Discussion
4. DISCUSSION

4.1 Zinc deficient diet:

Three ppm (part per million) zinc available in the diet is the approximate minimal level necessary for the maintenance of pregnancy in rats, without available zinc store (Apgar, 1969).

In this study zinc deficiency was produced in dams by giving zinc deficient diet containing 0.3 ppm zinc from gestation day 12 to term completion, no apparent maternal deficiency sign was produced in the dam, while zinc deficiency was teratogenic in fetuses (Fig. 5) shows hydrocephalous fetuses from severe zinc deficient group.

The aim of this study was to produce prenatal zinc deficiency, by use of zinc deficient diet, and to compare it with a diet which inhibits absorption of zinc such as cereals which are rich in phytic acid. Presence of high amount of phytic acid in food can cause mild zinc deficiency in certain conditions. In this study administration of a zinc deficient diet as late as 12 days to term completion resulted in pregnancy to term in some dams. The fetuses from dams which maintained pregnancy to term were further studies.

Clinical manifestations ranging from mild to severe zinc deficiency have now been recognized in humans. Zinc is required for many biological functions, including DNA, RNA
and protein synthesis, (Sandstead et al., 1969) cell division, (Taylor, 1982) and gene expression, (Miller, et al., 1985 and Kluge, 1987). It is also required for the activity of many enzymes in biological system. Large pools of zinc exist in some tissues of the body (muscle and liver), but this zinc is not readily mobilized. During zinc deficiency there is either a small decline in the concentration of zinc in these tissues or no changes at all (Hurley, et al., 1971). Severe zinc deficiency during pregnancy results in embryonic death, small fetuses and malformation (Hurley 1966).

Sensitivity of the fetuses to zinc deficiency is partly due to the need for zinc during neurogenesis and for normal formation of the central nervous system. Biochemically, the teratogenicity of zinc deficiency is widely ascribed to impaired nucleic acid synthesis during embryonic development (Dreosti et al 1972) resulting in asynchrony of histogenesis and organogenesis for normal morphogenesis (Hurley et al 1972). Considerable evidence suggests that diminished activity of the zinc dependent enzyme, thymidine kinase, may be an important factor responsible for reduced mitotic activity, (Duncan et al 1975). since this enzyme is widely recognized to represent a rate limiting step in DNA biosynthesis.
Female rats are more willing to copulate with male rats whose faeces they have come in contact with. Absorption of zinc, as of certain other minerals from the gastrointestinal tract, is decreased by phytic acid. Therefore, the rat faeces was considered as an important source of micronutrient. If the diet consumed by rats was rich in phytic acid, it was noted that dams fed peas soaked in water from gestation day 0 and individually housed failed to deliver on term completion. On the other hand, the dams which were housed along with a male produced pups at term, suggesting that the absence of male rats during gestation period resulted in mild zinc deficiency and inability of the dams to deliver at terms. It was concluded that mineral content's of male rat faeces is of vital importance for the normal development of fetuses and for parturition in dam.

4.2 Plan of work

The role of zinc deficiency on the prenatal development of the fetal rat brain has been evaluated. Combined histomorphological, histochemical, quantitative biochemical and ultrastructural investigation have been carried out on fetal rat brain (Day 15 to Day 21) and the dams. The effects of zinc neurotoxicity in the adult male rat have been considered with estimations of zinc concentration in various regions of the control and intoxicated rat brain. Zinc-zincon reaction (Hasan, 1977) has been utilized for histochemical demonstration of zinc in the rat brain.
4.2 Morphological studies

4.2.1 Light microscopy:

It allows revealing only pronounced changes of the neurons and neuroglia but the details of these changes escape the grasp of the researcher. They can only be studied with the aid of electron microscopy. In practice, however, there is a wide gap between the electron and light microscopic studies. Electron microscopic findings are hardly comparable with the light microscopic finding on Paraffin section of the same material because of the differences in preparation techniques. In order to avoid that, a combined light microscopic studies of plastic section and electron microscopic study of the same material was conducted. Samples from the control and experimental material were fixed in mixture of 3% paraformaldehyde and 3% glutaraldehyde, then in osmium, thereafter dehydrated and embedded in epoxy resins (araldite) which are most commonly used media for embedding tissues for electron microscopic study. In this way the blocks were prepared. Semi-thin plastic sections (0.5 -1 micrometer) were obtained first and studied under the light microscope, after the conventional staining with toluidine blue as well as the special staining technique described by Richardson (1960). The data obtained from the semi-thin sections are a bridge, between the light and electron microscopic studies and they permit comparison between the pictures of the same preparation seen by means
of both types of microscope. This is the right way to compare the appearance of a cell under the light and electron microscopes. In this connection, it is worthwhile to emphasize that in the light microscopic studies of the semi-thin sections it is also feasible to identify the basic pattern of the changes in the neurons. It follows from the data shown in Figs. 45, 46, 47 that the pictures of changes in the cells in paraffinized and semi-thin sections basically coincide. Also, it is easier to orientate the areas of interest in semi-thin sections for an in-depth detailed evaluation by subsequent examination of the ultra-thin sections obtained from the same block. Clearly, the morphological changes 'lag behind' in time from biochemical, physiological and clinical signs of overt zinc deficiency on the neurons or neuroglia in the various regions of the developing fetal rat brain. In particular, one of the most important indications in light microscopic studies is the state of nucleus and perikaryal Nissl substance. It is worthwhile to mention a classification of nerve cells based primarily on nucleus-cytoplasm relationship, the structure and distribution of Nissl substance in the cytoplasm (Einarson, 1960). According to this classification the following types of cells are distinguished:

1. Somatochromic nerve cells: With a wide band of cytoplasm around the nucleus, with well-developed Nissl granules. Such
nerve cells in their turn are subdivided into (a) strychochromic cells in which the Nissl substance consists of large polygonal, triangular and other kinds of corpuscles (b) archichromic cells in which Nissl bodies are arrange as a network (c) Cryochromic cells in which Nissl bodies are small granules evenly scattered within the cytoplasm.

2. Karyochromic: Cells in which the nucleus is surrounded by a narrow ring of cytoplasm with lesser amount of Nissl granules in the latter, these may be sub-divided into (a) hypokaryochromic and (b) hyperkaryochromic cells great diversity in the content of Nissl bodies in the nerve cells in health was emphasized in the quantitative studies by Pachenberg (1963).

It should be noted that pathomorphological changes in the nerve cells in any form of trace metal deprivation / toxicosis have some general tendencies, though each of them causes only some of the features of the pathology of the neurons. To identify the basic patterns of pathomorphological changes in developing nerve cells in zinc deficiency syndrome, combined light microscopic (both paraffined and semi-thin section plastic embedded material) and ultrastructural studies were utilized.

4.2.2. Electron Microscopic Studies

The typical picture of zinc deficiency may be delineated as follow on the background of homogeneous, devoid
of Nissl granules cytoplasm, which took darker colour than normal. The pyknomorphous nucleus can be identified clearly, the nucleus may become irregular, invaginated, or rod-shaped. There are also cells in a state of acute swelling. The polymorphism of changes in the structure is striking. A common form of damage to nerve cells is their homogenizing change pyknotic cells are also common, in zinc intoxicated rat brain. A frequent pattern of changes is the presence of multiple lipid and lipofuscin inclusion in the perikarya.

In electron microscopic studies, it is essential to observe very thoroughly the methodical approach to the experiments, since the artifacts which appear during fixation, dehydration, embedding, ultra-microtomy and staining may both simulate or disguise the pathocytological changes. Sometimes such methodological artifacts may be described as signs of cell pathology. Preparations of the brain tissue for electron microscopic studies have their own peculiarities and problems which have been tackled within special monographs (See 2.8.1).

4.2.2.1 Zinc deficiency

Quantitative biochemical studies (Table-3) indicated that zinc deficiency reduces the DNA synthesis in fetal rat brain. The loss of DNA during zinc deficiency in developing fetal rat brain where the cell divisions follow very closely upon each other, there is a strong possibility that a mistake
may occur during replication, the cells with wrongly replicated DNA are eliminated by necrosis (Ernst, 1962). The good fixation of neighbouring cells in the vicinity of necrotic cells, however, seems to eliminate the possibility of fixation artifact and post-mortem autolysis.

In severe zinc deficiency, different types of isolated necrotic cells were observed situated far apart from each other, while in case of mild zinc deficiency necrotic cells were placed close together (Fig.68-71).

Figures 20,21,24,46,65 show that the cell proliferation in the form of mitotic cell division exists in the control and experimental fetal rat brain. Severe zinc deficiency was produced in dams from day 12 of gestation. In this case, the proliferating cells in the cortex were usually in the form of isolated cells. As the zinc deficiency produced its effect on proliferating cells, therefore, the necrotic cells were located far from each other.

On the other hand, in the case of mild zinc deficiency where zinc deficiency were produced from gestation day 0, the mass of proliferating cells was affected and caused the formation of large necrotic regions in the cerebral cortex of the fetuses (Fig.68-71).

Cerebellar morphology is adversely affected by severe postnatal zinc deficiency (Dvergsten, 1981). The number and
migration of granule cells are also depressed. Purkinje, basket and stellate cell dendritic arborizations are deformed and suppressed and parallel fibres are decreased (Dvergsten, 1981).

In the present study dealing with severe prenatal zinc deficiency from gestation day 12 to term completion, the morphology of the cerebellum was markedly affected. The granule cell layer was differentiate into outer and inner granule cell layer in the control group fetuses (Fig.10,12) while the cerebellum from fetuses belonging to severe zinc deficiency group showed a uniform cell population density, (Fig. 11,13) pointing to depressed migration of the granule cells during fetal rat brain development in severe zinc deficient group.

4.2.2.2 Ultrastructural changes in zinc intoxicated rat brain

4.2.2.2.1 Neurons

Ultrastructural changes in zinc intoxicated in rat brain were marked by intranuclear inclusion (Fig.89,90).

4.2.2.2.2 Neuropil

It has been suggested that zinc plays a key role in the structure and function of biomembranes (Bettger et al 1981). The neuropil in zinc intoxicated animals shows signs of degeneration in the form of splitting of myelin membrane (Fig.94) and formation of electron dense granules, lipofuscin
In general, zinc intoxication increases the formation of electron dense material, especially in the neuropil.

In biochemical studies, the concentration of zinc in the intoxicated rat brain was reduced. As zinc is important for the maintenance of the structure and function of biomembranes, its reduction in the brain might have caused damage to myelin membranes and formation of electron dense bodies in nerve fibers. Some degenerating nerve fibers showed formation of lipofuscin material in the vicinity of myelin sheath.

4.2.3 Quantitative biochemical studies

4.2.3.1 Zinc intoxication

Table-1 shows that the concentration of zinc was reduced in all region of brain in experimental rats, receiving 5 mg/kg body weight zinc intraperitoneally. Based on this observation, and that of Ebadi et al (1984), that intraperitoneal administration of zinc sulphate did not stimulate the synthesis of any zinc binding protein in the rat brain. It is concluded that peripherally administered zinc gets bound rapidly to zinc binding protein and was unavailable to be transported to the CNS. The peripheral zinc binding protein acts like a buffer and has stronger affinities to bind with zinc ions than the zinc binding
protein present in the brain. The difference in the binding capacity is of significant importance in protecting CNS from accidental sudden increase in the concentration of peripherally administered zinc.

4.2.3.2 Protein

Quantitative biochemical studies shows that the protein concentration falls with increasing gestation period. The concentration of protein was less in the fetuses in experimental group at gestation day 15 as compared with control group (Table-2).

4.2.3.3 RNA and DNA

The level of RNA decreased with increasing gestation period. RNA concentration was significantly reduced in the fetal rat brain in experimental group (Table-4).

The level of DNA increased with increasing gestation period. DNA concentration was reduced in experimental fetal rat brain as compared to control group (Table 3).

Reduction in the synthesis of protein, RNA and DNA caused by zinc deficiency is of significant importance as the developing fetal rat brain is in "high metabolic state". Zinc-dependent enzymes, which are involved in the synthesis of protein, DNA and RNA, must have limited the metabolic process of the cell. The fetal rat brain weight was reduced in fetuses of severe zinc deficient group Fig.7 shows the difference in the size of the cerebral hemisphere in the control and experimental group.
4.2.3.4 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an enzyme which scavenges the superoxide radical, a toxic species generated by metabolic reduction of oxygen in respiring cells (Grankvist, 1979). This enzyme has been shown to inhibit lipid peroxidation and protect against pathologic changes resulting from DNA damage, proteins and sulphydryl oxidation by the superoxide radical (Fridovich, 1975).

In this study, the level of SOD in the control fetal rat brain increased with increased gestation period from gestation day 15 to gestation day 21. The level of (SOD) was significantly increased in fetuses from dam kept on zinc deficient diet from gestation day 12 to gestation day 15.

The conditions that lead to peroxidative damage often result in increased expression of SOD.

4.2.3.5 Alkaline phosphatase (AP)

Histochemical observation reveals that alkaline phosphatase is present in the rat brain. Quantitative biochemical studies reveal marginal decrease in alkaline phosphatase activity in the fetal rat brain from severe zinc deficiency group. The two enzymes (SOD and AP) studied require zinc for their activity.

The possibilities of biochemical alteration during feeding of low zinc diet are many since zinc is a cofactor in...
a large number of enzymes which play an important role in the stabilization of protein structure and may be integral part of nucleic acid. The ingestion of zinc deficient diet was accompanied by changes in the activity of zinc dependent enzymes in the zinc deficient developing rat brain.