SUMMARY AND CONCLUSIONS
Lead (Pb) has been known to have effects on wide range of molecular and cellular mechanisms in the mammalian brain, including energy metabolism. Pb poisoning is many faceted monsters that have been around since antiquity. Pb is a highly neurotoxic agent causing functional and structural abnormalities in the brain. The nervous system is the primary target of low level Pb-exposure and the developing brain appears to be especially vulnerable to Pb neurotoxicity. Interference of Pb with the development and differentiation of the central nervous system (CNS) and impairment of cognitive processes by direct pharmacological interaction of Pb with specific sites that are important in neurotransmission are the two distinct mechanisms of Pb neurotoxicity.

Exposure to Pb in the developmental period can lead to a number of behavioural changes in children. Since children are much more sensitive than adults to learning impairments following low-level Pb-exposure, the developmental toxicity of Pb has become a significant area of research in recent days. The apparent sensitivity of the developing brain to Pb-neurotoxicity suggests that there is a ‘window of vulnerability’ to low-level Pb-exposure, which results in long-lasting, possibly irreversible cognitive deficits and neurochemical changes.

Nutritional factors play an important role in Pb-poisoning. Pb toxicity can be reduced by supplementation of certain essential metals. The literature suggests that supplementation with essential metal like Ca\textsuperscript{2+}, Zn\textsuperscript{2+}, Fe\textsuperscript{2+} individually plays a major role in reducing the Pb-burden to a great extent. However, there were no studies to know how they act when they were given in combination. Hence the present study is designed to examine the protective effects of combining supplementation of Ca\textsuperscript{2+}/Zn\textsuperscript{2+}/Fe\textsuperscript{2+} against Pb-induced alterations on behavioral and neurochemical aspects and to examine the interrelationships between the behavioral alterations, disturbances in neurotransmitter systems and the antioxidant defense systems in Pb-exposed developing brain.

Wistar strain albino rats were used for the present study. Rats (90 days, 140 ± 10 g) were purchased from Sri Venkateswara Traders, Bangalore and maintained in the animal house of Department of Zoology, Sri Venkateswara University. Rats were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk, and provided filtered tap water ad libitum and standard rat food (purchased from Sai Durga Agencies, Bangalore, India). They were maintained in a well controlled environment (temperature
24 ± 2°C, 12-hour light and 12-hour dark cycle, humidity 55 ± 15%). The protocols and experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India (CPCSEA, 2003) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India.

One male and two female rats were housed in the same cage every evening, and sperm plug in the female rat vagina was examined next morning. The day when sperm plug was confirmed was designated as day of gestation. The pregnant rats were randomly divided and lactationally exposed to 0.2% Pb by adding Pb-acetate to deionized drinking water of the mother. All pups were pooled on postnatal day 1 (PND 1) and new litters consisting of eight males were randomly selected and placed with each dam. Pb-exposure was continued up to PND 21 and stopped at weaning. Control rats received only deionized water without Pb. A separate group was maintained with nutrient metal mixture supplementation (Ca$^{2+}$+Zn$^{2+}$+Fe$^{2+}$) as 0.02% in 0.2% Pb-water given to the mothers up to PND 21 and stopped at weaning.

Group I : Control (Received normal water)
Group II : 0.2% Pb
Group III : Metal mixture supplementation (Ca$^{2+}$+Zn$^{2+}$+Fe$^{2+}$) as 0.02% in 0.2% Pb.

The doses of Pb, essential metal supplementation were selected on the basis of previously published studies (Prasanthi et al., 2006). Behavioral studies were conducted in young age (PND 21, 45) adults (4 Months old) old age (12, 18 Months old) rats (Prasanthi et al., 2006, Flora et al., 2012). For biochemical studies, rats were scarified by cervical decapitation on PND21, PND 45 and at 4, 12, 18 Months age. After decapitation, the brain was removed onto ice-cold glass plate and different brain regions (cerebral cortex, hippocampus, and cerebellum) were quickly dissected and stored at -80°C until use.

Open-field behavior (crossings, rearings, sniffings, groomings), locomotor activity, exploratory behavior (No. of dipping, dipping duration) and hole board test were decreased in experimental animals as compared to control animals, the effect of Pb on these tasks was reduced in nutrient supplemented group animals. The activity was found
to be greater in PND 45, 4 Months rats as compared to young (PND 21) and aging (12 Months and 18 Months old) rats.

Pb interferes with the development and differentiation of the CNS, impairs cognitive processes by direct pharmacological interaction with specific sites that are important in neurotransmission. The impairment in the behavioral tasks is presumably due to the known disruptive effects Pb on the developing hippocampus.

In the present study, the specific activity of AChE was determined in the crude synaptosomal fractions of cerebral cortex, hippocampus and cerebellum of control, Pb-exposed and Ca\textsuperscript{2+}/Zn\textsuperscript{2+}/Fe\textsuperscript{2+} supplemented rats. The results showed that the specific activity of AChE activity was significantly inhibited by Pb-exposure. Among the three brain regions, the specific activity of AChE in crude synaptosomes was found to be high in hippocampus followed by cerebellum and cerebral cortex and the activity increased with age. However, supplementation with nutrient metal mixture (Ca\textsuperscript{2+}, Fe\textsuperscript{2+} and Zn\textsuperscript{2+}) reversed the Pb induced inhibition on AChE activity.

The affinity of Pb for the free sulphydryl groups in enzymes and proteins and its binding can alter their correct function. Pb disturbs the normal development of the brain, causing reductions in cellular development in the cerebellum, cerebral cortex and hippocampus. The alterations in AChE activity even after the Pb-exposure was withdrawn are due to the hippocampal damage and cholinergic deficiency. The decrease in the activity of AChE observed in this study indicates the reduction in cholinergic synapses in the Pb-exposed rat brain.

In our present study, exposure to Pb resulted in a significant decrease in the specific activity of MAO in the three brain regions of young rats (PND 21, PND 45) adult (4 months old) and aging (12 Months and 18 Months old) rats. Among the three different age points, the decrease in MAO activity was more pronounced in young rats (PND 21, PND 45) than the adult (4 months old) and aging (12 Months and 18 Months old) rats. In control rats, the MAO activity was increased from PND 21 to 4 months and then decreased in aging rats. The effect Pb is more in PND 45 as compared to PND 21, adult (4 months old) and aging (12 Months and 18 Months old) rats. Among the three brain regions, the activity level of MAO was found to be higher in cerebral cortex as compared to hippocampus and cerebellum in all the age groups.
The decrease in MAO activity observed in Pb-exposed brain regions may be due to the high affinity of Pb for sulfhydryl groups in enzymes which may be one of the reasons for increase in monoamines at low concentrations.

Pb-exposure alters the monoaminergic system during development of the central nervous system. Monoamine levels in various brain regions were reported to respond differently to the same dose of Pb. Pb, a potentially neurotoxic metal, produces central nervous system disorders by interfering with the metabolism of neurotransmitters and the synaptic transmission requiring Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions for transmitter carrier or synaptosomal membrane integrity. The susceptibilities of different brain areas to Pb-exposure could be related to local differences in their formation and maturation as well as the development of neurotransmitter systems. Pb, even at low concentrations has the ability to increase the basal release of the neurotransmitters from the presynaptic nerve endings which occur both in the peripheral nervous system and central nervous system. This may be the reason for the increased monoamine levels at low level exposure.

In the present study the monoamine (dopamine, epinephrine, nor epinephrine, and serotonin) levels increased in experimental animals as compared to control animals. These activity levels were reversed in nutrient supplemented animals. Among the three brain regions cerebral cortex documented higher levels of dopamine, nor epinephrine and serotonin, followed by hippocampus and cerebellum. These levels showed marginal increase in PND 21 to 4 months and then decrease in aging (12 Months and 18 Months old) rats. Higher levels of epinephrine documented in hippocampus followed by cerebral cortex and cerebellum. These levels showed marginal increase from in PND 21, PND 45, 4 months and 12 months old rats and then decreased in 18 Months old rats.

Decrease in MAO activity observed in the present study can be attributed to the simultaneous increase in monoamines in 0.2% Pb-exposed rats. The action of Pb on the metabolism of neurotransmitters can be attributed to its action on three general types of sub cellular processes such as interference with the mechanisms resulting Ca\(^{2+}\) distribution in nerve terminals, general anaerobic effects that may occur as a result of the impairment of energy production or the inhibition of enzymes, transferases etc., involved in the synthesis and storage of neurotransmitters resulted in the decrease of catecholamine levels at high dose of Pb-exposure. Decrease in neurotransmitters observed in this study could be related to decrease synthesis as Pb has been shown to
inhibit the activity of tyrosine hydroxylase. These effects indicate that the brain neurotransmitter studied may be responsible for the increased brain excitability and behavioral changes induced by Pb in animals and humans.

In terms of specific effects on the nervous system, Pb seems to play a significant role in the disruption of cholinergic and catecholaminergic processes. Although conflicting evidence exists for its exact mechanism, a general consensus among researchers indicates that Pb plays two key roles in the operation of the catecholaminergic and cholinergic systems by blocking the opening of voltage sensitive $\text{Ca}^{2+}$ channels and entering the neuronal intracellular environment.

Pb induces oxidative stress, mainly by disturbing the antioxidant defense of the body. Significant age dependent alterations with Pb-exposure were observed in all the selected enzymes of detoxification mechanism. Pb exposure altered the activity of antioxidant enzymes superoxide dismutase (SOD), xanthine oxidase (XOD), catalase (CAT), Glutathione (GSH), Glutathione peroxidase (GPx), Glutathione reductase (GR) and increased Glutathione S-transferase (GST), lipid peroxidation and protein oxidation. Among the different regions of brain, cerebral cortex seem to be more susceptible to Pb-toxicity following hippocampus and cerebellum. Supplementation with nutrient metal mixture ($\text{Ca}^{2+}$, $\text{Fe}^{2+}$ and $\text{Zn}^{2+}$) recovered the activity of all the above enzymes, lipid peroxidation and protein oxidation.

Oxidative damage associated with the presence of Pb in the brain indicates a possible role of free radicals in the pathogenesis of Pb-toxicity by disrupting the delicate prooxidant / antioxidant balance that exists within mammalian cells. Inhibition of 5-aminolevulinic acid (ALA) dehydratase by Pb leading to the accumulation of ALA, a potential endogenous source of free radicals, direct interaction of Pb with biological membranes, production of LP, increase of intracellular levels of $\text{Ca}^{2+}$ impairing mitochondrial fractions, decrease in free radical scavenging enzymes and glutathione induced by Pb may be some of the reasons for Pb-induced oxidative stress.

Pb is known to produce oxidative stress in animals manifested as an increase of LP in different regions of brain tissue. Inhibition of antioxidant enzymes and interactions with sulfhydryl groups of proteins may play an important role in Pb poisoning. Pb-induced decrease in free radical scavenging enzymes is mainly attributed to the high
affinity of Pb for sulfhydryl groups or metal co-factors in these enzymes and molecules. Pb binds to the –SH groups of CAT, SOD, XO leading to inhibition of these enzyme activities. CAT and SOD forms the first line of defense against ROS and the decrease in their activities contribute to the oxidative insult in the tissue. SOD requires Cu and Zn$^{2+}$ for its activity and was found to be decreased in Pb administered mice as Pb competes and replaces Cu and Zn$^{2+}$ in their binding sites. CAT possesses heme as the prosthetic group. The reduced absorption of Fe by Pb in the gastrointestinal tract and inhibition of heme biosynthesis may lead to a decrease in CAT activity. Pb has a very high affinity for thiol groups and therefore decreases SOD, XO and CAT activity levels. The decrease in the activity levels of enzymes which protect neuronal cells against oxidative stress may be responsible for increased levels of free-radical damage in rats brain, or that these enzymes themselves are susceptible to inactivation by free radical molecules which increase in brain. Impaired antioxidant defenses may be due to the inhibitory effects of Pb on various enzymes which make the cells vulnerable to oxidative attacks.

Pb-induced cytotoxicity increases oxidative stress, which is well marked by the enhanced LP and PO levels. The enhancement of LP observed as a result of Pb administration could be due to the formation of free radicals through an exhaustion of antioxidants leading to oxidative stress. The effect of Pb on LP is not a direct effect but these changes could rather be due to an indirect effect of Pb on free-radical scavenging enzymes as Pb does not undergo oxidation-reduction cycle.

In our results from AAS (atomic absorption spectroscopy) in experimental animals high levels of Pb concentration was documented in cerebral cortex followed by hippocampus and cerebellum. These results were found to be reversed when the animals were supplimented with nutrient metal mixture (Ca$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$). Higher Pb levels were observed in young rats (PND 21, PND 45) followed by adult (4 months old) and aging (12 Months and 18 Months old) rats.

Young rats were found to be more vulnerable to Pb than adult and aging rats due to underdeveloped BBB in young rats. Therefore, Pb crosses the BBB more easily and accumulates in the tissues resulting in higher impairment in behavioral performances. Therefore, greater decrese in the enzyme activity lends were recorded in PND 21 and PND 45 (AChE, SOD, XO, CAT, GSH and MAO). The gradual recovery of AChE, and antioxidant enzyme activities in four month rats can be attributed either to spontaneous
reactivation of the enzyme or the denovo synthesis of the enzyme molecule or both. Adult and aging rats may be less susceptible to Pb due to compensatory mechanisms that occur with age besides well developed BBB. The susceptibilities of different brain areas to Pb-exposure could be related to local differences in their formation and maturation. It is known that the differential sensitivity to Pb neurotoxicity in different brain regions is not due to a preferential Pb accumulation, but is possibly due to alteration of biochemical or cellular processes that are uniquely associated with, or greatly enhanced in a particular region.

There are a number of mechanisms through which Pb poisoning can disrupt neurological functioning. Many of the biological dysfunctions produced by Pb appear to be associated with the metal’s ability to mimic or inhibit the action of Ca\(^{2+}\). A number of calcium dependent processes are sensitive to Pb. Appropriate Ca\(^{2+}\) levels are essential to maintain and activate nerve cell function, but Pb causes derangements of Ca\(^{2+}\) flux in the extra and intra cellular spaces which induce central nervous system degeneration. Pb competes with Ca\(^{2+}\), inhibiting the release of neurotransmitters and interferes with the regulation of cell metabolism by binding to second messenger Ca\(^{2+}\) receptors, blocking Ca\(^{2+}\) transport by Ca\(^{2+}\) channels and Ca\(^{2+}\), Na\(^{+}\) pumps. It is believed that Pb competitively blocks the opening of voltage–sensitive Ca\(^{2+}\) channels. As a result Ca\(^{2+}\) cannot enter the cell causing the inhibition of presynaptic, evoked neurotransmitter release. Usually the secretion of neurotransmitters and neurohormones is triggered by a rise in intracellular Ca\(^{2+}\). Once, inside, Pb may act in a mimetic role and will activate the Ca\(^{2+}\) mediated synaptic vesicle release mechanisms. Pb may facilitate the activation of protein kinase II through use of Ca\(^{2+}\)-calmodulin pathway. The overall effect is an increase in spontaneous neurotransmitter release. Exposure to Pb has been reported to result in abnormal Ca\(^{2+}\) metabolism. Excessive Ca\(^{2+}\) influx may enter into the mitochondria, resulting in the production of free radicals. Ca\(^{2+}\) relieves the Pb-burden on the production of free radicals but to certain extent by reducing the absorption and accumulation of Pb in the gastrointestinal tract.

Potential mechanisms of interaction between Pb and Zn\(^{2+}\) include an inhibition of Pb gastrointestinal absorption by Zn\(^{2+}\). Indirect evidence includes the observation that supplemental Zn\(^{2+}\) decreased the concentrations of Pb in blood and in tissues in Pb-exposed rats. A protein’s precise shape is crucial to what it does, and even a subtle
disruption can affect its function. When Pb takes the place of Zn\(^{2+}\) the proteins shape, this is because Pb forms different chemical bonds than Zn\(^{2+}\) does. Zn\(^{2+}\) forms four, equally separated chemical bonds, while Pb forms three and these are at different angles. Pb also knocks out Zn\(^{2+}\) from a protein that helps form molecules of heme, which among other things, carries oxygen in the blood. Zn\(^{2+}\) displacement occurs even with relatively low levels of Pb in the body. Oral exposure to Pb and Zn\(^{2+}\) decreased peripheral nerve conduction and tissue distribution of metals. The simultaneous administration of Zn\(^{2+}\) reduced tissue accumulation of Pb and Pb-induced biochemical alterations. The protective effect of Zn\(^{2+}\) against Pb toxicity could be attributed to a decrease in metal absorption in the gastrointestinal tract. Zn\(^{2+}\) could be competing for and effectively reducing the availability of binding sites for Pb.

Iron deficiency is a common nutritional problem among the children at risk for Pb toxicity; it is now general practice to supplement the diets of these children with iron as well as calcium. The relationship between iron deficiency and the impaired cognitive and behavioral development seen in children with excess Pb exposure is complex in the iron deficiency in itself may impair early mental development. Iron deficiency affects regional monoamine metabolism, in part likely through iron-dependent enzymes such as tryptophan hydroxylase (for serotonin) and tyrosine hydroxylase (for dopamine and norepinephrine). Changes in monoamine regulation in various brain areas (eg, substantia nigra, lateral and reticular thalamic nuclei, and zona incerta) are directly proportional to the degree of iron deficiency.

Based on the observations of this study, an interrelation is proposed between the behavior, cholinergic system, monoamines, antioxidant enzymes, LP, PO and free radicals. The alterations caused by Pb-exposure on behaviorl responses (Open field behavior, Locomotor activity and Exploratory behavior and Hole board tasks) may be due to inhibitory effect of Pb on AChE activity and decrease in neurotransmitter (dopamine, norepinephrine and serotonin) levels. Decrease in AChE, antioxidant enzymes, MAO activities could be due to Pb-induced enhancement of LP, PO and free radicals as free radicals destroy –SH groups or may act as inhibitors of the enzymes. The decrease in neurotransmitters can be related to the inhibition of ATP synthesis as Pb interfere with cellular energy metabolism leading to dysfunction of energy dependent neuronal events such as neurotransmitter uptake. The generation of H\(_2\)O\(_2\) and free radical
production by oxidative stress could also be due to Pb inhibited dopamine release. Binding of Pb to –SH groups leading to inhibition of antioxidant enzymes could be implicated in oxidative stress and production of superoxide ion, hydrogen peroxide and hydroxyl radical. The decrease in the activity of antioxidant enzymes by oxidative stress of Pb leads to increase of LP and production of free radicals in an age dependent manner.

The supplemented nutrient metal mixture containing Ca$^{2+}$/Zn$^{2+}$/Fe$^{2+}$ were basically compete for similar binding sites as that of Pb, decreases Pb gastrointestinal absorption and tissue accumulation. Thus Ca$^{2+}$/Zn$^{2+}$/Fe$^{2+}$ replace Pb in the body and reduce the Pb-burden.

The findings of the present study thus showed age and brain region dependent neurochemical alterations, where young animals (PND 21, PND 45) were found to be more vulnerable to Pb and so also hippocampus, the principle regions for cognitive dysfunctions in children exposed to Pb. The behavioral alterations were also found to be maximum in young animals (PND 21, PND 45) compared to other age groups. The present study most importantly showed that combination therapy with an essential metal mixture (Ca$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$) containing as more efficient than their individual therapeutic effects.