Chapter 4

Gestational exposure to lead (Pb) acetate: low protein as a response modifier of neurotoxicity (Prenatal/Postnatal period)
Heavy metals including lead (Pb), arsenic (As) and cadmium (Cd) have been regarded as both environmental contaminants and potential neurotoxicological hazards (Brender et al., 2006; Fowler et al., 2004; Jadhav et al., 2007). Exposure to these metals in utero and in infancy is associated with risk of impaired cognitive development (Hu, 2000), subclinical brain dysfunction (Lanphear et al., 2005), and behavioral abnormalities (Tsai et al., 2003; Wright et al., 2006). Studies with single exposure to metal have demonstrated that Pb, As or Cd infiltrate the immature blood-brain barrier (BBB) and accumulate in developing brain (Lidsky and Schneider, 2003; Wang et al., 2007a; Xi et al., 2010). Pb uptake through the BBB is reported to disrupt calcium transport mechanism (Marchetti, 2003) and promotes activation of mitogen-activated protein (MAP) kinases in apoptotic glial cells (Posser et al., 2007). The sequestration of Pb at the level of the choroid plexus undermines brain growth and affects learning/memory and cognitive functions of developing CNS (Marchetti, 2003).

Pb is one of the first heavy metal poisons identified and the developing nervous system is particularly vulnerable to its toxic effects (Kasten-Jolly et al., 2012). Pb exposures during gestation and early postnatal have been found to cause neurotoxicity in children, since these Pb exposures (blood Pb concentrations <30 mg/dl) have lasting effects on neuronal function (Bellinger, 2000; Needleman, 2004; Chen et al., 2007; Surkan et al., 2007; Bellinger, 2008; Al-Saleh et al., 2008). Exposure to Pb during embryonic life causes changes in the expression of developmental genes that may last for a lifetime and adversely affect the exposed individual (Sanchez-Martin et al., 2013). Recent studies showed that gestational Pb exposure during early life impaired learning and memory in the offspring (PND 21 rats) as evidenced by decreased water maze performance. The expression of insulin-like growth factor 1 and 2 in the hippocampus of pups also decreased in Pb exposed group (Li et al., 2014).

Several evidence suggest that gestation through lactation is a sensitive period for exposure to low Pb levels leading to lifelong cognitive deficits (Basha
and Reddy, 2010; Ryzhavskii et al., 2008; Tang et al., 2009). Developmental Pb exposure causes behavioral and cognitive deficits in children and animals and reduces activity-dependent synaptic plasticity in the hippocampus of rodents (Gilbert et al., 2005; Nie et al., 2011). Pb-induced impairments in behavior are not confined to any particular sensitive period, as deficits have been described following gestational exposure, early postnatal exposure, post-weaning and adult exposures, and lifetime exposures (Schneider et al., 2012). Several studies have shown that Pb exposure during pre- and neonatal period at levels below ‘safe level’ (10µg/dl) in offspring blood caused disruption of pro/antioxidant balance in the developing rat brain. Impairments in executive functions, are one of the hallmark effects, even at low Pb levels in whole blood (Canfield et al., 2003), and suggest the involvement of glutamatergic, cholinergic and dopaminergic neurotransmission (Basha et al., 2012; Jones and Miller, 2008; Szczerbak et al., 2007). Perinatal Pb-exposure is known to affect several aspects of dopamine neurotransmission in a dose-related and brain region-specific manner (Cory-Slechta, 1995). The hippocampus area is particularly sensitive to postnatal Pb exposure since it induces astrogliosis and increases the expression of GFAP (Selvin-Testa et al., 1994). Pb is known to alter the expression of human fetal astrocyte genes and also the second messenger and transcription factors involved in the induction of VEGF genes (Hossain et al., 2000, LaBreche et al., 2011). The presence of Pb also affects expression levels of numerous proteins that are involved in inflammation or apoptosis. Pb was found to increase GFAP levels as well as inflammatory protein levels such as cytokines (interleukine-1β and 6) and tumor necrosis factor-alpha (TNF-α) (Struzynska et al., 2007) and expression of GFAP (Kasten-Jolly et al., 2011).

Studies have described the interrelationship of socioeconomic status and Pb exposure and SES plays a key role in modifying the risk that Pb poses to children (Bellinger, 2008). However, despite appreciable worldwide improvement in life expectancy, adult literacy, and nutritional status, an estimated 780million people in low-income countries lack sufficient food. Children exposed to prenatal or early postnatal MN are known to exhibit cognitive deficits and enhanced risk of developing psychiatric disorders, and schizophrenia. Deficits in learning and
memory, enhanced responses to stress and altered sensitivity to psychotropic 
drugs have been documented in adult animals exposed to prenatal or neonatal 
MN. Perinatally undernourished rats showed decreased number of hippocampal 
neurons and are due to the deleterious effects of early nutrient restriction on cell 
proliferation (Matos et al., 2011). The way in which Pb exposures during different 
developmental periods and nutritional status affect the brain development are 
not well understood and complex interactions between Pb exposure, brain 
maturation, and early-life experience will influence multiple biological and 
behavioral indices.

Our understanding on the neurotoxic implications of Pb exposure during 
gestation under protein deficiency conditions is rather limited. Since 
undernourishment as well as protein deficiency (low protein diets) have specific 
effects on brain development during the critical stages (both during gestation and 
early postnatal period), this study aimed to assess the role of low protein diet as 
a response modifier of Pb-induced behavioral alterations, oxidative impairments, 
mitochondrial dysfunctions and neurotoxic implications. In the first study, 
implications of Pb exposure during gestation were studied in maternal brain 
regions and fetal brain and the results are presented in Section A. In a satellite 
study, implications of gestational and lactational Pb exposure were assessed in 
PND 21 day rats and the results are presented in Section B.

2.0 OBJECTIVE

The primary objective of this study was to assess the extent to which low protein 
diet consumption during gestation would render the dams susceptible to Pb-
mediated oxidative impairments and neurotoxicity. Further, neurotoxic 
implications following lactational Pb exposure on oxidative dysfunctions in brain 
of rats (PND 21) were also examined.
3.0 EXPERIMENTAL DESIGN

SECTION -A

3.1 Low Protein diet as a response modifier of Pb-induced neurotoxic implications following gestational exposure (GD-1 to GD-19)

3.1.1 Relevance of Pb exposure during gestation
The present study was designed to understand the effect of gestational Pb exposure on oxidative stress and mitochondrial dysfunctions in both maternal and fetal brain. GD16 to GD-19 of gestation is a period of key brain structure development, including hypothalamic nuclei, hippocampus, striatum and frontal cortex. Gestation through lactation is a sensitive period when exposure to low levels of Pb causes long lasting cognitive deficits in offspring.

3.1.2 Preparation of lead (Pb) acetate (PbA)/ Dosage selection
The criterion of selection of lead (Pb) acetate (500ppm) was based on the preliminary studies. Pb acetate solution was prepared by dissolving Pb acetate trihydrate in deionized water (0.5mg/ml)

3.1.3 Preparation of Diet
Compositions of normoprotein (17%) and low protein diet (8.5%) have been presented in the materials and methods section.

3.1.4 Pregnant rats for the study
Details related to the mating schedule and timing of pregnancy is described in Materials and Methods section.

3.1.5 Gestational Pb exposure (GD-1 to GD-19)
The sperm-positive females (GD-0) were individually housed, and were designated into 4 groups (n=6) as follows:

Group I: Normoprotein diet control (NPD-17%)
Group II: Normoprotein diet + PbA 500ppm in drinking water from GD-1 to GD-19)
Chapter 4

Group III: Low protein diet Control (LPD-8.5%)
Group IV: Low protein diet + PbA 500ppm in drinking water from GD-1 to GD-19

During the experimental period, control dams received deionized drinking water and the treatment group received deionized drinking water with Pb starting from gestation day 1-19. Daily feed intake, body weight and water consumption were measured.

3.1.6 Behavioral assessment: motor activity

On GD-17, dams were subjected to open field locomotor phenotype assay (as described in Materials and Methods).

3.1.7 Biochemical studies

Terminally, pregnant dams (on GD-19) were sacrificed under mild anesthesia; whole blood was drawn by cardiac puncture in heparinized tubes. Fetal resorptions were recorded. Embryo and placenta were separated and weighed. Fetal brain was excised from fetuses. Maternal brain was excised and placed in 0.9% ice-cold NaCl solution and cleaned with the physiological saline solution to completely remove blood cells, blotted on filter paper.

3.1.8 Analysis of Pb markers in whole blood

Hemoglobin (Hb) levels, ALAD activity was determined. Blood Pb levels were measured as described in Materials and Methods.

3.1.9 Biochemical analysis in brain regions

The maternal brain was placed on ice pack and sub-dissected into cortex (Ct), cerebellum (Cb), hippocampus (Hc) and striatum. Cytosol and mitochondria were prepared from both maternal brain regions and whole fetal brain (as described in Materials and Methods) and the following biochemical markers were determined in brain regions.

Oxidative stress induction in maternal brain regions and fetal brain

Induction of oxidative damage: Markers of oxidative stress viz., ROS, HP level was estimated in the cytosolic fractions of brain regions. The degree of lipid peroxidation was determined as malondialdehyde (MDA) levels in all the brain
regions. Reduced GSH levels were quantified in all the brain regions. Extent of protein oxidation was also determined in all the brain regions.

**Antioxidant enzymes**: Activities of antioxidant enzymes/detoxifying enzymes *viz.*, SOD, GPx, and TRR were determined in the cytosolic fractions.

**Mitochondrial functions**: Mitochondrial functional enzymes, NADH-cyt C reductase (complex I-III), MTT reduction and SDH activity levels were measured.

**Cholinergic function/dopamine levels**: Cholinergic function was measured by AChE activity. Dopamine levels were measured in maternal striatum and fetal brain by HPLC method.

**Histopathological evaluation**: Histopathological examination of maternal hippocampus of control and Pb treated rats was conducted.

**Western blot analysis**: Protein expression of glial fibrillary acidic protein (GFAP) and vascular endothelial growth factor (VEGF) were studied in GD-19 fetal brain.

### SECTION -B

#### 3.2 Protein as a response modifier of lead (Pb) acetate induced neurotoxicity in rats: postnatal study

##### 3.2.1 Pb dosage/Diet preparation

For Pb dosage and diet preparation refer Section-A, 3.1

##### 3.2.2 Perinatal Pb exposure (PND 21 day study):

Animals and care (detailed in Materials and Methods section).

For experimental groups refer Section A, 3.1.3. Females of the experimental group (n=3) received from the first day of gestation 500ppm lead (Pb) acetate in drinking water; control group (NPD/LPD) pregnant females (n=3) received deionized drinking water until weaning of the offspring. Born pups (males) stayed with their mothers and were fed by them.

During feeding of pups, mothers of the experimental group were still receiving PbA in drinking water *ad libitum*. Pups were weaned in postnatal day 21 (PND 21). During the experimental period, feed intake, body weight and water intake of
maternal dams were recorded. Body weight of pups was recorded. At PND 21 day, pups were subjected to open filed motor activity and motor coordination test.

3.2.3 Behavioral assessment: Open field test and Rotarod test

At PND 21, offspring’s were subjected to open field locomotor phenotype assay in open field box (as described in materials and methods). At PND 21, offspring’s were subjected to motor coordination on rotarod (as described in materials and methods).

3.2.4 Biochemical studies

Terminally, dams and PND 21 pups were sacrificed under mild anesthesia; whole blood was drawn by cardiac puncture in heparinized tubes. Maternal and offspring brain was excised by decapitation and placed in 0.9% ice-cold NaCl solution and cleaned with the physiological saline solution to completely remove blood cells, blotted on filter paper.

3.2.5 Analysis of Pb markers in whole blood

Hemoglobin (Hb) levels, ALAD activity and blood Pb levels were measured in maternal and offspring whole blood as described in materials and methods.

3.2.6 Biochemical analysis in brain regions (PND 21)

Further, placing the PND 21 day pup brain on ice pack, brain was sub-dissected into cortex (Ct), cerebellum (Cb), hippocampus (Hc) and striatum. Cytosol and mitochondria were prepared from both maternal and whole fetal brain (as described in materials and methods) and the following biochemical markers were determined in brain regions.

Induction of oxidative damage (cytosol): Markers of oxidative stress viz., ROS, HP level was estimated in the cytosolic fractions of brain regions. The degree of lipid peroxidation was determined as malondialdehyde (MDA) levels in all the brain regions. Reduced GSH levels were quantified in all the brain regions. Nitrite levels were quantified by measuring NO levels. Extent of protein oxidation (protein carbonyl levels) was determined in all the brain regions.
Antioxidant enzymes: Activities were determined as described earlier.

Induction of oxidative damage (mitochondria): HP level was estimated in the cytosolic fractions of brain regions. Nitrite levels were quantified by measuring NO levels and the extent of protein oxidation (protein carbonyl levels) was determined in all the brain regions.

Mitochondrial functions: Were studied as described in 3.1.8.

Cholinergic function: Cholinergic function was measured by AChE activity.

Histopathological evaluation: Histopathological examination of PND 21 hippocampus (CA1, CA2, CA3, CA4 and DG regions) of control and Pb treated rats was conducted by hematoxylin and eosin staining.

4.0 RESULTS

SECTION A

4.1 Protein as a response modifier of Pb-induced neurotoxic implications following gestational exposure (GD-1 to GD-19)

4.1.1 Effect on gestational parameters

No significant change was evident in daily food and water intake among both NPD and LPD rats consuming Pb acetate (500ppm) in drinking water (Data not shown). Further, there was no significant effect on the body weight of dams that consumed PbA in drinking water (Table 4.1). However, Pb consumption in normo and low protein group of dams caused significant reduction in the number of implants. Further, Pb in LPD group also caused significant reduction in the placental (28%) and fetal body weight (28%) (Table 4.1).

4.1.2 Effect of Pb intoxication markers and open field activity

Pb consumption resulted in significant decrease in the hemoglobin levels among dams of both the dietary groups (Fig 4.1A). Dams fed LPD per se showed a marginal decrease in the ALAD activity. Pb consumption among both NPD and NPD dams resulted in significant reduction in the ALAD activity levels (Fig 4.2B).
The blood Pb concentrations determined among both dietary groups showed higher levels of Pb among LPD fed dams (NPD control: 2.7 ± 1.0µg/dl; vs NPD+Pb: 19.2 ± 1.4µg/dl; LPD control : 2.91 ± 0.3µg/dl vs , LPD+Pb: 23.8 ± 0.4µg/dl) (Fig 4.1C). Further, Pb consumption among both the dietary groups increased the ambulatory activity (Fig 4.1D).

**Status of oxidative impairments in maternal cortex and striatum**

**4.1.3 Modulation of Lipid peroxidation: Oxidative markers**

Significant decrease in the ROS levels in the Ct is evident among LPD dams compared to NPD. Further, Pb exposure in LPD dams significantly enhanced the ROS levels in both Ct and St (Fig 4.1A). Likewise, Pb exposure in LPD dams further increased the MDA levels in both Ct and St (Fig 4.2B). Further, Pb exposure in both the dietary groups significantly increased the protein carbonyl levels in Ct. However; the extent of increase in the protein carbonyl levels among LPD dams was relatively higher compared to the NPD group (Fig 4.2D).

**4.1.4 Perturbations in the activity of antioxidant enzymes**

Significant increases in the SOD levels in Ct were evident in LPD alone group. However, Pb administration in NPD group further increased the SOD levels in Ct and decreased the activity levels in St (Fig 4.3A). Further, significant decrease in the GPx activity levels was evident in LPD alone group. Likewise Pb caused significant decrease in the GPx activity levels in St (Fig 4.3B). However, in Pb exposed LPD dams, the activity levels were further increased (Fig 4.3B). Interestingly, among LPD dams, Pb elevated the TRR activity levels in Ct and St (Fig 4.3C).

**4.1.5 Activities of ETC enzymes - mitochondrial function**

Increased complex I-III activity was evident in LPD alone group, while Pb caused increased mitochondrial complex I-III activity levels in Ct (Fig 4.4A). However, Pb caused elevated levels of complex I-III activity in St in both the dietary groups (Fig 4.4A). Mitochondria of Pb exposed LPD rats exhibited a significant reduction in the formation of formazan on exposure to MTT in St compared to Pb exposed NPD group (Fig 4.4B). Interestingly, SDH activity levels in LPD alone
rats were increased in Ct. However, the levels were decreased upon Pb exposure in NPD groups, while the levels were further enhanced in Ct of LPD group (Fig 4.4C). Interestingly, the activity levels were further decreased in LPD group in St (Fig 4.4C).

**Status of oxidative impairments in maternal cerebellum/ hippocampus and fetal whole brain**

### 4.1.6 Effect on ROS, glutathione levels

Among NPD fed dams, Pb caused significant increase in ROS levels in Cb. However, the levels were decreased in Cb of Pb exposed LPD group (Fig 4.5A). Interestingly, ROS levels fetal brain was increased among Pb exposed LPD group (Fig 4.5A).

### 4.1.7 Effect on MDA levels and protein carbonyls

Pb consumption resulted in higher degree of increase in MDA levels in Cb of LPD dams (Fig 4.6A), compared to the NPD group (Fig 4.6A). On the other hand, Pb consumption increased the MDA levels in Hc among both dietary groups (Fig 4.6B). Likewise, Pb caused elevated MDA levels in fetal brain among LPD fed dams (Fig 4.6A). However, Pb exposure increased the protein carbonyl levels in fetal brain among both the dietary groups (Fig 4.6B).

### 4.1.8 Effect on antioxidant enzyme activities

Data on the effect of Pb exposure on antioxidant enzymes activities in maternal and fetal brain are presented in Fig 4.7 and Fig 4.8. Pb consumption in LPD dams increased the CAT activity in Cb compared to NPD dams (Fig 4.7A). However, among LPD group, Pb caused elevated levels of CAT activity in fetal brain (Fig 4.7A). Interestingly, significant increase in SOD levels was observed in Cb among dams of LPD group compared to NPD. However, Pb caused decreased SOD levels in Cb of LPD dams (Fig 4.7B). Likewise, Pb consumption in NPD dams resulted in decreased SOD activity, while the activity levels were further increased in LPD dams (Fig 4.7B). Interestingly, with Pb consumption caused marginally increase in SOD levels in fetal brain of NPD dams, while the levels were further increased in LPD dams (Fig 4.7B).
Further, Pb consumption caused an elevation in the GPx activity levels in Cb among both the dietary groups (Fig 4.7C). However, Pb decreased the activity levels in Hc among both the dietary groups (Fig 4.7C). While, GPx activity levels were increased in LPD alone dams compared to NPD in fetal brain, Pb caused increased levels in fetal brain of only NPD group (Fig 4.7C).

While Pb consumption in NPD dams caused an elevation in the activity levels of GR in Hc, decreased activity levels were evident in LPD group (Fig 4.8A). In contrast, Pb caused increased activity levels of GR in fetal brain in both the dietary groups (Fig 4.8A). Further, increased TRR activity was evident in Cb of LPD alone group. Pb caused increased TRR activity in NPD dams and activity levels were further increased in LPD group administered with Pb (Fig 4.8B).

Pb consumption resulted in increased TRR activity levels in Hc in both the dietary group (Fig 4.8B). Interestingly, Pb caused increased GST activity levels in Hc of NPD rats. However, the activity levels were further decreased in LPD dams (Fig 4.8C). Interestingly, Pb consumption further increased the GST activity levels in fetal brain of both the dietary groups (Fig 4.8C).

**4.1.9 Effect on mitochondrial function**

Pb caused significant increase in the complex I-III activity in the Cb among both the dietary groups (Fig 4.9A). Interestingly, the activity levels were further increased in Hc of LPD dams compared to Pb treated NPD dams (Fig 4.9A). Likewise, Pb consumption among LPD dams resulted in increased complex I-III activity (Fig 4.9A) and decreased MTT reduction in fetal brain (Fig 4.9B). Further, Pb caused increased SDH activity levels in Cb among LPD dams (Fig 4.9C). While, the SDH levels in Hc was decreased with Pb administration among both the dietary groups (Fig 4.9C), the levels were further increased in fetal brain among LPD dams (Fig 4.9C).

**4.1.10 Effect on cholinergic function and dopamine levels**

AChE activity levels in LPD alone group were increased compared to NPD control. While, Pb consumption increased the AChE activity in Hc of NPD dams, the activity levels were decreased in Hc of LPD dams (Fig 4.10A). However, Pb
consumption caused increase in the dopamine levels in St of both the dietary groups (Fig 4.10B).

4.1.11 Effect on Pb-induced histological alterations in the hippocampus

Histoarchitecture of cornu ammonis areas- CA1, CA2, CA3, CA4 and DG of the hippocampus of LPD control and Pb treated rats are presented in Fig 4.11 and 4.12. The hippocampi of the LPD control group showed a normal architecture and damaged cells were almost nonexistent in cornu ammonis and DG regions. However, the number of damaged neuronal cells among Pb treated group was markedly increased in CA3, CA4 and DG region (Fig 4.11). In contrast, the damaged cells in both NPD control dams and Pb treated dams were almost nonexistent in hippocampi regions (Fig 4.12).

4.1.12 Effect on the expression of VEGF and GFAP in fetal brain

Expression of glial fibrillary acidic protein (GFAP) in LPD alone group was decreased (by 1fold) compared to NPD control (Fig 4.13A). Interestingly, Pb consumption increased the GFAP expression (1fold) compared to LPD dams and further increased the expression (19%) compared to Pb administered NPD dams (Fig 4.13A). Likewise, the expression of vascular endothelial growth factor (VEGF) was increased (by 40%) in LPD alone group compared to NPD. However, Pb consumption in NPD group increased the expression levels (1fold) compared to NPD control (Fig 4.13B). Likewise, Pb further increased (39%) the expression in LPD dams compared to Pb administered NPD group (Fig 4.13B).

SECTION B

4.2 Differential effect of dietary Protein levels on Pb-induced neurotoxic implications in brain regions of PND 21 rats

4.2.1 Effect of Pb consumption on growth of PND 21 rats

There were no significant differences in body weight of dams during gestation and lactation (Data not shown). However, significant reduction (NPD -29%; LPD-19%) in the body weight of pups was observed at PND 0 (Fig 4.14A). Terminally, in PND 21 pups lactational Pb exposure further reduced the body
weight in both the dietary groups. Interestingly, in LPD group lactational Pb exposure further reduced (33%) the pup weight compared to Pb exposed NPD group (Fig 4.14A).

4.2.2 Effect on motor activity and motor coordination (PND 21)

Pups of LPD control group exhibited a significant decrease in the crossing activity compared to NPD controls. Pb intake among NPD pups caused a marked increase in the crossing (44%) performance (Fig 4.15A) suggesting hyperactivity phenotype. Lactational Pb exposure also caused a robust increase in the crossing (7 fold) activity among LPD pups (Fig 4.15A).

Further, control pups of LPD group showed reduced exploratory activity compared to NPD group (Fig 4.15B). Pb exposure caused a decrease in rearing (72%) activity. Further, pups born to LPD dams showed significant delay in motor development (18%) as assessed by rotarod analysis (Fig 4.15C). However, lactational Pb exposure in LPD pups, the rotarod performance was significantly different and the pups fell off the rod quicker than the LPD control pups (Fig 4.15C).

4.2.3 Pb markers in in blood of maternal and PND 21 rats

Pb exposure caused a significant decrease (18%) in the hemoglobin levels in both maternal and offspring blood (Fig 4.16A&B). Further, in maternal blood, Pb administration reduced the ALAD (50-54%) activity levels among both the dietary groups (Fig 4.16C). Likewise, in the offspring blood reduced ALAD (24%) activity was observed in LPD control group. However, lactational Pb exposure further reduced the activity levels in the offspring blood among both the dietary groups (Fig 4.16D).

There was a significant difference in Pb concentrations between maternal and offspring blood. Interestingly maternal LPD group exposed to Pb showed increased BLLs compared to Pb exposed NPD group (Fig 4.16E). Likewise lactational Pb exposure in LPD rats further increased the BLLs compared to NPD rats (Fig 4.16F).
Effect of Pb on biochemical parameters in PND 21 rat brain

4.2.4 Effect on oxidative stress markers in brain regions: cytosol

Data on the effect of lactational Pb exposure on the status of oxidative stress markers is presented in Fig 4.17 and 4.18. Pb exposure marginally increased the ROS levels in Ct in NPD groups, while the levels were further decreased in LPD group (Fig 4.17A). However, Pb exposure in LPD rats caused an elevation in ROS levels in St compared to NPD rats (Fig 4.17A). Interestingly, lactational Pb exposure further decreased the ROS levels in Cb and Hc in both the dietary groups, while the ROS levels were further increased with Pb exposure in St of LPD group (Fig 4.17C).

However, increased MDA levels were evident in St among LPD alone group compared to NPD controls (Fig 4.17E). Further, Pb exposure increased the MDA levels in Ct of LPD rats compared to NPD group (Fig 4.17C). Further, Pb exposure increased the MDA levels in Cb and Hc in both the dietary groups (Fig 4.17D). Likewise, Pb exposure in both the dietary groups marginally increased the HP levels in St (Fig 4.17E). However, increased HP levels were evident in Cb and Hc of LPD alone rats. Pb exposure increased the levels only in Hc of NPD group (Fig 4.17F).

Elevated GSH levels were evident in both Ct and St of LPD alone rats (Fig 4.18A). However, Pb exposure decreased the GSH levels in Ct and St of both the dietary groups. Interestingly, the levels were decreased in Pb exposed LPD group compared to Pb exposed NPD rats (Fig 4.18A). Likewise, Pb caused significant decrease in the GSH levels in Cb and Hc LPD group (Fig 4.18B).

Further, Pb caused an increase in the NO levels in NPD group, while the levels were further decreased in Pb exposed LPD group (Fig 4.18C). However, the NO levels were decreased in LPD alone group and Pb exposed NPD group in St (Fig 4.18C). Interestingly, protein carbonyl levels were increased in Pb exposed LPD group compared to NPD group in Ct, St and Hc (Fig 4.18F).
4.2.5 Effect on antioxidant enzyme activities

The effect of lactational Pb exposure on the status of antioxidant enzyme activities is presented in Fig 4.19 and 4.20. Increased SOD levels were evident in Ct and St of LPD alone rats compared to NPD group. Pb exposure in NPD rats increased the SOD levels, while the levels were marginally decreased among LPD rats (Fig 4.19A). Further, SOD levels were increased in St of Pb exposed LPD rats compared to Pb exposed NPD rats (Fig 4.19A). Interestingly, Pb caused increased SOD levels in NPD group, while the levels were further decreased in Cb and Hc of LPD group (Fig 4.19B).

Further, Pb caused decreased GPx activity levels in Ct of NPD group, while the levels were further increased by Pb in St of LPD rats (Fig 4.19C). However, Pb-induced increase in GPx levels were marginally decreased by Pb in Hc of LPD rats (Fig 4.19D). Interestingly, Pb-induced decrease in GR levels in St of NPD rats were further increased by Pb in LPD rats (Fig 4.19E). Further, Pb exposure increased the GR levels in Cb and Hc of both NPD and LPD rats (Fig 4.19F).

Low protein diet further enhanced the TRR activity levels in Ct (Fig 4.20A) and Cb (Fig 4.20B). Likewise, GST activity levels were further decreased in St (Fig 4.20C) and Hc (Fig 4.20D) of Pb exposed LPD rats compared to Pb exposed NPD rats (Fig 4.20F).

4.2.6 Effect on mitochondrial oxidative stress markers

HP levels were significantly diminished in Ct of LPD alone rats, while the levels were increased by Pb exposure (Fig 4.21A). Pb further increased the HP levels in St of both the dietary groups, while the levels were further increased with Pb exposure (Fig 4.21A). Pb caused increased HP levels in Cb of both the dietary groups (Fig 4.21B). However, decreased HP levels were evident in Hc of LPD alone rats. Further, Pb decreased the levels in NPD group, while the levels were further enhanced in Hc of LDP group (Fig 4.21B).

Likewise, Pb exposure further decreased the NO levels in Ct, St (Fig 4.21C) and Hc (Fig 4.21D) of LPD rats compared to NPD group. Pb exposure further increased the protein carbonyl levels in Ct of both the dietary groups (Fig
4.21E). However, decreased protein carbonyl levels in LPD alone group were further increased by Pb exposure in St (Fig 4.21E). Likewise, Pb in both the dietary groups increased the protein carbonyl levels in Cb (Fig 4.21F).

4.2.7 Effect on mitochondrial function

Increased complex I-III activity levels were observed in LPD alone group. However, Pb marginally decreased the complex I-III activity levels in St of NPD and LPD rats (Fig 4.22A). The activity levels were increased in Cb and Hc of LPD rats, while the levels were decreased by Pb exposure in both Cb and Hc (Fig 4.22B). Further, mitochondria of Pb exposed LPD rats exhibited significant reduction in the formation of formazan on exposure to MTT in Hc (Fig 4.22D).

4.2.8 Effect on cholinergic function

Decreased AChE activity levels were evident in St of LPD alone rats. Further, Pb caused decreased activity levels in St of NPD rats. However, Pb exposure in LPD rats further increased the AChE activity in St (Fig 4.23A). Further, a marginal decrease in AChE activity in Cb of LPD alone and Pb exposed NPD rats were evident (Fig 4.23B). Interestingly, AChE levels were further decreased by Pb exposure in Cb of LPD rats (Fig 4.23B).

4.2.9 Effect on Pb-induced histological alterations: (PND21 hippocampus)

The hippocampi of the NPD control group had a normal architecture and damaged cells were absent in cornu ammonis and DG regions. However, the number of damaged neuronal cells among Pb treated group was markedly increased in CA3 and DG region (Fig 4.26). Interestingly, the damaged cells in CA3, CA4 and DG region were significantly increased in LPD control rats. However, Pb exposure in LPD group further increased the number of damaged neuronal cells in CA3, CA4 and DG region of the hippocampus (Fig 4.27).
Fig 4.1

Effect of lead (Pb) acetate exposure during gestation (GD-1-19) on Hb, ALAD, blood lead level and open field activity among rats fed either on Normoprotein (NPD) or low protein (LPD) diets.

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey's test.

- a significant against 0 ppm (NPD control) at p<0.05
- b significant against 0 ppm (LPD control) at p<0.05
- c significant against 500 ppm (NPD+500ppm) at p<0.05

A-Hemoglobin levels; B-ALAD activity;
C-Blood Pb levels; D-Ambulatory activity (open field test)
### Table 4.1

**Effect of gestational Pb exposure on body weight, fetal weights and placental weights of dams fed on either normo (NPD) or low protein (LPD) diet**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoprotein (Pb acetate (ppm))</th>
<th>Low protein (Pb acetate (ppm))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>Body weight (g) on GD1</td>
<td>198.5 ± 8.4</td>
<td>199.0 ± 4.00</td>
</tr>
<tr>
<td>Body weight (g) on GD19</td>
<td>277.5 ± 5.5</td>
<td>257.5 ± 7.5</td>
</tr>
<tr>
<td>number of implants/litter</td>
<td>10.55 ± 0.8</td>
<td>6.79 ± 0.26</td>
</tr>
<tr>
<td>Placental weight/litter</td>
<td>0.31 ± 0.05</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Fetal weight/litter</td>
<td>0.78 ± 0.09</td>
<td>0.75 ± 0.03</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19.

Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- superscript a significant against 0 ppm (NPD control) at p<0.05
- superscript b significant against 0 ppm (LPD control) at p<0.05
- superscript c significant against 500 ppm (NPD+500ppm) at p<0.05
**Fig 4.2**

Effect of gestational lead (Pb) exposure on the status of antioxidant enzyme activities in brain regions (maternal) of rats on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19.

Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- **a** significant against 0 ppm (NPD control) at p<0.05
- **b** significant against 0 ppm (LPD control) at p<0.05
- **c** significant against 500 ppm (NPD+500ppm) at p<0.05

**A**-Reactive oxygen species; **B**-Malondialdehyde;
**C**-Hydroperoxides; **D**-Protein carbonyls
Fig 4.3

Effect of gestational Pb exposure on the status of antioxidant enzyme activities in brain regions (maternal) of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

a significant against 0 ppm (NPD control) at p<0.05
b significant against 0 ppm (LPD control) at p<0.05
c significant against 500 ppm (NPD+500ppm) at p<0.05

A- Superoxide dismutase; B- Glutathione peroxidase; C- Thioredoxin reductase
Fig 4.4

Effect of gestational Pb exposure on the mitochondrial function in maternal brain regions of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- significant against 0 ppm (NPD control) at p<0.05
- significant against 0 ppm (LPD control) at p<0.05
- significant against 500 ppm (NPD+500ppm) at p<0.05

A-Complex I-III MTT reduction; B- MTT reduction; C- Succinate dehydrogenase
Fig 4.5

Effect of gestational Pb exposure on oxidative stress markers in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- a significant against 0 ppm (NPD control) at p<0.05
- b significant against 0 ppm (LPD control) at p<0.05
- c significant against 500 ppm (NPD+500ppm) at p<0.05

A- Reactive oxygen species; B- Glutathione
Fig 4.6

Effect of gestational Pb exposure on oxidative stress markers in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

\[ a \] significant against 0 ppm (NPD control) at p<0.05
\[ b \] significant against 0 ppm (LPD control) at p<0.05
\[ c \] significant against 500 ppm (NPD+500ppm) at p<0.05

\textbf{A}-Malondialdehyde; \textbf{B}- Protein carbonyls
Fig 4.7

Effect of gestational Pb exposure on antioxidant enzyme activities in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19.

Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

\(^a\) significant against 0 ppm (NPD control) at p<0.05

\(^b\) significant against 0 ppm (LPD control) at p<0.05

\(^c\) significant against 500 ppm (NPD+500ppm) at p<0.05

A-Catalase; B-Superoxide dismutase; C-Glutathione peroxidase;
Fig 4.8

Effect of gestational Pb exposure on antioxidant enzyme activities in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

a significant against 0 ppm (NPD control) at p<0.05
b significant against 0 ppm (LPD control) at p<0.05
c significant against 500 ppm (NPD+500ppm) at p<0.05

A-Glutathione reductase B-Thioredoxin reductase; C-Glutathione-s-transferase
Fig 4.9

Effect of gestational Pb exposure on mitochondrial complex activities in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- Significant against 0 ppm (NPD control) at p<0.05
- Significant against 0 ppm (LPD control) at p<0.05
- Significant against 500 ppm (NPD+500ppm) at p<0.05

A-Complex I-III; B-MTT reduction; C-Succinate dehydrogenase
Fig 4.10

Effect of gestational Pb exposure on AChE and dopamine levels in maternal and fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one- way analysis of variance followed by post hoc Tukey’s test.

- a significant against 0 ppm (NPD control) at p<0.05
- b significant against 0 ppm (LPD control) at p<0.05
- c significant against 500 ppm (NPD+500ppm) at p<0.05

A- Acetylcholinesterase; B- Dopamine (maternal striatum); C- Dopamine (Fetal brain)
Fig 4.11

Effect of gestational lead (Pb) acetate exposure on maternal (GD-19) hippocampal histoarchitecture of rats fed on normo protein (LPD) diet
**Fig 4.12**

Effect of gestational lead (Pb) acetate exposure on maternal (GD-19) hippocampal histoarchitecture of rats fed on low protein (LPD) diet

<table>
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<tr>
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<tr>
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</tbody>
</table>
Fig 4.13

Effect of gestational lead (Pb) exposure on the expression of vascular endothelial growth factor and glial fibrillary acidic protein in GD19 fetal brain of rats fed on either normo (NPD) or low protein (LPD) diet

Values are mean ± SE (n=6/group).

Pb acetate was provided in drinking water during gestation days GD 1-19. Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

a significant against 0 ppm (NPD control) at p<0.05
b significant against 0 ppm (LPD control) at p<0.05
c significant against 500 ppm (NPD+500ppm) at p<0.05

A-Glial fibrillary acidic protein; B-Vascular endothelial growth factor
Fig 4.14

Effect of Pb acetate exposure on body weight gain of pups during postnatal period of 3 weeks

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

a significant against NPD control) at p<0.05
b significant against LPD control at p<0.05
c significant against NPD+500ppm at p<0.05
Fig 4.15

Effect of Pb acetate exposure on motor activity (open field) and motor coordination test (rotarod) measured among postnatal 21 d rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

a significant against NPD control at p<0.05
b significant against LPD control at p<0.05
c significant against NPD+500ppm at p<0.05
Fig 4.16

Effect of Pb acetate exposure on hemoglobin, ALAD and blood Pb levels in maternal and PND 21 rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

\( \text{a} \) significant against NPD control at p<0.05
\( \text{b} \) significant against LPD control at p<0.05
\( \text{c} \) significant against NPD+500ppm at p<0.05

A, B-Hemoglobin levels
C, D-ALAD activity
E, F-Blood Pb levels
Fig 4.17

Status of oxidative stress markers in brain regions of postnatal 21 d rats following perinatal Pb exposure

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days)
Data analysed by one- way analysis of variance followed by post hoc tukey test.

- a significant against NPD control at p<0.05
- b significant against LPD control at p<0.05
- c significant against NPD+500ppm at p<0.05

A, B- Reactive oxygen species; C, D- Malondialdehyde; E, F- Hydroperoxides
Fig 4.18

Effect of perinatal Pb exposure on glutathione levels, nitric oxide levels and protein carbonyls in brain regions of postnatal 21 d rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one- way analysis of variance followed by post hoc Tukey's test.

- **a** significant against NPD control at p<0.05
- **b** significant against LPD control at p<0.05
- **c** significant against NPD+500ppm at p<0.05

**A, B**-Glutathione; **C, D**- Nitric oxide; **E, F**-Protein carbonyls
Fig 4.19

Effect of perinatal Pb exposure on antioxidant enzyme activities in brain regions of postnatal 21 d rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one- way analysis of variance followed by post hoc Tukey’s test.

- a significant against NPD control) at p<0.05
- b significant against LPD control at p<0.05
- c significant against NPD+500ppm at p<0.05

A, B-Superoxide dismutase; C, D-Glutathione peroxidase; E, F-Glutathione reductase
Fig 4.20

Effect of perinatal Pb exposure on antioxidant enzyme activities in brain regions of postnatal 21 d rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21days).
Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- $^a$ significant against NPD control at p<0.05
- $^b$ significant against LPD control at p<0.05
- $^c$ significant against NPD+500ppm at p<0.05

A, B-Thioredoxin reductase; C, D-Gluthathione-s-transferase
Fig 4.21

Effect of perinatal Pb exposure on the oxidative stress markers in the mitochondrial brain regions of postnatal 21 d rats

Values are mean ± SE (n=6/group).

Pre and neonatal Pb exposure (PND 21 days)
Data analysed by one-way analysis of variance followed by post hoc Tukey’s test.

- **a** significant against NPD control) at p<0.05
- **b** significant against LPD control at p<0.05
- **c** significant against NPD+500ppm at p<0.05

**A, B**-Hydroperoxides; **C, D**-Nitric oxide; **E, F**-Protein carbonyls
**Fig 4.22**

Effect of perinatal Pb exposure on the mitochondrial complex I-III activity and MTT reduction in brain regions of postnatal 21 d rats

A. B-Complex I-III; C, D-MTT reduction

**Fig 4.23**

Effect of perinatal Pb exposure on acetylcholinesterase activity and in brain regions of postnatal 21 d rats

Values are mean ± SE (n=6/group).
Pre and neonatal Pb exposure (PND 21 days).
Data analysed by one- way analysis of variance followed by post hoc Tukey’s test.

- a significant against NPD control at p<0.05;
- b significant against LPD control at p<0.05
- c significant against NPD+500ppm at p<0.05.

A, B-Acetylcholinesterase
Fig 4.24

Effect of Pb acetate exposure (drinking water) In PND 21 rats hippocampal histoarchitecture (H&E stain)
Fig 4.25

Effect of Pb acetate exposure (drinking water) in PND 21 rats hippocampal histoarchitecture (H&E stain)
5.0 DISCUSSION

Gestational exposure to Pb and Low protein as response modifier

Neurodevelopment begins in utero and continues throughout the perinatal period and this period of rapid development is highly vulnerable to Pb toxicity. Hence, in the present study, Pb exposure included both gestation and lactation since these periods represent critical windows for Pb neurotoxicity. In the gestational model, Pb intoxication caused significant reduction in the number of implants among both the dietary groups. However, Pb intoxication in LP group resulted in significant reduction in the placental and fetal weight. Dietary protein is an important source of essentials amino acids (e.g. cysteine and methionine), which can serve as intracellular antioxidants and decrease in dietary protein content could potentially lead to oxidative stress (Wang et al., 2002). Evidences suggest that chronic consumption of diet deficient in protein has adverse effect on the CNS leading to morphological, neurochemical, and behavioral alterations (Camargo et al., 2008; Fukuda et al., 2002). In animal models, prenatal LP malnutrition increased oxidative damage to lipids and proteins in cortex, hippocampus and cerebellum and the consequent decrease in essential amino acids may alter the antioxidant system and the redox state in the hippocampus (Morgane et al., 2002; Bonatto et al., 2005; Feoli et al., 2006; Tatli et al., 2007).

Findings from this study are consistent with previous reports wherein malnutrition has been demonstrated to alter the activity of enzymes and interfere with protein synthesis and protein structure as with the incorporation of lipids into various brain structures. Omission of a single essential amino acid from the maternal diet can have deleterious effects on the fetal brain development (Alamy and Bengelloun, 2012). Essential amino acids are a major component of the dietary protein and can serve as an intracellular antioxidant. Therefore, its restriction may lead to an increase in oxidative damage by diminishing antioxidant defences of tissue (Bonatto et al., 2005). It is also well established that oxidative stress induction is one of the major mechanisms involved in Pb-induced neurotoxicity in animal system and occupationally exposed workers (Adonaylo and Oteiza, 1999; Pande and Flora, 2002). In the present model, significant increase in ROS and lipid peroxidation levels were evident in maternal
Ct and St of low protein group exposed to Pb. Pb administration further increased the lipid peroxidation (Cb) and protein carbonyl levels (Ct) in low protein group. Also, Pb increased the ROS, MDA and protein carbonyl levels in fetal brain of low protein group compared to Pb administered normoprotein group. Evidence suggests that earlier the dietary insult, the more severe and permanent are its effects (Morgane et al., 1992). It is pertinent to note that the gestational period comprises most of the process of neurogenesis, whereas in the postnatal period myelinization, dendritic proliferation and synaptogenesis take place (Alamy and Bengelloun, 2012).

In the present study, in LP alone group, elevated SOD activity levels in Ct/Cb and diminished GPx levels in Ct were evidenced in the maternal milieu compared to NP group. These findings are consistent with recent observations of elevated activities of CAT, SOD, GST and reduced GR activity levels in both cortex and cerebellum of rats subjected to protein malnutrition (Adebayo et al., 2013). Interestingly, Pb exposure is reported to alter antioxidant activities by inhibiting functional SH groups in several enzymes (such as ALAD, SOD, CAT, GPx, and glucose-6-phosphate dehydrogenase (G6PD) (Hsu and Guo, 2002). GPx, CAT, and SOD are potential targets for Pb toxicity since these antioxidant enzymes depend on various essential trace elements for proper molecular structure and activity. Since Pb-associated reduction in selenium uptake may increase the susceptibility of the cell to oxidative stress, an antagonistic effect between selenium and Pb was found to affect GPx activity that requires selenium as a cofactor (Hsu and Guo, 2002). In the present study, Pb intoxication in LPD group further increased the CAT activity in maternal Cb/ Fb, while SOD levels were further elevated in Fb. Likewise, Pb intoxication further increased the GPx activity and decreased the SOD levels in maternal St of LPD group. GPx, a multifunctional selenoprotein, plays a role not only in antioxidative defense, but also in expression regulation of redox sensitive genes (Brigelius-Flohe and Flohe, 2003), apoptosis (Imai and Nakagawa, 2003) and embryogenesis (Bosiacka et al., 2012). Previous studies in rats have shown that, Pb-administration significantly decreased the hepatic Cu/Zn-SOD and GPx activity levels (Liu et al., 2010).
Thioredoxin reductase reduces oxidized thioredoxin (Trx), the electron donor for the ribonucleotide reductase-catalyzed conversion of ribonucleotides to deoxy-ribonucleotides. This enzyme plays a crucial role in protection against oxidative stress by removing hydrogen peroxide and organic hydroperoxides. In the present model, Pb intoxication in LP group further increased the TRR activity in Ct and St of NP group. Pb in both the dietary group increased the GR and TRR levels in fetal brain.

Pb is absorbed through the placenta during pregnancy and passes into milk during lactation period, reaching the developing brain where it preferentially impacts the functionality and morphology of the hippocampus (Zhang et al., 2004, 2009). In previous in vitro experiments, mitochondria and cytoplasm isolated from cerebellar granule cells from rats subjected to pre/neonatal Pb exposure showed decreased membrane potential and an increase in ROS (Bosiacka et al., 2011). Mitochondria play a critical role in redox equilibrium and are directly related to the oxidative stress state in cells. In the present study, in the maternal milieu, LP diet increased the complex I-III activity and decreased SDH activity in St compared to NP group. Pb further increased the complex I-III in Hc and St and decreased the MTT reduction in LPD dams. In the fetal brain, Pb intoxication in LP group increased the complex I-III, SDH activity and decreased the MTT reduction. These findings corroborate the previous data described under Pb-induced neurotoxicity in animals (Wang et al., 2010).

Both VEGF and TGF-β pathways known to be important for neural stem cell proliferation and differentiation during ischemia (Sun et al., 2010) and developmental Pb-exposure causes significant perturbations (Bouton et al., 2001; Barbeito et al., 2010; Kasten-Jolly et al., 2011). In the present study, expression of VEGF increased in GD-19 fetal brain of LP group and Pb intoxication further increased the expression of VEGF. This is consistent with earlier studies where, Pb altered the expression of human fetal astrocyte genes and defines the second messenger and transcription factors involved in the induction of one of these genes, VEGF/VPF (Hossain et al., 2000). VEGF is a dimeric secretory protein containing an amino-terminus secretory sequence (Tischer et al., 1991; Leung et al., 1989) and exerts its action via high-affinity
binding to phosphotyrosine kinase receptors Flt-1 and Flk-1 (De Vries et al., 1992; Quinn et al., 1993). Compelling evidence indicates that VEGF is a fundamental regulator of physiological and pathological angiogenesis (Ferrera and Henzel, 1989). Overexpression of VEGF can contribute to progression of several disorders such as intracerebral hemorrhage (Cheng et al., 1997), development of brain edema (Bates and Curry, 1996) and disruption of blood-brain barrier, pathological processes seen in acute Pb toxicity. In addition neurons have been found to express high-affinity VEGF receptors and thereby might be influenced directly to VEGF disregulation.

Glial fibrillary acidic protein is an intermediate filament (IF) protein that is expressed by numerous cell types of the CNS including astrocytes, and ependymal cells. Astrocytes are the major glial cell population within the CNS which play important physiological roles in brain functions. Astrocytes react to various neurodegenerative insults rapidly, leading to vigorous astrogliosis (Reier, 1986; Eng et al., 1992). The hippocampus area is particularly sensitive to postnatal Pb exposure since it induces astrogliosis (Selvin-Testa et al., 1994, 1997), increases the expression of the astrocyte marker glial-fibrillary acidic protein (GFAP) (Selvin-Testa et al., 1994). Hence activation of astrocytes has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer’s disease, inflammatory demyelinating diseases, acute traumatic brain injury etc. (Eng et al., 1994). Although activated astrocytes secrete different neurotrophic factors for neuronal survival, it is believed that rapid and severe activation augments/initiates an inflammatory response, leading to neuronal death and brain injury (Tani et al., 1996). After severe activation, astrocytes secrete various neurotoxic substances and express an enhanced level of GFAP, which is considered a marker protein for astrogliosis (Eng et al., 1994). A large body of experimental evidence suggests that astrocytes have a greater metabolic plasticity than neurons (Belanger et al., 2011). In the present study, expression of GFAP in fetal brain of LP alone group was decreased (by 1 fold) compared to NP group. Interestingly, Pb intoxication increased the GFAP expression (by 1 fold) in fetal brain of LP group, which corroborates with recent microarray results which showed that Pb exposure
significantly increased expression of GFAP (Kasten-Jolly et al., 2011). Previously Pb exposure was shown to cause increased GFAP levels and inflammatory protein levels interleukine-1β and 6 and TNF-α (Struzynska et al., 2007).

**Lactational exposure to Pb: Implications of LP diet among PND 21 rats**

It is well known that a hypoproteic diet leads to low body weight and brain weight (Del Angel-Meza et al., 2001). The wide range of defects induced by protein deficiency on the brain includes reduction in brain weight and size, neuronal differentiation, synaptic potentiation, nerve myelination, neurotransmitter production and decreased velocity of impulse conduction (Ranade et al., 2012). PMN especially during lactation causes significant disturbances in the CNS, with lower nucleic acid levels, as well as alterations in neurotransmitter levels (Adebayo et al., 2013). Previously, the effect of PMN on neurotransmitters related to learning, motor co-ordination, stress and other behavioral changes have been reported (Soares et al., 2008). In the present study, Pb intoxication during gestational period resulted in decreased body weight in pups among both NP and LP groups. Lactational exposure to Pb reduced the body weight in both the dietary groups among PND 21 rats. It is speculated that PMN leading to the decrease rate of growth and loss of body weight may be due to the increase in the generation of ROS as well as excessive breakdown of tissue proteins (Adebayo et al., 2013).

Pb poisoning is shown to intensely affect cerebral cortex/basal ganglia, brain areas involved in motor control (Moreira et al., 2001; Prasanthi et al., 2010; Ramesh et al., 2001) and locomotor activity in open field is often used to assess Pb-induced behavioral phenotype. In the present study, lactational Pb exposure increased the ambulatory/crossing activity of PND 21 rats of both the dietary group in open field box. Interestingly, LP diet further enhanced the hyperactivity response which corroborated our earlier findings in PP rats exposed to Pb in drinking water (Kumar and Muralidhara, 2014). Although protein deficiency affects the cerebrum, brainstem and spinal cord, brain regions such as the cerebellum seem to be more affected than others due to malnutrition during the developmental stage (Ranade et al., 2011). In the present study, pups born to protein deficient mothers showed significant delay in motor development (as
assessed by rotarod analysis). Interestingly, pups of LP group, the rotarod performance were significantly affected and the pups fell off the rod quicker than the LP control pups. This is consistent with observations where in consumption of LP diet by the mother caused significant motor deficits in the offspring which correlated well with cerebellar pathology (Ranade et al., 2011).

Pb-induced inhibition of d-ALAD accounts for the accumulation of its substrate delta-aminolevulinic acid (d-ALA) that can be rapidly oxidized to generate free radicals (Liu et al., 2011). In the present study, lactational Pb exposure in LP group of both maternal and offspring blood caused significant decrease in the hemoglobin levels and ALAD activity. Also, Pb exposure in LP group further increased the blood Pb levels in both maternal and offspring milieu. The zinc requiring enzyme δ-aminolevulinic acid dehydratase catalyses the second step in the heme biosynthesis pathway, forming the prophobilinogen by condensation of two molecules of δ-aminolevulinate. It is the inhibition of δ-aminolevulinic acid dehydratase that is presumed to account for the anemia of lead poisoning.

Pb exposure has recently been shown to disturb the aminergic system in the cerebral cortex, cerebellum, and hippocampus and to contribute to cognitive and behavioral impairments in rats (Devi et al., 2005). Pb exposure during early life can alter granule cell neurogenesis and morphology in the hippocampus of young adult rats, which provides a cellular basis for the deficits in synaptic plasticity and learning documented in Pb-exposed animals (Verina et al., 2007). Exposure to Pb during early development persistently inhibited neurogenesis and altered the pattern of differentiation of new cells in the dentate gyrus of rat hippocampus, which could, at least partly, contribute to behavioral and cognitive impairments observed in adulthood (Jaako-Movits et al., 2005). Other studies have also shown weaker nerve growth factor gene expression in the hippocampus of adult rats exposed to Pb after weaning or during lifetime (Cory-Slechta., 2010).

In the present study, lactational Pb intoxication in LP group (PND 21 rat) caused relatively higher ROS levels in Cb, Hc and St compared to the NP group suggesting the enhanced oxidative stress induction in vivo. Further in LP group
of PND 21 rat pups, GSH levels were depleted in Ct and St, while lipid peroxidation was increased in St and hydroperoxide levels were increased in Cb and Hc. This is consistent with previous reports of increased lipid peroxidation and decreased GSH levels in cortex/ cerebellum in weanling rats maintained on 5% casein for 13 weeks (Adebayo et al., 2013). However, in the present study lactational Pb exposure in LP group (PND 21 rats) further increased the lipid peroxidation (Ct, Cb and Hc), hydroperoxides (Hc), while GSH levels decreased (Ct/St) compared to normoprotein group. It has been shown that Pb causes induction of oxidative stress markers and depletion of cellular glutathione content, associated with nuclear κB and AP-1 activation (Korashy and El-Kadi, 2008). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent GR. In addition, GR catalyzes the GSH-dependent reduction of H$_2$O$_2$ and other peroxides. Previous studies reported that oxidative damage occurs following PM resulting in brain dysfunction and we found increased MDA levels in both cortex and cerebellum of PM rats confirming the involvement of free radicals in CNS pathology (Tatli et al., 2007).

In the present model, lactational Pb intoxication caused differential effects and significant perturbations in enzymic antioxidants in brain regions. This is consistent with previous reports of wherein both prenatal and postnatal treatment of Pb (50-500 ppm) was shown to cause significant perturbations in antioxidant enzyme activities (SOD, GPx and GR) in hippocampus, cerebral hemispheres and cerebellum of rat at PND 7, 14, 21 days (Babu et al., 2007). Enhanced GR and GPx levels and diminished levels of ubiquinol have also been demonstrated in Pb intoxicated animals which were speculated to reflect a protective response to an increased oxidative damage associated to Pb neurotoxicity (Adonaylo and Oteiza, 1999).

In the present study, NO levels were decreased in St of LP group compared to NP group. Further, lactational Pb exposure decreased the NO levels in cytosol and mitochondria of Ct/St and increased the protein carbonyl levels in Ct, St and Hc. NO plays an important role in the function of brain processes that involve synaptogenesis, cerebral blood flow, neuroendocrine secretion, and neuro- transmission (Estrada and Murillo-Carretero, 2005). NO
has been shown to play an important role in cell signaling, neurotransmission, cell protection, and regulatory effects in various cells (Nava-Ruiz et al., 2011; Ahamed and Siddiqui, 2007). Thus, the interference with NO production might be one of the subcellular mechanisms explaining neurotoxicity mediated by environmental toxins. In the case of Pb its chemical similarity with Ca\(^{2+}\), is an important factor to consider for interaction with NO dependent processes (Liu et al., 2013). Several workers have demonstrated that Pb exposure could decrease NO production (Kim et al., 2011). Several studies have reported a decrease in NOS activity and NO production in animals exposed to Pb (Nava-Ruiz et al., 2011; Liu et al., 2012; Liu et al., 2013). Pb also affects processes involved in NOS expression. It is possible that the Pb cation negatively acts on Ca\(^{2+}\)-dependent transcription elements for nNOS (the cyclic-AMP-response element-binding protein, CREB; cyclic adenosine monophosphate (cAMP) and eNOS) to decrease protein expression (Nava-Ruiz et al., 2011).

Evidence suggests that cholinergic system plays important role in the modulation of synaptic plasticity (Segal and Auerbach, 1997; Shinoe et al., 2005). Pb competes with calcium (Ca\(^{2+}\)) and this may account for its disruption of cholinergic functioning and alterations in other transmitter systems. Pb-exposure mainly affects cholinergic system by reducing acetylcholine (ACh) release and turnover rates. Earlier Pb-exposure has been shown to exert a direct effect on AChE activity in the developing cerebellum leading to alterations in motor coordination (Reddy et al., 2003; 2007). In the present study, decreased AChE levels were observed in Cb and St of LP group. However, lactational Pb exposure further increased the AChE levels in St and decreased the levels in Cb. This is consistent with the earlier studies where, increased AChE levels were evident in cortex and cerebellum in young rats (1month) exposed to Pb during lactation (Reddy et al., 2007).

Vulnerability of hippocampus to malnutrition is well documented (Cardoso et al., 2013). Pb exposure during early life is known to alter granule cell neurogenesis and morphology in the hippocampus of young adult rats, which provides a cellular basis for the deficits in synaptic plasticity and learning (Verina et al., 2007). Psychological and nutritional stresses during gestation may be
involved in fetal programming as shown in several experimental models (Mesquita et al., 2010a, b). Several studies have demonstrated that nutrient deficiency during gestation and/or the first days of postnatal development results in long-lasting structural abnormalities in the hippocampus of the offspring (Andrade et al., 1991; Diaz-Cintra et al., 1991). In the present study, Pb intoxication in the LP group increased the damaged neuronal cells (CA1, CA3 and DG region) in maternal hippocampus. In hippocampus of PND 21 rats, the damaged neuronal cells were increased in LP alone group and Pb exposure further increased the incidence of damaged cells. This is consistent with the earlier studies, which showed that chronic protein deprivation induces a significant increase of the number of PV-IR neurons in the granule cell layer and in the hilus of the dentate gyrus (Cardoso et al., 2013). Previous studies have also shown that hippocampi of pups (PND21) exposed to Pb showed degenerating neurons which were markedly increased in cornu ammonis CA1, CA3, and dentate gyrus (Chang et al., 2012).

Taken together, these data clearly suggest that Pb intoxication during gestational stages in rats maintained on low protein diet (LPD) appears to cause higher degree of oxidative impairments in the maternal brain regions and fetal brain. The increased susceptibility may be largely due to the preexisting compromised antioxidant defenses caused by chronic protein deficient diet which appears to be very vital during the gestational period. Further, a similar trend of enhanced oxidative stress and toxicity was also discernible in the lactational Pb exposure model as evidence in the brain region of PND 21 d rats clearly suggesting the role of low protein diet in enhancing the Pb mediated neurotoxic response.
6.0 SUMMARY

1. Pb caused significant reduction in the number of implants in both the dietary groups, while significant reduction in the placental/fetal weight was evident among LP group; Although Pb intoxication resulted in decreased hemoglobin levels among dietary groups, blood Pb levels were increased in LP group.

2. Significant increase in ROS and TBARS levels were evident in maternal Ct and St of LP in Cb and protein carbonyl levels (Ct) in LP group, while fetal brain showed increased ROS, MDA and protein carbonyl levels.

3. Low protein diet alone increased SOD activity levels in Ct and Cb and decreased GPx levels in Ct in maternal milieu compared to NP group.

4. Pb intoxication in LPD dams caused significant perturbations in the activity levels of antioxidant enzymes in maternal Cb region and fetal brain. SOD levels were further increased by Pb in fetal brain of LP group.

5. Pb intoxication further increased the GPx activity levels and decreased the SOD levels in maternal St of LP group. Low protein further increased the GPx activity in St compared to Pb administered normoprotein group.

6. Pb intoxication in LP group further increased the TRR activity in Ct and St of NP group. Pb in both the dietary group increased the GR and TRR levels in FB. Pb administration in LPD dams further decreased the GST activity in Hc, and increased the activity levels in FB.

7. In the maternal milieu, LP diet alone increased the complex I-III activity and decreased SDH activity in St compared to NP group; Pb significantly affected the mitochondrial function in LPD dams as evident by increased complex I-III (Hc/ St) and decreased MTT reduction. In the FB, enhanced activity of complex I-III/ SDH activity were accompanied with decreased MTT reduction.

8. Expression of VEGF increased in GD-19 fetal brain of LP group, while Pb intoxication further increased the expression. Expression of GFAP in fetal brain of LP alone group was decreased by 1 fold compared to NP group and Pb intoxication increased the GFAP expression by 1 fold in FB of LP group.
9. Lactational Pb intoxication increased the ambulatory/crossing activity of PND 21 rats of both the dietary group in open field box. Interestingly, low protein further increased the hyperactivity response.

10. Pups born to mothers of LP group showed significant delay in motor development as assessed by rotarod and lactational Pb exposure caused higher degree of motor deficits.

11. Lactational Pb intoxication in LP group of both maternal and offspring blood caused significant decrease in the hemoglobin levels and ALAD activity. Pb intoxication in LP group further increased the blood Pb levels in both maternal and offspring milieu.

12. Lactational Pb exposure in LP group (PND 21 rat) further increased the ROS levels in Cb, Hc and St compared to NP group. Elevated lipid peroxidation (Ct, Cb and Hc), hydroperoxides (Hc) and decrease GSH levels (Ct/St) were evident in LP group compared to NP group.

13. In LP group of PND 21 rat pups, GSH levels were diminished in Ct and St, while LPO was increased in St and HP levels were increased in Cb/ Hc.

14. NO levels were decreased in St of LP group compared to NP group. However, lactational Pb exposure further decreased the NO levels in cytosol and mitochondrial regions of Ct and St. Pb exposure further increased the protein carbonyl levels in Ct, St and Hc.

15. Decreased AChE levels were observed in Cb and St of LP group. However, lactational Pb exposure further increased the AChE levels in St and decreased the levels in Cb.

16. Mitochondrial complex I-III activity levels were increased in Hc of low protein fed PND 21 rat brain compared to normo protein group. However, lactational Pb exposure decreased the complex I-III activity levels (Cb/Hc) and MTT (Hc) reduction.

17. In PND 21 rat hippocampus, the damaged neuronal cells in CA3, CA4 and DG were increased in low protein group. Interestingly, lactational Pb exposure further increased the damaged cells in CA3, CA4 and DG.