CHAPTER IV

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

During the tenure of the present investigation, the toxic effects of sodium fluoride ingestion for varied durations (7, 15, 30, 45 and 60 days) was studied in order to evaluate time related changes on the structure and physiology of reproductive organs of adult, female albino mice (*Mus musculus*) of Swiss strain. Sodium fluoride (NaF) was administered orally at a dose of 5 mg/kg body weight/day. The dose used was based on the LD$_{50}$ value of fluoride, i.e. 51.6 mg F/kg body weight in female mice (Pillai et al., 1987). Oral administration was preferred in view of water being the main source of fluoride among the human population in endemic areas.

The various parameters studied at the end of treatment were body weight, uterus, ovary weights, histology and ultrastructure of these organs. In addition, the concentration of glycogen and phosphorylase activity in the uterus, levels of serum catecholamines and blood glucose were carried out to investigate the alterations in carbohydrate metabolism. The levels of DNA, RNA, DNA/RNA and RNA/Protein ratios were evaluated to investigate the effects on nucleic acid metabolism in uterus and ovary. Similarly, certain specific parameters viz., cholesterol, activities of 3β and 17β hydroxysteroid dehydrogenases and serum E$_2$, FSH and LH levels were investigated to study the impact of NaF on ovarian functions. To evaluate ovarian cell injury, the activities of some antioxidant enzymes vis., lipid peroxidase, superoxide
dismutase, catalase, glutathione peroxidase and levels of glutathione and ascorbic acid were evaluated. The serum electrolyte concentrations \((\text{Na}^+, \text{K}^+ \text{ and } \text{Ca}^{2+})\) and the tissue burden of fluoride as well as fluoride levels in the serum and urine were also investigated. Fertility rate and cyclicity were studied during the course of the investigation.

In a different set of experiments, the treatment was withdrawn after 45 days of \(\text{NaF}\) ingestion to study the reversible effects if any, upon withdrawal of treatment.

In view of fluoride induced toxic effects, the therapeutic action of some agents viz., calcium, vitamins (C, D and E) and aminoacids (glycine, glutamine) 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, ovary and uterus of \(\text{NaF}\) treated mice revealed an enhancement, which indicates that the 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 \(\text{Mg}^{2+}, \text{Ca}^{2+}\), \(\text{Al}^{3+}\) etc. (Krishnamachari, 1986), urinary flow and pH as well as previous exposure to fluoride (Whitford et al., 1976; Schiffl and Biswanger, 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 consumption of high fluoride intake. The retained fluoride in the serum, thus would affect the general body metabolism in these individuals,
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 7, 15 and 30 days did not produce any significant alterations in the above mentioned parameters. NaF was found to be effective from the 45th day onwards and the effects were more pronounced after 60 days of treatment. 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). Fluoro-silicate, a fluoride derivative was also found to cause loss of appetite in cattle (Egyed and Shlosberg, 1975). Reports from our laboratory have revealed loss of body weight in mice and rats after ingestion of 10 mg/kg bodyweight fluoride for 30 days (Chinoy and Sequeira, 1989a; Chinoy et al., 1992a; 1993a). A similar decline in body weight occurred in rabbits fed with 40 mg/kg body weight fluoride for 30 days (Chinoy et al., 1991c). 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).

The results of the present study corroborate the above data as a significant decline in the body weight was obtained after 45 and 60 days of 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.

The uterine weight was declined, whereas, the ovarian weight was found to be unaltered throughout the treatments. The low uterine weights might be due to decline in protein levels alongwith reduced metabolic activity as observed in the present study.
The histological and ultrastructural studies on ovary of mouse treated with 5 mg/kg body weight NaF revealed structural alterations after 30 days. The effects were more pronounced with the concomitant increase in the duration of fluoride treatment. NaF (30 days) brought about vacuolisation in the stromal tissue and corpora lutea follicular atresia and pyknosis in follicular cells. After 45 days NaF treatment, disintegration and dense vacuolisation in the stroma, follicular atresia and atrophy of corpus luteum was observed. The degenerative changes were more pronounced after 60 days.

Ultrastructural studies revealed disruption of mitochondria with lack or fragmentation of cristae, indentation of the nuclear membrane, vacuoles and lipid droplets in the cytoplasm of granulosa cells. Histopathological studies in rabbit ovary in experimental fluorosis had also revealed marked necrosis of follicular tissue, congested and atrophic follicles with interstitial oedema (Shashi, 1990). Thus, the above data clearly elucidates alterations in ovarian structure which would influence its functions.

Fluoride has been reported to inhibit protein synthesis in Hela cells (Vesco and Colombo, 1970). Shashi et al. (1987) revealed significant decline in acidic, basic and total proteins in rabbits treated with NaF for 100 days. A decline in protein levels occurred in various soft tissues of rodents treated with different doses of NaF for 30 to 70 days (Chinoy and Sequeira, 1989a; Chinoy et al., 1991b,c; 1993a; 1994b,c; 1995; 1997a; Narayana and Chinoy, 1994 a,b). The results of the present study corroborate with the above data as a significant decline in the levels of total proteins in uterus, ovary and serum was obtained after 45 and 60 days of treatment. This might related to the impairment of polypeptide chain initiation by fluoride ions (Godchau
and Atwood, 1976). Weak incorporation of aminoacids into proteins (Helgeland, 1976) or possibly inhibition of DNA synthesis by fluoride in cells in vitro has also been reported (Holland, 1979). However, testicular and epididymal protein profile of rats showed induction of some new proteins after fluoride treatment which were not present in the control animals (Chinoy et al., 1995; 1997a). The decline observed in protein levels of uterus and ovary in the present study would affect the activities of its various enzymes and reduce their secretions.

The ovarian 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 ovary 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 of the mice ovary were carried out which revealed that fluoride treatment caused structural disorganisation and rupture of mitochondrial cristae. Similar results were reported in rabbits fed with fluoride (Chongwan and Daijei, 1988). It therefore follows that the mitochondrial enzymes would be affected by NaF treatment. Similarly, fluoride treatment caused a decrease in SDH activity in various reproductive tissues and muscle of rodents (Chinoy and Sequeira, 1989a; Chinoy et al., 1991a,c; 1992a; 1993a). The decrease in SDH might be similar to that of isocitrate dehydrogenase (Dousset et al., 1987), another TCA cycle enzyme which leads to accumulation of citric acid. Bogin et al. (1976) reported that LDH and isocitrate dehydrogenase levels were reduced in various tissues of mice treated with 100 ppm NaF.
The ovaries have two interrelated functions viz., gametogenesis and steroidogenesis (Knobil and Neill, 1988).

In the present study, the exploration of intermediary enzymes in the steroidogenic pathway after fluoride treatment revealed a decline in the activities of 3β hydroxysteroid dehydrogenase (HSD) (which converts dehydroepiandrosterone into androstenedione) and 17β hydroxysteroid dehydrogenase (which converts androstenedione into testosterone). These results were correlated with a decrease in the circulating serum estradiol levels and a hypercholesterolemic effect in the serum as well as accumulation of cholesterol in the ovary, indicating that the metabolism of cholesterol might be altered. These results are also supported by the ultrastructural studies in the ovary which revealed extensive lipid droplets in the granulosa cell cytoplasm. The lipid droplets represent the storage site for cholesterol and other secretory products (Nicosia, 1980). Some earlier studies (Narayana and Chinoy, 1994a) have also reported changes in testicular cholesterol levels with a decrease in the activities of 3β and 17β hydrosteroid dehydrogenases and circulating testosterone levels in rats as well as a decline in the testosterone levels of human populations in endemic areas of North Gujarat (Chinoy et al., 1992b). The above data corroborate the present results in female mice.

The principal action of estradiol is the manifestation of oestrus, changes in the reproductive tract and mammary glands, and to regulate the secretion of gonadotropins. In the present study, NaF administration caused an enhancement alterations in the serum concentrations of circulating FSH and LH, indicating changes in the hypothalamo-pituitary-gonadal axis leading to altered ovarian folliculogenesis, steroidogenesis and other functions. These changes thus caused a decline in the serum
estradiol levels in the present study. Tokar and Savchenko (1977) also obtained low testosterone levels with high FSH and LH in individuals afflicted with fluorosis. Similarly, other workers have also reported reduction in testosterone levels in fluorotic individuals (Chinoy et al., 1992b; Susheela and Jethnandani, 1996).

Furthermore, the histomorphometric studies revealed a significant change in corpus luteum diameter and other histological features of the ovary. Target organ structure and functions which are dependent on circulating estrogen levels were adversely affected after fluoride intoxication. The possible impact of fluoride may be on the estrogen receptor sites, viz., by altering the concentration or configuration of the receptor, the action of estrogen on the target organ might be inhibited. Therefore, it is important to investigate these aspects in order to understand the exact mechanism of action of fluoride at the receptor level, especially in the target sites.

The effect of fluoride on serum lipids are controversial. Townsend and Singer (1977) observed decrease in serum cholesterol in guinea pigs exposed to fluoride. On the other hand, fluoride treatment to rabbits with 100 mg/kg body weight for 100 days resulted in a 2-3 fold increase in cholesterol and triglycerides in the brain (Shashi, 1992). Similarly, Vatassery et al. (1980) reported an increase in serum cholesterol in guinea pigs which had received deionized water containing 25 ppm fluoride for 13 weeks. However, according to Singer and Armstrong (1971), an increase in fluoride intake did not influence serum cholesterol. The serum cholesterol levels did not show any significant variation after 30 days fluoride administration to rats (Chinoy and Sequeira, 1989a) which elucidates that short term fluoride intake may not cause hypo/hypercholesterolemia in these animals. However, chronic exposure for 70 days resulted in a decrease in serum cholesterol in rats (Narayana and Chinoy, 1994a).
Ascorbic acid depletion is considered as an index for steroidogenesis in the ovary and is involved in overcoining stress. NaF brought about a significant decline in total ascorbic acid levels in the ovary indicating that fluoride treatment causes stress in the animals leading to rapid utilization of the vitamin. This suggests that the stored ascorbic acid is rapidly oxidized in the ovary under fluoride induced stress and converted to its dehydroform which consequently showed an increase as obtained in the present study. According to Lewin (1976) maintenance of proper levels of ascorbate is essential for preventing the conversion of adrenalin and noradrenalin to adrenochrome, which are highly toxic compounds acting as protent inhibitors in many enzymatic reactions.

Glutathione (GSH) is involved in the mechanism of detoxification of various xenobiotics. In the present study, GSH significantly decreased in ovary after 45 and 60 days NaF administration, which suggests its rapid oxidation. The significantly suppressed GSH levels would further aggravate the toxic effects of NaF. The depleted GSH by NaF treatment strongly suggests that, like several exogenous compounds, fluoride might also largely be dependent on glutathione for detoxification (Chinoy, 1978). Reports from our laboratory (Chinoy et al., 1995; 1997a,b) have also revealed depleted glutathione levels in sperm suspensions of rats and guinea pigs treated with fluoride.

It is known that free radicals and lipid peroxidation may play an important role in the mechanism of fluorosis (Sun et al., 1994; Bian et al, 1994). It is known that glutathione (GSH) could inhibit peroxidation, scavenge free radicals and protect cell membranes (Patel, 1987).

Fridovich (1976) found that glutathione peroxidase (GSHPx), catalase and
superoxide dismutase (SOD) accomplish their antioxidant functions through detoxification. The superoxide anion can acquire an electron to become superoxide anion radical (O$_2^-$) which in turn initiates the oxidation of polyunsaturated fatty acids. The free radicals bring about the process of lipid peroxidation (Fridovich, 1976). Superoxide dimutase has been recognised to play an important role in the defence mechanism of the body against the harmful effects of oxygen free radical in biological systems, whereas, the two related enzymes viz., GSHPx and catalase scavenge the dismutation of superoxide radicals (Fridovich, 1976). In the present study, fluoride administration at a dose of 5 mg/kg body weight for 45 and 60 days inhibited the activities of superoxide dismutase, glutathione peroxidase and catalase in the ovary and increased ovarian lipid peroxidation, thus rendering the tissue susceptible to injury. Production of the superoxide anion O$_2^-$ is caused by an incomplete oxygen oxidation. These radicals are liable to react with several molecules, provoking their destabilization. The most important consequences are the denaturation of the proteins and peroxidation of membrane lipids, with an increased permeability of the cell membrane (Subramaniam et al., 1994). Reports by Sun et al. (1994) have revealed increased levels of LPO in liver, kidney and testis with decrease in the GSHPx and SOD activities in fluorotic mice in agreement with the results of the present study. Epidemiological studies of patients with dental and skeletal fluorosis from endemic regions in China also revealed an inhibition of GSHPx and SOD activities in blood with increased lipid peroxidation in serum (Bian et al., 1994).

In the recent years, there have been a number of studies relating to the metabolic control mechanisms in uterine tissue, particularly in terms of ovarian steroid influence under differing physiological conditions (Valadares et al., 1968; Singhal and
Valadares, 1970; Lea et al., 1970). Investigations have also been made in the rat and mouse uterine tissue which relate to the hormonal conditions (Finn and Martin, 1970) and metabolic requirements of egg implantation (Surani and Heald, 1971).

The uterus is the site of implantation of the blastocyst. Therefore, it is necessary to maintain the internal milieu of the uterus in the normal state conducive for implantation of the blastocyst and its further development into the embryo. Sodium fluoride treatment at a dose of 5 mg/kg body weight for 30, 45 and 60 days caused vacuolisation in the endometrium, myometrium and serosa with pyknosis in the myometrium and endometrium. The atrophy and confluence of the endometrial glands with pyknosis of their nuclei was also observed.

These investigations were supported by ultrastructural studies on uterus which revealed structural alterations in the cytoplasmic organelles of the epithelial cells. The nucleus showed indentation. The cytoplasm was packed with secretory vacuoles and empty spaces surrounding the spherical shaped mitochondria. The rough endoplasmic reticulum and Golgi complex were scanty. Due to histological and ultrastructural change in the uterus, its metabolic status was also affected by fluoride as evidenced by dramatic changes in the uterine carbohydrate and nucleic acid metabolisms (Described in subsequent part of discussion).

Effect of NaF on Carbohydrate Metabolism:

Underwood (1977) reviewed the effects of fluoride on carbohydrate metabolism. Fluoride is known to act as an inhibitor of glycolysis either by enolase mediated inhibition (Suttie et al., 1974) or decrease in the activity of isocitrate dehydrogenase. The present study revealed marked alterations in the glycolytic
pathway with a gradual accumulation in the levels of uterine glycogen from day 7 up to day 60 of treatment. Similar enhancement in glycogen was reported in different soft tissues of rats and mice (Chinoy and Sequeira, 1989a; Chinoy et al., 1993a; 1994b,c; Patel et al., 1994). In support of these results, Dousset et al. (1987) found a decline in glycogen turnover and citrate accumulation in rats fed with NaF. In the present study too, the turnover of glycogen seems to be affected as is evident from the decrease in the activity of phosphorylase in uterus of NaF treated mice. This might be the main causative factor for the accumulation of glycogen in uterus as also reported earlier for other tissues (Chinoy et al., 1994b; Patel et al., 1994). The fluoride induced decline in the activity of glucose-6-phosphate dehydrogenase (Carlson and Suttie, 1966) in rats would also affect the glycogen metabolism.

As a result of decreased glycogen breakdown, a significant decline was observed in the blood glucose levels of fluorotic mice similar to those reported earlier in chicks exposed to atmospheric fluoride in the vicinity of a fluoride emitting industry (Pillai and Mane, 1985) as well as in fishes (Chitra and Ramana Rao, 1980) maintained in fluoridated water. Recent studies carried out by Bennis et al. (1993) on goats from fluoride endemic areas revealed a similar decline in plasma glucose levels.

Catecholamines are known to regulate the carbohydrate metabolism wherein epinephrine promotes glycogenolysis. The serum of fluoride treated mice showed an enhancement in the levels of epinephrine and nor-epinephrine (present study) in agreement with the work of Cheon and Distefano (1973) and Chinoy and Narayana (1992) in rats. The increase in adrenal epinephrine and nor-epinephrine would also influence carbohydrate metabolism. The increase could be attributed to their rapid synthesis due to stress caused by fluoride intake. The enhanced catecholamine levels...
would have a stimulatory action on the sympathetic nervous system and might influence the hypothalamo-gonadal axis (Damber and Janson, 1978), and as discussed in the earlier part of this chapter. In the present study too, FSH and LH levels were increased. However, the increased epinephrine level failed to cause glycogenolysis in uterus of NaF treated mice, suggesting a probable alteration in hormone receptor interaction and signal transduction. Therefore receptor level studies are necessary to be carried out in fluoride treated animals to investigate the exact site of fluoride action.

The glycogen concentration of the uterus has long been implicated as an important nutritional reserve for the many energy consuming reactions that take place in this dynamic proliferating tissue. The energy requirements of the uterine smooth muscle contractile processes as well as the metabolic events surrounding nidation are believed to be satisfied in a major part by a rapid mobilization of glucose from the uterine glycogen reserve (Demers et al., 1972). Hence its decreased utilization would affect the normal functioning of the uterus and would alter its internal milieu.

Effect of NaF on nucleic acids:

Fluoride has been found to produce primary damage by injuring the genetic material of the cell they enter (Muller, 1961). Several human conditions including ageing, cancer and arteriosclerosis have been associated with DNA damage and its misrepair (Rubin et al., 1983). Fluoride has been reported to cause depression in DNA and RNA synthesis in the cultured cells (Strochkova et al., 1984). Earlier reports have revealed that fluoride ingestion (100 mg/kg body weight) to rabbits resulted in a decrease in DNA and RNA levels in the ovary (Shashi, 1994) in corroboration with
the present data, wherein, a decrease was observed in DNA and RNA levels in uterus and ovary of fluorotic mice. This might be due to the inhibitory action of fluoride on DNA or due to alteration in the synthesis of RNA. The enzymes responsible for RNA synthesis or repair need to be studied in the future. It is known that the inhibition of DNA synthesis might result in delayed mitotic and meiotic cycles including chromosomal breakages (Vorishilin et al., 1973). Ensley et al. (1982) have investigated the interaction between nucleic acid and fluoride and proposed that fluoride could completely disrupt the thymine-adenine link in DNA duplex. Tsutsui et al. (1984) found that sodium fluoride induced morphological and neoplastic transformation, chromosome aberrations, sister chromatid exchanges and unscheduled DNA synthesis in cultured Syrian hamster embryo cells. These authors further suggested that the DNA damaging activity of fluoride may be weak or insufficient to induce detectable DNA damage (Tsutsui et al., 1984) during a short treatment time or else inhibition of protein synthesis by fluoride may retard the progression of DNA repair following DNA damage. It has been known for sometime that fluoride inhibits a number of metalloproteins (Chinoy and Sequeira, 1989a) including DNA polymerase of E. Coli (Lehman,1963). Studies on fluorotic human population in endemic areas of North Gujarat, India, have revealed an increase in frequency of sister chromatid exchange as compared to the control indicating that fluoride might have a genotoxic effect (Sheth et al., 1994).

The DNA/RNA ratio declined in the uterus, whereas, it remained unaltered in the ovary. This decrease might be due to a significant decline in RNA concentration and probably inhibition of DNA synthesis which might lead to the alteration in transcription process.
The RNA/Protein ratio was also significantly decreased in the ovary and uterus which could be related to the significant decline in protein levels. Thus, it is likely that the process of transcription and translocation would be affected in NaF treated mice. Further, detailed studies in this direction are called for in the future.

The above mentioned changes (in the uterus) might not be conducive for nidation or implantation of the fertilized ovum in the uterus of female mice. It is known that the mechanism of egg implantation requires proper interaction between estrogen and progesterone (Knobil and Neill, 1988) and a slight disturbance in this hormonal balance may lead to instability of pregnancy. In the present study, the serum estradiol levels were significantly declined in NaF treated mice in support of the above mentioned observations.

The results obtained thus demonstrated that fluoride ion has adverse effects on the female reproductive system. Very high concentrations of fluoride (70-800 ppm) in the diet interferes with reproduction (Shashi, 1990). It has been reported that large amounts of dietary fluoride affects reproductive performance causing delayed oestrus, failure to conceive repeatedly, decreased birth weights and lower viability (Hodge and Smith, 1965). 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.

Previously, it was considered that fluoride is unable to cross the placental barrier and this fact explained the common observations that temporary teeth did not show mottling. But there are some controversial reports (Roholm, 1937) which showed mottling of temporary teeth in breast fed children in abnormally high fluoride areas. Durbin Wallace (1954) found that the rat foetus contained fluoride which was approximately half the concentration of the maternal blood. The fetal fluoride
concentration was controlled by the degree of calcification of the fetal skeleton. On the other hand, Teotia et al. (1979) reported that the passage of fluoride through the placenta and its accumulation in fetal bone was directly proportional to the amount of fluoride ingested by the mother.

The evidence of infertility due to fluoride intoxication in experimental animals presented by previous workers, has thus been confirmed by the histopathological studies on the ovaries and uterus and decline in the fertility rate as observed in the present study. The study further revealed a reduction in the number of implantation sites, the Female Fertility Index, Gestation Index, Live Litter Size and Live Birth Rate, which might be due to irregularity in cyclicity and prolongation of the cycle. Hence the administration of NaF for 45 and 60 days prolonged the duration of the diestrus stage. Lengthening in the duration of diestrus stages in treated mice caused a suppression in the duration of oestrus, metaestrus and proestrus stages. The stages of oestrus cycle and their interconversions are mainly governed by the female sex hormone, estrogen and progesterone mainly synthesized in the ovary. It is known that the levels of these hormones are controlled by the secretions of pituitary gonadotropins (Knobil and Neill, 1988) through feedback mechanism and further the level of gonadotrophins are controlled by the estrogens and progesterone. In the present study, this feedback system was affected which could be a contributory factor altering the duration of oestrus cycle in adult female mice.

Sodium fluoride treatment also brought about dramatic alterations in serum electrolyte levels with significant enhancement of sodium and potassium ions. McIvor et al. (1985) reported a fluoride induced potassium efflux from cells. 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., 1993b; 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 to 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. The increased amount of potassium in serum suggests cell deterioration since potassium level acts as an indicator for cell damage (Mc Ivor 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 recently 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 brought about significant structural and functional alterations in the ovary and uterus thus leading to fertility impairment of the mice.
WITHDRAWAL STUDIES ON FLUORIDE INDUCED TOXIC EFFECTS

In view of the fluoride induced toxic effects reported above, in a different group of animals, NaF was fed for 45 days and the treatment was withdrawn afterwards for another 45 and 60 days (Group III). During this period, the animals were maintained on standard diet and water ad libitum.

Upon withdrawal of treatment, recovery was obtained in several parameters, viz., the body and organ weights, activities of 3β, 17β hydroxysteroid dehydrogenases, superoxide dismutase, catalase and lipid peroxidase in ovary, uterine glycogen levels, serum catecholamine and electrolyte levels. However, a partial recovery was observed in the levels of protein, DNA and RNA in the ovary and uterus, serum and ovarian cholesterol, levels of TAA, RAA, glutathione and the activity of glutathione peroxidase in the ovary, blood glucose level, serum estradiol, FSH and LH as well as the fertility rate and cyclicity. The tissue burden of fluoride and morphology of the ovary and uterus was also partially recovered from fluoride induced toxicity after 45 days of withdrawal of treatment. This might be due to delayed sequestration of fluoride from the body, as evident by high serum fluoride levels in the present study, even after 45 days withdrawal of treatment. However, the recovery was pronounced after 60 days in the above mentioned parameters. Earlier studies had revealed that the fluoride induced effects were significantly recovered after withdrawal of treatment to rats, mice and rabbits (Chinoy and Sequeira, 1989 a,b; 1992; Chinoy et al., 1991c, 1995; Patel et al., 1994; Narayana and Chinoy, 1994 b). This might be due to difference in the animal species, the duration of exposure and the dose used. In the present study, the results revealed that a longer period was needed for a significant recovery.
II. BENEFICIAL EFFECTS OF ASCORBIC ACID (AA) AND CALCIUM (Ca\(^{2+}\)) ON FLUORIDE INDUCED EFFECTS:

To evaluate the beneficial role of ascorbic acid in overcoming the fluoride induced effects, a group of animals were administered ascorbic acid (AA) orally for 45 days during the NaF withdrawal period. The parameters investigated were same as those under NaF treatment.

The result 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. Pandit and Narayan 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, 1952, 1954; Chinoy et al., 1991c; 1993a; 1994c).

The participation of AA in cellular oxido-reduction reactions occurs via the formation of its free radical, mono dehydroascorbic acid (MDHA) which is a more powerful reducing agent than AA by virtue of possessing an unpaired electron, which subsequently gets oxidised to dehydroascorbic acid (DHA). DHA could be converted back to AA by glutathione (Chinoy, 1978). Commoner et al. (1954) have correlated the concentration of free radical (FR) and biological activity in tissues. This could be the main mechanism behind the recovery observed in various fluoride induced effects.
alterations after AA treatment. Ascorbic acid is also known to bind with macromolecules like proteins, nucleic acids (Chinoy, 1978) by charge transfer complex formation, which appears to be a very active source of energy for biological processes. This seemed to be another probable mechanism occurring for the mitigation of fluoride induced toxicity. Ascorbic acid is also known to activate adenyl cyclase but inhibit phosphodiesterase (PDE) resulting in high C-AMP levels (Pasternak, 1979). The increase in C-AMP, which is known as the "Second messenger" might have resulted in the recovery in the activities of several enzymes in the present study, viz., SDH, phosphorylase, 3β, 17β HSD, etc. By virtue of being a reducing agent, AA itself is known to activate several hydroxylating enzymes and those involved in the oxido-reduction reactions in various tissues (Kutsy, 1973, Chinoy, 1978). In addition, AA treatment also brought about a significant recovery in electrolyte concentrations viz., Na⁺, K⁺, and Ca²⁺.

In the present study, significant enhancement in serum catecholamines were obtained by NaF treatment but which were recovered to normal levels upon ascorbic acid administration. It is also known that the activity of 3β HSD (an enzyme required for adrenal steroidogenesis) was altered in the ascorbate deficient rats as compared to the controls (Blischke and Hertting, 1971). Das and Susheela (1991) have demonstrated low steroidogenesis by adrenals in fluoride treated rabbits as well as fluorotic human population. Therefore, the results emphasize that ascorbic acid does play a beneficial role in the amelioration of fluoride induced toxic effects.

Another group of animals were treated with NaF (5 mg/kg body weight) for 45 days and the treatment was withdrawn on day 46th. These animals were then fed calcium at a dose of 25 mg/animal/day for another 45 days. The results revealed a
significant recovery in fluoride induced alterations in all the parameters studied but which was on the whole, less than that obtained by AA in comparison.

It is well known that Ca\(^{2+}\) combines with fluoride forming an insoluble compound, calcium fluoride (CaF\(_2\)), thereby reduces its absorption. In the present study also, a significant reduction in serum fluoride levels were obtained by calcium administration, which could be a contributing factor in maintaining body and organ weights by allowing normal food intake, recovery of body metabolism as well as electrolyte concentrations. The recovery observed in the glutathione levels could also be due to an increase in its synthesis as calcium is known to activate many enzymatic reactions.

Calcium is known to activate several enzymes and enzyme systems. Earlier workers demonstrated that calcium ingestion to fluoride intoxicated mice, rats and rabbits brought about a significant regain in the NaF inhibited enzyme activities (Chinoy et al., 1991a; 1993a; 1994b,c; 1995). In the current investigation, the electrolyte concentrations such as Na\(^{+}\), K\(^{+}\) which were enhanced by NaF treatment also showed reversibility upon Ca\(^{2+}\) treatment. Moreover Ca\(^{2+}\) levels were found to be significantly recovered due to the extraneous administration of calcium. It has been shown that Ca\(^{2+}\) and c-AMP interact with each other for various metabolic reactions in different tissues (Pasternak, 1979; Alberts et al., 1989). The inhibition of phosphodiesterase (PDE) activity by Ca\(^{2+}\) leads to enhanced C-AMP levels as Ca\(^{2+}\) is a known inhibitor of PDE. In his extensive studies (Rasmussen, 1989) found that calcium-ion pump was activated by the formation of calcium-calmodulin complex. The stimulation of the calcium-ion pump by Ca\(^{2+}\)-calmodulin complex and calcium activated phosphorylation enables calcium efflux to compensate for the increased...
According to Machoy (1987), one of the possible mechanisms of enzyme inhibition in the fluorotic mice may be the calcium binding to fluoride in the catalytic centre. Therefore, the excess intake of calcium might have activated the above mentioned enzymes and helped in recovery of the carbohydrate metabolism. Calcium has been found to act on beta cells of Langerhans in the pancreas and control secretion of insulin, which in turn regulates glucose levels (Rasmussen, 1989). Therefore, the recovery in carbohydrate metabolism after calcium administration could also be due to this pathway.

To a different group of animals, NaF was given orally for 45 days at a dose of 5 mg/kg body weight. The treatment was withdrawn on 46th day and the animals were treated with AA and Ca²⁺ (15 and 25 mg/animal/day) for another 45 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 Ca²⁺ than by their individual treatments. This might be due to an additive/synergistic action of the two chemicals as reported earlier in mice rats and rabbits (Chinoy et al., 1991c; 1993a; 1994b,c; 1995).

It is known that phosphodiesterase (PDE) catalyzes the conversion of C-AMP to 5C AMP, thus decreasing the levels of C-AMP. However, both ascorbic acid and calcium are recognised as potent inhibitors of PDE. Thus, it is suggested that the increased levels of C-AMP might lead to recovery of all parameters studied. A significant regain of fertility by the synergistic action of AA and Ca²⁺ was also obtained.

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). Studies conducted on diet surveys indicated that inadequate calcium and ascorbic acid have been related to severity of endemic fluorosis (Srirangareddy and Srikantia, 1971). Experimental studies on monkeys proved the absolute necessity for Ca^{2+} and Vitamin C in the prevention of fluorosis (Srikantia, 1974).

The present study confirms that calcium and ascorbic acid have significant role in 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. Therefore, 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 VITAMIN D ON FLUORIDE INDUCED EFFECTS

A group of animals were administered sodium fluoride (NaF) (5 mg/kg body weight) for 45 days. The treatment was then withdrawn from day 46 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 45 days.

The results revealed that a significant recovery from NaF induced effects occurred following administration of vitamin E which was almost same as that produced by ascorbic acid and calcium in combination. On the other hand, Vitamin D ingestion resulted in partial recovery in all the NaF induced effects to a variable extent.
Moreover, on administration of vitamins E and D alone and in combination the results revealed significant recovery from NaF induced toxic effects. The recovery was almost same as that produced by Vitamin E and combined administration of ascorbic acid and calcium.

Vitamin E has come under much scrutiny for its possible therapeutic roles in numerous disease states especially those involving oxidation related events (Phelps, 1987) and is proved to be the most potent biological antioxidant form of the Vitamin (Farrell, 1980; Burton et al., 1985; Burton and Ingold, 1989). Vitamin E reduces cell injury (Massey and Burton, 1989) and impedes the formation of oxidised low density lipoproteins (LDL) and their postulated atherosclerotic effects (Burton, 1990). It has also been related to changes in calcium homeostasis in the tissue (Meerson et al., 1982).

Various adverse health affects of vitamin E deficiency in vertebrates are well documented, including disorders of the reproductive organs (Nelson, 1980). Many of these effects are similar to those associated with fluoride intoxication (Burgstahler, 1985). In rats, the main symptoms of Vitamin E deficiency are degeneration of the testis, abnormalities of gestation, regression in the ovary and changes in ovulation (Marks, 1975). Chinoy and Sharma (1997) have reported that ingestion of Vitamin E to fluorotic male mice brought about a significant recovery in NaF induced reproductive failure. Administration of α-tocopherol (Vitamin E) to rats has also been reported to provide significant protection against fluoride induced chromosome damage to their bone marrow cells. α-tocopherol-mediated prevention of cell injury is specially due to its maintenance of sulfhydryl groups of membrane binding proteins (Jones et al., 1983).
Membrane lipids and proteins are the targets of the free radicals and one of the mechanisms by which these active species damage cells is through lipid peroxidation. Biological membranes are readily susceptible to peroxidation damage since they are rich in polyunsaturated fatty acids in their phospholipid. The accumulation of lipid peroxides introduces hydrophilic moieties into the hydrophobic phase of the membrane and damages it. The importance of Vitamin E for protecting the integrity of lipid structures (especially membranes) in vivo is known since it is an antioxidant that has been found in plasma, red cells and tissues (Cheeseman et al., 1984).

Reduced glutathione (GSH) and other sulfhydryl groups protect the cell membrane against free radical attack (Patel, 1987). Free radical produced in the membrane, rapidly reacts with α-tocopherol and the α-tocopheryl radicals thus formed in the membrane are regenerated to α-tocopherol by cytosolic GSH and Vitamin C. The total thiol content was well maintained in α-tocopherol co-administered rats (Subramaniam et al., 1994).

Vitamin E has also been shown to prevent the in vivo toxicity of drugs like paracetemol in rat liver (Walker et al., 1974). Vitamin E has also been found to function as a cancer preventive agent (London, 1985). Hence, the protective effect of Vitamin E shown in this study may be of great significance in amelioration of fluoride induced toxicity.

Earlier reports have revealed that elevated levels of Vitamin D mitigated the symptoms of fluorosis. Studies carried out by Gupta et al. (1996) have revealed that the treatment of Vitamin D showed a significant improvement in the skeletal, clinical fluorosis and biochemical parameters in children consuming water containing 4.5 ppm of fluoride, corroborating the work by Chinoy and Sharma (1997) who revealed a
significant recovery on Vitamin D ingestion in fluoride induced alterations in various organs of male mice. However, only partial recovery was observed in the reproductive functions supporting our present study.

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 bone. A secondary action may promote the renal excretion of phosphorus which would result in mobilization of skeletal calcium. Within the kidney, vitamin D increased the clearance of phosphate (Marks, 1975). In addition to these effects on calcium and phosphorus, Vitamin D increases tissue citrate levels and consequently increases citrate excretion. These effects are brought about by alterations in the level of calcium binding proteins. Parathyroid hormone mobilizes calcium from bone and has no effect on its intestinal absorption. This action may be secondary to a phosphaturia and favours the production of rickets and other abnormalities (Marks, 1975). Vitamin D is capable of stimulating the incorporation of nucleotide precursors into messenger RNA fractions of the intestinal mucosa. To date, no assessment has been made as to whether this represents an increased biosynthesis of m-RNA, t-RNA or r-RNA. Andersen et al. (1988) reported Vitamin D deficiency in fluorotic pigs. In cattle and sheep severe Vitamin D deficiency during gestation leads to the birth of weak, dead or deformed calves (Marks, 1975).

The above reports thus elucidate that Vitamins E and D could mitigate fluoride induced effects.
IV and V. 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 female mice daily for 45 days. The effects of withdrawal upon cessation of NaF ingestion and administration of amino acids, viz. glycine and glutamine alone and in combination were also investigated.

The results revealed significant recovery from NaF induced toxic effects following administration of amino acids in the withdrawal period. On the other hand, another group of mice were administered simultaneous glycine and/or glutamine alongwith NaF (45 and 60 days).

The results revealed that administration of aminoacids, glycine and glutamine individually and in combination alongwith NaF helped in maintaining status quo of all parameters as compared to control, thus elucidating their ameliorative role.

Aminoacids 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. Some of the amino acids protect against fluoride toxicity, where glycine and glutamine were found to be the most potent amongst them. Reports by Chinoy and Mehta (1997) revealed that glycine and glutamine were both beneficial in recovery of fluoride induced changes in various organs of mice. The basis for the protective role is not yet clear, but it may involve slight changes in intracellular fluoride concentrations, or some as yet undetermined 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 than NaF alone. The conversion of glyxoylate to glycine by transamination has been demonstrated in several systems (Meister, 1965) which could be correlated 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). The basis for the protective effect is not yet clear. It might be through inhibiting absorption and retention of fluoride in the body.

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. Hence a protein supplemented diet would be beneficial while a protein deficient diet, would aggravate fluoride toxicity (Chinoy and Mehta, 1997b). This aspect merits further detailed investigation.

Thus, in conclusion, sodium fluoride has definite effect on reproduction. However, the fluoride induced effects could be effectively reversed by withdrawal of treatment and subsequent supplementation of Vitamins C, D and E, calcium and amino acids. Thus, fluoride induced effects are transient and reversible by the use of the
above mentioned therapeutic agents. Thus mitigation of fluoride induced toxicity in endemic areas all over the world could be possible. Hence these studies have important implications for amelioration of fluorosis in endemic regions.