CHAPTER - II

Developing Blood-Brain Barrier: A Vulnerable Target Following Neonatal Exposure to Certain Xenobiotics
A. Functional Impairment of Blood-Brain Barrier Following Exposure to Selected Pesticides
I. **INTRODUCTION:**

Development of the unique properties of brain microvasculature is a consequence of tissue-specific interaction between endothelial cells of extraneural origin and the developing brain cells (Farrell and Risau, 1994). Since the development of BBB starts during late gestation and continues up to early postnatal life, an exposure to environmental toxicants, drugs, pathogens, etc. during this critical period may interfere with the normal development and maturation of this barrier. Furthermore, the functional integrity of the BBB is disturbed during certain diseased and clinical conditions also (Knudsen, 1994).

Pesticides are the most important environmental toxicants, widely used for the control of agricultural pests and for household purposes. These are potentially hazardous to non-targeted organisms (Kyvic and Bente, 1995) and produce a variety of biochemical alterations (Srinivas et al., 1993; Wu and Casida, 1995) leading to pathological disorders including neurological dysfunctions (Cavanagh, 1973; Kim, 1975; Srinivas, 1989). Neurotoxicity of such substances may occur due to their ability to cross the barrier, there by causing functional damage (Kyvic and Bente, 1995). Pesticides have been reported to cause toxic neuropathies, seizures, paralysis, neuronal degeneration, lesions in CNS, impairment in the neuronal energy metabolism, etc. Sometimes, these exposures are fatal also. Pesticides are classified in different groups according to their structure and mode of action.
In the present study, pesticides belonging to structurally and functionally different groups, namely, quinalphos (QP: organophosphates), cypermethrin (CP: pyrethroids) and lindane (LD: organochlorine) were used.

1.1. Quinalphos-an organophosphate pesticide:

Organophosphates, increasingly used important pesticides, have been reported to induce deleterious effects on biological system of human and non-targeted organisms (Tracey and Gallagher, 1990). Organophosphate pesticides are usually esters of pentavalent phosphoric acid. Quinalphos (O,O-diethyl O-2 quinoxalinyl phosphothioate) is a broad spectrum organophosphorus insecticide, extensively used for pest control. Generally, toxic effects observed following exposure to organophosphates result from inhibition of the enzyme acetylcholinesterase. Although QP is a weak inhibitor of acetylcholinesterase but is biotransformed into its corresponding oxygen analog which is potent inhibitor of this critical enzyme (Forsyth and Chambers, 1989). QP is readily absorbed and metabolized to 2-hydroxy quinoxaline (free or as its conjugates) which are excreted in urine (87%) and bile (13%) with in a short period of time.

1.2. Cypermethrin-a pyrethroid pesticide:

Pyrethroid insecticides are widely used in agriculture and home to control a variety of insects. The selection of pyrethroids for such a variety of uses attributes to their limited soil persistence and relative safety due to a high insect/mammalian toxicity ratio (Elliott, 1976). Extensive studies in adult animals have revealed that the primary target site for pyrethroids in both insects and mammals is neuronal sodium channels (Casida et al., 1983; Lund and Narahashi, 1983; Vijverberg and Vanden-Bercken, 1990; Narahashi, 1992). After modification by pyrethroids, sodium channels continue to perform many of their normal functions retaining their
selectivity for sodium ions and link with membrane potential. However, the kinetics of sodium channel are drastically changed (Narahashi, 1985). A further characteristic of pyrethroid is that although they have a very high affinity for active sodium channels, they have little effect on inactive or closed channels. The pyrethroids are thus also known as open channel blocker (Jacques et al., 1980). Pyrethroids also acts via antagonism of γ-aminobutyric acid (GABA) mediated inhibition modulation of nicotinic cholinergic transmission, enhancement of noradrenaline release or action on calcium ions (Lawerence et al., 1983). Pyrethroids can be classified into two main types on the basis of structure. Pyrethroids that contain a cyano substituent on the α-carbon of the phenoxy-benzyl moiety have been classified as type II; pyrethroids which lack this α-cyano moiety are classified as type I (Dorman and Beasley, 1991).

Cypermethrin is a type II pyrethroid. Its chemical name is: (R,S) α-cyano-3-phenoxy benzyl-2,2-dimethyl (1R1S)-cis trans-3 (2,2 dichloro vinyl) cyclo propane carboxylate. There are eight isomeric forms. Metabolism of CP occurs with hydroxylation and cleavage at ester bond. This occurs over hours but there is much more slower elimination of CP sequestered in body fat with a half life of 18 days for cis isomer or 3-4 days for trans isomer (Crawford et al., 1981).
1.3. Lindane- a chlorinated hydrocarbon pesticide:

All chlorinated hydrocarbon pesticides are aryl carbocyclic or heterocyclic compounds of molecular weight ranging from about 291 to 545.

Lindane (LD) has been used for protection of seeds, treatment of poultry and livestock and for control of household insects. For humans, LD is used as scabicide, and pediculocid usually as lotions, creams and shampoos. Chemical name of LD is benzene hexachloride. The acronym for benzene hexachloride is BHC. LD is defined as not less than 99% pure γ-BHC. Metabolism of LD involves not only phase I pathway such as oxidation by cytochrome P-450 but also phase II pathway such as conjugation of alcohol and phenol products to form glucuronides (Grover and Sims, 1965). The different isomers of BHC have opposite pharmacological actions. LD is stimulant of nervous system, causing violent epileptic form convulsions that are rapid in onset and generally followed by death or recover with in 24 hr (Woolley et al., 1985). LD also causes hypothermia and anorexia in rats. Chronic administration of LD causes a reduction in number of muscarinic receptors in cerebellum and diencephalon perhaps following alterations in cholinergic transmission (Fonseca et al., 1986). The main site of action of LD appears to be at synapse with both excitatory and inhibitory effects (Joy and Albertson, 1985).

Although the information about the adverse consequences of the pesticide exposure is available in case of humans and experimental animals, there is very scarce knowledge about the effects of low-level pesticide exposure on the functional integrity of the BBB in developing/growing individuals. Therefore, the present study was aimed to investigate the effects of single and repeated low-level exposure of selected pesticides, belonging to chemically different groups, on the functional integrity of BBB during development. The study was extended to compare the pesticide induced changes in developing versus adults and to evaluate the BBB
permeability after the withdrawal of the treatment, in order to find out whether the changes are reversible and if so up to what extent.

2. **EXPERIMENTAL DESIGN:**

Rat neonates were divided into four groups and were orally exposed to different doses of QP, CP and LD. Control animals were given corn oil (1 mL/100 g) in an identical manner. Treatment schedule for each group and the doses selected for the treatment of three pesticides are given below:

### 2.1. Doses

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>1/100(^{th}) of LD(_{50})</th>
<th>1/50(^{th}) of LD(_{50})</th>
<th>1/25(^{th}) of LD(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinalphos</td>
<td>0.25 mg/kg/d</td>
<td>0.5 mg/kg/d</td>
<td>1 mg/kg/d</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>2.5 mg/kg/d</td>
<td>5 mg/kg/d</td>
<td>10 mg/kg/d</td>
</tr>
<tr>
<td>Lindane</td>
<td>1 mg/kg/d</td>
<td>2 mg/kg/d</td>
<td>4 mg/kg/d</td>
</tr>
</tbody>
</table>

### 2.2. Treatment schedule:

**Group I:** Twelve rat pups (PND 10) per group were orally exposed through gastric intubation to QP, CP and LD at a dose of 1/50\(^{th}\) of LD\(_{50}\) and the BBB permeability was assessed in six animals after 2 hr and in rest of animals after 3 days of pesticide exposure. Respective control groups received corn oil (1 mL/100g) in an identical manner.

**Group II:** Ten-day-old rat pups were divided into following sub-groups (50 pups per group) and were exposed to QP, CP and LD at the dose of 1/50\(^{th}\) of the LD\(_{50}\) respectively. Duration of the pesticide exposure was as follows:

(i) Pesticide exposure from PND 10-13.
(ii) Pesticide exposure from PND 10-17.
(iii) Pesticide exposure from PND 10-30.
(iv) Pesticide exposure from PND 10-17 and withdrawal of
the treatment from 17th day of age up to PND 30, 45 and 60. Respective controls received corn oil in an identical manner.

Group III: Another group of 10-day-old rat pups were orally exposed to QP, CP and LD at a further low dose of 1/100th of LD50 unto 17 days of postnatal age. Control animals were treated with corn oil.

Group IV: Growing (PND 15) and adult rats (160±20 g) were exposed to these pesticides, so as to compare the age-dependent changes in the BBB permeability properties. Rat pups were orally exposed to these pesticides at 1/50th of LD50 dose and BBB permeability was assessed 2 hr after the treatment. Adult rats were exposed to the same pesticides at 1/25th of LD50 for three consecutive days and the BBB permeability was assessed 2 hr after the last dose. In another set of experiment, adult rats were given pesticides in combination with piperonyl butoxide (PB, 600 mg/kg, i.p.), a cytochrome P-450 inhibitor, which enhances the activity of pesticides by decreasing their metabolism. A group of animals were also given oil in combination with PB to serve as respective controls.

2.3. Blood-brain barrier permeability assessment:

Blood-brain barrier permeability was assessed in age-matched controls and in the pesticide treated animals by using a micromolecular tracer dye sodium fluorescein, according to the method of Gulati et al. (1985) as described in the Methodology section.

3. RESULTS

3.1. Body and brain weight:

The brain and body weight of the pesticide treated pups were found to be comparable to respective controls and there were no sign of overt toxicity in the experimental animals.
3.2. Permeability pattern at different ages of development:

The data presented in Fig. 1 show the BBB permeability pattern of micromolecular dye sodium fluorescein during postnatal development (PND 10-45) and during adulthood. The BBB permeability to sodium fluorescein was found to be maximum at PND 10 (1.279±0.114), which further decreased with increasing age. The three time points observed with sharp decreases in BUI were observed (i) from PND 13-15 (1.150±0.062 to 0.843±0.026), (ii) from PND 17-30 (0.840±0.067 to 0.414±0.016) and (iii) from PND 30-45 (0.414±0.016 to 0.272±0.01). The BUI at PND 45 was found to be comparable to the adult values.

3.3. Effect of single treatment of pesticides on BBB permeability at 2 hr and 3 days of exposure:

The data in Fig. 2 showed the effects of selected pesticide exposure on the BBB integrity after 2 hr and 3 days of exposure. Single exposure of QP, CP and LD (1/50th of LD50) were found to significantly increase the BUI at 2 hr by 97% (p<0.001), 37% (p<0.01) and 72% (p<0.001), respectively. The BBB assessment on the 3rd day of single exposure of these pesticides on 10-day-old rats showed a complete recovery in CP treated pups; however, residual increase in BBB permeability was evident in QP (28%, p<0.01) and LD (23%, p<0.01) treated pups.

3.4. Blood-brain barrier permeability assessment following repeated exposure of pesticides:

Effects of daily exposure of the three pesticides from PND 10 to 30 on the functional integrity of BBB during developmental stages are shown in Fig. 3-5. In QP treated pups, the BBB permeability was found to be significantly increased at all the studied time points i.e. at PND 13, 17 and 30 by 43% (p<0.01), 130% (p<0.001) and 35% (p<0.05), respectively, as compared to age-matched controls. CP exposure to the developing rats also significantly increased the BUI for sodium fluorescein at all the selected time intervals. It was increased by 71% (p<0.001), 80% (p<0.001) and 61% (p<0.001) respectively at 13, 17 and 30 days of postnatal ages as compared to the age-matched controls. The BBB permeability in LD exposed pups was increased at PND 13 and 17 by 35% (p<0.01) and 50% (p<0.01), respectively. However, no significant increase in the sodium fluorescein permeability was observed at PND 30.
Fig. 1: Brain uptake index of sodium fluorescein at different ages of development and during adulthood. Values represent mean ± SE of BUI for six animals in each age group.
Fig. 2: Effect of single treatment of pesticides (1/50th of LD_{50}) at PND 10 on BBB permeability following 2 hr and 3 days of exposure. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. * p<0.05, ** p<0.01, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (↑) = increase.
Fig. 3: Effect of quinalphos exposure (1/50th of LD₅₀) through oral intubation on BBB permeability at different ages and following its subsequent withdrawal. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student's 't' test. * p<0.05, ** p<0.01, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (†) = increase.
Effect of cypermethrin at the dose of (1/50th of LD₅₀) through oral intubation on BBB permeability at different ages and following its subsequent withdrawal. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. * p<0.05, ** p<0.01, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (†) = increase.
Fig. 5: Effect of lindane at the dose of (1/50th of LD_{50}) through oral intubation on BBB permeability at different ages and following its subsequent withdrawal. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student's 't' test. ** p<0.01. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (↑) = increase.
3.5. Blood-brain barrier permeability assessment following withdrawal of pesticide treatment:

In order to study the recovery of pesticide induced changes, a group of animals which were treated from PND 10 to 17, were withdrawn from the treatment from PND 18. The BUI was measured at PND 30 in all experimental groups and at PND 45 and 60 only in case of CP. BBB permeability which was increased by QP and LD treatment from 10 to 17 days of postnatal age completely recovered following 13 days of withdrawal i.e. from PND 18 to 30. However, in case of CP, residual increase (27%, p<0.01) in BBB permeability to sodium fluorescein was observed when CP treatment was withdrawn for 13 days (PND 18 to 30) following exposure from PND 10 to 17. CP induced changes in BBB permeability following the treatment from PND 10 to 17, were still evident (22%, p<0.01) after the withdrawal of treatment for 37 days i.e. from PND 18 to 45. The increase in the BBB permeability due to CP exposure from PND 10 to 17 was completely recovered after a withdrawal period of 43 days (PND 18 to 60) and BUI recorded at this time point was comparable to control values.

3.6. Dose-dependent effect of pesticide exposure on BBB permeability:

The data presented in Fig. 6 show a very low dose (1/100th of LD50) effect of these pesticides on the BBB integrity following exposure from PND 10 to 17. QP and CP increased the permeability by 20% (p<0.05) and 28% (p<0.05), respectively, while LD did not affect the permeability.

3.7. Age-dependent effect of pesticide exposure on BBB permeability:

The age-dependent effects of pesticide on the BBB permeability are shown in Fig. 7. Pesticide treatment (1/50th of LD50) given to 15-day-old rat pups increased the BUI to sodium fluorescein by 48% (p<0.001), 15% (p<0.05) and 20% (p<0.05) following 2 hr of QP, CP and LD exposure, respectively. However, the adult rats treated with these pesticides at a dose of 1/25th of LD50 for 3 days did not show any significant effect on BBB permeability. Furthermore, when these pesticides were given in combination with cytochrome P-450 inhibitor PB to the adult rats, the BBB permeability was observed to be quite normal as their values were comparable to that of control animals. (Fig. 8).
Fig. 6: Effects of different doses of pesticides (1/50\textsuperscript{th} and 1/100\textsuperscript{th} of LD\textsubscript{50}) on BBB permeability following repeated exposure from PND 10-17. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student's 't' test. * p<0.05, ** p<0.01, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (↑) = increase.
Fig. 7: Age-dependent effects of pesticides exposure on BBB permeability in neonates and adults. Neonates (PND 15) were once exposed to pesticides for two hours at the dose of 1/50th of LD50, and adult were exposed to pesticides daily for three days at the dose of 1/25th of LD50. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student's 't' test. * p<0.05, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (†) = increase.
Fig. 8: Blood-brain barrier permeability following pesticides exposure alone (1/25th of LD$_{50}$) and in combination with PB (600 mg/kg, i.p.) for three days in adults. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. Values are arithmetic mean ± SE of six animals of each group.
4. **DISCUSSION:**

Increasing use of pesticides have imposed threat to human health (Jamal, 1995), among them immature individuals are at higher risk of damage (Sheets et al., 1994; Srinivas et al., 1993). Neonates are highly vulnerable to the toxic challenges as compared to adults because of functionally inefficient excretory mechanism and poorly developed drug metabolizing system (Gange and Brodeur, 1972; Benke and Murphy, 1975). A variety of neurological disorders have been reported in neonates and adults due to acute or chronic exposures to pesticides (Gaines and Linder, 1986; Dorman and Beasley, 1991; Kyvic and Bente, 1995) and there are chances of permanent damage to the developing nervous system due to toxic exposures. The developing organism is remarkably dynamic, with constantly changing metabolic profile, which may be responsible for the different sensitivities to various toxicants that are exhibited during developmental period. The developing CNS is more prone to toxicant-induced damage because of higher brain uptake of xenobiotics due to immature BBB system (Gabbiani et al., 1967). Altogether such age dependent differences leads to a developing organism at the higher risk.

Our data clearly show the progressive tightness of BBB to micromolecular tracer sodium fluorescein up to PND 45. This is in agreement with earlier studies by Butt et al., 1990; Saunders and Mollagard, 1991 and Schulze and Firth, 1992. They reported that although BBB to large proteins exists in fetus, progressive tightness of the barrier to other smaller molecules occur postnatally. High uptake of sodium fluorescein was observed during early stages of development, this may be due to the presence of immature tight junctions during development (Kniesel et al., 1996).

The result of our study demonstrate an age-dependent differences in the susceptibility of rats to low-level of pesticide exposure. Single oral exposure of these pesticides at a dose of 1/50th of LD₅₀ to 10-day-old rat pups enhanced the BBB permeability to the micromolecular tracer sodium fluorescein following 2 hr of exposure by 97%, 37% and 72% with QP, CP and LD, respectively. Repeated exposure of low-level pesticides from PND 10 to 30 also altered the BBB function by increasing the BUI for sodium fluorescein at various time points with the maximum changes observed at PND 17 with all the three pesticides. The study by Srinivas et al. (1993) has also reported a higher magnitude of changes in the BBB permeability of neonatal rats to trypan blue dye following acute and subacute exposure of an organocarbamate herbicide, thiobencarb.

Pesticides are mainly lipophilic in nature and are likely to cross the BBB,
thereby causing functional changes. Since the developing BBB is immature, both structurally and functionally, pesticide exposure at an early life may therefore be more toxic as compared to adults. Widdowson et al. (1996) have also demonstrated the influence of age on the passage of paraquat through the BBB in rats. They reported an increased entry of paraquat into the brain of neonates that have poorly developed BBB as compared to adult brain. The study of Abraham et al. (1996) has similarly shown the vulnerability of the developing BBB, as evident by the time- and dose-dependent increases in the BBB permeability to both micromolecular (sodium fluorescein) and macromolecular (Evans blue) tracers following intracarotid injection of TNF-α. Thus, during neonatal infections, an altered BBB function may lead to vasogenic brain edema. The results of our experiments have shown maximum increase in the BUI with QP (130%) at PND 17 following daily exposure from PND 10. However, when treatment was extended up to PND 30, there was only 35% increase in BUI. Furthermore, QP-induced changes were recovered fully at PND 30 when the treatment was withdrawn from PND 18 following an exposure from PND 10-17. In another set of experiment, we found that a further low dose of QP (1/100th of LD50) exposure from PND 10 to 17 increased the BUI by 20%, as compared to the controls. This shows the dose-dependent effect of QP on the developing BBB. The extent of damage to the BBB was maximum at PND 17, with less changes observed at later periods. It may be due to the development of certain metabolic enzymes with the advancing age, that are involved in the detoxification of this pesticide (Gange and Brodeur, 1972; Benke and Murphy, 1975). QP is an organophosphate pesticide, mainly causes neurotoxicity by the anticholinesterase property. Study by various investigators (Benke and Murphy, 1975; Pope et al., 1991) have shown that organophosphates are generally 2 to 9 folds more toxic to immature (6-23 day-old) rats as compared to adult ones. Petrali et al. (1991), have reported structural and functional disruption of BBB in young rats exposed to soman, an organophosphate compound. Soman exposure increased the extravasation of macromolecule Evans blue-albumin and uptake of HRP in cerebellum and cerebral cortex.

Cypermethrin, another pesticide belonging to pyrethroid group used in our study was also found to adversely affect on the functional integrity of the BBB in the developing individuals after single and repeated exposure, but the effect was more pronounced in repeatedly exposed rats. Rat pups exposed to CP from PND 10 to 30 showed a significant increase in BUI for sodium fluorescein at PND 13, 17 and 30 as compared to the age matched controls. CP induced changes took a longer period for full recovery. CP took 43 days for complete recovery when the treatment was stopped on PND 18 following an exposure from PND 10 to 17. This
shows that CP induced changes are long-lasting and may also lead to other abnormalities at later life. Hayes, (1982) have reported that pyrethroid poisoning in infants which was due to inefficient hydrolysis of the pyrethrum esters in very young children. A study by Sheets et al. (1994), has also reported an age-dependent differences in the susceptibility of rats towards an acute dose of deltamethrin. They observed that neonates are considerably more sensitive than adults. This is in accordance with our study which shows that the adult animals are quite resistant to the pesticide exposure (1/25th of LD₅₀ for three days) whereas neonatal BBB integrity was altered even after a single exposure (1/50th of LD₅₀) at PND 10 and 15.

An organochlorine pesticide LD was also found to adversely affect the developing BBB function, but this pesticide appears to be somewhat less toxic to BBB system in comparison to QP and CP. Single exposure of LD (1/50th of LD₅₀) given to 10-day-old rat pups increased the BBB permeability by 72% 2 hr after the exposure. However, repeated exposure of this pesticide from PND 10 to 30 increased the BUI at PND 13 (35%) and at PND 17 (50%). A continuous exposure of LD from PND 10 to 30 had no effect on BBB permeability. An age-dependent effect on the functional integrity of the BBB was observed with LD also, in which neonates were found to be sensitive, where as adults had no effect. Adult rats were resistant to all the three pesticides studied. The exposure of these pesticides alone or in combination with PB (cytochrome P-450 inhibitor), which enhances their insecticidal activity by decreasing metabolism of insecticides (Casida et al., 1983), had no effect on BBB permeability. A study by Kim et al. (1988), has shown that 2,4-dichlorophenoxyacetic acid (2,4-D) an organochlorine herbicide accumulated in the brain of rabbits and mice without causing any generalized increase in the BBB permeability. Thus, the functional inefficiency of the developing BBB allows some toxic agents to freely enter the brain that could have never entered the mature brain. It appears from our study that the three pesticides studied had adverse effects on the developing BBB to varying extent.

The results of present study clearly demonstrate that the developing BBB is highly sensitive to a low-level QP, CP and LD exposure as evident by the increased BBB permeability to micromolecular tracer, sodium fluorescein following single and repeated exposures. Pesticides induced alterations in the integrity of BBB had persistent effects after single and repeated exposures especially in the case of CP, which required a longer period for complete recovery. Mature BBB was found to be highly resistant to all the three pesticides as compared to the developing individuals. Therefore, it may be concluded that a low-level pesticide exposure during critical period of development had adverse impact on the immature BBB that may lead to neurological dysfunctions immediately and at later life.
B. Functional Impairment of Blood-Brain Barrier Following Exposure to Selected Heavy Metals
1. **INTRODUCTION:**

Metals have always been occurring as an intrinsic component of earth’s crust and long been known for their broad spectrum utility. Recent advancement in metal based industries have resulted into increased levels of trace elements in the environment which led to inadvertent exposure of humans and ecosystem to a wide variety of metallic elements. This may sometimes resulted in toxicity to mankind and serious disbalance in the ecosystem as whole (Foulkes, 1986). Rapid industrialization and unchecked effluent discharge and emissions have alarmingly increased the contamination of various heavy metals like lead (Pb), mercury (Hg), chromium (Cr), cadmium (Cd), aluminium (Al), manganese (Mn), tin (Sn), etc. in the environment. The existing information on the metal toxicity has been derived from the animal experimentation and human case reports. Out-break of Itai-Itai disease in Japan following the consumption of rice containing high levels of Cd, the Minamata disease caused after eating methyl mercury contaminated fish and Pb poisoning in children who licked toys painted with Pb based paints are some of the landmarks of environmental pollution due to metals (Friberg, 1985; Foulkes, 1986). Exposure of some of these heavy metals to humans is of great concern due to their potential toxicity in the biological systems. Therefore, we have included some of these heavy metals namely Al, Pb, Cd and one of the organotin compounds in our study to explore the mechanism of their toxicity in central nervous system.
Aluminium is widely used in many household items, pharmaceutical products, foods, water purification, etc. The reports are available indicating the risk of Al exposure to developing and growing individuals through the consumption of Al-containing infant formula, diet, parenteral fluids, antacids and soil (Andreoli et al., 1984; Stanek et al., 1988; Calabrese et al., 1989). A high level of brain Al has been reported in senile dementia of Alzheimer’s type (Perl and Brody, 1980), Amyotrophic lateral sclerosis and Parkinsonian dementia of Guam (Perl et al., 1982). Cd is a ubiquitous environmental pollutant and its exposure to general population occurs through water, food and air. Workers are exposed to relatively higher amounts of Cd in occupational situations and in those residing near the industrial areas (Zwennis-Willem et al., 1992). Cigarette smoking have been reported to be an important source of non-occupational Cd exposure. Anosmia is very common finding in the Cd exposed workers and behavioural deficits like loss of memory, learning disability, poor I.Q. performance along with disturbed motor and peripheral abilities in the prenatally exposed children have been reported (Adams and Crabtree, 1961; Pihl and Parkers, 1977; Bonithon-Kopp et al., 1986). Pb is recognized as an environmental and occupational hazard that has a significant impact on the health and development in many species. The major source of Pb exposure in infants and young children living in non-contaminated areas are the diet and drinking water. Pb poisoning has been associated with numerous biological effects including neurological and gastrointestinal disorders, calcium metabolism disruption and anemia (Pounds et al., 1991; Schanne et al., 1992). During infancy the CNS is especially susceptible to the toxic effects of Pb. Several reports have shown that Pb exposure during growth period is connected with intellectual impairment and behavioural deficits (Davis and Sevensgaard, 1987; McMichael et al., 1988; Needleman et al., 1990; Needleman, 1995). In young children the onset of sign of Pb encephalopathy, which can lead to permanent neurological impairment and sometimes death have been associated with blood Pb levels in excess of 80-100 µg/ dL reported by U.S Environmental protection agency (1977). Tin combines with carbon containing materials to form organotin compounds. These compounds are used in making plastics, food packages, plastic pipes, pesticides, wood preservatives and rodent repellent. Tri-substituted organotins are useful biocides in agriculture and industry. They function as fungicide, bactericides, antihelminthics and rodent repellents. Triethyltin (TET), the most toxic of the alkyl tin compounds is used industrially both as a catalyst and a biocide. The sign of toxicity of TET are motor dysfunction and paralysis (Richman and Bierkamper, 1984).
All these metals are reported to be neurotoxic via different mechanisms by a number of investigators. Neurotoxic effects of these metals may differ in growing and adult animals (Wong and Klaassen, 1982). One of the determining factors which make these metals to be neurotoxic is related to their ability to cross blood-brain barrier (BBB). Several metals are reported to alter the functional integrity of BBB which may ultimately cause CNS damage. Acute high doses of Pb have been shown to cause swollen and edematous brain with haemorrhaging and necrosis, associated with increased BBB permeability (Goldstein, 1984). Long-term exposure of Cd has also been shown to alter the BBB permeability (Shukla et al., 1996a). The vulnerability of developing brain to the toxic effects of Cd may be due to immature BBB besides incomplete development of detoxification mechanism at this stage (Gupta et al., 1996). Al-induced disruption of BBB has been suggested to be an important event underlying Al encephalopathy. Aluminium has also been reported to increase the uptake of $[^{14}\text{C}]-\text{sucrose}$ and $[^{14}\text{C}]-\text{glucose}$ in different brain regions (Lipman and Tolchard, 1989; Favarato et al., 1992) and stimulate the incorporation of $[^{3}\text{H}]-\text{thymidine}$ in the cultured microvessels (Audus et al., 1988).

All these studies emphasize the risk of human metal exposure related to neurological and mental deficits. Some of these effects produced during early period may persist even during adult life, which may be of considerable importance. Based on the above mentioned reports, further investigations were carried to study the effects of certain heavy metals namely Al, Pb, Cd and TET on the functional integrity of the blood-brain barrier.

2. **EXPERIMENTAL DESIGN:**

2.1. Experiment (i):

Five-day-old rat pups were orally exposed through intubation to Al lactate at the doses of 10 mg Al/kg/d and 30 mg Al/kg/d ($1/10^{\text{th}}$ and $1/4^{\text{th}}$ of NOAEL) up to 17 and 30 days of postnatal age.

2.2. Experiment (ii):

Seven-day-old rat pups were exposed to Pb as Pb acetate through oral intubation up to PND 10 at the dose of 10 mg Pb/kg/d and up to PND 21 at the dose of 50 mg/kg/day. Another group of 7-day-old rat pups were given Pb acetate i.p. at the
doses of 1 and 10 mg Pb/kg/d up to PND 21. Respective controls received sodium acetate in an identical manner.

2.3. Experiment (iii):

Pregnant rats were exposed to 10 ppm Cd as CdCl₂ ad libitum, in drinking water from zero day of pregnancy through gestation period and the neonates were exposed lactationally by continuing the exposure of nursing mothers to the same dose of Cd up to PND 30 after parturition. BBB permeability assessment of developing rats was done at PND 30 in Cd exposed and in age-matched control animals.

2.4. Experiment (iv):

Male adult mice were exposed orally to 10 and 20 ppm TET (as TET bromide) in their drinking water and BBB permeability was assessed after 20 days of exposure in treated as well as in control animals.

Permeability assessment was done according to the method of Gulati et al., (1982), with the help of sodium fluorescein dye and the brain uptake index (BUI), that is the percentage of serum fluorescein in the brain tissue was calculated for each animal.

3. RESULTS:

3.1. Effect of Al on BBB permeability:

Effects of Al exposure on brain and body weights have been shown in Table 1. Aluminium decreased the body weight by 23% (p<0.001) and brain weight by 26% (p<0.001), when given as Al lactate at a dose of 10 mg Al/kg/d from PND 5-17, whereas no change in relative brain weight was observed. However, at the dose of 30 mg Al/kg/day, body weight decreased by 46% (p<0.001), brain weight decreased by 26% (p<0.001), and relative brain weight increased by 34% (p<0.001), when Al was exposed from PND 5-17. In all other groups no significant change was found in body weight, brain weight and relative brain weight.

Aluminium did not affect the integrity of BBB when given for 13 days from PND 5 to 17 as Al lactate at a dose of 30 mg Al/kg/d; however, at a dose of 30 mg Al/kg/d from PND 5-17, it decreased the BBB permeability significantly by 36%
Table 1

*Effect of aluminum on body weight, brain weight and relative brain weight at different doses and different time periods of exposure*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body wt. (g)</th>
<th>Brain wt (g)</th>
<th>Relative wt. brain wt.x100/body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (17-d-old rat pup)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>29.36±0.462</td>
<td>1.56±0.056</td>
<td>5.372±0.272</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium (10 mg/kg/d, oral, PND 5-17)</td>
<td>22.72±0.488</td>
<td>1.16±0.012</td>
<td>5.14±0.0765</td>
</tr>
<tr>
<td>(23%)</td>
<td></td>
<td>(26%)</td>
<td></td>
</tr>
<tr>
<td>Aluminium (30 mg/kg/d, oral, PND 5-17)</td>
<td>15.88±0.447</td>
<td>1.153±0.018</td>
<td>7.192±0.132</td>
</tr>
<tr>
<td>(46%)</td>
<td></td>
<td>(26%)</td>
<td></td>
</tr>
<tr>
<td><strong>Control</strong> (30-d-old rat pups)</td>
<td>40±0.01</td>
<td>1.46±0.016</td>
<td>3.644±0.0417</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium (10 mg/kg/d, oral, PND 5-30)</td>
<td>39.8±0.828</td>
<td>1.43±0.021</td>
<td>3.478±0.078</td>
</tr>
<tr>
<td>Aluminium (30 mg/kg/d, oral, PND 5-30)</td>
<td>42.8±1.01</td>
<td>1.525±0.018</td>
<td>3.672±0.106</td>
</tr>
</tbody>
</table>

Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s 't' test. Significantly different when compared with corresponding control at *** = p<0.001. Values are arithmetic mean ± SE of six animals of each group. Values in the parentheses denote the percent change from the control. (!) = increase, (n) = decrease.
Effect of Al on BBB permeability on PND 17 and PND 30 when Al lactate was given through oral intubation from PND 5 at the doses of 10 or 30 mg Al/kg/day. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. * p<0.05, ** p<0.01, *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (†) = increase, (↓) = decrease.
(p<0.001). When exposure was prolonged up to PND 30, BBB permeability to sodium fluorescein was increased compared to the values obtained following relatively a short period of exposure. AI exposure as AI lactate from PND 5-30 increased the permeability by 39% (p<0.05), and 31% (p<0.01) at the dose of 10 and 30 mg AI/kg/d, respectively (Fig.1).

3.2. Effect of Pb on BBB permeability:

Lead exposure decreased the body weight by 25% (p<0.05), and brain weight by 15% (p<0.001), at the dose of 50 mg Pb/kg/day when exposed from PND 7-21 through oral intubation, whereas no significant change in relative brain weight was observed. In all other groups, Pb exposure did not affect the body weight, brain weight and relative brain weight significantly as shown in Table 2.

The data in Fig. 2 show the effect of Pb given as Pb acetate at the doses of 1 and 10 mg Pb/kg/d, when given i.p. for 16 days (PND 7-21). Pb increased the BUI for sodium fluorescein by 62% (p<0.001) at the dose of 10 mg Pb/kg/d, whereas no significant change was observed in BUI at the dose of 1 mg Pb/kg/d. When Pb as Pb acetate was given through oral intubation for 16 days (PND 7-21) at a dose of 50 mg Pb/kg/d, it increased the permeability by 42% (p<0.01) as shown in Fig. 3. No significant change was observed in the permeability following Pb exposure for 4 days (PND 7-10) at a dose of 10 mg Pb/kg/d (Fig. 3).

3.3. Effect of Cd on BBB permeability:

The body weight, brain weight and relative brain weight of Cd treated pups were found to be comparable to the respective controls and there were no sign of overt toxicity in experimental animals.

Fig. 4 shows the effect of 10 ppm Cd on BUI of 30-day-old rats following exposure through gestational, lactational, and postnatal periods. No significant change was observed in BUI for sodium fluorescein at PND 30 following low level Cd exposure.

3.4. Effect of TET on BBB permeability:

No significant change in body weight, brain weight and relative brain weight was found in TET treated animals as compared to control animals.

Exposure of 10 ppm and 20 ppm TET as TET bromide for 20 days in drinking
Table 2

**Effect of lead exposure at different doses through different routes and for different time periods on body weight, brain weight and relative brain weight**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body wt. (g)</th>
<th>Brain wt (g)</th>
<th>Relative wt. brain wt x100/body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong>&lt;br&gt;(10-d-old rat pups)</td>
<td>13.52±0.732</td>
<td>0.912±0.029</td>
<td>6.858±0.534</td>
</tr>
<tr>
<td><strong>Experimental</strong>&lt;br&gt;Lead (10 mg/kg/d, oral, PND 7-10)</td>
<td>15.3±1.016</td>
<td>0.928±0.030</td>
<td>6.656±0.42</td>
</tr>
<tr>
<td><strong>Control</strong>&lt;br&gt;(21-d-old rat pups)</td>
<td>34.16±2.713</td>
<td>1.514±0.026</td>
<td>4.432±0.22</td>
</tr>
<tr>
<td><strong>Experimental</strong>&lt;br&gt;Lead (50 mg/kg/d, oral, PND 7-21)&lt;br&gt;(25%) ↓ *&lt;br&gt;(15%) ↓ ***</td>
<td>25.5±1.02</td>
<td>1.28±0.013</td>
<td>4.8±0.178</td>
</tr>
<tr>
<td><strong>Experimental</strong>&lt;br&gt;Lead (10 mg/kg/d, i.p, PND 7-21)</td>
<td>30.89±1.97</td>
<td>1.45±0.15</td>
<td>4.42±0.38</td>
</tr>
<tr>
<td><strong>Experimental</strong>&lt;br&gt;Lead (1 mg/kg/d, i.p, PND 7-21)</td>
<td>26.25±1.256</td>
<td>1.156±0.044</td>
<td>4.417±0.22</td>
</tr>
</tbody>
</table>

Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. Significantly different when compared with corresponding control at * p<0.05 and *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Values in the parentheses denote the percent change from the control. (↓) = decrease.
Fig. 2: Effect of Pb on BBB permeability at PND 21 when Pb as Pb acetate was given through intraperitoneal injection from PND 7 at dose of 1 or 10 mg/kg/day. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. *** p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (↑) = increase.
Fig. 3: Effect of Pb on BBB permeability at PND 10 and PND 21 when Pb as Pb acetate was given through oral intubation from PND 7 at dose of 10 or 50 mg/kg/day. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. ***p<0.001. Values are arithmetic mean ± SE of six animals of each group. Percent changes are in comparison to respective controls. (↑) = increase.
Table 3

Effect of cadmium and triethyltin treatment on body weight, brain weight and relative brain weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body wt. (g)</th>
<th>Brain wt (g)</th>
<th>Relative wt. brain wt. x100/body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (30-d-old rat pups)</td>
<td>33.5±1.27</td>
<td>1.359±0.047</td>
<td>3.998±0.0775</td>
</tr>
<tr>
<td><strong>Experimental</strong> 10 ppm Cd as CdCl₂ drinking water through gestational, lactational and post natal period</td>
<td>33±1.60</td>
<td>1.434±0.0</td>
<td>4.232±0.118</td>
</tr>
<tr>
<td><strong>Control</strong> (Adult mice)</td>
<td>21.8±0.60</td>
<td>0.426±0.0036</td>
<td>1.992±0.0496</td>
</tr>
<tr>
<td><strong>Experimental</strong> 10 ppm TET through drinking water for 10 days</td>
<td>21.3±0.75</td>
<td>0.441±0.0026</td>
<td>2.1±0.106</td>
</tr>
<tr>
<td><strong>Experimental</strong> 20 ppm TET through drinking water for 10 days</td>
<td>24.23±0.309</td>
<td>0.448±0.015</td>
<td>1.82±0.059</td>
</tr>
</tbody>
</table>

Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. Values are arithmetic mean ± SE of six animals of each group.
Fig. 4: Effect of Cd on BBB permeability at PND 30 when Cd was given as CdCl₂ through out gestation, lactation and postnatal period via drinking water at 10 ppm dose. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student's 't' test. Values are arithmetic mean ± SE of six animals of each group.
Fig. 5: Effect of Triethyltin (TET) on BBB permeability in adult mice following 20 days of treatment at the dose of 10 and 20 ppm in drinking water. Statistical analysis was done using one-way ANOVA and significance of the difference was evaluated by using Student’s ‘t’ test. Values are arithmetic mean ± SE of six animals of each group.
water in adult mice did not change the BBB permeability significantly, as compared to control mice (Fig. 5).

4. DISCUSSION:

The present study demonstrates the effect of heavy metals on developing BBB. The data have shown that repeated exposure of a very low level of Al at an early life increases the BBB permeability to micromolecular tracer sodium fluorescein in growing rats, suggesting the possible interaction of Al with the development and functional maturation of the BBB system. An increased permeability to another micromolecule $^{14}$C-sucrose has been reported in Al exposed rats following intraperitoneal administration (Favarato et al., 1992). However, there is a report that chronic exposure of Al (0.06 M) in drinking water to mice for 6 months did not alter the BBB integrity to macromolecular tracer Evans blue and HRP. *In vitro* studies with AlCl$_3$ (0.1-10 mM) on bovine brain endothelial cells showed an increased permeability of monolayer to micromolecule sodium fluorescein (Audus et al., 1988). Our study also demonstrated the increased permeability to sodium fluorescein in developing rats at a very low dose. Mechanism involved in the Al-induced increase in BBB permeability may probably be mediated through the neutralization of surface negative charges at the luminal and abluminal surfaces of the endothelial cells. Cationic molecules such as polyamines have been shown to increase the BBB permeability through this mechanism. Aluminium has also been reported to disrupt the tight junctional complexes by inhibiting Ca pumping ATPase. Thus our results emphasize the risk of Al exposure during developing period. Adverse consequences of Al exposure has also been reported in young children suffering from uremia, treated with large doses of Al-containing antacids to control hyperphosphatemia resulted in Al-induced encephalopathy (Andreoli et al., 1984). If Al-induced damage of BBB occurs at a very early life, it may be injurious not only during childhood but may lead to deleterious CNS function even at later life as well.

Our studies on Pb exposure demonstrated the functional impairment of BBB in neonates. The data show that Pb exposure through i.p. administration from PND 7-21 increased the BBB permeability at the dose of 10 mg Pb/kg/d. An oral Pb exposure from PND 7-21 also increased the permeability at a dose of 50 mg Pb/kg/d. It shows that i.p. exposure is more effective than oral exposure as very little
amount of ingested Pb is absorbed through oral exposure while total absorption of Pb occurs through i.p. route. Our result show that massive doses of Pb are required to produced BBB disintegration.

It has also been shown that low level oral Cd (10 ppm) through out gestation, lactational and postnatal period did not affect the BBB permeability in 30-day-old rat pups. Earlier studies on the mechanistic aspects of Cd toxicity have shown that exposure of this metal decreases the levels of various enzymatic and non-enzymatic antioxidants, increases the levels of lipid peroxides in different organs including brain regions (Shukla et al., 1988b; Shukla et al., 1989). Fetal and neonatal brain contains high levels of antioxidant substances and appears to be capable of modulating the removal of free radicals by normal cellular processes and as a consequence of exposure of environmental chemicals (Gupta et al., 1995). Since the fetal and neonatal brain microvessels antioxidant system is capable to cope up with the challenges of oxidative stress, the low level Cd exposure could not affect the BBB permeability. It has also been shown by Shukla et al. (1996) that 10 ppm oral Cd exposure for 30 days (from PND 21) did not cause any significant change in BBB permeability. However, a prolonged exposure up to 90 days increased the BBB permeability to a micromolecular tracer. They concluded that the exposure of oral Cd to growing rats for a prolonged time period may impair the antioxidative defenses in the brain microvessels and produce BBB dysfunction.

It is clear from present investigation that higher susceptibility of developing CNS to toxic injury from heavy metals than adult CNS may be due to their adverse effect on developing BBB. Thus, the toxic metals that have limited entry in mature brain could enter the developing brain freely following BBB dysfunction leading to a higher metal accumulation in different brain regions which may affect the cerebral functions (O’Callaghan and Miller, 1986). The severity of these effects may depend on the levels of metal ions to which the organism is exposed, as well as on the duration and route of exposure.

Therefore, it may be concluded that the higher vulnerability of developing CNS towards certain metals, if exposed in early life, may be associated with the disruption of BBB integrity.