CHAPTER IV
DISCUSSION

I. STUDIES ON THE EFFECTS OF FLUORIDE INGESTION TO ADULT MALE ALBINO MICE

During the tenure of the present investigation, the effects of sodium fluoride ingestion for varied durations (30 and 45 days) was studied in order to evaluate time related changes in 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 5 mg/kg body weight. The dose used was based on the LD$_{50}$ value of fluoride, i.e. 54.6 mg F/kg body weight in male mice (Pillai *et al.*, 1988). Oral administration was preferred in view of water being the main source of fluoride among the human population in endemic areas. The durations of the study were 30 and 45 days as one spermatogenic cycle requires a period of 30 to 32 days in mouse and the entire spermatogenic process as well as sperm maturation period in the epididymis is completed by 45-50 days.

The various parameters studied at the end of treatment were body weight and organ weights of testis, epididymides and vas deferens. In addition, certain specific androgen dependent parameters in testis viz., cholesterol, activities of 3β and 17β hydroxysteroid dehydrogenases and serum testosterone levels were investigated to study the impact of NaF on testicular functions. Similarly, in cauda epididymis and certain specific parameters of its spermatozoa viz., motility, count, viability, morphology, sperm
mitochondrial activity index, hyaluronidase and acrosin activities were studied. Further, succinate dehydrogenase (SDH) in cauda epididymides and gastrocnemius muscle, adenosine triphosphatase (ATPase) and sialic acid in caput and cauda epididymides were also investigated.

To find out the effects of fluoride on the protein metabolism, levels of protein were determined in testis, epididymides, vas deferens, liver, gastrocnemius muscle and kidney. The concentration of glycogen and phosphorylase activity in the vas deferens, liver and gastrocnemius muscle were determined to investigate the effects on carbohydrate metabolism. The levels of DNA and RNA in the testis and cauda epididymis were evaluated to study the impact on nucleic acid metabolism. In the kidney, creatinine levels were determined.

To evaluate free radical induced cell injury by fluoride, the activities of some antioxidant enzymes viz., superoxide dismutase, catalase, glutathione peroxidase as well as levels of lipid peroxides, glutathione and ascorbic acid were determined in the testis and liver. The serum electrolyte concentrations (Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+}), the tissue burden of fluoride as well as fluoride levels in the serum and urine were also investigated. Fertility rate as well as histology and ultrastructure studies were carried out during the course of the investigation. Apart from this, the levels of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) which are considered as specific markers of liver functions, were also investigated.

In a different set of experiments, the treatment was withdrawn after 45 days of NaF ingestion to study the reversible effects if any, upon cessation of treatment.
In view of fluoride induced toxic effects, the therapeutic action of some agents viz., calcium, vitamins (C, D and E), amino acids (glycine and glutamine) and protein supplemented diet were also explored in the light of earlier data.

TISSUE BURDEN OF FLUORIDE

In the present study, the analysis of fluoride levels in serum, urine, testis, cauda epididymis, liver and kidney of NaF treated mice revealed a significant enhancement which indicates that fluoride accumulates in these tissues and would affect their metabolism.

It has been reported that fluoride excretion depends on total daily consumption of fluoride, the degree of renal efficiency, and interaction of fluoride with Mg$^{2+}$, Ca$^{2+}$, Al$^{3+}$ etc. (Krishnamachari, 1986), urinary flow and pH as well as previous exposure to fluoride (Whitford et al., 1976; Schiffl and Biswanagar, 1980). Several workers have established a good correlation between fluoride intake and urinary loss. Therefore, Hodge and Smith (1970) opined that measurement of the urinary fluoride could be regarded as the best indicator for intake level of the element. The high serum fluoride levels were also due to high fluoride intake. The transportation of fluoride in the body takes place via the blood. Intestinal transport of fluoride is positive and regulated by plasma fluoride levels. Susheela (1985) has reported a significant increase in the urine, skeletal muscle, liver and kidney F$^-$ levels following NaF ingestion to rabbits and by Mathews et al. (1996) in fluorotic individuals of North Gujarat. Similarly, Chinoy and Patel (1998a) found an enhancement in the levels of fluoride in the serum, uterus and ovary of mice. The retained
fluoride in the serum, thus would affect the general body metabolism probably by altering soft tissue functions.

EFFECT ON BODY AND ORGAN WEIGHTS

The results revealed that a low dose of NaF (5 mg/kg body weight) for a period of 30 and 45 days produced significant alterations in the above mentioned parameters. A reduction in growth rate of rats supplemented 100 ppm fluoride through drinking water for two months (Saralakumari et al., 1988) and a 40% reduction in body weight upon administration of low-protein fluoride diet to mice has also been reported (Yu and Hwang, 1985). The earlier observation of Simon and Suttie (1968) also demonstrated a decline in growth rate in rats probably due to drastic curtailment of food intake. A consistent reduction in body weight in mouse by 5.2 mg/kg body weight fluoride for 35 days was also reported by Pillai et al. (1988). Studies carried out from our laboratory have revealed decline in body weight by sodium fluoride treatment in mice and rats (Chinoy and Sequeira, 1989a; Chinoy et al., 1991c: 1992b; 1993b) and rabbits (Chinoy et al., 1991a). Singh et al. (1963) also obtained a general decrease of body weight in fluoride exposed individuals in Punjab.

The results of the present study corroborate the above data as a significant decline in the body weight was obtained after 30 and 45 days of the treatments. The mechanism by which the growth rate is inhibited by fluoride could be due to low food intake as well as reduction in protein levels.

On the contrary, Schwarz and Milne (1972) showed an increase of 30% in the...
daily weight gain of rats on fluoride supplemented diets using a controlled isolator system and highly purified amino acids. Yu and Driver (1978) reported no significant change in body weight of chicks exposed to fluoride.

In the present study, the organ weights of testis, caput and cauda epididymides and vas deferens were decreased which might be due to loss of electrolytes (Chinoy, 1991a), low protein levels (Chinoy and Sequeira, 1989a) and low metabolic activity. Ramesh et al. (1997) found that fluoride administration caused a decrease in the weight of heart of rats.

EFFECTS OF FLUORIDE ON SOFT TISSUES

HISTOLOGY

NaF at a dose of 5 mg/kg body weight for 30 and 45 days to mice (administered orally) resulted in severe alterations in histology of testis. The process of spermatogenesis was disturbed. Denudation of germinal epithelial cells with pyknosis of germinal cell nuclei and absence of spermatozoa in the lumen was observed. The Leydig cells also did not appear to be normal. Vacuolization of interstitium was observed specially near the Leydig cells, which also appeared to have vacuoles in their cytoplasm. Kour and Singh (1980b) reported a lack of maturation and differentiation of spermatocytes in testis of mice treated orally with 10, 500 and 1000 ppm fluoride for 2 months. After three months of treatment, spermatogenesis had ceased and the seminiferous tubules had become necrotic. The earlier studies from our laboratory had revealed that the spermatogenic cells had pyknotic nuclei and were sloughed off in the lumen of the seminiferous tubules.
leading to disorganization and denudation of the seminiferous epithelium resulting in complete absence of spermatozoa in the lumen following fluoride administration for 30 days in mice (Chinoy and Sequeira, 1989b) and rats (Chinoy et al., 1991c). Shashi (1990) observed similar effects and increase in the amount of interstitial tissue as well as necrosis of seminiferous tubules which led to cessation of spermatogenesis in rabbits treated with 5, 10, 20 and 50 mg/kg body wt/day fluoride for 100 days. Thus, the present data clearly corroborates with earlier work and elucidates that fluoride ingestion results in arrest of spermatogenesis, which was further confirmed by low sperm count obtained in cauda epididymis. This might be due to the fact that fluoride may exert effects on the paracrine functions of testis.

Histological studies on epididymis following NaF ingestion in the present investigation revealed that the epithelium showed nuclear pyknosis and in the lumen absence of spermatozoa were observed in caput epididymis. The tubular diameter increased and apical tubular degeneration was conspicuous. The cauda epididymal tubules also showed degeneration and epithelial cells had pyknosis of nuclei and loss of stereocilia. The epithelial cell height of epididymal tubules was decreased. The spermatozoa appeared clumped together. This data corroborates with earlier observations obtained in epididymis of mice treated with sodium fluoride (Chinoy and Sequeira, 1989b). A single microdose vasal injection of NaF given to rats (Chinoy et al., 1991c) as well as prepubertal rats treated with NaF (Chinoy et al., 1994b) also manifested similar changes in epididymis viz., degeneration and confluence of tubules, vacuolization of epithelial cells, pyknosis of their nuclei, increase in the interstitium and hyalinization.
In vas deferens, NaF treatment brought about vacuolization in epithelial cells with pyknosis of nuclei and reduction in stereocilia. The lamina propria was increased in thickness and lumen contained cell debris but absence of spermatozoa. These observations corroborate with earlier studies following NaF treatment to mice and rats (Chinoy and Sequeira, 1989b; Chinoy et al., 1991c). Ultrastructural studies on NaF treated mice vas deferens also revealed alterations (Chinoy and Sharma, 1999b).

Sodium fluoride in acutely toxic doses produced hepatocellular necrosis and few enlarged cells with pycnotic nuclei. The histology of liver revealed vacuolated cytoplasm. This study is in agreement with the observations of Haber (1973) who reported mid-zonal liver cell necrosis by fluoride ions in patients receiving fluorinated anesthetics. Kour et al. (1981) also obtained necrosis, fatty deposition, congested central veins and sinusoids by sodium fluoride in drinking water (10, 500 and 1000 ppm) administered to mice. The present findings have been further substantiated by Chinoy et al. (1993b) who reported zonal necrosis in fluorotic animals. In fluoride treated (40 and 80 ppm) mud-skippers, the liver revealed ruptured cell membrane, fatty infiltration and cytoplasmic vacuolization within 48 to 72 h of exposure (Shaikh and Hiradhar, 1987).

The ultrastructural studies using an EM900 Zeiss Electron microscope also revealed alterations in the ultrastructure of the testis, cauda epididmis and liver of treated mice in corroboration with earlier work (Sharma and Chinoy, 2000). The above mentioned alterations in the histology and the ultrastructure of these organs might be related to the tissue burden of the fluoride.
EFFECTS ON STEROIDOGENESIS

In the present study, the investigation of intermediary enzymes in the steroidogenic pathway after fluoride treatment revealed a decline in the activities of 3β hydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase. These results were correlated with accumulation of cholesterol in the testis and a decrease in the circulating serum testosterone levels. Some earlier studies (Narayana and Chinoy, 1994a) have also reported similar changes as well as a decline in circulating testosterone levels in rats and human populations in endemic areas of North Gujarat (Chinoy et al., 1992a). Susheela and Jethanandani (1996) also showed that testosterone concentrations decrease in males suffering from skeletal fluorosis. The above data corroborate the present findings. These results suggest that fluoride does interfere with cholesterol metabolism and testicular androgenesis.

EFFECTS ON PROTEIN METABOLISM

Fluoride has been reported to inhibit protein synthesis in Hela cells (Vesco and Colombo, 1970). Shashi et al. (1987) revealed a significant decline in acidic, basic and total proteins in rabbits treated with NaF for 100 days. Similar changes were observed in various soft tissues of rodents treated with different doses of NaF for 30 days (Chinoy and Sequeira, 1989a; Chinoy et al., 1991a,b,c; 1992b; 1993a,b; 1994b,c; 1995; 1997a; Patel and Chinoy, 1997; Chinoy and Sharma, 1998). The results of the present study corroborates with the above data as a significant decline in the levels of total protein in testis, epididymides, vas deferens, liver, muscle and kidney were obtained after 30 days.
of NaF treatment. Lack of adequate protein turnover would have an adverse effect on the enzymes, receptors and secretions. Thus one of the factors responsible for arrest of spermatogenesis in the current study might be due to lack of available proteins necessary for cell division, growth and differentiation of germ cell during spermatogenesis. The testicular and epididymal protein profile 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). This might be a response to the stress imposed by NaF.

EFFECTS ON OXIDATIVE AND ENERGY METABOLISM

Succinate dehydrogenase (SDH) is an oxidative enzyme involved in the Krebs cycle. In the present study, a significant decrease was observed in the activity of SDH in testis, epididymides and gastrocnemius muscle of fluoride treated mice. This would affect the conversion of succinate to fumarate and may cause a block in the Krebs cycle. Moreover, SDH is a mitochondrial enzyme and its decreased activity indicates a possible alteration in mitochondrial structure and function as a result of fluoride ingestion. To support this observation, ultrastructural studies in the present investigation in testis, cauda epididymis and liver as well as report of Chinoy and Patel (1998b) in ovary of fluorotic mice revealed alterations in mitochondrial structure. Some other reports are also available suggesting fluoride induced alterations in mitochondrial structure in different tissues of fluorotic animals (Chongwan and Daijei, 1988; Pang et al., 1996). Pang et al. (1996) carried out an in vitro study on rat skeletal muscles and reported that NaF decreased the
activities of SDH, Mg\(^{2+}\) ATPase and cytochrome oxidase. A decrease in the SDH activity was also reported by Chinoy and Sequeira (1989a) and Chinoy et al. (1991b) in testis of mice. The muscle SDH is an oxidative enzyme involved in the contractile mechanism of muscle fibers (George and Berger, 1966). Earlier studies carried out in our laboratory (Chinoy 1991b; 1992; Chinoy et al., 1991b; 1993b; 1994b,c and Patel et al., 1994) have reported decrease in the activity of SDH in gastrocnemius muscle of mice and rats by NaF in agreement with work of Bogin et al. (1976) in mice. Detailed studies on ultrastructure of muscle after NaF treatment are called for.

The activity of the enzymes SDH and adenosine triphosphatase (ATPase) reflect the state of oxidation and energy metabolism of a tissue. In the present study, the ATPase activity showed a significant decline in caput and cauda epididymides in treated mice. ATPase plays a role in sperm motility and metabolism and energy supply. The restricted energy supply for the spermatozoa as a result of decrease in ATPase would affect its motility. A similar inhibition 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; 1993b). A direct action of fluoride on the motile apparatus of sperm which inhibits the dynein ATPase in cilia was reported by Blum and Hayes (1984).

The above results clearly elucidate that fluoride affects the energy and oxidative metabolisms of reproductive and non-reproductive organs.

Sialic acid, a sialomucoprotein is essential for the maintenance of structural integrity of sperm membrane, besides aiding in maturation. The sialic acid levels were
EFFECTS ON CARBOHYDRATE METABOLISM

Glycogen is considered as one of the main fuel for muscle contraction. Its accumulation or decreased utilization under NaF treatment would affect the normal functioning of muscle. The current study revealed marked accumulation of glycogen in the vas deferens, liver and muscle. Earlier work carried out in our laboratory reported similar effects in different soft tissues (vas deferens, uterus, liver and muscle) of rats and mice (Chinoy, 1992; Chinoy and Sequeira, 1989a; Chinoy et al., 1991b; 1993b; 1994b; 1995; Chinoy and Patel 1996; Patel and Chinoy, 1997; Chinoy and Sharma, 1998; Patel et al., 1994) and fishes (Chinoy et al., 1994a; Shaikh and Hiradhar, 1985). The increase in glycogen could be correlated with the decrease in the activity of phosphorylase in these organs as obtained in the present study and as reported earlier by others (Chinoy, 1992; Chinoy et al., 1991b; 1993b; 1994b; 1995; Chinoy and Patel, 1996; Patel and Chinoy, 1997; Chinoy and Sharma, 1998). Thus, the phosphorylase activity and the levels of glycogen of a tissue are correlated. The fluoride induced decline in the activity of glucose-6-phosphate dehydrogenase in rats would also affect the glycogen metabolism (Carlson and Suttie, 1966).

EFFECTS ON NUCLEIC ACIDS

Fluoride has been reported to cause depression in DNA and RNA synthesis in the
cultured cells (Strochkova et al., 1984). Shashi (1993) found decrease in DNA and RNA levels in thyroid gland of rabbits of both sexes in acute and chronic fluoride intoxication (5, 10, 20, 50 kg/kg body weight for 100 days) and in rabbit ovary by treatment of fluoride (100 mg/kg body weight) (Shashi, 1994). Similar changes were observed in ovary, liver and muscle of NaF or NaF + AlCl₃ treated mice (Patel and Chinoy, 1998; Chinoy and Patel, 1999; Patel and Chinoy, 2000; Memon and Chinoy, 2000a). A decrease was also observed in DNA and RNA levels in testis and cauda epididymis of fluorotic mice (present work) in corroboration with the above data. This might be due to the inhibitory action of fluoride of DNA. It is known that the inhibition of DNA synthesis might result in delayed mitotic and meiotic cycles including chromosomal breakages (Vorishilin et al., 1973). Treatment of Hela cell monolayers with toxic concentrations of fluoride inhibited DNA, RNA and protein synthesis (Holland, 1979). Thus, it is likely that process of transcription and translocation would be affected in NaF treated mice. Further, detailed studies in this direction are called for in the future.

EFFECT OF NaF ON SPERM METABOLISM AND FERTILITY RATE

In the present study, the low sperm motility obtained may be correlated with its reduced metabolic activity by fluoride. Chinoy and Narayana (1994) revealed that human spermatozoa lost their motility \textit{in vitro} in the presence of 250 mM NaF within 20 minutes incubation, while Schoff and Lardy (1987) demonstrated that bovine sperm treated with 30 mM fluoride became immobile within two minutes. Earlier studies carried out by Chinoy and associates (Chinoy et al., 1991a,c; 1992b; 1994b; 1995; 1997b; Chinoy and
Sequeira, 1992; Chinoy and Sharma, 2000; Narayana and Chinoy, 1994b) found similar effects on spermatozoa by NaF treatment to rodents.

A reduction in the cauda epididymal sperm count in mice, rats and rabbits have been reported by Chinoy and co-workers following fluoride ingestion (Chinoy and Sequeira, 1992; Chinoy et al., 1991a; 1992b; 1994b; Narayana and Chinoy, 1994b). Similar results were obtained in the current investigation. The decrease in sperm density could be correlated with testicular spermatogenic arrest following NaF ingestion in mice as obtained in the present study as well as by others (Chinoy and Sequeira, 1989b; Kour and Singh, 1980b). These changes suggest a clear relationship between fluoride intake and testicular damage which hampers spermatogenesis.

After NaF treatment the spermatozoa exhibited loss of acrosome integrity and deflagellation which resulted in increase in the number of abnormal forms. However, this treatment did not bring about any change in nuclear integrity as observed by acridine orange staining of cauda epididymal spermatozoa. Sperm mitochondrial activity index was also inhibited. A significant decline in percentage of live sperms occurred following fluoride treatment.

Sodium fluoride caused head and tail abnormalities of epididymal spermatozoa in corroboration with earlier observation in rat and rabbit spermatozoa (Narayana and Chinoy, 1994b; Chinoy et al., 1991a). The head to head agglutination process could be due to changes in sperm acrosomal or plasma membrane proteins causing stickiness and their clumping (Chinoy et al., 1991a). As epididymal proteins are important sperm antigens, it is likely that the NaF treatment caused configurational or qualitative changes
in sperm surface protein/antigens. This aspect needs to be investigated further.

NaF caused a significant inhibition of sperm acrosomal membrane bound enzymes, viz., hyaluronidase and acrosin in the present study. These are two main acrosomal enzymes required for the acrosome reaction before fertilization. Hyaluronidase, a lysosomal enzyme has a role in the dispersion of the cumulus oophorus and thus facilitates sperm penetration (Zaneveld et al., 1973). The reduction in hyaluronidase activity obtained after fluoride treatment might therefore be associated with lower penetrating and fertilizing ability of the sperm. Similarly, the sperm acrosomal acrosin activity was also affected by NaF treatment. The elevation in proacrosin activity but decrease in free acrosin and acrosin-acrosin inhibitor complex, in NaF treated mice suggest a block in auto-activation of proacrosin. The results of the present study are in agreement with those reported in rat spermatozoa upon administration of fluoride at a dose of 10 mg/kg body weight for 50 and 70 days (Narayana and Chinoy, 1994b).

The above mentioned alterations in sperm morphology and metabolism might be the outcome of altered and hostile internal milieu of the epididymis of NaF treated mice as the epididymal micro-environment is important for sperm maturation and maintaining them in a viable, motile state. The present findings are in agreement with results obtained by Chinoy and co-workers (Chinoy and Sequeira, 1989b; 1992; Chinoy et al., 1991a,c; 1994b). The reduction in sperm count, motility, viability and changes in their metabolism led to the inhibition of fertility of treated mice. Similar loss of fertility in NaF treated 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, 1998b.
1994b). Neelam et al. (1987) reported infertility among married men in a highly endemic area in India.

The results obtained thus demonstrated that fluoride ion has adverse effects on the male reproductive system. Very high concentrations of fluoride (70-800 ppm) in the diet interferes with reproduction (Shashi, 1990). A study by Messer et al. (1973) revealed a definite decline in the reproduction of mice when fed 100-200 ppm fluoride for 5 weeks.

Patel and Chinoy (1998) found irregular oestrus cycle with prolonged duration of the diestrous stage which inturn severely affected the fertility rate following NaF treatment to female mice. These authors also reported absence of implantation sites in NaF treated females when mated with control males.

Occupational exposure to organic fluoride induced abnormal menstruation, increased frequency of miscarriages and pregnancy complications among female workers of fluorine factories (Zhang et al., 1993). Epidemiological study of gynecological problems in female workers in a superphosphate manufacturing plant offers a direct evidence of fluoride effects in human pregnancies (Kuznestova, 1969a,b).

**FLUORIDE AND FREE RADICALS**

Free radicals are highly reactive species that have an unpaired electron. Cellular damage caused by these oxygen derived species has been speculatively implicated in the aetiology of a range of diseases such as atherosclerosis, cancer, Parkinson’s disease and other neurodegenerative disorders. It has also been suggested that many of the degenerative changes associated with ageing may be due to the cumulative effects of free
Free radicals and lipid peroxidation play an important role in fluorosis (Sun et al., 1994). Fluoride is known to stimulate the so called respiratory burst and the production of superoxide radicals in neutrophils of humans, rabbits and guinea pigs. The high reactivity of superoxide radicals may lead to chemical modification and impairment of proteins, lipids, carbohydrates and nucleotides in living cells (Rzeuski, 1998). High fluoride concentrations are reported to inhibit superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in blood in people living in areas endemic to fluorosis in China (Li and Cao, 1994; Bian et al., 1994; Dai et al., 1999). Similarly, fluoride administration inhibited the activities of superoxide dismutase, glutathione-peroxidase (GSH-Px) and catalase in the ovary and testis and increased their lipid peroxidation, thus rendering the issue susceptible to injury (Chinoy and Patel, 1998a; Chinoy and Sharma, 1998). The results of the present study in testis and liver of NaF treated mice corroborate the above data. NaF + AlCl₃ treatment to mice also resulted in similar changes as described above in the brain (cerebral hemisphere) (Memon and Chinoy, 2000b; Chinoy and Patel, 2000).

The most important consequences of injury are the denaturation of proteins and the peroxidation of membrane lipids, with an increase in the permeability of the cell membrane (Subramaniam et al., 1994). The depleted GSH by NaF treatment strongly suggests that, like several compounds, fluoride might also be largely dependent on GSH for detoxification (Suketa and Mikami, 1977), since, fluoride induced lipid peroxides could be reduced by oral intake of glutathione and selenium in rats (Liang et al., 1999;
Similarly, some herbal antioxidants have also been used in reducing free radical damage in case of chronic fluorosis in China (Liu, 1999).

Hence, from the above data, it was concluded that fluoride appears to cause an imbalance in the antioxidant defence system by inhibiting some related enzymes and by depressing the GSH levels, thereby enhancing the free radical mediated peroxidation of lipids. This could be the possible mechanism for the fluoride induced free radical toxicity.

NaF treatment for 45 days brought about significant increase in total ascorbic acid (AA) levels in the testis and liver. Chinoy et al. (1993a) have also reported enhanced levels of AA in liver and adrenal gland of fluoride treated rats. Adrenal gland is known to be involved in the stress mechanism and helps in overcoming it by an increased utilization and mobilization of AA from the bound form (Chinoy, 1978). It has been reported that ingestion of inorganic fluoride in rats caused promotion of ascorbic acid (AA) synthesis (Venkateshwarlu and Narayana Rao, 1957). The increase in AA level observed in the present study might be related to augmented synthesis of the vitamin in the liver, in order to overcome the imposed stress by fluoride.

The data of present investigation revealed decreased levels of creatinine in the kidney in agreement with report of Chinoy et al. (2000) in NaF treated mice muscle. However, the levels were elevated in the serum by NaF which suggests muscle damage, since the alterations in serum or urine creatinine levels are indicative of impairment of muscle function. The levels of creatinine in the serum of workers exposed to air borne fluoride at an aluminium plant in China were also significantly decreased (Yu et al.,
In the present study, the levels of serum glutamate oxaloacetate transaminase (SGOT) and serum pyruvate transaminase (SGPT) which are considered as specific markers for liver function, showed significant increase in NaF treated animals suggesting alteration in liver structure and function. The increase in levels of serum enzymes are usually associated with damage or death of the cell. Malfunctioning of cellular activity is associated with disturbance in the function of cell membrane and as a result, the enzyme enters the serum and its levels become high. Transaminases are known to be increased in animals and humans due to fluorosis (Tsunoda et al., 1985; Chinoy et al., 1992a). The results of current investigations (including the histology and ultrastructure) confirms the hepatocellular damage and change in liver function by fluoride as reported by others in liver of different animal models (Chitra et al., 1983; Chinoy et al., 1991c; 1993a; 1994a).

EFFECTS OF NaF ON ELECTROLYTES

Sodium fluoride treatment brought about alterations in serum electrolyte levels with significant enhancement of sodium and potassium ions in mice. Increased serum electrolyte (sodium and potassium) levels were observed earlier in rats intoxicated with fluoride and in sera of human population residing in endemic areas (Chinoy and Narayana, 1992; Chinoy et al., 1992a; 1994d). Suketa et al. (1977) and Suketa and Mikami (1977) observed changes in physiological mobilization of ions such as sodium, potassium, magnesium and calcium by a single oral dose (50 mg/kg body weight) of
sodium fluoride in rats. The changes observed in sodium and potassium would affect the electrolyte balance of several tissues of the body and may cause loss of water from the cells and tissues which would affect the extracellular and intracellular water. McIvor et al. (1985) reported a fluoride induced potassium efflux from cells. The increased amount of potassium in serum suggests cell deterioration since potassium level acts as an indicator for cell damage (McIvor et al., 1985). Hyperkalemia would also affect nerve and muscle functions.

The role of calcium is known in several vital processes viz., blood coagulation, muscle contraction, impulse transmission in nerve, sperm motility and as a second messenger in signal transduction (Alberts et al., 1989). The levels of calcium in the serum of fluoride fed mice revealed a significant decline as compared to control. The decrease in serum calcium might have occurred as a result of alterations in thyroid and/or parathyroid gland functions (Teotia et al., 1978). Rabbits chronically exposed to fluoride revealed ectopic calcification in the aorta (Susheela and Kharb, 1990). Similarly, Suketa et al. (1977) reported fluoride induced renal calcification. Therefore, the risk of kidney stone formation is likely in fluorotic populations in endemic areas.

The present study thus elucidates that NaF treatment brought about significant structural and functional alterations in the soft tissues.

WITHDRAWAL STUDIES

In view of the fluoride induced toxic effects reported above, in a different group of animals, NaF was fed for 30 and 45 days and the treatment was withdrawn afterwards.
for another 30 and 45 days. During this period, the animals were maintained on standard diet and water *ad libitum*.

The data of the current investigation revealed that upon withdrawal of treatment, insignificant recovery was obtained in most of the parameters studied. Chinoy and co-workers (Chinoy et al., 1991a; 1995; Chinoy and Sequeira, 1992; Narayana and Chinoy, 1994b; Patel and Chinoy, 1997; Chinoy and Sharma, 1998; Chinoy and Patel, 1998a) have reported partial or incomplete recovery in several biochemical parameters in various organs after the withdrawal of NaF treatment for one or two months. Therefore toxic effects induced by fluoride were found to be partially reversible after cessation of fluoride treatment.

II. **BENEFICIAL EFFECTS OF ASCORBIC ACID (AA) AND CALCIUM (Ca) ON FLUORIDE INDUCED EFFECTS**

To evaluate the role of ascorbic acid in overcoming the fluoride induced effects, a group of animals were administered ascorbic acid (AA) orally during the NaF withdrawal period. The parameters investigated were same as those under NaF treatment.

The results showed that ascorbic acid administration during the NaF withdrawal period manifested significant recovery in all the parameters studied. This would be attributed to the active detoxification of the toxicant by ascorbic acid (Chinoy, 1978). Many epidemiological and experimental studies have shown that dietary factors such as protein, calcium, vitamin C etc., could modify the toxic effects of fluoride in experimental animals (Chinoy, 1991a,b; Chinoy et al. 1994b). Pandit and Narayana Rao (1940) reported
that green vegetables containing ascorbic acid mitigated the effect of fluoride in monkeys, while, deficiency of vitamin C is a contributing factor in the aggravation of fluoride toxicity. A number of the ensuing studies have demonstrated mitigation of fluorosis in experimental animals and fluorotic human population by the administration of vitamin C (Wadhwani, 1954; Chinoy, et al., 1991a; 1993b; 1994b,c; 1995: 1997a,b; Narayana and Chinoy, 1994b; Patel and Chinoy, 1997; Chinoy and Sharma, 1998; Mehta and Chinoy, 2000).

Ascorbic acid (AA) is an important biologically active antioxidant which is widely distributed in animal cells (Chinoy, 1978). Ascorbic acid is known to inhibit phosphodiesterase (PDE) (Pasternak, 1979) and thereby increase C-AMP levels. The increase in C-AMP, a second messenger, might have resulted in the recovery in the activities of several enzymes in different tissues. Ascorbic acid itself is known to activate several hydroxylating enzymes and those involved in the oxido-reduction reactions in various tissues (Chinoy, 1978). Antioxidative preparations containing glutathione, B carotene and superoxide dismutase have been used for the cure of endemic fluorosis and arsenism (Qiu and Sun, 1999). Therefore, the results emphasize that an antioxidant like ascorbic acid does play a beneficial role in the amelioration of fluoride induced toxic effects.

The mechanism of action of calcium (Ca) is that it combines with fluoride to form an insoluble compound, CaF$_2$, thereby reducing its absorption. Calcium activates several enzymes, whereas, both calcium and ascorbate are known as inhibitors of phosphodiesterase (PDE) and enhance C-AMP levels (Pasternak, 1979; Rasmussen, 1989).
Ameliorative role of Ca for mitigation of fluoride induced toxicity in mice, rats, rabbits has already been reported by Chinoy and co-workers (Chinoy et al., 1994b; 1995; 1997a,b; Chinoy and Sharma, 2000). Farley et al. (1983) also reported that long term calcium therapy was beneficial for osteoporosis. In the present study also, a significant reduction in serum fluoride levels and recovery in several parameters was obtained by calcium administration, which would be a contributing factor in maintaining body and organ weights by allowing normal food intake, recovery of body metabolism as well as electrolyte concentrations.

Therefore, these results clearly indicate that calcium has an important role in alleviating the fluoride toxic effects. Thus, it could be a very beneficial agent against fluorosis.

To a different group of animals, NaF was given orally for 30 days at a dose of 5 mg/kg body weight. The treatment was withdrawn on 31st day and the animals were treated with AA and Ca (15 and 25 mg/animal/day) in combination for another 30 days. The results showed significant recovery in all the parameters affected by NaF treatment. The extent of recovery was however more pronounced by the combined treatments of AA and Ca than by their individual treatments. This might be due to an additive/synergistic action of the two chemicals as reported earlier in mice, rats, guinea pig and rabbits (Chinoy et al., 1991a; 1993b; 1994b,c; 1995; 1997a,b; Narayana and Chinoy, 1994b). Experimental studies on monkeys proved the absolute necessity for calcium and vitamin C in the prevention of fluorosis (Srikantia, 1974). Studies conducted on diet surveys indicated that inadequate calcium and ascorbic acid have been related to severity of
endemic fluorosis (Sriranga Reddy and Srikantia, 1971). Extensive studies carried out earlier have demonstrated that calcium and vitamin C deficiency especially under fluoride toxicity, poor nutrition and hard labour exaggerate the endemic fluorosis (Siddiqui, 1955).

The present study confirms that calcium and ascorbic acid have significant beneficial role in amelioration of fluoride toxicity and manifest a synergistic effect in recovery of NaF induced alterations. It is well known that due to excessive amounts of fluoride in drinking water and foods, a large part of the globe has been crippled by fluorosis. Vitamin C and calcium deficiency might therefore cause aggravation of fluorosis in fluoride endemic areas. Hence, it is very necessary to recommend these two therapeutic agents, atleast in children, so as to prevent this health hazard.

III. BENEFICIAL EFFECTS OF VITAMIN E AND D ON FLUORIDE INDUCED EFFECTS

A group of animals were administered sodium fluoride (NaF) (5 mg/kg body weight) for 30 days. The treatment was then withdrawn from day 31 and administered vitamin E and vitamin D alone and in combination at a dose of 2 mg/animal/day and 0.002 μg/animal/day for another 30 days.

The results revealed that a significant recovery from NaF induced effects occurred following administration of vitamin E or vitamin D alone and in combination. Moreover, the recovery was almost same as that produced by ascorbic acid or calcium alone and in combination.

Vitamin E (α-tocopherol) has therapeutic roles in numerous disease states
especially those involving oxidation related events (Phelps, 1987). Isomers of tocopherol function as biological antioxidants and free radical scavengers (Burton and Ingold, 1989; Burton, 1990; Basu and Dickerson, 1996; Chinoy and Sharma, 1998). The deficiency of vitamin E in experimental animals results in reproductive failure (Nelson, 1980), necrotizing myopathy, liver and kidney damage and neurological abnormalities. Vitamin E deficiency is also associated with impaired mitochondrial oxidative metabolism and activity of microsomal cytochrome P450 dependent mixed function, oxidases and hence the metabolism of mitochondria will be affected (Bender, 1992). These effects are similar to those associated with fluoride intoxication (Burgstahler, 1985).

Chinoy and Sharma (1998) have reported that ingestion of vitamin E to fluorotic male mice brought about a significant recovery in NaF induced reproductive failure. Vitamin E also reduces cell injury and has a therapeutic role as a potent biological antioxidant (Chinoy and Sharma, 1998).

Chinoy and Patel (1999) have reported that vitamin E administration to fluoride fed female mice brought about significant recovery in several parameters including the activities of SOD, and levels of lipid peroxides in ovary, as well as serum calcium and potassium levels.

The chief function of vitamin D is to promote the intestinal absorption of calcium and phosphorus and thus maintain an optimal blood concentration of these elements for calcification of the bones. Within the kidney, vitamin D increased the clearance of phosphate (Marks, 1975). Administration of vitamin D during NaF withdrawal period to mice was found to be effective in recovery of NaF induced effects, thus elucidating its
beneficial role (Chinoy and Sharma, 1998; Sharma and Chinoy, 2000; Chinoy et al., 2000). Similar results were also found in female mouse after vitamin D administration during the withdrawal period (Chinoy and Patel, 1998a).

The above reports thus elucidate that vitamins E and D could mitigate fluoride induced effects.

IV. BENEFICIAL EFFECTS OF AMINO ACIDS (GLYCINE AND GLUTAMINE) IN MITIGATION OF FLUORIDE INDUCED EFFECTS

Sodium fluoride was administered at a dose of 5 mg/kg body weight orally to male mice daily for 30 days. The effects of withdrawal upon cessation of NaF ingestion and administration of amino acids viz. glycine or glutamine alone and in combination were investigated.

The results revealed that administration of amino acids, glycine or glutamine individually and in combination in the withdrawal period helped in maintaining status quo of all parameters as compared to control, thus elucidating their ameliorative role.

Amino acids are important biologically active antioxidants. Suttie et al. (1974) reported that addition of various amino acids to the growth media in concentrations in excess of those present in the media enhanced the growth of fluoride treated mouse fibroblast cells. Reports by Chinoy and Patel (1996) and Patel and Chinoy (1998) revealed that supplementation of amino acids (glycine and/or glutamine) alone and in combination manifested an ameliorative influence in all NaF induced effects in ovary and uterus of mice. The recovery was more pronounced when both glycine and glutamine were...
administered together. The basis for the protective role may involve slight changes in intracellular fluoride concentrations, or some metabolic alterations.

Glycine occurs in a relatively high proportion in proteins belonging to the collagen and elastin categories. The recovery obtained by glycine ingestion might be due to its role in various important physiological functions. Glycine acts as a conjugating agent and renders toxic metabolites more soluble and thus facilitates their excretion. The levels of fluoride in the tissue were significantly lower in groups administered glycine along with NaF (Chinoy et al., 1996). The conversion of glyoxylate to glycine by transamination has been demonstrated in several systems (Meister, 1965) which could be related with the amelioration of fluoride induced toxicity. Glycine may be metabolically converted to pyruvate which is an important metabolite in the process of glycolysis, while glutamine, on deamination, is converted to ketoglutarate which is an intermediate metabolite in citric acid cycle (Harper, 1965). Glutamine is needed for the growth of mammalian cells in tissue culture in concentrations considerably higher than other amino acids (Meister, 1965). Thus, the ingestion of the amino acids glycine and/or glutamine was beneficial in promoting the recovery from fluoride induced toxicity. The ameliorative effect of the amino acids was probably due to their roles in various physiological functions (Harper, 1965).

V. EFFECTS OF PROTEIN SUPPLEMENTATION AND DEFICIENCY ON FLUORIDE INDUCED TOXICITY

In the present study, the effects of sodium fluoride at a dose of 5, 10 and 20
mg/kg body weight/animal for 30 days fed along with a control protein diet, protein-deficient diet and protein-rich diet respectively were investigated. These studies were undertaken to determine if a protein supplemented diet would have ameliorative effects in reducing fluoride toxicity.

The results of the present study revealed that fluoride treatment administered to mice fed a protein-deficient diet aggravates the toxic effects. On the contrary, feeding a protein-rich diet definitely has a beneficial influence in reducing NaF induced toxicity in various organs.

Sriranga Reddy and Srikantia (1971) have reported that in experimentally produced fluorotic monkeys, administration of a low protein diet appeared to accelerate the development of reflection of bones and a higher incidence of rarefaction was observed in these animals. The results obtained in the present study corroborate the above mentioned data and suggest that a protein supplemented diet would be beneficial, while, a protein-deficient diet would aggravate fluoride toxicity. Therefore, these results have important implications the world over and especially in developing countries where protein malnutrition and the occurrence of fluorosis co-exist. More detailed studies in this direction are therefore solicited in future.

The present study supports many epidemiological and experimental studies which have shown that dietary factors such as proteins, vitamins and amino acids could modify the toxic effects of fluoride.

Thus, it is concluded that sodium fluoride has a definite effect on reproduction as well as other soft tissue functions. Fluoride induced effects were not completely reversed
by withdrawal of treatment. However, supplementation of vitamins C, D, E, calcium, amino acids and a protein-rich diet were more conducive for recovery from fluoride toxicity. Thus, fluoride induced effects are transient and reversible by the use of the above mentioned therapeutic agents. Hence, mitigation of fluoride induced toxicity in endemic areas could be possible all over the world. These studies have important implications for amelioration of fluorosis in endemic regions and as such are a significant contribution in the field.