CHAPTER IV

DISCUSSION
PART - I

IN VIVO STUDIES

The in vivo studies on effects of sodium fluoride and aluminium chloride on male mice (Mus musculus) and the ameliorative effects by some vitamins and calcium.

The present investigations were carried out to explore the in vivo effects of administration of sodium fluoride (NaF) and/or aluminium chloride (AlCl₃) on the structure and physiology of some reproductive and non-reproductive organs of adult male albino mice (Mus musculus) of Swiss strain. Sodium fluoride (NaF) was administered orally at a dose of 10 mg/kg body weight and aluminium chloride at a dose of 200 mg/kg body weight. The dose used was based on the LD50 value of fluoride, i.e. 54.6 mg F/kg body weight in male mice (Pillai et al., 1988) and aluminium chloride i.e. 4 g/kg body weight in male mice (Chinoy and Bhattacharya, 1996; 1997). Oral administration was selected, since drinking water is the major source of fluoride and aluminium is found in major foods, beverages and pharmaceuticals as well as mineral water, etc.

The various parameters studied at the end of treatment were body weight and organ weights of testis, epididymides, vas deferens, liver and brain. In addition, some specific parameters in testis viz., cholesterol, 3β and 17β hydroxysteroid dehydrogenase activities and serum testosterone levels were investigated to study the alteration in
steroidogenesis. Similarly, certain specific parameters of cauda epididymal spermatozoa viz., sperm motility, count and viability were studied. The androgen dependent parameters like sialic acid in the epididymis was also investigated. To study the effects of fluoride and aluminium on oxidative/energy metabolism, activities of succinate dehydrogenase in testis, epididymides, liver and gastrocnemius muscle as well as of adenosine triphosphatase in epididymides were investigated.

In order to investigate the effects of fluoride and aluminium on carbohydrate metabolism, the concentration of glycogen and phosphorylase activity in the vas deferens, liver and gastrocnemius muscle were determined. The levels of DNA and RNA in testis, epididymides, liver, gastrocnemius muscle and cerebral hemispheres were evaluated to study the impact on nucleic acid metabolism. To find out the effect of fluoride and aluminium on the protein metabolism, levels of protein were determined in testis, epididymides, vas deferens, liver, gastrocnemius muscle, cerebral hemisphere and serum. To evaluate free radical induced cell injury by fluoride and/or aluminium, the activities of some antioxidant enzymes, viz., superoxide dismutase, catalase, glutathione peroxidase and levels of lipid peroxides, glutathione and ascorbic acid were determined in the testis, cauda epididymis, liver, gastrocnemius muscle and cerebral hemispheres. Some marker enzymes for liver toxicity viz., serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) were investigated. Cholinesterase activity in liver and gastrocnemius muscle was also investigated. Fertility rate was studied during the course of the investigation.

In a different set of experiments, the treatments were withdrawn after 30 days of
NaF + AlCl₃ ingestion in order to study the reversibility of the induced effects if any, upon cessation of treatment.

In view of fluoride induced toxicity, the therapeutic effects of calcium and vitamins (C and E) were also explored in the light of earlier work.

Aluminium and fluoride individually are considered potential health hazards. Studies on their combined toxicity have come up in recent years and there is a great deal of controversy as to whether these chemicals together have a synergistic effect or are antagonistic to each other. Aluminium is known to decrease the intestinal absorption of fluoride and to increase its excretion in humans, thereby reducing its toxicity (USDH & HS, 1997). However, Allian et al. (1996) have demonstrated that the absorption of aluminium could be enhanced by fluoride in rats and mice. Yet another study suggests that the formation of aluminium and fluoride complexes may alter the distribution of body aluminium and thus play a role in the development of various diseases associated with aluminium toxicity (Stevens et al., 1987). Reports by Dai et al. (1994) have shown that the combined effects of aluminium and fluoride aggravated toxicity in blood and femur bone of male chicks. According to Banks and Kastin (1983) neuroendocrine function may be more severely impaired when aluminium is combined with fluoride and may thereby be the mechanism for the altered behaviour. Xiao et al. (1992) in their study on activities of fifteen enzymes in jejunal mucus membrane observed that fluoride (F) alone chiefly inhibited the metal activated enzymes, while, aluminium (Al) mainly depressed the activities of enzymes of energy metabolism. Al and F both together demonstrated a strong antagonism and inhibited these enzymes more significantly. Aluminium fluoride mimics
the action of many neurotransmitters, hormones and growth factors. They also affect the activity of a variety of phosphatases, phosphorylase and kinases (Strunecka and Patocka, 1999). Although, much work has been done on the toxic effects of fluoride and aluminium administered alone, there is still need to study their combined toxicity and if it is reversible or otherwise by the use of therapeutic agents.

In the present study, treatment of NaF, AlCl₃ and NaF + AlCl₃ brought about reduction in body weight of male mice. Previous reports from our laboratory have also found decline in the body weight of male and female mice and rats due to ingestion of fluoride and aluminium (Chinoy and Sequeira, 1989a; Chinoy et al., 1991a,b,c; 1992b; 1993b; Chinoy and Bhattacharya, unpublished observation) in mice, rats and rabbits.

A 19% decrease in maternal body weight gain was observed in pregnant Sprague-Dawley rats fed 13, 26 or 52 mg Al/kg/day as aluminium nitrate via gavage on gestation days (USDH & HS, 1997). Nephrectomized rats injected subcutaneously with Al, F or both in combination for 30 days showed a significant impaired body weight regulation (Stevens et al., 1987). Singh et al. (1963) also obtained a general decrease of body weight in fluoride exposed individuals in Punjab. The results of the present study are in agreement with the above results. A reduction in the body weight of animals treated with aluminium and/or fluoride could be possibly due to low intake of food.

A significant decline in the weight of testis, epididymis, vas deferens, liver and brain was observed in the present study following the treatments of NaF or AlCl₃ alone and in combination for 30 days. Similar results were found by fluoride treatments at different doses and duration in male and female rodents (Chinoy and Sequeira, 1989a;
Chinoy and Patel, 1996; 1998; Chinoy and Sharma, 1998; Chinoy et al., 1991c; 1992b; Patel and Chinoy, 1997). Agrawal et al. (1996) showed decrease in testicular weight in rats by 50 mg Al/kg body weight for 5-15 days. Significant decrease in testicular and epididymal weights occurred in male mice given 100 or 200 mg/kg/day of aluminium nitrate (Llobet et al., 1995). However, no change in weight of brain, liver and spinal cord of mice was obtained by 500 and 1000 μg aluminium lactate in the diet (Golub et al., 1995).

The decline in the organ weights as obtained in the present study may be attributed to an increase in tissue accumulation of aluminium and/or fluoride and decreased food intake. The decline in tissue protein levels (as described in the later part of the discussion) could also have contributed to the decrease in tissue weights.

The changes in the body and organ weights in this study underlines the necessity for seeking other, more sensitive indices of metabolic disturbances caused by chronic exposure of fluoride and aluminium which are discussed further in this chapter.

EFFECT ON PROTEIN METABOLISM

The treatments of sodium fluoride and aluminium chloride alone and in combination to mice resulted in decline in protein levels in all the tissues investigated viz., testis, vas deferens, epididymis, liver, muscle, brain and serum of male mice. Similar results were noted by Kathpalia and Susheela (1978) in various tissues and organs of rabbits treated with fluoride. Shashi et al. (1987) also reported a significant decline in acidic, basic and total proteins in stomach of rabbits treated with NaF for 100 days.
Several studies by Chinoy and co-workers have obtained a decline in protein levels of various soft tissues and serum of rats, mice, rabbits and guinea pigs treated with NaF at different doses and duration (Chinoy, 1991a,b; 1992; Chinoy and Sequeira, 1989a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Patel, 1999; Chinoy and Memon, 2000; Chinoy et al., 1991a; 1993a,b; 1994c; 1995; 1997a,b; Patel et al., 1994). The testicular and epididymal protein of rats showed reduction of some proteins, loss of others but induction of some new proteins which were not present in the control animals after fluoride treatment (Chinoy et al., 1995; 1997a).

Fluoride affects the rate of cellular protein synthesis (Holland, 1979) and impairs polypeptide chain initiation (Godchau and Atwood, 1976). These factors might be responsible for the decline in protein levels in a wide range of tissues by fluoride ions.

Jagannatha Rao (1992) reported that aluminium ion preferentially interacts with amino acid based on the number of -COOH moiety and ionizable R-groups. In plasma, aluminium is predominantly bound to transferrin and albumin wherein binding with albumin is weaker than transferrin (Greger, 1993). This binding may make proteins less available to the tissue for the normal processes in the body and hence result in various other disturbances. The present study is in agreement with the above data regarding fluoride or aluminium induced decrease in protein levels of all the tissues studied. Similar mechanism might be responsible for the reduced protein concentration in combined treatment of fluoride and aluminium in this study as well as reported earlier (Chinoy and Patel 1999; Chinoy and Memon, 2000). It is evident that the overall decline in protein levels could be the outcome of its disturbed metabolism due to the treatment of NaF +
A1C13 which could subsequently lead to decline in activities of several enzymes in the affected tissues, their secretions as well as probably the various receptors in the cells.

Decline in serum protein by NaF and/or AlCl₃ treatments would affect the osmotic balance and cause water retention or other adverse effects in the body, since, it is regulated by plasma proteins (Carola et al., 1992; Chinoy and Patel, 1999; Chinoy and Memon, 2000).

EFFECTS ON CHOLESTEROL METABOLISM AND STEROIDOGENESIS

Increase in cholesterol levels in testis and serum was noted in the present investigation by fluoride and/or aluminium treatments indicating alterations in its metabolism. These were correlated with decrease in the activities of 3β hydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase which would affect testicular steroidogenesis. Studies by Chinoy and associates (Chinoy and Bhattacharya, 1996; 1997; Chinoy and Patel, 1998b; Chinoy and Memon, 1998) have shown that NaF, AlCl₃ or NaF + AlCl₃ treatments to male and female rats and mice also caused similar changes in their testis and ovary suggesting alterations in steroidogenesis. This was accompanied by a decrease in serum testosterone and estradiol levels which could affect the structure and functions of all androgen and estrogen dependent reproductive organs (Chinoy, 1992; 1996; Narayana and Chinoy, 1994a; Chinoy and Mehta, 1999a).

A hypercholesterolemic effect was noted following chronic exposure of aluminium (25 mg/kg body weight) in the brain of monkeys (Sarin et al., 1997). Rao and Susheela (1979) also noted a decrease in the activity of 3β HSD in adrenal gland of rabbits treated
with fluoride. This implies that in fluoride toxicity, adrenal steroidogenesis would also be impaired.

Shashi (1992a,b) reported that fluoride interferes with the lipid metabolism in brain of fluoridated rabbits as well as influenced metabolism of total lipids, triglycerides and cholesterol in their liver. The high levels of lipids in experimental animals may be in response to fluoride toxicosis which strongly indicates an imbalance between the synthesis and utilization of lipids.

Different dietary concentrations of aluminium given to broiler chickens for 35 days resulted in an increase in serum cholesterol levels but a decrease in those of triglycerol (Szilagyi et al., 1994). A similar increase in serum cholesterol was noted in monkeys treated with aluminium and in guinea pigs following fluoride treatments (Vatassery et al., 1980; Sarin et al., 1997). However, a decrease in serum cholesterol of guinea pigs was found by Townsend and Singer (1977) by fluoride treatment.

In the present study, serum cholesterol levels were also found to increase following fluoride, aluminium and their combined treatments which induced a hypercholesterolemic condition. This could lead to atherosclerosis or cardiac problems. Susheela and Kharb (1990) have reported ectopic calcification of rabbit aorta treated with NaF. Studies in fluorotic individuals of Mehsana District of North Gujarat, India, revealed no significant changes in serum cholesterol or serum testosterone ruling out the possibility of hypo/hypercholesterolemia at earlier stages of the affliction (Chinoy et al., 1992a; 1994b; Mathews Michael et al., 1996).
EFFECTS ON OXIDATIVE METABOLISM

In the present study, significant decrease was observed in the activities of SDH in testis, epididymis, liver and gastrocnemius muscle of fluoride and/or aluminium ingested male mice. A similar decline was found in various tissues of fluoride treated male and female rodents (Chinoy, 1991a,b; 1992; Chinoy and Sequeira, 1989a; Chinoy and Mehta, 1999a; Chinoy et al., 1991a,b; 1993a,b; 1994a,b,c) as well as in liver and muscle of NaF, AlCl₃ and NaF + AlCl₃ treated mice (Chinoy and Bhattacharya, 1996; 1997; Chinoy and Patel, 1999).

Succinate dehydrogenase is an oxidative enzyme involved in Kreb cycle. The decrease in its activity may affect the conversion of succinate to fumarate and may cause a block in the Krebs cycle altering the energy metabolism of the tissue. Moreover, SDH being a mitochondrial enzyme, a decline in its activity indicates a possible alteration in mitochondrial structure and function. Some reports are available suggesting fluoride-induced alterations in mitochondrial structure in different tissues of fluorotic animals (Chongwan and Daijei, 1988; Pang et al., 1996; Chinoy and Patel, 1998; Sharma and Chinoy, 1999). Ultrastructural studies on liver, ovary and uterus in fluoride treated male and female mice revealed structural disorganization of mitochondria and rupture of cristae (Chinoy and Patel, 1998b; Sharma and Chinoy, 2000). An immediate decrease in oxygen consumption may occur in the presence of fluoride which may also induce alterations in the energy metabolism of mitochondria and the whole cell (Elsair and Khelfat, 1988).

Aluminium chloride or aluminium citrate treatment caused their accumulation in the cytoplasm and mitochondria of liver cells and those of proximal tubule in kidney.
(Spencer et al., 1995) as well as in serum, kidney, testis and epididymis of mouse (Chinoy and Bhattacharya, unpublished observations). This may affect the SDH activity and cause alterations in energy metabolism of these tissue. AlCl₃ treatment for 90-100 days in rats showed changes in mitochondrial respiratory activity in liver, brain and heart, probably due to the significant increase in Al concentration in these tissues (Swegert et al., 1999). Aluminium is mainly known to depress the enzyme of energy metabolism (Xiao et al., 1992) and hence SDH may be affected by aluminium. These authors have also reported that some enzymes are more significantly affected by Al and F together than by their individual treatments. Hence, it is evident that fluoride and aluminium cause similar effects on SDH activity in different tissues.

In the present study, the ATPase activity showed a significant decline in caput and cauda epididymides of fluoride and/or aluminium treated mice. ATPase is a hydrolytic enzyme involved in the contractile process of muscle and plays a role in spermatozoa motility and metabolism and their energy supply. The restricted energy supply for the sperm as a result of decrease in ATPase would affect their motility. In the present study, the motility of spermatozoa was significantly reduced after the treatment. A decline in the levels of ATPase in muscle, kidney and reproductive organs of mice, rats and rabbits treated with NaF are also known (Chinoy, 1991a,b; 1992; 1995; Chinoy and Sequeira, 1989a; Chinoy et al., 1991a,b; 1993a; Chinoy and Sharma, unpublished observation). A direct action of fluoride on the motile apparatus of spermatozoa which inhibits the dynein ATPase in cilia was reported by Blum and Hayes (1984). The study conducted by Jagannatha Rao (1992) on the effect of aluminium on the brain cells of the rat revealed
inhibition of membrane bound Na, K⁺, ATPase activity. An in vitro kinetic study by Jagannatha Rao (1990) on the effects of aluminium salts on synaptosomal, ATPase (Na⁺, K⁺, Mg²⁺ and Ca²⁺ dependent) revealed their significant inhibition. The intracellular Na⁺, K⁺ balance is necessary for ATPase activity. Memon and Chinoy (1999) showed a decline in ATPase activity in epididymis of mice treated with aluminium chloride alone and together with fluoride. Thus, the decrease in ATPase activity would be responsible for decline in spermatozoa motility and consequently fertility rate.

Sialic acid is a sialomucopolysaccharide which is essential for the maintenance of the structural integrity of spermatozoa membranes, besides aiding in their maturation. The sialic acid levels were reduced by fluoride or aluminium alone and in combination in epididymides of mice in the present study. This data corroborates with earlier reports (Chinoy and Sequeira, 1989a; Chinoy et al., 1994a).

EFFECT ON CARBOHYDRATE METABOLISM

In the present study, treatment with fluoride or aluminium alone and in combination resulted in significant accumulation of glycogen but inhibition of phosphorylase activity in the liver, gastrocnemius muscle and vas deferens of male mice. Earlier work on liver, muscle, vas deferens and uterus of rats, mice and mudskippers treated with NaF, AlCl₃ and NaF + AlCl₃ support the present work (Chinoy, 1991a,b; Chinoy and Patel, 1996; Chinoy and Bhattacharya, 1996; 1997; Chinoy and Sharma, 1999; Chinoy and Patel, 1999; Chinoy et al., 1993b; 1994a; 2000). Most of these studies had correlated the histology and ultrastructural changes with the biochemical data and
suggested tissue toxicity by the treatments. The above data suggests that carbohydrate metabolism was significantly affected in all these tissues by the treatments.

Increase in glycogen with decreased SDH and blood glucose levels in various tissues of mudskipper was noted by Shaikh and Hiradhar (1985). According to them, the stress-induced glycogen synthesis from non-carbohydrate sources mediated by increased secretion of adreno-corticoids during stress, might have led to the increase in the glycogen concentration. The fluoride induced decline in the activity of glucose-6-phosphate dehydrogenase in rats would also alter the glycogen metabolism (Carlson and Suttie, 1966).

It is evident that alterations in carbohydrate metabolism would affect the muscle contractility as glycogen is an important fuel source for contraction. The secretions of uterus and vas deferens would be altered thus affecting their internal milieu. The glycogen turnover in the liver might also be hampered affecting liver functions as evident by the increase in SGOT and SGPT (Chinoy, 1991a; 1992; Chinoy and Sharma, 1999; Chinoy and Patel, 1999; Chinoy et al., 1993b; Singh, 1984; Flora et al., 1991). Chinoy et al. (1992) also reported elevated levels of serum transaminase in fluoride endemic populations in villages of Mehsana District of North Gujarat, India, suggesting impaired liver functions.

Besarabova (1960) suggested that inhibition of glycolysis and phosphorylation are the most significant toxic reactions of aluminium containing compounds. AlCl$_3$ caused a significant depression of synaptosomal production of glucose in rat brain (Johnson and Jope, 1986). This may be due to decrease in glycogen breakdown and hence in glucose
levels by aluminium. Contradictory to the above statements, a decline in hepatic glycogen of rats was observed (Sugawara et al., 1988) by aluminium treatment.

A decline in the levels of catecholamines in cerebellum, cortex and mix-brain of rats at different doses of aluminium treatment were reported (Moshtaghe et al., 1996). Since catecholamines regulate carbohydrate metabolism, the above mentioned changes might have occurred. Reports from our laboratory (Chinoy, 1992; 1996; Chinoy and Narayana, 1992; Chinoy and Patel, 1996) have also revealed alterations in catecholamines in fluorotic population of North Gujatat.

Cholinesterases are enzymes which hydrolyze esters of choline and their activity reflects on the neuronal status, which in turn influences the metabolic rate of an animal. In the present study, a decrease in activity of acetylcholinesterase (AChE) was observed by NaF, AlCl₃ or NaF + AlCl₃ treatments in cerebral hemisphere of brain, gastrocnemius muscle and liver of male mice. Similar results were found by Chinoy and Patel (1999) in liver and gastrocnemius muscle of female mice. Sarm et al. (1997) have also reported a decrease in activity of acetylcholinesterase in brain of the aluminium treated monkeys and suggested that loss of membrane integrity by aluminium (75 mg/kg body weight), might be responsible for this decline in the enzyme activity in monkey’s brain.

NaF is also known to inhibit AChE activity in all tissues of fresh water crab at sublethal concentration (Reddy and Venugopal, 1990). Similar results were found in fluoride treated mice muscle (Chinoy et al., 1991b) and brain (Laxmivani and Reddy, 2000). Six week old female Wistar rats receiving 8, 30, and 60 mg F⁻/L showed lowest AChE activity in serum by the highest dose suggesting inhibition of the enzyme by
fluoride (Machoy Mokrzynska et al., 1999). The inhibition of cholinesterase by fluoride ions in human serum, red blood cells and rat brain have been reported (Cimasoni, 1996). However, Szilagyi et al. (1994) showed no change in the serum AChE activity of broiler chickens treated with different doses of aluminium.

Fluoride is known to have great affinity to bind with calcium, which has an indispensable role in neurotransmission. The decrease in AChE may be due to interference of fluoride with the receptors and neurotransmission (Laxmivani and Reddy, 2000). Lu et al. (2000) reported a low intelligence quotient (IQ) in Chinese children (10-12 years) exposed to high levels of fluoride since birth.

Farnell et al. (1985) reported that aluminium inhibits synaptosomal uptake of transmitters and precursors including choline, glycine, GABA, L-glutamate, nor adrenaline and serotonin. It also depresses acetylcholinesterase in neuroblastoma and is a non-competitive inhibitor of neuronal acetylcholinesterase.

The activities of synaptosomal enzyme ATPase and AChE which help in chemical transmission of nerve impulse are inhibited by aluminium chloride and by complex of fluoride and aluminium (AlF₃) (Jagannatha Rao, 1990). The above results revealed that the toxic effects of fluoride or aluminium alone or in combination may lead to altered release and/or utilization of acetylcholine, thus, affecting the transmission of nerve impulse in liver, brain and gastrocnemius muscle and hence affect their functions.

FREE RADICALS AND ANTITOXICANTS

Free radicals are highly reactive species that have unpaired electrons, e.g. the hydroxy and superhydroxy radicals. Cellular damage caused by the reactive oxygen species (ROS) has been implicated in the etiology of a range of diseases, such as
atherosclerosis, cancer, Parkinson’s disease and other neurodegenerative disorders. The macromolecules like protein, nucleic acids, lipids and carbohydrates are at risk of oxidative damage due to their destabilisation by increase in ROS. The most important consequences are nucleic acid destabilization, lipid peroxidation and change in permeability of the cell membrane (Subramaniam et al., 1994). Important enzymes involved in curbing these harmful effects include superoxide dismutase (SOD) which catalyzes dismutation of superoxide radical leading to the formation of hydrogen peroxide which inturn is detoxified by the enzyme glutathione peroxidase (GSH-Px) and catalase (Rzeuski et al., 1998).

In the present study, fluoride and/or aluminium caused a decrease in the activities of free radical scavenging enzymes viz., SOD, GSH-Px and catalase but increased the lipid peroxidation in testis, cauda epididymis, liver, muscle and brain of male mice in corroboration with earlier studies in male and female rodents treated with NaF, AlCl$_3$ and NaF + AlCl$_3$ (Chinoy and Patel, 1998a; Chinoy and Patel, T. 2000b; Sharma and Chinoy, 1998; Memon and Chinoy, 2000a).

The above data is in agreement with those of others (Sun et al., 1994; Liang et al., 1999; Li et al., 1999) in liver, kidney, brain, heart and serum of fluoride treated rats and Chainy et al. (1993) in brain of aluminium treated chicks.

Verstraeten et al. (1998) found that Al$^{3+}$ induced changes in the physical properties of membrane and accelerated lipid oxidation rates in myelin sheath of mice brain. Sun and Sun (1974) reported that brain being rich in polyunsaturated fatty acids is highly susceptible to lipid peroxidation. Aluminium also alters calcium flux and...
homeostasis which may lead to increase in lipid peroxide formation (Mundy et al., 1997).

The increase in lipid peroxides and decrease in activity of free radical scavenger enzymes by aluminium and/or fluoride as well as the tissue burden of these chemicals may have led to cellular injury in the present work as also reported earlier (Chinoy and Patel, T. 2000; Chinoy and Bhattacharya, unpublished observation).

In people living in areas endemic to fluorosis, the high fluoride concentration was reported to inhibit SOD and GSH-Px activities resulting in accumulation of large amounts of free radicals and peroxides causing cell damage (Li and Cao, 1994; Bian et al., 1994).

EFFECTS ON ASCORBIC ACID AND GLUTATHIONE

Ascorbic acid (AA) is known to be a powerful reducing reagent which helps in activating several enzymes and acts as an antioxidant for detoxifying several toxic substances (Kutsky, 1973). Ascorbic acid depletion is considered as an index for steroidogenesis and is also involved in overcoming stress (Chinoy, 1978). A decrease in levels of total and reduced ascorbic acid (TAA and RAA) concomitant with an increase in the dehydroascorbic acid (DHA) was obtained in testis, cauda epididymis, liver, muscle and brain of male mice administered with fluoride or aluminium alone and in combination for 30 days in the present study. Earlier studies from our laboratory (Chinoy and Patel, 1998a; Chinoy and Patel, 2000; Sharma and Chinoy, 1998; Memon and Chinoy, 2000a) have also revealed similar effects on various tissues of NaF and/or Al treated male and female mice. It is thus evident that both aluminium and fluoride caused disturbances in the utilization and probably synthesis of ascorbic acid leading to change in its metabolism.
which might be influenced by decrease in glutathione (GSH) levels in the present study, as it (GSH) is involved in the mechanism of detoxification of various xenobiotics (Meister and Anderson, 1983), inhibition of lipid peroxidation by scavenging free radicals (Liang et al., 1999; Satsangi and Dua, 2000), as well as reducing dehydroascorbic acid to the reduced form.

EFFECTS ON NUCLEIC ACIDS

In the present study, a decrease in concentration of DNA and RNA was obtained in testis, cauda epididymis, liver, muscle and brain by fluoride and/or aluminium treatments in male mice. Earlier reports support the present work, wherein, treatments of fluoride (100 mg/kg body weight) to rabbits resulted in a decrease in DNA and RNA levels in the ovary (Shashi, 1994). Chinoy and co-workers (Chinoy and Patel, D., 1998a,b; Chinoy and Patel, T., 1999; Patel and Chinoy, 1997; 1998; Patel, T. and Chinoy, 2000; Memon and Chinoy, 2000b) also showed similar results in several tissues of NaF, AlCl$_3$ and NaF + AlCl$_3$ treated male and female rodents.

The reduction in DNA and RNA might be due to the inhibitory action of fluoride on their synthesis as in cultured cells (Muller, 1961; Strockova et al., 1984; Tsutsui et al., 1984). The inhibition of DNA synthesis may result in delayed meiotic and mitotic cycles (Vorishilin et al., 1973). Shashi et al. (1994) reported that inhibition of protein synthesis might retard the progress of DNA repair following its damage.

Recent studies showed that aluminium potentially inhibits neuronal RNA polymerases and alters m-RNA pool size. It also inhibits DNA polymerase. This indicates
that aluminium has a potential to alter genetic information (Jagannatha Rao et al., 1993). Aluminium preferentially binds within the nuclear compartment, particularly in heterochromatin and DNA bases (Crapper-Mchachlan et al., 1989; Crapper-Mchachlan and Farnell, 1986). Karlik et al. (1980) observed pH and concentration dependent interactions of aluminium with DNA. These authors indicated that the DNA helix may be hyperstabilized in the presence of aluminium and the binding of Al to chromatin proteins may alter the charge distribution along the surfaces of these molecules resulting in an increased affinity for DNA.

AlF$_4^-$ a phosphate analogue, binds to the nucleotide binding site of H1 histones. This adduct formation seems to abolish the nucleoside triphosphate hydrolysis capability of H1 and interferes with the expression of certain cell specific genes. It may also bind to other proteins like protein kinase and G-protein and alter their functions (Tarkka et al., 1993).

The above discussion reveals that fluoride or aluminium alone and in combination are capable of causing changes in nucleic acid metabolism.

EFFECT ON SPERMATOGENESIS

Kour and Singh (1980b) reported that fluoride ingestion in mice for a period of two to three months resulted in a lack of differentiation and maturation of spermatocytes in most of the seminiferous tubules. Chinoy and co-workers (Chinoy, 1991b; 1992; Chinoy and Sequeira, 1989a; Chinoy et al., 1992b; 1994a; 1997a; Narayana and Chinoy, 1994a; Sharma and Chinoy, 1999) have reported alterations in the histo-architecture of
testis, affecting spermatogenesis, steroidogenesis as well as in several biochemical parameters in rodents treated with fluoride in doses ranging from 5-20 mg/kg body weight for 30, 45, 60 or 70 days. Shashi (1990) observed necrosis of seminiferous tubules which led to cessation of spermatogenesis in rabbits treated with different doses of fluoride for 100 days. The ultrastructural studies carried out in fluoride treated rabbits by Susheela and Kumar (1991) revealed disruption and degeneration of spermatogenic cells. The spermatozoa showed mitochondria with no cristae in mid piece region.

The above mentioned reports corroborate with the present studies showing decrease in number of spermatozoa due to disruption of spermatogenesis.

Llobet et al. (1995) found necrosis of spermatocytes and spermatids in the testis of mice treated intraperitonealy with 100 and 200 mg/kg/day aluminium nitrate for four weeks.

EFFECT ON METABOLISM OF SPERMATOZOA AND FERTILITY RATE

In the present study, the motility, count and viability of spermatozoa decreased significantly after the treatment of NaF, AlCl₃ and NaF + AlCl₃ to mice. The present findings are in agreement with results obtained by Chinoy and co-workers earlier in NaF, AlCl₃ and NaF + AlCl₃ treated mice, rats and rabbits (Chinoy and Sequeira, 1989a; 1992; Chinoy et al., 1991a; 1994a; 1995; 1997b; Chinoy and Bhattacharya, unpublished observations; Narayana and Chinoy, 1994b). Schoff and Lardy (1987) reported that bovine sperm treated with 30 mM fluoride became immobile within two minutes and flagella assumed a linear, rod like configuration. Human spermatozoa lost their motility *in vitro*.
in the presence of 250 mM NaF within 20 minutes incubation (Chinoy and Narayana, 1994).

Aluminium chloride has been reported to inhibit the upward movements of spermatozoa in cervical mucus and might have affected the motility of spermatozoa directly in various concentration gradients (Kaur, 1988).

The above mentioned alterations in spermatozoa metabolism might be the outcome of alterations in the biochemical profile of epididymides which would ultimately render their internal milieu hostile for spermatozoa maturation and survival in treated mice, as the epididymal microenvironment is important for maintaining spermatozoa in a viable, motile state.

The reduction in spermatozoa count, motility, viability and changes in their metabolism led to the decline in fertility of treated mice. Similar loss of fertility by NaF, AlCl₃ and NaF + AlCl₃ treatment in male mice, rats and rabbits have also been reported (Chinoy, 1991a,b; Chinoy and Sequeira, 1992; Chinoy and Sharma, 1998; Chinoy et al., 1992b; Narayana and Chinoy, 1994b; Memon and Chinoy, 1999).

WITHDRAWAL

In view of the fluoride and aluminium induced toxic effects reported above, in a different group of animals, NaF and aluminium chloride were fed together for 30 days and the treatments were withdrawn from day 31st for another 30 days. During this period, the animals were maintained on standard diet and water *ad libitum*.

Insignificant recovery was shown by withdrawal of NaF + AlCl₃ treatment in most
of the parameters. However, some parameters showed partial or significant recovery. The differences in the recovery patterns of the parameters studied may be due to varying tissue response to the toxicity of these chemicals in combination. Earlier study conducted by Chinoy and co-workers on withdrawal of fluoride or aluminium treatments for different durations revealed almost similar data (Chinoy, 1992; Chinoy and Sequeira, 1992; Chinoy and Patel, 1996; 1998a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Patel, 1999; Chinoy et al., 1991a; 1994a; 1995; 1997a; Patel and Chinoy, 1997). However, none of the studies observed a complete recovery. In the present study too, complete recovery did not occur after the withdrawal period of 30 days in any of the parameters. To bring about a faster and full recovery, certain therapeutic agents were used to reverse the induced toxic effects.

**INDIVIDUAL ROLE OF ASCORBIC ACID (VITAMIN C), CALCIUM OR VITAMIN E (α-TOCOPHEROL) IN REVERSAL OF INDUCED TOXICITY**

In order to evaluate the beneficial effects of ascorbic acid, calcium and vitamin E, they were administered alone and in combination to the animals during withdrawal period. Ascorbic acid, calcium and vitamin E were given at doses of 15 mg, 25 mg, 2 mg/animal/day respectively.

The results showed that ascorbic acid, calcium or vitamin E administration during withdrawal period brought about a significant recovery in all the parameters studied. All the therapeutic agents individually produced almost similar effects but ascorbic acid was comparatively more beneficial than calcium and vitamin E. However, combined treatment
had an additive or synergistic effect for complete recovery of the tissues to more or less control state.

Many epidemiological and experimental studies have shown that dietary factors such as protein, calcium, vitamin C, D and E etc., could modify the toxic effects of fluoride or aluminium induced toxicity (Chinoy and Patel, D. 1996; 1998a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Patel, T. 1999a; Chinoy et al., 1991a; 1993b; 1994a; 1995; 1997a,b; Narayana and Chinoy, 2000; Sriranga Reddy and Srikantia, 1971; Yolken et al., 1976; Parker et al., 1979; Burgstahler, 1985; Clark et al., 1989; Susheela, 1999; Satasangi and Dua, 2000; Khandre et al., 2000)

Ascorbic acid (AA) is an important biologically active antioxidant which is widely distributed in animal cells (Chinoy, 1978). AA participates in cellular oxido-reduction reactions via formation of its free radical, monodehydroascorbic acid (MDHA) which is a more powerful reducing agent than AA by virtue of possessing an unpaired electron, which subsequently gets oxidized to dehydroascorbic acid (DHA). DHA could be converted back to AA by glutathione (Chinoy, 1978). Ascorbic acid binds to proteins, nucleic acids and other macromolecules by charge transfer complex formation (Chinoy, 1978). Besides being an antioxidant, ascorbic acid is known to activate adenylate cyclase but inhibit phosphodiesterase, hence causing an increase in C-AMP levels (Pasternak, 1979) which being a second messenger, is known to activate several enzymes, and influence cell metabolism.

In the present study, most of the enzymes viz., SDH, ATPase, phosphorylase, 3β and 17β hydroxysteroid dehydrogenases, cholinesterase, SOD, GSH-Px, catalase etc., were
found to recover from the fluoride and aluminium induced toxicity by ingestion of ascorbic acid alone. Blaskhe and Hertting (1971) reported that deficiency of ascorbic acid alters 38 HSD activity in rats. Low dietary supplement of ascorbic acid is known to increase the adverse effects of fluoride in monkeys (Sriranga Reddy and Srikantia, 1971). According to the Satasangi and Dua (2000), ascorbic acid mobilizes aluminium and reduces its tissue burden in liver, kidney and blood.

Calcium is also known to activate several enzymes. Earlier studies have demonstrated that calcium ingestion by rodents during withdrawal period resulted in recovery of fluoride or aluminium induced toxicity in several parameters (Chinoy and Patel, 1998; Chinoy et al., 1991a; 1994a; 1995; 1997a; Patel and Chinoy, 1997). Calcium also inhibits phosphodiesterase similar to ascorbic acid and hence increases the concentration of C-AMP and thus metabolism. Calcium and C-AMP interact with each other for various metabolic reactions in different tissues (Pasternak, 1979). The activities of peptidases, phosphatases etc., are known to be activated by calcium ions and are inhibited by added fluoride. One of the possible mechanism of enzyme inhibition may be due to the binding of calcium with fluoride or aluminium in the catalytic center (Machoy, 1987; WHO, 1997). Therefore, external supplementation with calcium might help in recovery of various tissues from induced toxicity.

Vitamin E has come under much scrutiny for its possible therapeutic roles in numerous disease states especially involving oxidation related events (Phelps, 1987). One of the group of animals treated with fluoride and aluminium were fed vitamin E (α-tocopherol) during the withdrawal period. The results revealed a significant recovery in
all the parameters studied following Vitamin E administration. Vitamin E deficiency in experimental animals has been shown to result in a variety of conditions affecting neuromuscular, vascular and reproductive systems (Marks, 1975). Basu and Dickerson (1996) reported that other conditions, such as myopathy, liver necrosis, testicular degeneration are known to respond to vitamin E. The NaF, AlCl3 and NaF + AlCl3 treated male and female mice showed a significant recovery in all tissues after treatment with vitamin E (Chinoy and Sharma, 1998; Chinoy and Patel, 1999; 2000; Chinoy and Memon, 1999; 2000). Burgstahler (1985) also showed curbing of dental fluorosis in rats by supplementation of vitamin E.

Vitamin E is a proven antioxidant with a property of scavenging free radicals and hence maintaining the integrity of lipid structures in vivo (Cheeseman et al., 1984). The oxygen free radicals being highly reactive species, may attack the double bonds of polyunsaturated fatty acid initiating a chain reaction, leading to complete destruction of membrane integrity and hence cellular function. This chain reaction is thought to be inhibited by α-tocopherol by breaking it and by reacting with free radicals and converting itself into α-tocopheroxyl radical which is not harmful (Basu and Dikkerson, 1996). This α-tocopheroxyl radical thus formed is reverted back to α-tocopherol by cytosolic vitamin C (Subramanian et al., 1994).

COMBINED ADMINISTRATION OF VITAMIN C, CALCIUM AND VITAMIN E

Ascorbic acid, calcium and vitamin E alone are known for amelioration of fluoride and aluminium toxicity as also obtained in the present study. In combination they played
a crucial role in reversal of toxicity induced by fluoride or aluminium. The recovery was almost comparable to control in all the organs/tissues studied as described earlier. This may be due to several factors and their interaction in the biological system. Previous studies have shown that vitamin C and calcium have a synergistic effect in bringing about a pronounced recovery in fluoride induced toxicity (Chinoy et al., 1991a; 1993b; 1994a; 1995; Chinoy and Patel, 1998a). Their mechanism of action has been described in earlier part of the discussion. Deficiency of calcium and vitamin C caused aggravation of the toxic manifestation of fluoride (Siddiqui, 1955; Sriranga Reddy and Srikantia, 1971), whereas, calcium and ascorbic acid have a role to play in curbing fluoride induced toxicity (Sriranga Reddy and Srikantia, 1971; Chinoy and Patel, 1998a; Chinoy et al., 1991a; 1994a; 1995; 1997a,b; Patel and Chinoy, 1997).

Farries et al. (1985) reported that lower levels of calcium in a system causes a depletion of cellular α-tocopherol. It is known that the cell calcium content affects the intracellular metabolism of α-tocopherol and its esters, which may subsequently govern the outcome of a toxic challenge (Pascoe and Reed, 1987). The results of the present study revealed that calcium, vitamin E and vitamin C act together in vivo to mitigate the induced toxicity.
PART - II

IN VITRO STUDIES

At present, there is a growing interest concerning the potential genotoxic effects of fluoride and aluminium. Only a limited amount of information is available on this subject and the published reports are inconclusive.

The results of the present investigation revealed that fluoride and/or aluminium treatment produced a significant increase in the frequency of sister chromatid exchanges (SCEs) and chromosomal aberrations in peripheral blood lymphocyte cultures of male volunteers between the age group of 20 to 25 years. The mean sister chromatid exchanges, SCE/chromosome and SCE/cell increased significantly as compared to controls. Some spontaneous aberrations were recorded in controls and an increase was observed in chromosomal and chromatid type aberrations after the addition of fluoride and aluminium either alone or in combination. Similar type of results supporting the present work in animals and humans have been reported (Manna and Das, 1972; Jachimczak and Skotarozak, 1978; Mohamed and Chandler, 1982; Roy et al., 1990; 1991; Sheth et al., 1994; Wu and Wu, 1995). Thompson et al. (1985) on the other hand, found no increase in SCE frequency or chromosomal aberrations in peripheral blood lymphocyte cultures with fluoride. Significant increase in chromosome aberrations, SCE and unscheduled DNA synthesis was also found in hamster embryo cells, exposed to NaF concentrations of 75 and 125 ppm (34 and 56 ppm F) (Tsutsui et al., 1984). These findings support the observations of the present study. AlF$_4^-$ a phosphate analogue, which binds to the nucleotide binding site of H1 histones might affect the expression of certain cell specific
genes, leading to impaired chromatin function and thus, increased chromosomal aberration (Tarkka et al., 1993).

Cross-linking agents like aluminium chloride frequently produce clastogenic effects due, presumably to conformational distortions that inhibits proper DNA replication.

The results of the present study on the proliferative kinetics revealed that the replicative index declined as compared to the control. The percentage M1, M2 and M3 also showed changes in fluoride, aluminium and their combined treated groups. He et al. (1983) reported that NaF and fluoroacetamide influence the cell cycle kinetics, chromosomal aberrations and SCE frequencies in cultured red Muntjac cells. Cell cycle analysis revealed an inhibitory effect of NaF on cell proliferation with doses above 1.0 mM in CHO cell line in vitro. The percentage of M1 cells increased while that of M2 and M3 decreased significantly by NaF (Li et al., 1987b). There was a significant lag in the cell cycle observed in this study also, which is in agreement with the above reports.

Blair et al. (1989) reported that micromolar levels of aluminium reduced $^3$H thymidine incorporation in a transformed cell line, which indicates that aluminium may impede cell cycle progression. Prolonged treatments with Al-Salts in rats also caused a dose-dependent inhibition of dividing cells of bone marrow (Roy et al., 1991). The activity of DNA and RNA polymerases are also impaired by aluminium. These factors might also lead to inhibition of proliferation and hence reduce the replicative index.

The present study revealed increase in number of chromatid and chromosomal aberrations following the treatments of fluoride, aluminium and their combination. Thomson et al. (1985) and Luchnic et al. (1985) reported that increase in aberration
frequency was not caused by a direct mutagenic effect of NaF but rather represented ‘pseudomutagenesis’ due to the inhibition of repair processes by NaF. Emsley et al. (1982) reported that fluoride forms a strong hydrogen bond in base pair of thymidine adenine which could disrupt the structure of DNA. It has also been shown to inhibit many enzymes in vitro including DNA polymerase (Wiseman, 1970) which could directly damage DNA. These factors might be resulting in the gaps and breaks observed by its treatments.

Aluminium is known to alter m-RNA pool size which indicates that it may cause genetic malfunctioning (Jagannatha Rao et al., 1993) by increasing the number of aberrations.

Significant increase was observed in the frequencies of binucleates with micronuclei (MN) and the total number of micronuclei in fluoride and/or aluminium treated cultures. The micronuclei were observed in control subjects also indicating that chromosome elimination in micronucleus is a common phenomenon. In some individuals a single binucleate had more than one micronucleus in treated groups, suggesting multiple chromosome or chromatid elimination, which could be attributed to multiple chromosome or chromatid lagging at anaphase due to non-disjunction. The above data corroborates with those of Liu (1992) and Zhang and Meng (1999) who found a significant increase in the number of micronuclei in fluoride intoxicated individuals than in control population suggesting that an increase in MN may cause a higher chromosome malformation frequency (Lu Wenqing, 1991).

Increased micronuclei in the present study might be the result of increased non-
disjunction and lagging at anaphase. This may cause aneuploidy and increase the incidence of abnormality in the populations getting exposed to these chemicals.

In the present study, a significantly higher frequency of aneuploidy was observed after fluoride, aluminium and their combined treatments which might have resulted from microtubular malfunctioning (Ford, 1984). NaF (190 ppm) had mutagenic action on the mitotic chromosome of onion root tips, causing anaphase lags and bridging, tetraploid nuclei and multipolar anaphases which is a result of non-disjunction and could further lead to other genotoxic manifestation. Bonhaus et al. (1980) reported that aluminium at concentration as low as 0.1 mM inhibits microtubule formation in vivo and in vitro in Protozoa. Carlier et al. (1988) noted that AlF$_4^-$ was responsible for slowing down of the microtubular turnover and which might result in non-disjunction and aneuploidy.

The normal ends or telomeres of human chromosomes sometimes appear to be fused end to end, and such arrangements are called telomeric associations (Fitzgerald and Morris, 1984). Increase in telomeric associations have been found in virus infected cells (Moorhead and Saksela, 1963), senescent fibroblast cell lines (Benn, 1976) and in a variety of leukaemias as well as solid tumours (Saltman et al., 1989; Hastie et al., 1990). Morgan et al. (1986) suggested that telomeric association may function as a mechanism for the development of chromosome rearrangements that may play a role in human neoplasia.

It has been demonstrated that telomeric association is one mechanism that can initiate chromosome instability (Sawyer et al., 1996) and leads to a variety of balanced and unbalanced chromosome, rearrangements (Mondello et al., 1995).
In the present study, a significant increase in the frequency of chromosomal and chromatid telomeric associations was observed following an exposure to aluminium and fluoride alone and in combination. Thus, it is suggested that fluoride and/or aluminium may lead to chromosome instability, cell ageing and neoplasia.

A number of reports have suggested that NOR associations play a significant role in non-disjunctional events leading to aneuploidy (Mirre et al., 1980). In the present study, the NOR associations were observed to be significantly higher for fluoride and/or aluminium. These results suggest a relationship between Al and/or F and non-disjunction which could be correlated with increase in incidence of aneuploidy in F endemic areas (Takahashi, 1998).

Ascorbic acid has a role in maintaining the growth and integrity of leucocytes (Basu and Dickerson, 1996) and has antioxidant and detoxification properties (Chinoy, 1978). Satsangi and Dua (2000) showed that aluminium-induced genotoxic effects were not manifested by concurrent addition of ascorbic acid in the medium, indicating its protective effects. In vivo studies conducted in rodents showed that ascorbic acid (AA) was capable of reducing fluoride and/or aluminium induced toxicity and caused recovery in various tissues (Chinoy and Patel, D, 1998; Chinoy and Patel, T, 1999a; Chinoy and Memon, 2000a; Chinoy et al., 1991a; 1993b; 1994a; 1995; 1997a,b; Colomina et al., 1994; Roy and Sharma, 1993). In the present study, AA showed some protective effects when added to the cultures alongwith NaF and AlCl$_3$ at '0' hour, and revealed a significant decrease in the frequencies of sister chromatid exchanges, chromosomal aberrations, micronuclei, telomeric and acrocentric associations and number of aneuploid
The following hypothesis proposed might explain the mechanism of action of AA.

It is suggested that the binding of AA initially to DNA, may not allow aluminium fluoride complex to bind, thus preventing any damage to DNA. Hence, the stabilization and protection of DNA may occur in vitro by AA. Aluminium or fluoride alone and in combination are known to increase lipid peroxidation in vitro, whereas, some chemotherapeutic approaches have been proposed for the use of free radical scavenging agents and antioxidants such as vitamin C.

Thus, the results of the present study suggests that Al and/or F have genotoxic effects which can be revealed by AA. Further studies are required to confirm the ameliorative effects of AA on chromosomes.