DISCUSSION
CHAPTER IV

DISCUSSION

The current study was commenced to investigate the toxic effects of sodium fluoride (NaF) in vivo in brain (cerebrum and cerebellum) and blood (serum and cells) of adult male Wistar rats (Rattus novergicus) in Part I. In Part II, in vitro effect of fluoride on human red blood cells (RBCs) was done. In these studies, ameliorative efficacy of melatonin and/or amla was investigated. Withdrawal studies were also carried out for comparison in this study.

Fluoride is one of the few chemicals that have been shown to cause adverse effects in people and animals mainly exposed through drinking-water though it has beneficial effects at low concentrations. This ranges from mild dental fluorosis to crippling skeletal fluorosis as the level and period of exposure raise. Crippling skeletal fluorosis is a notable cause of morbidity in a number of regions of not only India but also the world.

PART I: IN VIVO STUDIES

In the present in vivo study, Wistar rats (Rattus novergicus) weighing 250-300 g were used to investigate the toxic effects of sodium fluoride (NaF) and its amelioration by melatonin and amla. Sodium fluoride, low (5 mg/kg b.w.) and high (10 mg/kg b.w.) doses were administered orally for 60 days to rats to explore the biochemical changes in blood and cerebral hemisphere, cerebellum of brain in rats. The doses selected were based on the LD50 value of fluoride (Pillai et al., 1987; 1988). As drinking water is the main source of fluoride toxicity, the mode of administration was preferred as oral in the present study. Antioxidants, melatonin (10 mg/kg b.w.)
and amla (20 mg/kg b.w.) were used for detecting their ameliorative effects against fluoride induced toxicity. In the later part of in vivo study, unlike the above set of experiments, the treatment of NaF ingestion was withdrawn for 30 and 60 days to study its reversibility effects, if any, upon termination of treatment.

The various indices studied at the end of each treatment after 60 days were gravimetric data and histopathology of brain. In addition, serum, cell count and tissue biochemical indices like total proteins, total lipids, cholesterol, acetyl cholinesterase (AChE) and sialic acid were also recorded. Besides, transaminases (SGPT & SGOT) in serum and prooxidant indices like succinate dehydrogenase (SDH), adenosine triphosphatase (ATPase), and antioxidant indices like lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) in brain (cerebrum and cerebellum) of Wistar rats were evaluated. Likewise, fluoride levels in the brain tissue were also estimated in all groups of animals.

**Gravimetric indices**

**Body and Brain**

In this study, NaF treatment brought about a reduction in body and brain weights of rats, which could be ascribed to very low food consumption, altered protein and energy metabolism (Chinoy, 1991a) and electrolyte imbalance (Das and Susheela, 1991). In our study, in addition to the weight loss, F⁻ treatment showed induced lethargic appearance, yellowish fur and teeth and rigid arms movement in ingested rats. In accordance with our records, the body and brain weights and somatic index of brain of mice decreased significantly in NaF treated groups (Reddy et al., 2009). Oral administration of sodium fluoride for 30 days caused significant reduction in body weight of mice that can be attributed to lower food consumption resulting in
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decreased protein synthesis and lower energy metabolism (Trivedi et al., 2012). A consistent decline in body weight of rats by 5.2 mg/kg b.w. fluoride for 35 days was also reported by Pillai et al. (1988). Similar results were also reported in animal body weight reduction by other researchers (Saralakumari et al., 1988) in rats and (Vani and Reddy, 2000) mice by fluoride treatment. Studies carried out in our laboratory have also revealed decline in body weights by fluoride treatment in mice and rats (Chinoy and Sequeira, 1989a; Chinoy et al., 1991b; 1992a; 1993a), rabbits (Chinoy et al., 1991a), rat pups and dams (Verma and Sherlin, 2002). Our findings are in conformity with others (Sharma et al., 2007; Tiwari and Pande, 2009; Vasanth and Narshimhacharya, 2012), who have attributed the reduction in body weights to reluctant food intake. Reports from our laboratory have further revealed that NaF for a period of 7, 15 and 30 days, did not produce any significant alterations in the gravimetric data, but it was effective from the 45th day onwards and the toxic effects were more pronounced after 60 days treatment (Patel and Chinoy, 1997). Another recent study from our laboratory on reproductive and other organs also supports present findings on gravimetric data (Chawla and Rao, 2012; Rao and Bhatt, 2012). A similar decline in body weight occurred in rats and pups when the female mother rats were fed with fluoride at chronic level (200 ppm) for two generations (Basha and Sujitha, 2011). Fluoride accumulation in tissues and bones might influence the growth of animals (Yoshitomi et al., 2006, Shi et al., 2009). Trabelsi et al. (2001) has reported reduction in weights of cerebral hemisphere and cerebellum which might be due to effect of F− on cell proliferation in the external germinal layer of mice brain. Similarly F− intoxication for 30 days caused a significant decrease in absolute weights of CH, CB and MO regions of brain in mice (Trivedi et al., 2011; 2012). Likewise, Samanta and Bandyopadhyay (2011) reported significant decrease in weights of albino rat
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kidney, liver, heart and brain and reason stated, may be a direct effect of fluoride water on vital organs. This might be due to inhibitory effect of fluoride on protein synthesis and/or breakdown of protein. Other investigators have also reported reduction in absolute weight of cerebrum, cerebellum, hippocampus and other regions of the brain in experimental animals exposed to F⁻ (Chinoy, 2002). Srivastava et al. (1989) were also quoted decreased weights in vital organs after fluoride water ingestion to mammals. All these factors could account for reduction in body and organ absolute weights of the brain regions. A reduction in food consumption by NaF treatment (Pillai et al., 1987; Paul et al., 1998) indicates loss of appetite in treated animals, which could be accounted for decreased protein concentration in soft tissues and the failure of the animals to gain weight. Similar changes were also observed in various soft tissues of rodents treated with different doses of NaF for 30, 40, 60 and 70 days (Chinoy, 1991a,b; 1992; 1995; 2002; Chinoy and Sequeira, 1989a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Memon, 2000; 2001; Chinoy and Patel, 1999; 2000; Chinoy et al., 1991a,b; 1992b; 1993a, b; 1994a,b,c,d; 1995; 1997a). This might be associated to the possible inhibition of DNA synthesis by fluoride treatment. Inadequate protein turnover could also have an adverse effect on the concentrations of enzymes, receptors, structural proteins and various secretions in the body. Hence, the cause for such inhibition of growth by fluoride feeding could be attributed to less intake of food, reduction in protein levels and slow down of metabolic activities as noticed in this study.

After administration of antioxidants like melatonin and amla alone at the doses of 10 and 20 mg/kg b.w. respectively to treated rats, body and organ weights did not reveal any significant variations compared to control groups. However, the treatment was withdrawn for 30 and 60 days after sodium fluoride ingestion for 60 days to study
the reversible effects. But much recovery was not observed in withdrawal groups of animals.

**Haematological indices**

The blood performs various vital functions in the body; viz. carries oxygen, enzymes, hormones, minerals and food forms and drugs etc. It excretes toxic substances from the body, absorbs essential nutrients from gut, and maintains internal homeostasis. In blood, the fluoride ions are asymmetrically distributed in between plasma and the blood cells, so that the plasma concentration is approximately twice as high as that associated with the cells (Whitford, 1996).

In the current study, haematological parameters like total red blood corpuscles (RBC) count, white blood corpuscles (WBC) count, haemoglobin (Hb), erythrocyte sedimentation rate (ESR), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) and platelets count were investigated in all groups.

The results revealed a significant decline in total RBC count, haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), whereas enhancement was observed in total WBC count (white blood cells) count and erythrocyte sedimentation rate (ESR) (Chinoy and Shah, 2004b).

This reduction in Hb, RBC, MCV, MCH, and MCHC with an increase in the WBC and ESR indicated alterations in haemopoietic tissue and their differentiation. Bone marrow cell number could also be affected and are in agreement with the data of others studied in mice and rats (Pillai et al., 1998; Mathews et al., 1996; Banupriya et al., 1997; Chinoy, 2002; Eren et al., 2005). The changes in erythrocytes count and
haemoglobin concentrations have been correlated with cytotoxic effects of sodium fluoride on erythropoiesis. Due to decline in the total RBC count and haemoglobin concentration, concomitant decrease in MCH an MCHC was observed in the present study.

NaF induced a decline in total erythrocytes count, haemoglobin percentage and hematocrit values, where as total leucocytes count elevated in experimental groups by numerous others (Pillai and Mane, 1985; Banu Priya et al., 1997; Bharti and Srivastava, 2009; Zabulyte et al., 2007). Karadeniz and Altintas (2008) also reported that fluoride (40 mg F/l) exposure caused alterations in RBC, hemoglobin and hematocrit values. Similarly, Samanta and Bandyopadhyay (2011) investigated fluoride associated significant decreases in RBC, hemoglobin and hematocrit values.

Bouaziz et al. (2006) accounted that ingestion of sodium fluoride caused anemic conditions in experimental animals, which may be due to deficiency of folic acid, vitamin B12 and copper, thereby causing disruptive effects in RBCs. F⁻ causes enhanced production of superoxide radicals and lipid peroxidation that lead to alterations in RBCs cell membrane function and structure. Fluoride gets accumulated on the surface of erythrocytes in turn the membrane loses calcium content which results in the formation of echinocytes. The life span of these echinocytes is less than the normal erythrocytes and hence early destruction of the erythrocytes in form of echinocytes causes anemic conditions in animals and human (Susheela, 2001; Suwalsky et al., 2004). The blood acts as a transport medium for fluoride. About 75% of blood fluoride is present in the plasma, the rest mainly in or on the red blood cells. Human exposed chronically to toxic levels of fluoride through drinking water, showed significant increase in lipid peroxidation of RBC membrane, cholesterol and
phospholipids (Saralakumari and Rao, 1991). Choubisa et al. (1996) found decreased RBC and haemoglobin in human living in an area of endemic fluorosis. The reduction in Hb after repeated administrations of NaF may be an indication that it would lead to anemia in rats. Similar findings were reported in sheep (Mohiuddin and Reddy, 1989), buffalo (Swarup and Singh, 1989) and cows (Singh and Swarup, 1994). Decrease in PCV was observed by Donald et al. (1979), Swarup and Singh (1989) and Gujrathi et al. (1991) during fluorosis in cattle and buffaloes.

Maheswaram et al. (2008) reported increased WBC count in a dose dependent manner, in collaboration of our observation. This enhancement of WBC could be attributed to a stimulation of immune system in response to tissue damage caused by sodium fluoride. However, the intoxication of sodium fluoride on total WBC count and erythrocyte sedimentation rate (ESR) has been observed to be significantly increased after treatment with sodium fluoride due to leucocytosis. Leucocytosis is an outcome of proliferation of haemopoitic cells leading to progressive infiltration in peripheral blood (Sharma et al., 2007). Similarly an increase in eosinophil level in fluorotic cattle (Hoogstratten et al., 1965; Hillman et al., 1979; Jagadish et al., 1998; Gujrathi et al., 1991) was observed in support of our data.

Sodium fluoride increases the ESR due to the decreased total RBC count as the total count depends on the rouleux formation of erythrocytes. Further the rouleux formation is an indication of increase in the density of its mass which along with reduced erythrocyte count, is responsible for increased erythrocyte sedimentation rate (Agarwal and Chaurosia, 1989). Connor and Fromm (1975) reported a decreased RBC count due to hemolysis as a consequence of sodium fluoride toxicity in support of our results. Banu Priya et al. (1997) also observed decreased concentration of Hb and Ht, RBC count; increased leucocytes count with fluoride toxicity which agrees
with the results reported by Vijaya Bhaskara Rao and Vidyunmala (2010). The decreased hematocrit levels may be attributed to a decrease in size of erythrocytes due to stressful conditions. Vijaya Bhaskara Rao and Vidyunmala (2010) showed inverse relation between F concentration and hematocrit values. The results coincide with the reports of (Mittal and Flora, 2007). Sharma et al. (2004), Sharma et al. (2006a,b) and Agrawal (2009), who observed increased leucocytes count following fluoride water (3, 4.5, 5.8, and 6 ppm) exposure to rats for 15, 30, 60, and 120 days.

Fluoride depleted the energy reserves and the ability of blood cells to phagocytose foreign agents. The excessive ingestion of fluoride hampers haemopoiesis, alters blood parameters and affects absorption, excretion, distribution and retention of several minerals (Machalinski et al., 2000; Singh et al., 2002). According to the observations of Agalakova and Gusev (2008), fluoride has adverse effects on Na\(^+\) and K\(^+\) transport across the rat erythrocyte membrane. High fluoride concentration may disturb the anion channel of the erythrocyte membrane, which leads to hemolysis, and swelling of cells (Lima-filho et al., 2004). This may attribute to decline in total erythrocyte count, hemoglobin and hematocrit values.

The reduction in hemoglobin levels, RBC counts and others, but an increase in the WBC count by NaF treatment documented in our study are in justified with data of above authors in mice and rats (Pillai et al., 1988; Banupriya et al., 1997; Chinoy, 1992; 2002) and in fluorotic people of Mehsana district of North Gujarat, India (Chinoy et al., 1994a and Mathews et al., 1996) in Orissa, India.
Biochemical indices

Brain is the principle organ of the central nervous system in human and animals. It is the most amazing complex organ which plays a crucial role in the information system of the body. In a typical human, it is estimated to have 15-33 billion neurons in cerebral cortex (the largest part of brain) (Pelvig et al., 2007). It operates thoughts, emotions, and motion of organs. It acts like a super computer by storing and recalling the information of memories.

The brain is the boss of the body which runs the whole show to be played by the body not only at work but also in sleep. The vertebrate brain has three main parts viz., cerebral hemisphere (CH), cerebellum (CB) and brain stem (BS) with important glands like pituitary and hypothalamus.

Free Radicals and Antioxidants

Reactive oxygen species (ROS) are implicated as an important pathological mediators in many disorders. Increased ROS generation and enhanced lipid peroxidation are considered responsible for the toxicity of a wide range of metabolic products (Halliwell and Gutteridge, 1989). Numerous authors have reported relationship between F' and oxidative stress raised by free radicals (Guan et al., 1989; Rzeuski et al., 1998; Chinoy and Patel, 1998a; Chinoy and Sharma, 1998; Yur et al., 2003; Bober et al., 2006).

Oxygen is necessary and also toxic for human life, depending on the concentrations. The structure of O$_2$ is responsible for this paradox because it favors the reduction of oxygen in single electron steps. This stepwise reduction slowdowns the direct combination of oxygen with organic compounds and allows the cell to oxidize fuels through the action of dehydrogenase, which eventually couples the reducing power of oxygen to ATP generation in the electron transport chain. On the
other hand, the generation of oxygen radicals and other reactive oxygen species (ROS) are capable of causing cell injury. Some of the disease states associated with free radical injury is atherogenesis, Parkinson's disease, bronchitis, Duchenne muscular dystrophy, acute renal failure, cerebrovascular disorders, cervical cancer, etc. Proteins, membrane lipids, carbohydrates, and nucleic acids are subjected to cellular damage caused by oxygen radicals (Marks et al., 1996).

Dismutation of superoxide anion to hydrogen peroxide and $O_2$ by superoxide dismutase (SOD) is often called the primary defense against oxidation stress. SOD isoenzymes are present in the cytosol and mitochondria of the cell. The major routes involved in decomposition of hydrogen peroxide to water are by catalase and glutathione peroxidase. Catalase is found principally in peroxisomes, and to a lesser extent in the cytosol and microsomal fractions of the cell. Glutathione peroxidase is one of the body's principle means of protection against oxidative damage. It catalyses the reduction of hydrogen peroxide and lipid peroxides by glutathione (GSH), wherein GSH serves as an electron donor (Marks et al., 1996). Thus, free radicals are highly reactive species that have an unpaired electrons e.g. hydroxyl radical ($OH^*$), superoxide ($O_2^*$) and hydrogen peroxide ($H_2O_2$).

**Antioxidant indices (LPO, SOD, CAT)**

In the present study, an increased lipid peroxidation (LPO) levels and declined superoxide dismutase (SOD) and catalase (CAT) activities in brain regions by sodium fluoride treatment might be a result of either over production or accumulation of ROS resulting from the loss of antioxidants and indicated damage to membrane unsaturated fatty acids. In this study, lipid peroxidation was assayed as a marker of oxidative damage to the lipids in the brain of rats treated with sodium.
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fluoride. This suggests an increased peroxidation of lipids concomitantly increased TBARS level loosing cellular function in cerebrum and cerebellum regions of brain to affect their neural inputs and outputs leading to behavioral changes in fluoride treated rats. In support of our observations, Inkielewicz et al. (2006) quoted similar findings that elevated levels in lipid peroxidation and decrement in the activities antioxidant enzymes SOD and CAT in liver, kidney, brain and serum of fluoride intoxicated rats.

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids or cellular membranes undergo reaction with oxygen to yield lipid hydro peroxides. The reaction occurs through a free radical chain mechanism initiated by the removal of a hydrogen atom from a PUFA by a reactive free radical followed by a complex sequence of propagative reactions. Fluoride has been demonstrated in vitro and in vivo to cause increased lipid peroxidation in erythrocytes of human (Saralakumari and Rao, 1991) and in brain, RBC and liver of 100 ppm fluoride treated mice and rats (Flora et al., 2009; Shivarajashankara et al., 2003). Similarly, Vani and Reddy (2000) and Godkar and Godkar (2003) also reported that fluoride enhances lipid peroxide and inhibits antioxidative enzymes in brain, liver, kidney, heart and blood of fluoride mice (Flora et al., 2009). Accumulated literatures demonstrated that the enhanced lipid peroxidation and decreased activities of antioxidant enzymes play crucial role in membrane structural lesions, systemic dysfunctions and cell apoptosis; interestingly, excessive F⁻ is highly related to an increased superoxide free radicals and lipid peroxidation in rabbits (Liang et al., 2012). The excessive production of reactive oxygen species overwhelming the antioxidant potential of the cells is represented by reduced cytoplasmic thiols (e.g. glutathione) and antioxidant enzymes such as SOD, CAT and GPx (Barbier et al., 2010; Jancinto-Aleman et al., 2010; Inkielewicz-Stepniak and Czarnowski, 2010;
Basha et al., 2011; Chen et al., 2012). As persistent oxidative stress causes apoptosis (Trachootham et al., 2008), exposure to F frequently results in the induction of cell death, both in vivo and in vitro (Barbier et al., 2010; Jancinto-Aleman et al., 2010; Gutowska et al. 2010; Wang et al., 2010). Researchers consider that the reason for negative effects of F on the brain traces back to its role in oxidative damage. Low levels of exposure over a period of 10 weeks to mice can increase free radical loads and reduce the crucial antioxidant glutathione, along with other key antioxidant enzymes, including catalase and superoxide dismutase (Chouhan and Flora, 2008; Basha et al., 2011).

In support of our findings, Madhusudhan et al. (2010) reported declined activities of antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH.Px) and catalase (CAT) as well as decreased levels of glutathione (GSH) in the brain of NaF treated rats. These results are in agreement with those of Qiu and Sun (1999) in kidney, liver, bone and brain of fluoride treated rats and the reports of our laboratory on fluoride and/or arsenic poisoning in mice (Chinoy, 2002; Chinoy and Sharma, 2000; Chinoy and Patel, 1999; Chinoy et al, 2004a; Memon and Chinoy, 2000; Jhala et al., 2004; Rao and Bhatt, 2012). Similarly, Liu et al. (1999) observed that fluoride and their combination with other toxicants affected the activity of SOD and GSH-PX in liver and kidney of rats as well as in blood of rats and their offsprings. Blaszczyk et al. (2008) documented that in rat liver, enzymes of the antioxidative system, such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were significantly reduced after NaF exposure. Bharti and Srivastava (2009) also declared that fluoride administration significantly increased brain MDA levels compared with control group, while SOD levels decreased in F treated group. Similarly, Samanta and Bandyopadhyay (2012) reported that the
antioxidant enzymatic parameters such as superoxide dismutase (SOD), glutathione transferase (GST), and catalase significantly altered in brain tissue. Further, the studies carried out in our laboratory on fluoride toxicity in rodent models exerted effects on defense system leading to oxidative damage of tissue including brain. It is known that SOD is involved in removal of dismutase free radical in the body, whereas catalase helps in converting $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$ in cells and tissue. Hence, loss of antioxidant status in brain of treated rats in this study satisfied the altered brain functions due to fluoride ingestion. These studies are correlated with loss of normal histoarchitecture and $\text{F}^-$ burden in these neural areas of brain of ingested animals. Thus the investigations of the current study exhibit that $\text{F}^-$ increases free radical production and at the same time hamper the antioxidant enzymes viz., SOD and catalase, which perhaps make the cells much more susceptible to oxidative injury. In support of these data, Rao et al. (2009) and Purohit (2010) reported the loss of antioxidant enzymes including non-enzymatic components like GSH and ascorbate, making vulnerable to oxidative stress induced by other toxicants like arsenic, mercury and fluoride in a rat model.

Proteins

In the current study, the 60 days treatment of sodium fluoride (NaF) to rats resulted in a decrease in protein levels in both parts of the brain as well as in serum. The decline in the serum total protein levels in NaF treated experimental animals was also reported by others (Frost et al., 2003; Shashi, 2003; Zabulyte et al., 2007 and Rao et al., 2009). Fluoride seems to be responsible for inhibition of protein synthesis. The decrease in RNA content of rabbit brain observed during acute and chronic fluoride intoxication seems to be due to fluoride induced inhibition of protein synthesis by
Shashi et al. (1994). Fluoride has been reported to cause reduction in DNA and RNA and DNA/protein ratio, indicating probable disturbances in the process of transcription, translation, as well as mitotic cycles and chromosomal aberrations (Memon and Chinoy, 2000; Patel and Chinoy, 1997). Further decrease in protein levels could be attributed to their damage by singlet oxygen, often due to oxidation of essential amino acids, viz., methionine, tryptophan, histidine or cysteine residues (Halliwell and Gutteridge, 1989). Aldehydes, like malondialdehyde formed during lipid peroxidation can also react with –SH groups of protein to damage them (Halliwell and Gutteridge, 1989), eventually leading to their depletion. Thus, it is evident that the overall decline in protein levels could be the outcome of its disturbed synthesis due to fluoride treatment. Fluoride further is known to inhibit peptide bond formation to inhibit protein synthesis (Hoerz and McCarty, 1971; Shashi et al., 1987), which could subsequently lead to retard growth of animals fed with it.

Bano et al. (1996) have reported decrement in the soluble protein levels of mice on administration of NaF at a dose of 10mg/kg body weight for 30, 60 and 90 days. Similarly, total serum protein levels were decreased in adult rats after orally treated with NaF at the doses 10, 20 and 30 mg/kg b.w. for 90 days (Qujeq et al., 2002). Further, Grucka-Manczar et al. (2006) found significant changes in the levels of protein in rat serum and liver by fluoride (10 and 30 ppm) given in drinking water. Similar decrease in serum proteins was also accounted by Samanta and Bandyopadhyay (2012) on F⁻ intoxication in rats and concluded that excess fluoride water exposure caused altered blood cells and produce toxic effects on brain. Shashi et al. (1994) reported that fluoride has a specific effect on the synthesis of proteins in the brain, which may lead to degenerative changes in the cerebellar cortex. The decreased serum proteins as obtained in the present study would also cause
disturbance in osmotic balance of the body and might lead to oedema (Chinoy, 1991a,b; Chinoy et al., 1991c; 1992a; Chinoy et al., 1994a). One more possible reason might be due to its probable increased utilization in conversion to glucose during stress/toxic condition in the serum (Srivastava et al., 1981). The oxygen derived free radicals are also a major source for DNA damage, which can cause strand breaks and base alteration in the DNA. Therefore the reduction in protein content may be due to either direct effect of fluoride on protein synthesis or indirectly through DNA and RNA damage. The overall decrease in tissue proteins after the fluoride treatment would affect various enzyme activities and general growth of the body, which might have resulted a reduction in body and organ weights as listed in our study. Several reports from our laboratory are available to show that $F^-$ is an inhibitor for protein synthesis and their oxidation in animal tissues including human exposed to $F^-$ through drinking water (Chinoy, 1996; Bhatt, 2012; Vyas, 2012).

**Energy Status (SDH and ATPase)**

In the current study, the activities of SDH and ATPase were estimated. Succinate dehydrogenase (SDH) is an oxidative enzyme involved in the Krebs cycle. A significant decrease was observed in the activity of SDH in brain of fluoride treated rats. This would affect the conversion of succinate to fumarate and could cause a block in the Krebs cycle to produce ATP molecules. Moreover, SDH is a mitochondrial enzyme and its decrement in the activity indicates a possible alteration in mitochondrial structure and function as a result of fluoride ingestion. The net ATP productions probably reduced due to the alteration in mitochondrial function. According to the previous reports which are in consistence, the SDH activity was decreased in pectoralis and gastrocnemius muscles of mice (Chinoy et al., 1991c; Rao
et al., 2012) and in liver and kidney of fluoride treated golden hamsters (William and Sullivan, 1969; Chinoy et al., 1995; Missiaen et al., 1988). As SDH is a mitochondrial enzyme involved in oxidative metabolism, fluoride may cause changes in the structure and function of mitochondria (Chinoy and Patel, 1998). Earlier studies carried out by Rao et al. (2010), had also reported a decline in its activity in three regions of brain alternating their respective functions.

In the present investigation, SDH and ATPase levels were decreased significantly in brain of NaF treated rats. ATPase is important in the cell to convert ATP to ADP and iP. The later is necessary to phosphorylation of proteins necessary for metabolic processes. But in our study this enzyme is reduced in the brain regions reflecting their lowered energy metabolism by fluoride ions (Purohit, 2010). In support of our findings, the work from our laboratory and others have reported similar reduction in SDH and ATPase levels in various tissues at different dose and durations in different animal models (Chinoy and Sequeira, 1989a; Chinoy and Mehta, 1999a, b; Chinoy and Shah, 2004c; Chawla et al., 2008; Rao et al., 2009; Vani and Reddy, 2000; Mathur, 2012; Vyas, 2012; Bhatt, 2012). Rao et al. (2012) also reported the reduction in the activities of SDH and ATPase levels that led to alter energy status in the brain regions of mercury intoxicated rats leading to loss of neural functions. In this study, thus the losses of enzyme activities are justified by F⁻ feeding.

**Cholesterol and Lipids**

In this work, total cholesterol and total lipids were estimated. Dorfman et al. (1963) reported that cholesterol is a primary cell constituent, present in the nervous tissues. The present research exhibited an increase in total lipids in both parts of brain and serum, but cholesterol levels elevated in brain and decreased in serum after
sodium fluoride exposure to the rats for 60 days. The reason could be attributed to fluoride induced alteration in the rate of TCA cycle (Szincz and Forth, 1988) as a result of inhibition of important enzymes in carbohydrate metabolic pathway. Hence, carbohydrates are channeled towards fatty acid synthesis through alternative pathway to increase lipid synthesis. Therefore, increased levels of total lipids in both parts of brain of rats would be possible. Thus, accumulation of lipids in brain region is justified. The phospholipids are important for neurotransmission of impulses which are altered and also contributory to their accumulation in our report. Cholesterol, which is transported into the blood as lipoproteins, serve as a stabilizing component of cell membrane and as a precursor of bile salts and steroid hormone in our study. The elevated levels of cholesterol in the brain are also reported by other investigators from our laboratory earlier (Purohit, 2010). In agreement with our results, Sharma et al. (2004, 2006a) have reported decreased cholesterol level in serum after fluoride (3, 4.5, and 6 ppm) water treatment for 15 and 30 days. Tao et al. (2006) also investigated a decline in serum cholesterol content in sodium fluoride (150 mg/kg b.w.) treated pigs. Similarly, decrement in rat serum cholesterol and phospholipid were revealed by Samanta and Bandyopadhyay (2011). All the above data corroborate our findings of the present investigation.

**Transminases (SGPT and SGOT)**

The present study revealed elevated levels of SGOT and SGPT in serum of NaF treated rats. This might be related to the extensive alterations in histology of liver of treated animals. These changes might have accompanied by the release of hepatocellular enzymes and therefore caused increased levels of SGOT and SGPT in serum. The increased levels of SGPT and SGOT in serum can be related to cytotoxic effect of liver after NaF treatment to
mice (Zabulyte et al., 2007). Previous studies have also reported an increased serum transaminases in fluorotic rats and mice (Chinoy, 1991a; 1992; Chinoy and Patel, 1999; Chinoy et al., 1993a, b; Kour et al., 1981; Tsunoda et al., 1985), and endemic populations in villages of Mehsana district of North Gujarat, India (Chinoy et al., 1992a). Similarly, Barot (1998) and Mukherjee et al. (2003) also reported increased levels of transaminases in people residing in fluoride endemic areas of India. Likewise, Sharma et al. (2007b) reported that SGPT and SGOT levels increased significantly in serum of population subjected to fluoride (5.8 ppm) endemic areas of Rajasthan and animals treated with water having 5.8 ppm (Agrawal and Sharma, 2008). Trivedi et al. (2007a) also observed a significant increase in these levels in blood serum following sodium fluoride (6, 12 mg/kg b.w.) treatment to mice for 30 days. Correspondingly, all these data are in agreement with our present study that, fluoride affected functions of liver (Vyas, 2012). Liang et al. (2012) recorded significantly increased SGPT activity in serum of rabbits treated with high dose NaF, which is supportive with the results of earlier investigators (Blaszczyk et al., 2011; Kanbut et al., 2009). Samanta and Bandyopadhyay (2011) exhibited loss of SGPT and SGOT activity in liver indicate liver toxicosis.

**Acetyl cholinesterase (AChE) & Sialic acid**

In this study, the Acetyl cholinesterase (AChE) activity, which is a candidate biomarker for neurotoxicity and levels of sialic acid were documented in Wistar rats. AChE index is widely used for the detection of F⁻ toxic alterations in the brain tissue. Oral administration of NaF decreased AChE activity, a neurotoxicity indicator in both parts of brain and serum of rats. The process of synaptic transmission takes place with the help of the neurotransmitter, acetylcholine at the neuromuscular/synoptic junctions. The acetylcholine is rapidly degraded into acetate and choline moieties by
cholinesterase whose activity reflects on the neuronal status, which in turn influences nervous system of the animal. In the present study, the activity of acetyl cholinesterase declined significantly in the brain and serum by NaF treatment in rats. Fluoride is known to bind with great affinity to calcium which has an indispensable role in neurotransmission. The decrease in acetyl cholinesterase may be due to interference of F⁻ and Ca²⁺ interaction with the events associated with ACh receptor and complex synaptic transmission (Vani and Reddy, 2000). Several studies carried out in fluoride treated rodents have elucidated similar decrease in cholinesterase activity (Chinoy, 1992; 2002; Chinoy and Memon, 2001). As a result of inhibition of acetyl cholinesterase the substrate acetylcholine does not get hydrolyzed resulting in its accumulation in the synaptic junction. This might affect muscle contraction and synaptic information (Kumar et al., 2009). All these data are in support of the present findings. Thus the toxic effects of fluoride may lead to an altered release and/or utilization of acetylcholine, affecting the transmission of nerve impulse and may lead to neurotoxicity in rodents. Cholinesterases are enzymes, which hydrolyze esters of choline. In the present study, the reduced enzyme activity in brain (cerebrum and cerebellum) and serum was justified by NaF treatment. Inhibition of cholinesterase activity was observed in rats exposed for 3 months to arsenic trioxide (WHO, 1984) and in brain of NaF treated mice as compared to control (Vani and Reddy, 2000) in light of our data.

Sialic acid is a marker parameter of NaF induced toxicity in different animal tissues. Its levels are reduced by fluoride in serum and brain of rats in the present study. In support of our data, Chinoy and Sequeira (1989a) and Chinoy et al. (1994a) from our laboratory, reported the decrement in sialic acid levels in NaF treated rodents. Sialic acid, gluco amino glycans (GAG) and their ratio are important markers for early detection of fluorosis.
symptom. Hence, reduction in sialic acid levels in serum might be an indicative of soft tissue fluorosis in our investigation.

**Fluoride levels**

In this study, fluoride levels were estimated in different regions of the rat brain. Fluoride accumulation in brain (cerebrum and cerebellum) also supported characteristic of neurotoxicity of it. Fluoride is known to pass blood-brain barrier for its accumulation in brain (Chinoy, 2002), which supported our data in this study. Chinoy and Mehta (1999a,b) have documented a significant enhancement of fluoride in mice serum urine, testis cauda epididymis, liver and kidney, which indicate F\(^{-}\) accumulation in these organs and would affect their metabolism, structure and functions. Since, the regions of the brain are devoid of a blood brain barrier including the median eminence of hypothalamus, it may be easier for fluoride to reach the hypothalamus. Chawla and Rao (2012) reported high levels of fluoride in the brain of mouse in support of the investigation.

In this study, the elevated fluoride levels in both the regions of brain in NaF treated rats revealed an increased neurotoxicity and altered internal milieu of brain. Whitford et al. (1990) and Krishnamachari and Lakshmaiah (1975) have also been reported that fluoride excretion depends on total daily consumption of fluoride, the degree of renal efficiency, and interaction of fluoride with other ions, urinary flow and pH as well as previous exposure to fluoride. Hodge and Smith (1970) reported that measurement of the urinary fluoride could be regarded as the best indicator for intake of the element. The retained fluoride in the serum, thus would affect the general body metabolism in these individuals, probably by altering soft tissue function including brain. Consumption of high fluoride intake leads to the high serum fluoride
levels. Patel and Chinoy (1997) from our laboratory have also reported increased tissue fluoride levels in animals treated with different fluoride concentrations in support of our data. This accumulation might result to neuro degeneration of brain regions affecting their functions in this study.

Histological studies

In the current research, histological studies were performed in cerebrum and cerebellum of rat brain. Fluoride is known to cross blood-brain barrier and accumulates in various regions of brain affecting its functions. It also affects neuro endocrines of C.N.S especially hypothalamus and pineal leading to less production of their hormones. Accordingly their manifestations are observed in their target tissues in F- exposed/fed rats.

The transverse section of cerebrum of control rat elucidated well developed cerebral cortex. The cerebrum consists of outer folded cortex region of gray matter covering the inner region of white matter. Neurons with particular functions in cerebrum are connected and arranged in form of six layers. The outermost four layers together are called as molecular layer consists of pyramidal cells and the sixth layer named as multiform comprising of fusiform cells. The transverse section of cerebellum of control rat showed a well developed molecular layer, Purkinje cell layer, granular layer and core of white matter.

The present study revealed alterations in the histological structure of rat cerebrum and cerebellum, resulting from sub-chronic administration of sodium fluoride. In correspondence of our studies, the histological structure of the cerebral hemisphere was disrupted particularly the purkinje cell layer (Shivarajashankara et al., 2002). In control rats, purkinje cells are present in a single layer between outermost
molecular and inner granular layers of the cerebellum. But in high dose treated group, perkinje cells are arranged in multiple layers rather than a single, which was evidenced by significant increase in the mean thickness of the purkinje cell layer in fluoride treated groups, compared to the controls. Similar neurodegenerative changes were also observed by previous investigators in brain of rats by fluoride treatment (Shivarajashankara et al., 2002). From our laboratory, Chinoy and Shah (2004b) have documented that histology of the cerebral hemisphere was altered by NaF and/or \( \text{As}_2\text{O}_3 \) treatment for 30 days revealing vacuolization and pyknosis of nuclei of neurons.

Other histopathological changes include, occurrence of neuron degenerative changes, chromatolysis, neuronal loss, fatty infiltration, and altered perkinje cells in cerebral hemisphere. It may be inferred, therefore, that during NaF intoxication, \( \text{F}^- \) crossed the blood-brain barrier to accumulate and damaged the nerve cells of brain. Other studies had also reported, swelling and irregular staining of the Nissl substance, and pycnosis of neurons in experimental animals subjected to \( \text{F}^- \) intoxication (Trivedi et al., 2012) in support our data; The other histopathological alterations indicate occurrence of neuron degenerative changes, chromatolysis, pycnotic nuclei, fatty infiltration, and vacuolization in cerebrum cerebellum and medulla oblongata.

It is well documented that \( \text{F}^- \) exposure can induce the loss of neuronal cell bodies and damage synaptic structures in different regions of brain as well as can cause an inhibition of enzyme activity leading to decrease in expression of membrane potentials. Rao et al. (2012) findings corroborate our observations that alterations in brain of NaF exposed rats. Thus, alterations in biochemical profiles can be correlated with histopathological changes of brain (cerebrum and cerebellum) regions of rats by fluoride poisoning, further supported by its accumulation in it.
Antioxidants

Melatonin

In order to study mitigative effect on fluoride induced toxicity in brain and blood of rats, the animals were supplemented with two antioxidants viz., melatonin and amla separately and the same parameters were performed as the case of fluoride treatment.

Melatonin, a biological antioxidant produced by pineal gland, is very well known to its antioxidant, anti-inflammatory and anti-hypertensive properties. It functions as an electron donor to highly toxic hydroxyl free radicals to detoxify them. In addition, melatonin has been recognized to protect cells, tissues and organs in both *in vivo* and *in vitro* experiments against oxidative damage provoked by variety of free radical generating agents and processes like CCl₄, cyanides, ionization radiation, glutathione depletion, etc. Melatonin as an antioxidant is more efficient in protecting membrane lipids, nuclear DNA and possibly cytosolic proteins from oxidative injury. It also has been reported to ameliorate the activities of enzymes viz., glutathione peroxidase, glutathione reductase, superoxidedismutase, glucose-6-phosphate dehydrogenase, and nitric oxide synthases, which improve the total antioxidative defense capacity of the organism. Though most of the studies have used pharmacological concentrations of melatonin to protect against free radical damage, in a few studies physiological levels of this indole have been proved to be beneficial against oxidative stress (Reiter et al., 2007).

In our study, melatonin supplementation revealed remarkable resurgence in gravimetry, SDH, and ATPase indices for energy metabolism, proteins, transaminases, lipids, antioxidant indices, AChE activity, F⁻ levels as well as histology are mitigated comparable to control values in brain and blood of treated rats.
in our study. Several reports have indicated the protective effect of melatonin against many well-known toxicants in accordance to our data. The antioxidant activity of melatonin was reported by El-Missiry et al. (2007) against gamma irradiation induced oxidative stress and tissue damage in vitro. Melatonin can significantly inhibit the production of reactive oxygen species (ROS) like superoxide free radicals, H$_2$O$_2$ and nitrite radical generation by activated macrophages, which play an important role in inflammation. Electron donation by melatonin is not only an aspect of direct radical scavenging, but additionally represents the basis for formation of the protective metabolites Nl-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and Nl-acetyl-5-methoxykynuramine (AMK). Recent reports on mitochondrial metabolism indicate that melatonin as well as AMK are capable of supporting the electron flux through the respiratory chain by preventing the breakdown of the mitochondrial membrane potential, and of decreasing electron leakage, thereby reducing the formation of superoxide anions (Hardeland, 2005), it was proved to prevent an incipient necrotic neuronal lesions by Mayo et al. (2003), suggesting that this hormone may provide a common protection against cell death, induced by high doses of the neurotoxins. All the above results confirm that, melatonin and its metabolites play an important role in amelioration of fluoride induced neurotoxicity and are useful for the fluoride exposed population as one of the most defensive neuroprotection molecules. In our laboratory, melatonin supplementation brought about a significant mitigation on fluoride induced toxicity in variety of tissues via its free radical scavenging cascade mechanism in animal models to confirm these data.

In vitro, work done on ameliorative effect of melatonin, there are plentiful evidences that indicate its radioprotective role (Vijaylaxmi et al., 2004; Kopjar et al., 2006). It is also suggested the ameliorative effect of melatonin on mercuric chloride
induced changes in cell cycle kinetics, SCEs and CAs in cultures for 24 hrs. Study by De Salvia et al. (1999) displayed clearly the reduction in frequency of induced SCEs and CAs due to oxidative stress generated by H₂O₂ and cyclophosphamide, after melatonin addition, corroborating our results. In support of our report, melatonin is able to reduce significantly sister chromatid exchange frequencies in human lymphocytes in vitro induced by lead- and 5-aminolevulinic acid (ALA), which are known to exert genotoxic effect via ROS generation, pointing towards the antioxidative effect of it (Ustundag and Duydu, 2007).

Melatonin has been shown to protect DNA from damaging effects of ethyl alcohol in brain, heart and skeletal muscle as well as in the liver (Mansouri et al, 2001). This study suggested that the protective action of melatonin is due to its ability to penetrate readily into mitochondria and thereby inducing anti oxidant enzymes. Bagchi et al. (2004) illustrated the mitigative action of melatonin against naphthalene induced oxidative stress and DNA damage in cultured macrophage. It reduces oxidative damage to all varieties of macromolecules under conditions where free radicals are produced. This antioxidant cascade greatly increases the free radical scavenging and antioxidant capacity of melatonin. Along with this, melatonin is highly efficient in reducing lipid peroxidation than the classical antioxidant like amla. Moreover, the peroxyl radicals, which are produced during the oxidation of polyunsaturated fatty acids, are also scavenged by melatonin, with efficiency greater than that of amla and twice as effective as trolox, a water-soluble vitamin E (Pieri et al., 1995), thus protecting the biomolecules from hydroxyl and peroxyl damage. All these functions of melatonin could attribute to its protective action against the fluoride induced haematological and neurotoxic effects, as observed in the present study.
**Amla**

Amal (Indian gooseberry), scientifically named as *Phyllanthus emblica* (syn. *Emblica officinalis*), Phyllanthaceae family is the richest possible natural source of vitamin 'C' (ascorbic acid). It is a medicinal plant with full of therapeutic properties. Medicinal plants are natural resources yielding valuable herbal products which are often used in the treatment of various ailments (Dulger and Gonuz, 2004). According to Tarwadi and Agte (2007), the topic of amla components is debatable, though it consists of maximum (445 mg/100 g) ascorbic acid. It also contains essential elements for keeping our body healthy and resistant to diseases and infections. This fruit has an ample amount of proteins, fats, mineral salts, carbohydrates, calcium, phosphorus and iron. It is not these components individual effect but their collective effect makes amla, one of the most antioxidant fruits. Another in vitro study shows that Amla extracts induce apoptosis and modify gene expression in osteoclasts involved in rheumatoid arthritis and osteoporosis (Letizia et al., 2008). The fruits of *Emblica officinalis* are rich in tannins. The fruits have 28% of the total tannins distributed in the whole plant. Tannins in amla include Phyllaemblicin B, emblicanin A (37%), emblicanins B (33%), punigluconin (12%) and pedunculagin (Sampath Kumar, 2012). The fruit also has Phyllemblin. Amla is capable of curing many diseases and ailments alone if it is taken in the right combination. Amla has antioxidant, cytoprotective (Bandyopadhyay et al., 2000) hepatoprotective (Gulati et al., 1995) anti-hepatitis, anti-cancer, anti-tumor activity (Jeena et al., 2001) and also have antimutagenic activity (Kaur et al., 2002). It is used for constipation, peptic ulcer and scurvy, immunomodulator (Xia et al., 1997). It is also useful for burning sensation in abdominal and cardiac regions and epigastric pain.
One week prior administration of Vitamin C (10mg/kg) and Gallic acid (20mg/kg) to sodium fluoride intoxicated rats revealed significant reduction in the thiobarbituric acid reactive substances and recovery in reduced glutathione, super oxide dismutase and catalase activity in brain tissue was noticed, compared to the treated rats (Nabavi et al., 2012). Ailani et al. (2009) documented the increased values of serum fluoride and urinary fluoride, were brought down with the supplementation of calcium, vitamin C and vitamin D. It is suggested that as the level of fluoride in drinking water increases the excretion of fluoride also increases in urine and supplementation with calcium, vitamin C and D further increases the renal clearance of fluoride. Mohammad et al. (2012) concluded that the TBARS levels in fluoride intoxicated rat neural tissues, significantly increased compared to the control group. Administration of silymarin and vitamin C reduced the levels similar to that of control groups. The elevated activities of serum AST and ALT in fenvalerate treated rats were significantly reduced in the animal groups treated with vitamin C (Hussein et al., 2012). Oral administration of vitamin C to Fenvalerate treated rats significantly restored total hemoglobin and glycosylated hemoglobin levels. Free radical reacts with lipids and causes per-oxidative changes that result in enhanced lipid peroxidation (Girotti, 1985). Supplementation with vitamins C and E during the withdrawal period cause significant recovery in liver parameters, which is attributed to their antioxidant action and their synergistic action (Nair et al., 2004), since the free radical scavenging property of vitamin E is enhanced in presence of vitamin C. Similarly, Chinoy et al. (2004d) concluded that ascorbic acid (vitamin C) and vitamin E are capable of completely, or almost completely, mitigating liver toxicity in mice induced by fluoride and aluminium. The mechanism of the ameliorative role of vitamin C and E in mitigating toxicity of fluoride and arsenic (Nair et al., 2004), fluoride and
aluminium (Chinoy and Patel, 2000; Chinoy et al., 2004c) toxicity in mice has been presented earlier. It is likely that both vitamins C and E act synergistically to activate several enzymes (Chinoy et al., 2004d; Chinoy and Sharma, 1998), since α-tocophero (vitamin E) interacts with vitamin C, which enhances its radical-scavenging activity (Bender, 1992).

Navabi et al. (2012) demonstrated increased lipid peroxidation and decreased superoxide dismutase activity in erythrocytes of sodium fluoride intoxicated rats; Rats pretreated with ascorbic acid for 7 days before intoxication by sodium fluoride showed significant reduction in the malondialdehyde level and elevation in superoxide dismutase activity and prevented change in catalase activity by toxicant. Besides, different antioxidants like ascorbic acid and vitamin E are also known as scavengers of these reactive oxygen species (Rice-Evans et al., 1991; Liu et al., 1995). Similarly, Najla et al. (2011) reported the reduced activities of SOD, CAT and GPX in liver and brain of lindane treated rats, as compared to controls. These changes were alleviated when the rats were pretreated with either vitamin E or C. Shivarajashankara et al. (2002) exposed their findings that malondialdehyde (MDA), the marker of lipid peroxidation, was elevated in the brain of rats treated with 100 ppm fluoride followed by decreased levels of GSH and ascorbate. However, the activity of glutathione peroxidase (GSH-Px) was elevated significantly in these fluoride-treated rats.

The mitigative effect of ascorbic acid was also investigated in reproductive and non-reproductive tissues of fluoride intoxicated animals and people exposed to higher levels of fluoride in drinking water (Chinoy et al., 1991a; Sharma et al., 2008). It is documented that the vitamin has exerted better prophylactic effect to trounce fluoride alone and combination in animals and human. In the present study, the protective role of amla extract was evaluated in body and organ (cerebrum and...
cerebellum) weights in addition with enzymatic and non-enzymatic indices following its supplementation with NaF to rats for 60 days. Vasant and Narasimhacharya (2012) have investigated the ameliorative efficacy of amla against fluoride intoxication in rats. In this study, amla exhibited mitigation in organ weights, LPO in the brain regions and most of the hematological indices. The levels of total proteins, cholesterol, lipids mitigated in serum as well as in brain regions and SGPT & SGOT activities in serum by it. AChE and sialic acid levels are mitigated in brain and serum. Energy metabolic parameters like SDH and ATPase were effectively ameliorated by ascorbic acid in the rat brain. SOD and catalase were remarkably mitigated by amla in the brain of F- treated rats. Further, Vasant and Narasimhacharya (2012) evaluated recovery in the levels of total proteins, SDH, ATPase due to the antitoxic affect of amla against fluoride toxicity in rats which are in agreement with our findings. Verma and Chakraborty (2008) documented that the administration of amla fruit aqueous extract (2 mg/day/animal) for 45 days along with ochratoxin caused significant amelioration in the achratoxin induced reduction in DNA, RNA and protein contents in the liver and kidney of mice. Our studies also potentially suggested that all ingredients of amla, expressed protection against fluoride induced toxicity due to their combined free radical scavenging properties.

Withdrawal studies

In this study, withdrawal effects were examined in rat brain and blood for all the indices mentioned in treated groups. In our withdrawal study, NaF was fed for 60 days and the treatment was withdrawn for another 30 and 60 days. The data of this investigation revealed that upon withdrawal of treatment, an insignificant/no recovery was obtained in most of the parameters studied. Same results are reported by others in
mice and rabbits (Chinoy and Sequeria, 1989a,b; Chinoy and Patel, 1998a; 1999; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Memon, 2001; Chinoy, 1991b; 1995; 1997a,b; 1994d; Jhala et al., 2004). The above data suggest a longer withdrawal period would be necessary as reversibility depends on duration, dose, response of tissues and other environmental factors. Hence, supplementation of antioxidant would yield better results, as presented in this study.

PART II: IN VITRO STUDY

Till date, a lot of research is available on the haemolytic effects of fluoride and its amelioration by natural antidotes. Like the other tissues of various organs in the body, a well known connective tissue (blood) and its components are also key target regions for most of the toxicants like fluoride, mercury, arsenic and lead etc. In the present study, the hemolytic effect of fluoride and its amelioration by melatonin and/or amla have been investigated in red blood corpuscles (RBC) of human peripheral blood. In addition, Percent hemolytic effect of fluoride, in the form of sodium fluoride (NaF) at different doses and percent amelioration of melatonin and/or amla in fluoride induced blood toxicity were accounted.

The results of our study demonstrate that NaF in the range of 50 to 500μg/ml in the RBC suspensions affected membrane permeability leading to influx of water into the cells thereby causing haemolysis. It might also be due to an increase in lipid peroxidation and oxidative damage. Moreover, F' can inhibit or activate various functions in the living systems in different ways. Neutrophils are affected by F' exhibiting increased oxygen intake and production of superoxide anions along with decreased phagocytic ability (Bober et al., 2000). Fluoride also affects erythrocyte membrane transport system, e.g. inhibits K⁺-Cl⁻ co-transport (O’Neill, 1991).
Grabowska and Guminska (1985) reported that the alterations in cation pump activity caused by F⁻ as a direct inhibition of Na⁺-K⁺-ATPase. This can be observed in erythrocyte shadows in people exposed to fluoride under both in vitro and in vivo conditions (Guminska et al., 1994). F ions in vivo have further been shown to decrease ATP concentration in erythrocytes (Guminska et al., 1985). Tan et al. (2002) and Qi et al. (1997) have observed that, under conditions of high oxidative stress in vivo and in vitro melatonin has proved superior to vitamins C and E in reducing oxidative damage. As observed in our study, addition of melatonin to RBC suspensions significantly reduced the rate of haemolysis compared to NaF alone sets in a dose dependent manner. Similarly Verma et al. (2006) also documented dose dependent increased haemolytic activity of fluoride in human erythrocytes. Further Alinezhad et al. (2011) investigated that fluoride induced haemolysis in rat RBC with increased doses of toxicant in light of our data. The in vitro and in vivo cytotoxic effects of fluoride in sperm cells have been demonstrated by various researchers in mice, rats and human. These authors elucidated that fluoride brings about an increased free radical formation by continuous breakdown of unsaturated fatty acids and proteins of membrane leading to its loss of permeability of cells. Hence, the cellular toxicity is induced losing their normal physiological stability of membranes in sperm cells (Chinoy et al., 1991a; Rao and Gangadharan, 2008). Further, Rao and Gangadharan (2008) reported that sperm cells in in vitro study, revealed alterations in energy metabolism and antioxidant indices followed by increased sperm anomalies. In vitro studies, in boar serum exposure with fluoride, loss of sperm acrosomal contents led to a loss of fertilizing capacity of sperm. Similarly addition of fluoride to human sperm in vitro induced sperm immobilization effect gradually. Many reports from our laboratory and others studied that fluoride ions are genotoxic in vitro. Human blood
culture studies reflected on genotoxic potential of fluoride ions by inducing changes in cell cycle kinetics, including mitotic index, frequencies of micronuclei, sister chromatid exchanges and DNA damage (Rao and Tiwari, 2006; Tiwari, 2008; Rao et al., 2010).

Melatonin supplementation (5 and 10 μg/ml) exhibits scavenging action at both physiological and pharmacological levels. It protects membrane lipids, nuclear DNA, and protein from oxidative damage induced by a variety of free radical generating agents (Reiter et al., 1995; Wakatsuki et al., 2001; Vijayalakshmi et al., 2004). Reduction in hemolysis by addition of melatonin could be attributed to scavenging hydroxyl radical, stimulating antioxidative enzymes, inhibiting capacity of prooxidative enzymes. Studies also reveal that melatonin eliminates the decomposition products of peroxynitrite (ONOO−) including OH•, NO2• and the carbonate ion radical (CO3•−) in the presence of physiological carbon dioxide concentrations (Zhang et al., 1999). There are also indications that melatonin is efficient in reducing oxidative stress. Recently, Bharti and Srivastava (2011) have reported that melatonin is capable of improving the serum Na+ level and therefore may prove beneficial in fluid electrolyte imbalance conditions. Gavella and Lipova (2000) found that the antioxidative property of melatonin suppresses experimentally induced lipid peroxidation in sperm membrane by protecting the integrity of the membrane and safeguarding against motor assembly deformities exerted by mercury ions (Ressmeyer et al., 2003). Possibly the efficiency of melatonin in reducing the oxidative stress may be due to it and its metabolites that are produced during its scavenging actions. These metabolites, i.e. cyclic 3-hydroxymelatonin (cyclic 3-OHM), N1-acetyl-N2-formyl-5 methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) also seem to be very efficient oxidative free
radical scavengers (Tan et al., 2003 and Reiter et al. 2004). Thus second and third generation metabolites of melatonin might well contribute to the ability of the parent molecule in protecting against oxidative burst. In this fashion, melatonin and its metabolites might be able to neutralize numerous adverse reactions exerted by certain toxicants like mercury, arsenic etc., (Avani and Rao, 2009). As accounted here, melatonin appears to be a potent antioxidant; therefore, its use under in vitro conditions provides significant protection against haemolysis induced by NaF.

Aqueous amla extract from Himalaya products (Bangalore) supplements at doses of 5 and 10µg/ml to fluoride at various concentrations exposed cultures reduced haemolytic activity of RBC being more at 10 µg/ml level, similar to melatonin. The ameliorative effect of this extract is dependent on its ingredients. In vivo studies carried out by Karadeniz and Altintas (2008) in mice found that panax ginseng application encountered anemia caused by fluoride administered through drinking water to mice. The antioxidant properties of ginsenosides like Rg I of panax ginseng are responsible for its protection. It is known to have stimulatory effects on immune system and bone marrow cell haematopoiesis. Protective effects of Primula heterochroma Strapt extract of various fractions are contributory to individual mitigative effect against fluoride induced haemolysis in rat erythrocytes. These extracts of plant contained polyphenolic flavonoids which are able to cause protective effect. All fractions viz., ethyl acetate, n-hexane and water possessed anti haemolytic action (Alinezhad et al., 2011) by inhibiting/scavenging free radicals generated by the toxicant, fluoride. Nabavi et al. (2012) mentioned that Silymanin, a potent antioxidant agent, isolated from milk thistle seeds of Silybum marianum exerted amelioration on fluoride induced oxidative stress in rat erythrocytes by stimulation of antioxidant enzymes like SOD, CAT and antioxidant non-enzymatic components like GSH by
counteracting LPO levels. This seed contains three main flavonolignans, main being silybirs to support our data that herbal products are important to alleviate fluoride induced toxicity in both in vitro and in vivo conditions. In support our observations, Verma et al (2006) demonstrated that black tea extract supplementation to fluoride added human RBCs, negated haemolytic activity of the toxicant. Tea extract is known to possess polyphenols of different types like catechine gallate that provide protection against fluoride exerted haemolysis by inhibiting free radical damage. Similar way, anticytotoxicity and antigenotoxic effects are counteracted by numerous antioxidants like melatonin, curcumin, andrographolesides, and vitamins in various in vitro and in vivo systems (Gangadharan, 2008; Tiwari, 2008, Gopalakrishnan, 2008). In our present study, amla extract (Emblica officinalis) contains ascorbate, flavonoids, Glutamic acid, proline, aspartic acid, alanine, lysine and polyphenols like emblicanin A and emblicanin B which are potential antioxidants. These antioxidant components, mainly ascorbate (highest one) could play a role in addition to others to exert alleviation on fluoride induced haemolysis in light of above results. Numerous reports are available that ascorbate acts as anti-stress factor and is known to participate in oxido-reduction and detoxification processes to stimulate cellular physiological activities. Combined co-supplementation of melatonin and amla to fluoride exposed RBCs, the anti haemolytic activity in our study was better, which could be attributed to the additive, synergistic and both affect these active components. Hence, melatonin and/or amla extract are potent antioxidants and counteract fluoride exerted haemolytic effect in human erythrocytes effectively.