.

The thermo nociceptive pains in lead administered rats was significantly decreased (Fig. 1b). Thermal pain response was increased in succimer protective group against lead as compared to lead alone treated group, which may be due to receptor damage in both fore and hind paws. The Randall-Selitto test serves as a tool to assess response thresholds to mechanical pressure stimulation (Eva Santos *et al.,* 2012a). The mechanically induced nociceptive pain in lead administered rats was significantly increased ($p<0.001$) to that of control, decreased ($p<0.01$) (Fig. 1c) in succimer protective group against lead as compared to lead alone treated group. The results of succimer group were found similar to that of control group. Lead damages the receptors present in the paws, and hence, it is expected to alter the normal receptor behavior.

Furthermore, lead exposure increased the lipid peroxidation (20.40% on day 1, 32.60% on day 15 and 32.64% on day 30) and oxidized glutathione (27.02%, 35.13% and 26.66% from day 1, 15 and 30 respectively) levels and decreased catalase (-32.00%, -23.07% and -23.33% from day 1 to 30 correspondingly), super-oxide-dismutase (-12.72%, -10.00% and -9.27% on day 1, 15 and 30) activity and reduced glutathione content (-7.46%, -10.00% and -20.77% on day 1, 15 and 30) compared to control rats ($p<0.01$) (Table 1). The lipid peroxidation levels in lead exposed rats increased as compared to control, whereas lead with succimer treated group showed lowered level of LPO (8.16%, 17.39% and 5.66% on day 1, 15 and 30, respectively) as compared to control rats. On the other hand, succimer alone treated rats showed in significant difference (2.04% on day 1, 0% on day 15 and -1.88% on day 30) in LPO with those of control rats. In accordance, the increase in lead level was accompanied with an increase in lipid peroxidation (Seddik *et al.,* 2010).

The present study is in an agreement with that of Seddik *et al.* (2010), who witnessed an amplification of lipid peroxidation in brain following lead exposure. Peroxidation of membrane phospholipids ultimately results in loss of membrane integrity following cell death. The decrease in the levels of glutathione reduced content was accompanied with an increase in glutathione oxidized levels in lead exposed brain tissue (Seddik *et*

Golgi –cox stain:

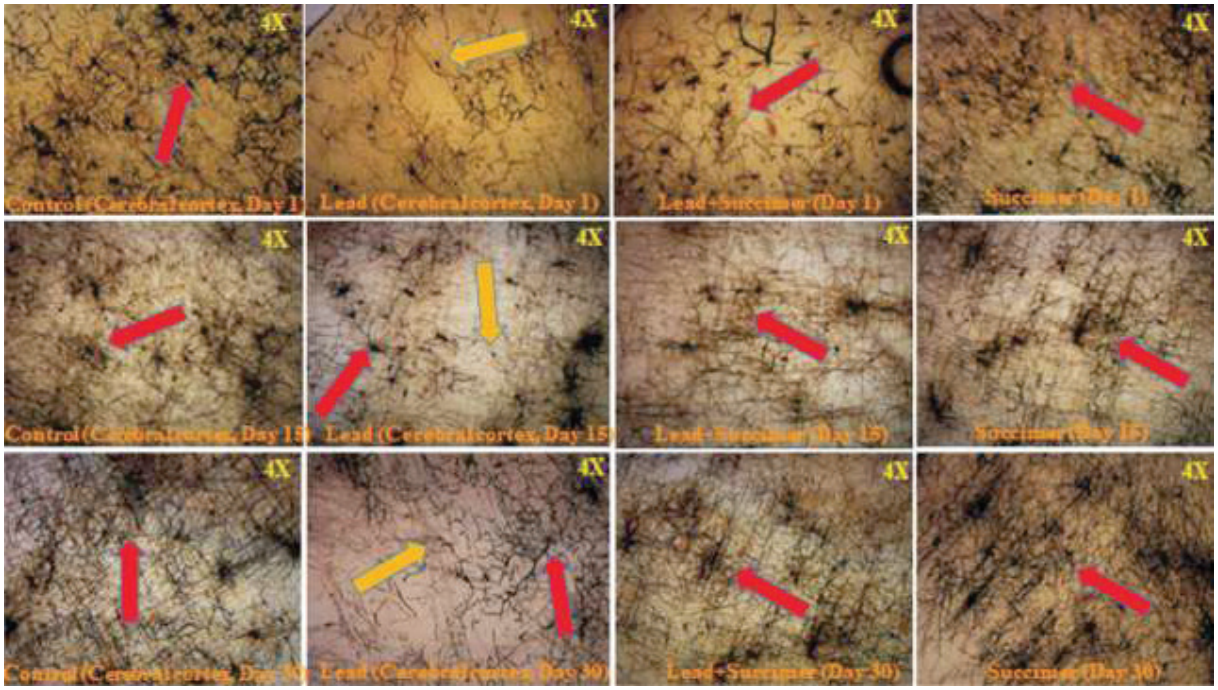

Fig. 2 : Succimer protective effects on the morphology (branches of soma, neural connections and networks) of rats exposed to lead. Slides (cortex region) observed under Lawrence digital microscope at 4X magnification. Horizontally first row 1st day, second row 15th day and third row 30th days. Red arrow – normal neural cells, yellow arrow – altered neurons.

al., 2010). The results of glutathione in reduced form in lead exposed rats were lower compared to control and lead with succimer treated group (Table 1). The percent content of GSH proportionately decreased in lead exposed rats from day 1 to day 30. Oxidized glutathione concentrations of lead alone treated rats increased over control (Table 1). GSSG levels were reverted on treatment with succimer. SOD is responsible for detoxification of potentially toxic and highly reactive free radicals to less toxic hydrogen peroxide (Adegbesan and Adenuga, 2007). In the present study, SOD activity decreased ($p<0.01$) in lead treated rats, to that of control (Table 1). The SOD activity decreased ($p<0.01$) progressively from day 1 to day 30 in which lead exposed rats, is in confirmation with the previous study of Uzbekov *et al.,* (2007).

As compared to control rats, the lead treated rats exhibited a decreased CAT activity (Table 1) ($p<0.01$). Lead with succimer group resulted in a reverted CAT activity. The percent decreased CAT activity in lead treated rats was more on day 1 and its activity gradually rose on day 15 and 30. In succimer alone treated rats, normal activity of catalase similar to control was observed. Catalase, an antioxidant enzyme, is responsible for the decomposition of hydrogen peroxide (Bhattacharya, 2015), therefore, decrease in catalase activity fails to eliminate free radicals, which is considered as a root cause of neuro-degeneration.

The current study showed that succimer had a significant protective effect on lead induced oxidative stress. It appears possible that succimer could decrease stress markers of oxidative stress, either by removing lead from the target tissue or by directly scavenging the reactive oxygen species through its sulfhydryl groups. Succimer is of four carbon molecules structure with two carboxyl groups and two sulfur groups. Heavy metals such as lead bind to the adjoining sulfur and oxygen atoms, whereas arsenic and mercury bind to both sulfur atoms, resulting in a pH dependent water-soluble compound (Raymond and Okieimen, 2011).

DMSA is primarily an albumin bound in plasma by disulfide bond with cysteine and a small part remains unbound. In fact other conventional chelating agents, such as D-penicillamine, have also been shown to scavenge ROS *in vitro* (Benov *et al.,* 1990). Thus, in this study, succimer treated rats showed reversed levels of LPO, GSH, GSSG and CAT, SOD activity against lead rats. A majority of the elimination of DMSA occurs within 24 hrs in the form of DMSA-cysteine disulfide conjugates (Rice, 1996). As a result of chelation therapy, lead excretion has shown to increase through urine. Further studies are needed to determine whether succimer exerts its beneficial effects solely through its ability to act as a metal chelator or

whether part of its protective effects can result from its potential as a thiol- antioxidant.

In addition, histological variations were also observed in the present study. In lead exposed rats, the morphology of neural cells *i.e.*, the number of dendrites arises from soma, connections between dendrites and telo-dendrites and neural networks (web of connections) decreased as compared to those of control (Fig. 2). Examination of Golgi cox stained sections of lead+succimer and succimer treated group showed apparent normal appearance of brain cells similar to control group. A similar result was reported by Patrick and Anderson (1995). Decreased neural branching of dendrites or re-arrangement of neural networking patterns was reported in lead treated rats (Alfano and Petit, 1982). In succimer treated group against lead showed increased neural connections and networks as compared with lead alone treated group. Succimer has scavenging properties against lead (Bjørklund *et al.,* 2017), and therefore it maintains the normal lipid profile of nerve cell members. Succimer, thus, maintains normal neural connections and stabilizes the density of neurons.

The developing brain of rats is more prone to lead toxicity due to poorly developed blood brain barrier, unable to provide protection towards an influx of toxicants, and incomplete formation of anti-oxidant status. Lead creates disturbances in the anti-oxidant status of brain tissue, as well as increase the oxidation of membrane lipids. Due to loss of lipid layer, myelin sheath gets disintegrated and as a result neural cells undergo necrosis. Since, loss in neuronal ultrastructure of brain becomes altered in terms of earlier mentioned characteristics (connections and networks) of neuron. Hence, it create disturbances in the brain tissues such as biochemical milieu and neurotransmitters function. Thus it finally results in neurobehavioral alterations.

Based on the observed results, it can be concluded that lead has more adverse effects on young pups than adults and administration of succimer can reverse neurotoxic effect through its anti-oxidative and chelation effects. Thus, succimer has potential therapeutic efficacy in amelioration of lead neurotoxicity, particularly during pre- and early post-natal exposure.

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