CHAPTER IV

DISCUSSION
PART - 1: IN VIVO STUDIES

The *in vivo* studies on effects of sodium fluoride (NaF) and arsenic trioxide (As$_2$O$_3$) on ovary and uterus of female mice (*Mus musculus*) and the ameliorative effects by some vitamins (C and E) and calcium were carried out.

The present investigations were undertaken to explore the *in vivo* effects of administration of NaF and/or As$_2$O$_3$ on the structure and physiology of reproductive organs of adult female albino mice (*Mus musculus*) of Swiss strain. The effect on fertility rate was also studied. Sodium fluoride was administered orally at a dose of 5 mg/kg body weight and arsenic trioxide at a dose of 0.5 mg/kg body weight. The dose used was based on the LD$_{50}$ values of fluoride, i.e. 51.6 mg F/kg body weight (Pillai et al., 1987) and arsenic trioxide i.e. 39.4 mg/kg body weight (Harrison et al., 1958: Chinoy, 1999a,b) in female mice. Oral administration was selected, since drinking water is the major source of both fluoride and arsenic.

The various parameters studied at the end of treatment were body weight, histology and histocytometry of ovary and uterus. In biochemical parameters, levels of protein were estimated in both organs to find out the effect of NaF and As$_2$O$_3$ on the protein metabolism. In addition, some specific parameters in ovary viz., activities of 3β and 17β hydroxysteroid dehydrogenases (HSD) and cholesterol levels along with those of estradiol in serum were investigated to study the alterations in steroidogenesis, if any.
In order to investigate the effects of fluoride and arsenic on carbohydrate metabolism, the concentrations of glycogen and phosphorylase activity in the uterus were determined.

To evaluate free radical induced cell injury by NaF and As$_2$O$_3$, the activities of some antioxidant enzymes, viz., superoxide dismutase, catalase, glutathione peroxidase and levels of lipid peroxides, glutathione and ascorbic acid were determined in the ovary.

Estrous cycle and fertility rate were studied during the course of the investigation.

In a different set of experiments, the treatments were withdrawn after 30 days of NaF + As$_2$O$_3$ ingestion and the mice were maintained on standard chow and water *ad libitum* in order to study the reversibility of the induced effects if any, upon cessation of treatment.

The therapeutic effects of calcium and vitamins (C and E) were also explored in the light of earlier work and in view of the widespread fluoride induced health hazards the world over.

Fluoride and arsenic are considered potential health hazards. Combined arsenic fluoride poisoning is an exceptional disease in the world. In most cases, this problem arose when deep wells, were installed to avoid the use of surface water which can be contaminated with biological pathogens. Due to this reason, after the 1970s, endemic fluorosis and arsenism appeared among the populations in some parts of the world. Studies on fluoride and arsenic combined toxicity have come up in recent years, to investigate whether these chemicals together have a synergistic effect in inducing toxicity or are antagonistic to each other.
In the present study, treatment of NaF and/or As$_2$O$_3$ brought about reduction in body weight of female mice. Previous reports from our laboratory have also found a decline in the body weight of rodents due to ingestion of fluoride (Chinoy and Sequeira, 1989a; Chinoy et al., 1991a,b; 1992b; 1993b). A consistent reduction in body weight was reported by Saralakumari et al. (1988) in fluoride treated (100 ppm in drinking water) rats. Singh et al. (1963) also obtained a general decrease of body weight in fluoride exposed individuals in Punjab, India.

This data suggests that the treatment causes a state of partial inanition due to reduced food intake.

**EFFECT ON OVARY**

The histological studies on ovary of mouse after combined treatment of fluoride and arsenic revealed structural alterations, necrosis and dense vacuolization in the stromal tissue, follicular atresia, pyknosis in the follicular cell nuclei and hemorrhage. Further, the diameter and number of primary, secondary and Graafian follicles were decreased. Structural alterations were also reported in mice ovary by fluoride treatment (Chinoy and Patel, D. 1998b) and after combined treatment of fluoride and arsenic (Chinoy, 1999b). Histological 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.
EFFECT ON UTERUS

The combined treatment (NaF + As$_2$O$_3$) resulted in changes in histology of the uterus. Vacuolization in the myometrium and endometrium with atrophy and confluence of the endometrial gland as well as pyknosis of their cell nuclei was observed. The earlier studies from our laboratory on uterus of mice corroborate with this data (Chinoy, 1999b). These changes would influence the growth of the organ as well as its enzymes, secretion, metabolism and ultimately change its internal milieu which is so necessary for nidation and implantation of blastocyst and its further development into the embryo.

EFFECT ON PROTEIN METABOLISM

The results of the present study demonstrated significant decline in the levels of total protein in ovary and uterus similar to results of Kathpalia and Susheela (1978). Shashi et al. (1987) also revealed a significant decline in acidic, basic and total proteins in rabbits treated with NaF for 100 days. Studies carried out by Bano et al. (1996) have reported decrease in the soluble protein levels on administration of NaF at a dose of 10 mg/kg body weight to mice for 30, 60 and 90 days. Chinoy and co-workers observed similar changes in various soft tissues of rodents treated with NaF in different doses for varied durations (Chinoy, 1991a,b; 1992; 2002; Chinoy and Sequeira, 1989a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Patel, T., 1999; 2001; Chinoy and Memon, 2001; Chinoy et al., 1991a,b,c; 1992b; 1993a,b; 1994c,d; 1995; 1997a,b; Patel et al., 1994). Polyacrylamide gel electrophoresis of proteins of testis and cauda epididymis, in NaF treated rats revealed disappearance of some proteins, induction of new
proteins and some proteins were found to be resistant to NaF (Chinoy et al., 1997a). This might be a response to the stress imposed by NaF.

The mechanism of action might be that fluoride affects the rate of cellular protein synthesis (Holland, 1979) and there is impaired polypeptide chain initiation by fluoride ions (Godchau and Atwood, 1976).

Arsenic is also known to alter sulfhydryl containing proteins and enzyme systems (Klassen et al., 1986). Combined effect of NaF (5 mg/kg body weight with two different dose of $\text{As}_2\text{O}_3$ (i.e. low dose 0.1 and high dose 0.5 mg/kg body weight) for 30 days revealed a decline in protein levels in several tissues. The effects were similar by low and high dose arsenic treatments but more severe by the latter (Chinoy, 1999a,b). Other studies (Chinoy and Nair, 2001; Chinoy and Shah, 2001; Chinoy et al., 2001a) also revealed decline in protein after treatment of arsenic alone or in combination with fluoride in muscle, kidney, testis and ovary of mice. Arsenite ($\text{As}^{3+}$) having a high affinity for thiol groups in proteins, can form complexes with vicinal thiols and sulfhydryl containing protein, hence it inhibits more than 200 enzymes (Aposhian, 1997; Roy and Saha, 2002). Decline in protein levels might be related to the possible inhibition of DNA synthesis by fluoride and arsenic. Lack of adequate protein turnover would also have an adverse effect on the available enzyme receptors and structural proteins. Thus one of the factors responsible for irregularity of estrous cycle in the current study might be due to lack of available proteins necessary for cell division, growth and differentiation of germ cells during oogenesis.
EFFECTS ON CHOLESTEROL METABOLISM AND STEROIDOGENESIS

Increase in cholesterol levels in ovary was noted in the present investigation by fluoride and/or arsenic treatment indicating alterations in its metabolism. These treatments also revealed a simultaneous decline in the activities of 3β hydroxysteroid dehydrogenase (3β HSD) and 17β hydroxysteroid dehydrogenase (17β HSD) which would affect ovarian steroidogenesis. Studies by Chinoy and associates (Chinoy, 1992; 1995; 1996; 1999b; 2002; Chinoy and Mehta, 1999a; Chinoy and Patel, T., 2001; Narayana and Chinoy, 1994a; Chinoy et al., 2001a) have shown that NaF, As₂O₃, or NaF + As₂O₃ treatments to male and female rats and mice also caused similar changes in their testis and ovary suggesting alteration in steroidogenesis. These results were correlated with decrease in circulating serum estradiol and testosterone levels and a hypercholesterolemic effect in the serum as well as accumulation of cholesterol in the ovary and testis indicating that its metabolism was affected, which could alter the structure and functions of estrogen- or testosterone-dependent reproductive organs. The changes in histology and histocytometry of the ovary and uterus in treated mice in the present study confirm the above observations. Rao and Susheela (1979) noted a decrease in the activity of 3β HSD in adrenal gland of rabbits treated with fluoride. This implies that in fluoride toxicity, adrenal steroidogenesis would also be impaired. Vatassery et al. (1980) also found increase in serum cholesterol in guinea pigs following fluoride treatment but Townsend and Singer (1977) observed a decrease.

Studies in fluorotic individuals of Mehsana district of North Gujarat revealed no significant changes in serum cholesterol levels or serum testosterone ruling out the
possibility of hypo/hyper cholesterolemia at earlier stages of affliction (Chinoy et al., 1992a; 1994a; Mathews et al., 1996).

**EFFECT ON CARBOHYDRATE METABOLISM**

Much of the history of fluoride and arsenic research indicates profound effects 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. Dousset et al. (1987) found a decline in glycogen turnover and citrate accumulation in rats fed with NaF. As$_2$O$_3$ also affects the carbohydrate metabolism.

In the present study, an accumulation in the levels of uterine glycogen was obtained probably due to the decrease in the activity of phosphorylase in uterus of NaF and/or As$_2$O$_3$ treated mice. This might be the main causative factor for the accumulation of glycogen in uterus. Earlier work carried out in our laboratory with treatment of NaF or As$_2$O$_3$ alone or in combination on vas deferens, muscle, liver, and uterus of rats and mice (Chinoy, 1991a,b; 1992; 1999a,b; 2002; Chinoy and Sequeira, 1989a; Chinoy and Patel, D., 1996; Chinoy and Sharma, 1998; Chinoy and Patel, T., 1999; 2001; Chinoy and Mehta, 1999a; Chinoy and Memon, 2001; Chinoy and Nair, 2001; Chinoy et al., 1991c; 1993b; 1994c; 1995) and in fishes (Chinoy et al., 1994b; Shaikh and Hiradhar, 1985) also revealed similar effects as obtained in the present study. The fluoride induced decline in the activity of glucose-6-phosphate dehydrogenase in rats would also alter the glycogen metabolism (Carlson and Suttie, 1966). According to Shearer et al (1971), high concentration of fluoride produced changes in the concentration of metabolic
intermediates indicative of inhibition of some glycolytic enzymes. Hence, they concluded that fluoride affects the carbohydrate metabolism mainly through inhibition of glycolytic pathway rather than its effect on citrate metabolism/tricarboxylic acid pathway.

The blood glucose level is an important marker for the carbohydrate metabolism involving glycogenolysis and gluconeogenesis. Hypoglycemic effects are exerted by a variety of chemical agents and drugs (De Bruin, 1976) and NaF as well as As$_2$O$_3$ might be amongst them. A decline in levels of blood glucose in mice by NaF treatment was reported (Chinoy, 1992; Chinoy and Patel, D., 1996). This hypoglycemia may be related to insufficiency of the adrenal cortex, reduced availability of glucose due to primary liver disease or be simply related to nutritional factors.

Catecholamines are also known to regulate the carbohydrate metabolism, wherein, epinephrine promotes glycogenolysis. The studies carried out by Chinoy and Patel, D. (1996) in NaF treated female mice and Chinoy and Narayana (1992) in fluorotic human populations revealed enhanced catecholamine levels in serum which would influence blood glucose. However, the effects of As$_2$O$_3$ alone and in combination with NaF have not been investigated, hence, studies in this direction are called for.

**EFFECT ON LIPID METABOLISM**

Lipid metabolism was altered in the male reproductive organs and brain of rabbits of both sexes during experimental fluorosis (Shashi, 1992a,b). Their results showed dose-dependent hypertriglyceridemia in brain along with hypercholesterolemia in testis of rabbits. Schiller et al. (1977) described blockage in conversion of pyruvate to citrate by
arsenic by affecting pyruvate dehydrogenase. The high levels of lipid in experimental animals may be in response to fluoride and arsenic toxicosis which strongly indicates an imbalance between the synthesis and utilization of lipid. These results are also supported by the ultrastructural studies in the ovary which revealed extensive lipid droplets in the follicular cell cytoplasm (Chinoy and Patel, D., 1998b).

EFFECT ON FREE RADICALS AND TISSUE INJURY

A free radical can be defined as an atom or molecule containing one or more unpaired electron in its outermost orbit, capable of free existence. \( \text{O}_3 \) has a tendency to form highly reactive oxygen species (ROS) such as the hydroxyl radical (OH') superoxide (\( \text{O}_2^- \)) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) during metabolism. The free radicals are unstable but gain stability either by donating or accepting an electron from some other atom or molecule. These radicals are liable to react with several substances and cause nucleic acid destabilization, lipid peroxidation and change in permeability of cell membranes (Subramaniam et al., 1994). These changes will occur unless ROS are kept under control by the innate antioxidant system of the body. In biological systems, a number of antioxidant defence mechanisms (enzymatic and nonenzymatic) operate to control the excessive levels of ROS (Srivastava, 1998; Peng et al., 2000) which might be generated by toxicants.

In the present study, fluoride and/or arsenic caused a decrease in the activities of free radical scavenging enzymes viz., SOD, GSH-Px and catalase but increased lipid peroxidation in ovary of female mice in corroboration with earlier studies with fluoride.
arsenic and aluminium in male and female rodents in various organs (Chinoy, 2002; Chinoy and Mehta, 1999c; Chinoy and Patel, D., 1998a; Chinoy and Shah, 2002; Memon and Chinoy, 2000; Sharma and Chinoy, 1998).

Increased lipid peroxidation in RBCs, brain, liver and modification of fatty acid composition of phospholipid in liver and kidney due to oxidative stress imposed by fluoride in rats were reported by Sivarajashankara et al. (2001a) and Shao et al. (2000). High fluoride concentration in endemic regions in China inhibited serum SOD and GSH-Px activities but increased blood lipid peroxide levels resulting in the accumulation of large amount of free radicals and peroxides causing cell damage in these people (Li and Cao, 1994). Similar results were obtained by Shivarajashankara et al. (2001b) in the blood of children who suffered chronic fluoride toxicity.

The toxicity of arsenic compounds may also be mediated by ROS generated during their metabolism through multiple pathways, but hydrogen peroxide and $O_2^-$ are the main ROS involved in arsenic induced free radical toxicity (Barchowsky et al., 1999; Pi et al., 2001) and thus may cause lipid peroxidation (Roy and Saha, 2002). Acute and chronic intake of arsenic increased lipid peroxides (LPO) in blood, liver, kidney and other organs of rats (Ramos et al., 1995; Flora, 1999).

Superoxide dismutase (SOD) which is thought to be essential for the protection of cells against ROS has been used experimentally and clinically as an antioxidant drug (Pi et al., 2002).
EFFECT ON ASCORBIC ACID AND GLUTATHIONE

Ascorbic acid has long been known as an antistress and detoxifying agent with antioxidant and detoxifying properties which could scavenge free radicals formed in the system (Chinoy, 1978; Basu and Dickerson, 1996). A decrease in levels of total and reduced ascorbic acid (TAA and RAA) occurred in the ovary in the present study, indicating fluoride and arsenic induced stress in the animals leading to rapid utilization of ascorbic acid. This suggests that the stored ascorbic acid is rapidly oxidized in ovary under fluoride and arsenic induced stress and converted to its dehydroform (i.e. dehydroascorbic acid) which consequently increased. Earlier work from our laboratory (Chinoy and Patel, D., 1998a; Chinoy and Mehta, 1999b; Sharma and Chinoy, 1998; Chinoy and Shah, 2002; Memon and Chinoy, 2000) has also reported similar effects on various tissues of NaF and/or arsenic or aluminium treated male and female mice. It is thus evident that fluoride and arsenic both caused disturbances in the utilization and probably metabolism of ascorbic acid which might also be influenced by reduction of glutathione levels in the present study.

Glutathione besides reducing dehydroascorbic acid to the reduced form, is also involved in detoxification of various xenobiotics and inhibit lipid peroxidation by scavenging free radicals (Anderson, 1996; Liang et al., 1999; Satsangi and Dua, 2000; Pi et al., 2002). GSH can react nonenzymatically with ROS or can act as a substrate in the GSH-Px mediated destruction of hydroperoxides (Anderson, 1996). GSH depletion could impair a cell’s defence against the toxic actions of many compounds and may lead to cell injury and death (Deneke and Fanburg, 1989; Anderson, 1996). The oxidation reduction
cycling of GSH is also central to the cellular response to oxidative stress. The balance between the oxidation of GSH to glutathione disulfide (GSSG) and the rapid reduction of GSSG by GSH reductase contributes to the maintenance of a cellular GSH:GSSG ratio of about 300:1 (Chung et al., 1991). Inhibition of GSH reductase diminishes the intracellular level of GSH. The depleted glutathione (GSH) levels by NaF treatment strongly suggests that like several compounds, fluoride might also be largely dependent on GSH for detoxification (Li et al., 1999). Other studies (Chinoy and Narayana, 1994; Chinoy and Patel, D., 1998a; Chinoy and Mehta, 1999c; Sharma and Chinoy, 1998; Chinoy et al., 1995; 1997a,b; Li et al., 1999) have also revealed reduced GSH levels in several organs of mice, rats and guinea pigs treated with fluoride and in endemic fluorotic patients (Dai et al., 1999).

Cellular toxicity of arsenic was also found to be inversely related to intracellular GSH levels and thus may be enhanced by GSH depletion (Pi et al., 2002). GSH or other non-protein sulphydryls, such as L-cysteine, play critical roles in the reduction of As (V) to As (III) in blood and in the methylation reaction of inorganic arsenic in liver (Pi et al., 2002). Acute administration of arsenic to rats produced a significant reduction in hepatic GSH (Maiti and Chatterjee, 2001). Similarly, chronic exposure of rats or mice to arsenic via injections caused up to 35% depletion in hepatic GSH, along with liver injury (Flora, 1999; Liu et al., 2000). Styblo and Thompson (1995) and Styblo et al. (1997) demonstrated that arsenicals and arsinothiols were potent inhibitors of the reduction of GSSG by GSH reductase. Wu et al. (2001) suggested that ingestion of arsenic contaminated well water may cause deleterious effects by increasing the level of reactive
oxidants and decreasing the levels of antioxidant capacity in plasma of individuals.

The above results corroborate with the present study in ovary suggesting that tissue stress and reduced detoxification mechanisms in the tissues induced by fluoride and/or arsenic could result in cell injury.

EFFECT ON REPRODUCTIVE ORGANS AND FERTILITY

In the present study, treatment with NaF, As$_2$O$_3$ and NaF + As$_2$O$_3$ caused a reduction in protein levels and in steroidogenesis due to decline in activities of 3β and 17β hydroxy steroid dehydrogenase which resulted in a decrease in serum estradiol levels with a concomitant increase in cholesterol. An increase in lipid peroxidation with simultaneous decline in antioxidant enzymes (i.e. SOD, GSH-Px and catalase) and in GSH as well as increase in dehydroascorbic acid occurred in the ovary of treated mice which resulted in toxic effects as mentioned earlier.

The uterus also showed decrease in protein levels and altered carbohydrate metabolism (i.e. the decline in the activity of phosphorylase and accumulation of glycogen) which would affect its secretion and thereby its internal milieu leading to fertility impairment. Studies from our laboratory have revealed that sodium fluoride at a dose of 5 mg/kg body weight for 45 and 60 days induced irregularity in cyclicity, complete loss of fertility and absence of implantation sites in female mice mated with control male mice (Patel, D. and Chhoy, 1998). The authors related the irregular cycle to the decrease in serum estradiol levels. Since the structure and internal milieu of the uterus is maintained by estrogens and progesterone, any change in these hormones will
lead to impaired fertility and irregular cyclicity. Fertility impairment in male mice and rats was also demonstrated by Chinoy and co-workers after NaF treatment (Chinoy 1991a,b; 1995; 2002; Chinoy and Sequeira, 1992; Chinoy and Sharma, 1998; 2000; Narayana and Chinoy, 1994b; Chinoy et al., 1991a,b; 1992; 1994d) and by fluoride and/or arsenic treatment in mice (Chinoy, 1999b; Chinoy et al., 2001a). Darmani et al. (2001) also reported decrease in fertility rate and absence of implantation sites after NaF treatment to the female mice. Studies by Messer et al. (1973) found retarded growth and impaired reproduction in mice given 100 and 200 ppm NaF. At higher doses they observed mortality.

Epidemiological study of gynecological problems in female workers of superphosphate manufacturing plant revealed irregularities in menstrual cycles and more frequent toxicosis during pregnancy due to fluoride exposure (Kuznetsova, 1969a,b).

WITHDRAWAL

The combined treatment of NaF and As\textsubscript{2}O\textsubscript{3} was withdrawn after 30 days and the animals were maintained on standard diet and water \textit{ad libitum} as in control groups for a month.

The data of the current investigation revealed that upon withdrawal of treatment, an insignificant recovery was obtained in most of the parameters studied. Earlier studies had reported that the fluoride induced effects were significantly recovered in some parameters after withdrawal of treatment to mice, rats and rabbits (Chinoy and Sequeira, 1989a,b; 1992; Chinoy and Patel, D., 1998a; Chinoy and Mehta, 1999a,b; Chinoy and
Sharma, 1998; 2000; Narayana and Chinoy, 1994b; Patel, D. and Chinoy, 1997; Chinoy et al., 1991a; 1995). This discrepancy might be due to difference in the animal species, the duration of exposure and dose used. However, withdrawal study of As$_2$O$_3$ alone or combined with NaF treatment shows insignificant recovery by Chinoy and co-workers in various organs of mice (Chinoy, 1999a,b; Chinoy and Nair, 2001; Chinoy and Shah, 2001; 2002; Chinoy et al., 2001a).

This difference in the recovery patterns of the parameters studied may be due to varying tissue response to the toxicity of these chemicals in combination. However, none of the studies observed a complete recovery. To bring about a faster and full recovery, certain therapeutic agents were used to reverse the induced toxic effects.

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

To evaluate the beneficial effects of ascorbic acid (AA), calcium (Ca) or vitamin E (Vit. E or $\alpha$-tocopherol), these antidotes were administered alone and in combination to the animals during withdrawal period. Ascorbic acid, calcium and vitamin E were given at the doses of 15 mg, 25 mg and 2 mg/animal/day respectively which brought about a significant recovery in all the parameters studied. All the three antidotes individually produced almost similar effects but ascorbic acid and vitamin E were comparatively more beneficial than calcium. Furthermore, their combined treatment had an additive or synergistic effect for complete recovery of the tissues to more or less control state.
Several studies have pointed out that certain dietary nutrients like protein, calcium, vitamins C, D and E could modify fluoride or arsenic induced toxicity (Wadhwani, 1954; Yu and Hwang, 1985; Chinoy, 1991a,b; 1992; 1999a,b; 2002; Chinoy and Mehta, 1999a,b,c; Chinoy and Memon, 2001; Chinoy and Patel, D., 1996; 1998a; Chinoy and Patel, T. 2001; Chinoy and Sharma, 1998; 2000; Chinoy and Shah, 2001; 2002; Chinoy and Nair, 2001; Mehta and Chinoy, 2000; Narayana and Chinoy, 1994b; Patel, D. and Chinoy, 1997; 1998; Roy and Saha, 2002; Sharma and Chinoy, 1998; 2000; Tewan and Chinoy, 2002; Chinoy et al., 1991a; 1994d; 1995; 1997a,b; 2001a; Patel et al., 1994).

Ascorbic acid exists in reduced (RAA) and oxidized (DHA) forms and hence can participate in maintaining the redox potential in the body. Ascorbic acid is oxidized to dehydroascorbic acid through a short lived intermediate, ascorbic acid free radical, monodehydroascorbic acid (MDHA) which is generally regarded as a more powerful reducing agent or antioxidant than ascorbic acid itself and thereby prevents the tissue damage (Chinoy, 1978). Ascorbic acid binds to proteins, nucleic acids and other macromolecules by charge transfer complex formation and ascorbic acid depletion in ovary is considered as an index for steroidogenesis and is also involved in overcoming stress (Chinoy, 1978). Besides being an antioxidant, ascorbic acid is known to inhibit phosphodiesterase, hence causing an increase in C-AMP levels (Pasternak, 1979). C-AMP being a second messenger is known to activate several enzymes, and influence cell metabolism. In the present study, most of the enzymes viz., phosphorylase. 3B and 17B hydroxy steroid dehydrogenases, SOD, GSH-Px, catalase etc., were found to recover from the fluoride and arsenic induced toxicity by ascorbic acid treatment alone. Blasckhe and
Hertting (1971) reported that deficiency of ascorbic acid alters 3β HSD activity in rats. Low dietary supplement of ascorbic acid is known to increase the adverse effects of fluoride in monkeys (Sriranga Reddy and Srikantia, 1971).

Calcium, like ascorbic acid has the property of activating several enzymes. Earlier studies have demonstrated that calcium could also bring about recovery of fluoride and/or arsenic induced toxicity (Yolken et al., 1976; Chinoy, 2002; Chinoy and Nair, 2001; Chinoy and Shah, 2001; 2002; Mehta and Chinoy, 2000; Ekambaram and Paul, 2001; Chinoy et al., 2001a). Calcium, like ascorbic acid, inhibits phosphodiesterase and hence increases the concentration of C-AMP. Calcium and C-AMP interact with each other for various metabolic reactions in different tissue systems (Pasternak, 1979). Calcium ion pump is activated by formation of calcium-calmodulin complex, which leads to calcium activated phosphorylation (Rasmussen, 1989). Calcium has been found to act on β-cells of Langerhans in pancreas and control insulin secretion (Rasmussen, 1989). Therefore, calcium given externally may help in recovery of altered carbohydrate metabolism in uterus.

One of the group of animals treated with fluoride and arsenic were supplemented with vitamin E during the withdrawal period and the results revealed a significant recovery in all the parameters studied in this group. Vitamin E has been proposed as a therapeutic agent for several disease conditions especially those which tend to produce oxidative stress in the body (Phelps, 1987; Jackson, 1987). In experimental animals, vitamin E deficiency has been shown to result in a variety of conditions affecting neuromuscular, vascular and reproductive systems (Marks, 1975). Other conditions such
as myopathy in striated and smooth muscles, liver necrosis, testicular degeneration are also shown to respond to vitamin E (Basu and Dickerson, 1996). Chinoy and co-workers (Chinoy and Sharma, 1998; Chinoy and Patel, T., 2001; Chinoy and Memon, 2001; Sharma and Chinoy, 1998; Chinoy and Nair, 2001; Chinoy and Shah, 2001; 2002; Chinoy et al., 2001a; Tewari and Chinoy, 2002) reported that male and female mice treated with different doses of NaF, As₂O₃ and NaF + As₂O₃ for varied durations and subsequently treated with vitamin E, showed a significant recovery in almost all tissues. Vitamin E supplemented rats showed curbing of dental fluorosis (Burgstahler, 1985). As described earlier, the oxygen free radicals being highly reactive species, may attack the double bonds of polyunsaturated fatty acids initiating a chain reaction, leading to complete destruction of membrane integrity and hence cellular function. This chain reaction is thought to be inhibited by vitamin E (α-tocopherol) by reacting with free radicals and converting itself into α-tocopheroxyl radical which is not harmful (Basu and Dickerson, 1996). This α-tocopheroxyl radical thus formed is reverted back to α-tocopherol by cytosolic vitamin C (Subramanian et al., 1994).

**COMBINED ADMINISTRATION OF VITAMIN C, CALCIUM AND VITAMIN E**

In the present study, administration of AA, Ca and Vit. E in combination showed a highly significant recovery of the induced toxicity in almost all the organs/tissues studied. This may be due to several factors working together in the biological systems and their interaction therein in the system. Previous studies have shown that vitamin C and calcium together have a synergistic effect in bringing about a pronounced recovery in
fluoride induced toxicity which was more significant than the recovery resulting from their individual treatments (Chinoy and Patel, D., 1998a; Chinoy et al., 1991a; 1993b; 1994c,d; 1995; 1997a; Patel, D. and Chinoy, 1997). It is known that PDE catalysis the conversion of C-AMP to 5'-AMP thus reducing the concentration of C-AMP. Ascorbic acid and calcium together are recognized as potent inhibitors of PDE as described earlier. Their action results in increased concentration of C-AMP which activates several enzymes and may have thus brought about a complete recovery of fluoride and arsenic induced toxicity in the present study. It is known that calcium and vitamin C deficiency will aggravate the toxic manifestations of fluoride (Sriranga Reddy and Srikantia, 1971) and that ascorbic acid and calcium have a role to play in curbing fluoride induced toxicity (Sriranga Reddy and Srikantia, 1971; Chinoy and Patel, D., 1998a; Chinoy et al., 1991a; 1993b; 1994c,d; 1995; 1997a; Patel, D. and Chinoy, 1997).

Lower calcium in a system causes a depletion of cellular α-tocopherol that is associated with cell-death (Fanss et al., 1985). It is known that the cell calcium content affects the intracellular metabolism of α-tocopherol and its esters, which may subsequently govern the outcome of a toxic challenge (Pascoe and Reed, 1987). The mechanism of calcium modulated de-estenfication of α-tocopheryl esters is nuclear. However, the present study shows a positive interaction between calcium and vitamin E along with vitamin C in vivo and hence, suggest the need for further assessment of the role of calcium-sensitive α-tocopherol metabolism.

The possible interaction of vitamins C and E in biological systems has been explored and some investigations have been performed to elucidate the synergistic...
beneficial effect by their combination (Chinoy and Sharma, 1998; Chinoy and Patel, D., 1998a; Sharma and Chinoy, 2000).

Vitamin E resides within the membrane and hydrophilic vitamin C is located in the aqueous region or cytosol. The present study showed that vitamin C and E alongwith calcium when administered in combination, caused very significant recovery in fluoride and arsenic induced toxicity. Miki et al. (1989) suggested that vitamin C in the aqueous phase was more accessible to the α-tocopheroxyl radical in the biomembrane and could show synergistic antioxidant effect. This mechanism may be responsible for reversal of the toxicity by combined administration of vitamin C and E alongwith calcium in the present study. Thus the interaction of these antidotes in the sodium fluoride and arsenic trioxide treated mice resulted in reversal of induced toxicity in all the parameters studied in ovary and uterus of female mice.

The above data elucidates that the arsenic and fluoride induced toxicity is transient and reversible by use of appropriate antidotes. These results have significance in the mitigation of toxicity manifestations in endemic populations the world over.

PART II: IN VITRO STUDIES

Although voluminous literature has been published regarding the influence of fluoride and arsenic on the environmental and biological systems, there is still a great deal of void and controversy about their genotoxic effects.

In vitro sister chromatid exchanges (SCEs) and chromosomal aberrations in metaphase plate are the most reliable methods to study the genotoxic effects of a given
Tsutsui et al. (1984a) demonstrated that sodium fluoride induced increase in SCEs in Syrian hamster embryo cells and NaF + AlCl₃ induced increase in SCEs in human lymphocytes respectively in culture (Chinoy et al., 2001b). A study carried out by Sheth et al. (1994) had reported an increase in the frequency of SCEs in fluoride endemic human populations of North Gujarat, India, as compared to the control population. Joseph and Gadhia (2000) also obtained the same results in a South Gujarat endemic population consuming more fluoride than another with low fluoride consumption. Wu and Wu (1995) also showed an increased frequency of SCEs in peripheral blood lymphocyte cultures of volunteers having a concentration of 4-15 mg/L of fluoride in drinking water in China.

Zanzoni and Jung (1980) reported that addition of inorganic trivalent arsenic elevates the SCE rate in human lymphocyte cultures and Burgdorf et al. (1977) obtained an elevated rate of SCEs, in lymphocytes of patients treated with arsenic. Similar results were also obtained in our laboratory in an in vitro study on human lymphocytes cultured with arsenic and fluoride alone or in combination (Nair et al., 2002).

The results of the present study revealed that fluoride or arsenic and their combined addition to the culture medium caused a significant increase in the frequency of SCEs in peripheral blood lymphocyte cultures of male volunteers between the age of 20 to 30 years. The SCEs/100 metaphase plates, SCE/chromosome and SCE/cell, increased significantly when compared to control.
Thompson et al. (1985) and Gadhia and Joseph (1997) however, found no increase in SCE frequency or chromosomal aberrations in peripheral blood lymphocytes cultures with fluoride.

The present increase in SCE suggests that fluoride and arsenic are genotoxic agent and may cause mutagenesis.

**EFFECT ON CELL CYCLE PROLIFERATIVE INDEX (CCPI)**

Lymphocytes proliferating *in vitro* represent a heterogenous population of cells. Cultures harvested 72 hours after stimulation or later, give a mixture of cells that have divided once (M1), twice (M2), thrice (M3) or more times (Perry and Wolff, 1974).

The results of the present study on the proliferative kinetics revealed a significant inhibition of proliferation as a result of which cell cycle proliferative index declined when compared to the controls. The number of M1, M2 and M3 metaphase plates also showed changes in fluoride, arsenic and their combined treated groups. He et al. (1983) reported that NaF and fluoroacetamide influence the cell cycle kinetics, chromosomal aberrations and SCE frequencies in cultured red Muntjac cells. The percentage of M1 cells increased while that of M2 and M3 decreased significantly by NaF (Li et al., 1987). A significant lag in the cell cycle was observed in this study which was similar to the above report. Arsenic also showed reduced mitotic activity and chromosomal alteration (Baron et al., 1975; Petres et al., 1977). Reports from our laboratory (Nair et al., 2002) also showed a decline in CCPI after As2O3, NaF or NaF + As2O3 addition to human lymphocyte cultures.

Hence, fluoride and arsenic caused a significant lag in cell cycle proliferation.
EFFECT ON ACROCENTRIC CHROMOSOME ASSOCIATION

The acrocentric chromosomes are sometimes seen at metaphase grouped together with their short arms in close proximity and such interactions of the nucleolar organizing regions are called "Satellite associations" (Miller et al., 1977) or acrocentric chromosome associations. Correlation between Ag-NOR counts and proliferation indices have been reported and it is likely that a correlation with ploidy could be substantiated (Underwood and Giri, 1988). The acrocentric variants might increase the likelihood of non-disjunction of chromosomes during cell-division, resulting in monosomy or trisomy. This could result from increased association of acrocentric chromosomes.

Present work also revealed significant increase in acrocentric chromosome association (viz. D-D; D-G and G-G) by NaF, As$_2$O$_3$ or NaF + As$_2$O$_3$, addition to human lymphocyte cultures.

This implies that exposure to such toxicants can increase the chances of non-disjunction of chromosomes during cell division in exposed populations.

EFFECT ON TELOMERIC ASSOCIATIONS

Fitzgerald and Morris (1984) reported that the chromosomes of human beings could join by the association of normal telomeric ends with minimal or no loss of material from either ends. Such anomalous rearrangement of telomeres has been referred to as telomeric associations by these authors.

In the present study, significant increase in the frequency of chromosomal and
chromatid telomeric association was observed following an exposure to fluoride and arsenic alone and in combination which could be attributed to multiple factors. Telomeric fusion is associated with rapid ageing or senescence. Loss of telomeric DNA during ageing \textit{in vivo} has been observed in peripheral blood cells and colon mucosa epithelia (Hastie et al., 1990) as well as in some types of human cancers (Yums, 1983; Pathak et al., 1994a,b). Increased telomeric fusions in the present study suggests that fluoride and/or arsenic may have a role to play in rapid ageing and also the individuals exposed to these chemicals may attain genetic instability associated with loss of chromosome during cell division.

**EFFECT ON CHROMOSOMAL ABERRATIONS**

Chromosome aberration studies are used to determine mutagenic and carcinogenic effects on chromosomes along with SCE. Nevertheless, they differ in several aspects. SCE are positively correlated with mutation rate and are compatible with cell survival, whereas, chromosome aberrations are mostly correlated with cell death (Venitt and Parry, 1984).

Mohammed and Chandler (1982) reported that fluoride was mutagenic and caused chromosomal damage even at a concentration of 1 ppm NaF in mice bone marrow, whereas, Jachimczak and Skotarczak (1978) reported that fluoride at 0.6, 6.0 and 60 ppm caused an increase in chromosome aberrations in human leucocytes when compared to control values in an \textit{in vitro} study. Fluoride and aluminium induced chromosomal aberrations were also observed by Chinoy et al. (2001b) in human lymphocyte cultures.
Fluoride has also been shown to inhibit many enzymes in vitro including DNA polymerase (Hellung-Larsen and Klenow, 1969) which could directly damage DNA. It is also known that fluoride forms a strong hydrogen bond (NH—F) with purine and pyrimidine bases (Clark and Taylor, 1981; Caspary et al., 1987) which could disrupt cellular macromolecules including DNA, resulting in the gaps and breaks. Tsutsui et al. (1984a,b) demonstrated the induction of gaps and possibly some breaks but no other chromosomal aberrations in cultures of Syrian hamster embryo cells and human diploid fibroblast cultures exposed to approximately 50-100 μg/ml of sodium fluoride. Scott (1985) also reported dose-related induction of chromosome gaps and breaks in cultured human fibroblasts after 48 h exposures to sodium fluoride at concentrations greater than 10 μg/ml.

Arsenic is also clastogenic and causes chromosomal aberrations. The clastogenic effect of trivalent arsenic was found in experimental studies of cultural human lymphocytes (Beckman and Nordenson, 1986). Laboratory evidence also incriminate chromosome breaks and other aberrations by arsenic (Oppenheim and Fishbein, 1965; Nakamuro and Sayato, 1981). The chromosomal aberrations were significantly increased in cultured human leucocytes and skin fibroblasts after arsenic treatment. The effect of trivalent arsenic was about 5 times greater than that of pentavalent arsenic (Nakamuro and Sayato, 1981). Smelter workers exposed to arsenic have also been found to have increased frequencies of chromosomal aberrations in short-term cultured lymphocytes (Beckman et al., 1977; Beckman and Nordenson, 1986).

In the present study, chromosomal and chromatid breaks and gaps were also
significantly increased after NaF, As₂O₃, or NaF + As₂O₃ was added to human lymphocyte cultures. This increase in chromosomal aberrations signifies loss of genetic material and may lead to alteration in gene expression.

**EFFECT ON MICRONUCLEI (MN) FORMATION**

The chromosomes or chromatid that fail to join with microtubule will, lag at the anaphase and may be retrieved back or eliminated. Ford et al. (1988) described a mechanism for chromosome or chromatid elimination. Displaced chromosome might lag at anaphase and these lagging chromosomes or chromatids could be enveloped in their own nuclear membrane at the time of nuclear membrane formation and appear as micronuclei which are subsequently eliminated. Although the etiology of lagging in mitosis is probably different from that in meiosis, the outcome of lagging may be the same.

In the present study, a significant increase was observed in the frequencies of binucleates with micronuclei (MN) and the total number of MN in fluoride and/or arsenic treated cultures. The increase was more by the combined treatment. MN was also observed in the controls showing that the chromosome elimination in micronucleus is a common phenomenon. In some individuals, a single binucleate had more than one micronucleus in treated groups suggesting multiple chromosome or chromatid elimination, which could be attributed to multiple chromosome or chromatid lagging at anaphase due to non-disjunction. The above data corroborates with those of Scott (1985) and Chinoy et al. (2001c) who demonstrated the induction of MN in cultured human fibroblasts and
lymphocytes respectively after sodium fluoride (greater than 10 μg/ml) and NaF + AlCl₃ respectively exposure to the cultures. Similar data was obtained in fluoride intoxicated individuals by Zhang and Meng (1999).

EFFECT ON ANEUPLOIDY

Aneuploidy is a gain or loss of chromosomes and has generally been attributed to the failure of chromosomes to disjoin at anaphase i.e. non-disjunction. Studies on human lymphocyte cultures have indicated the occurrence of aneuploidy in the mitotic cells. It is suggested that mitotic aneuploidy may reflect on the individual’s susceptibility to meiotic aneuploidy. This may be one of the reasons for increased incidence of Down Syndrome due to fluoride (Takahashi, 1998) and high rates of spontaneous abortions on exposure to arsenic (Akhtar Ahmad et al., 2001b). Non-disjunction, a precursor to aneuploidy, is an expression of spindle tubule dysfunction (Ford and Roberts, 1983) such as altered microtubule polymerization, structure and/or function. In the present study, a significant increase in aneuploidy occurred after fluoride, arsenic and their combined treatments of lymphocyte cultures. Increase in aneuploidy was also observed by Chinoy et al. (2001c) in lymphocyte cultures after addition of sodium fluoride and aluminium chloride.

Increased micronuclei in the present study might be the result of increased non-disjunction and lagging at anaphase. This may cause aneuploidy along with microtubular malfunctioning and increase the incidence of abnormality and cell death in the population getting exposed to these chemicals.
MECHANISM OF IN VITRO TOXICITY

Fluoride may not involve any direct damage to DNA caused by covalent binding or strand scission. Hence, it could be indirectly involved in disruption of normal DNA replication. The actual mechanism could be quite complex. The high electronegativity of fluoride ion allows it to interact with both organic and inorganic components of the cells, so its physiological effects could be expected to be more pronounced and varied than those caused by any other elemental ion. Thus fluoride has a great affinity for Ca$^{2+}$, Mg$^{2+}$ and phosphate (Bell et al., 1970), all of which are present in the culture medium and within the cells, so the active agent could be a magnesium-fluorophosphate, which is known to interfere with DNA polymerase and RNAase activities (Hellung-Larsen and Klenow, 1969; Srivastava et al., 1981). As mentioned earlier, fluoride ion also forms a strong hydrogen bond (NH---F) with purine and pyrimidine bases (Clark and Taylor, 1981; Caspary et al., 1987) which could disrupt the structures of both DNA and RNA and interfere with their synthesis or enhance the frequency of base-pair errors during DNA replication. Mutagenic activity of fluorides (NaF, KF, NaHF$_2$) was also demonstrated by Caspary et al. (1987). These few examples of known fluoride interactions with macromolecules, ionic or organic substances suggest that these effects could be dependent on the presence of other substances like arsenic during the treatment period.

The biochemical mechanism by which arsenicals exert their chromosome damaging properties is not yet clear, but arsenic is known to: (1) interfere with certain enzymic functions by blocking sulphydryl groups (Roy and Saha, 2002); (2) inhibit DNA repair enzymes (Jung et al., 1969); and (3) replace phosphorus in the phosphate group of DNA.
and form a weak bound in the DNA chain.

In conclusion, fluoride is known to interfere with protein synthesis and inhibit the enzyme involved in DNA synthesis (Imai et al., 1983) whereas, arsenic inhibits DNA synthesis and its repair (Sibatani, 1959; Jung et al., 1969) and probably as a result, the combined in vitro toxic effects were found to be more severe in the present study.

MITIGATING EFFECT OF ASCORBIC ACID (AA)

Ascorbic acid (AA) has a role in maintaining the growth and integrity of leucocytes and has antioxidant and detoxification properties as described earlier (Chinoy, 1978; Basu and Dickerson, 1996).

In vivo studies conducted in rodents showed that ascorbic acid was capable of reducing fluoride and/or arsenic as well as fluoride and/or aluminium induced toxicity in various tissues of rats, mice, rabbits and monkeys (Wadhwani, 1954; Yu and Hwang, 1985; Chinoy, 1991a,b; 2002; Chinoy and Patel, D., 1998a; Chinoy and Nair, 2001; Chinoy and Shah, 2001; 2002; Chinoy and Sharma, 1998; 2000; Chinoy et al., 1991a; 1994d; 1995; 1997a,b; 2001a; Narayana and Chinoy, 1994b; Patel, D. and Chinoy, 1997; Tewari and Chinoy, 2002). In the present study, when AA was added to the cultures along with NaF and As₂O₃ at ‘0’ hour, AA showed a significant protective effect by decreasing the genotoxicity. The following suggestions proposed might explain the mechanisms of action of AA.
I. Ascorbic acid is known to bind to the macromolecules like nucleic acids by forming a charge transfer complex \textit{in vivo} (Chinoy, 1978) which may also be true for \textit{in vitro} conditions.

II. It is suggested that the binding of AA initially to DNA, may not allow fluoride or arsenic to bind, thus preventing any damage to DNA. Hence, the stabilization and protection of DNA may occur \textit{in vitro} by AA.

III. Another possibility suggested is that fluoride and arsenic individually are known to affect many enzymes, related with DNA and RNA (Hellung-Larsen and Klenow, 1969; Roy and Saha, 2002), whereas, ascorbic acid is known to activate several enzymes. This mechanism may be true for the \textit{in vitro} system also, thus preventing any chromosomal damage by altered enzymatic activity in the cultures.

IV. Fluoride or arsenic alone and in combination induced genotoxicity in various cell lines was probably triggered by the generation of free radicals and increased lipid peroxidation \textit{in vitro}, whereas, some chemotherapeutic approaches have been proposed viz., the use of free radical scavenging agents and antioxidants such as Vitamins C, E, D as well as SOD to minimize the cytotoxicity and damage induced in normal tissues by free radicals (Nordenson and Beckman, 1991; Wang and Huang, 1994; Antunes and Takahashi, 1999; Chinoy, 2002; Roy and Saha, 2002). Selenium and superoxide dismutase have also been used for this purpose by some investigators (Nordenson and Beckman, 1991; Sun et al., 1999; Qui and Sun, 1999). Earlier studies from our laboratory have shown a protective effect of
vitamin C on chemical induced toxicity in vitro similar to the present study (Chinoy et al., 2001b,c; Nair et al., 2002; Rao et al., 1999; 2001).

There is still controversy about genotoxic effects of fluoride and arsenic but the work incorporated in this thesis is a significant contribution in explaining the genotoxicity of both fluoride and arsenic. However, further investigations are needed in this direction.
NaF + As$_2$O$_3$ TREATMENT ALTER STRUCTURE AND FUNCTIONS OF OVARY, UTERUS

Altered ovarian and uterine histology

Ovary
- Cholesterol↓
- 3β HSD↓
- 17β HSD↓

Steroidogenesis↓

Folliculogenesis, uterine endometrial growth↓

Uterine internal milieu altered

Non conducive for Nidation and implantation

Serum
- E$_2$↓

Altered cyclicity

Ovary free radical toxicity
- SOD↓
- TAA↓
- GSH Px↓
- RAA↓
- GSH↓
- DHA↑
- LPO↑
- Catalase↓

Total protein of ovary and uterus↓

Uterus glycogen↑
- phosphorylase↓

Less enzyme hormone secretion

Alterations in protein, lipid, carbohydrate metabolism and membrane permeability in uterus and ovary

FERTILITY RATE↓

IN VIVO TOXIC EFFECTS OF NaF AND/OR As$_2$O$_3$ IN PRESENT WORK

(↓ = decrease; ↑ = increase)
NaF + As₂O₃ TREATMENT

IN VITRO EFFECT ON HUMAN LYMPHOCYTES

- SCE RATE↑
  - SCE/Cell↑
  - SCE/Cs↑

- CELL CYCLE
  - CCPI↓
  - M₁↑
  - M₂↓

- TELOMERIC ASSOCIATION (TA)
  - Chr. TA↑
  - Ctd. TA↑

- CHROMOSOMAL ABERRATIONS
  - Chr. Break↑
  - Ctd. Break↑
  - Chr. Gap↑
  - Ctd. gap↑

- MICRONUCLEI (MN)
  - FORMATION MN↑

- ANEUPOLOYDY
  - Hypoploidy↑
  - Hyperploidy↑

Defects in DNA repair and predisposition to neoplasia

DNA synthesis↓

Loss of telomere

Loss of genetic material

Increased Chr./Ctd./acentric fragments lagging
Non-disjunction

Increased spontaneous abortion and abnormal offsprings

Cell compete for survival

Cell division↓

Ageing and apoptosis

Alteration in gene expression cell death

AGEING, CANCER, MUTATION, ABORTION, TERATOGENICITY

IN VITRO TOXIC EFFECTS OF NaF AND/OR As₂O₃ IN PRESENT WORK AND POSSIBILITY OF HUMAN HAZARDS

(Cs = chromosome; Chr. = Chromosomal; Ctd. = Chromatid; ↑ = increase; ↓ = decrease)
DIFFERENT MECHANISMS OF AMELIORATION OF TOXICITY BY ANTIDOTES IN THE PRESENT WORK

- Vitamin E (α-tocopherol)
  - Acts as an antioxidant
  - Decreases free radical toxicity
  - Prevents lipid peroxidation and atherosclerosis
  - Recovery in cell membrane function and ion exchange

- Vitamin C (Ascorbic acid)
  - Activates several hydroxylating enzymes
  - Recovery in enzyme activity
  - Recovery in activities of several enzymes and hence metabolism

- Calcium phosphate (Ca²⁺)
  - Inhibits phosphodiesterase
  - Form insoluble compound CaF₂
  - Increase in C-AMP
  - Decrease in absorption of fluoride

Mitigation of cellular functions

Cellular injury overcome and recovery

RECOVERY IN HISTOLOGY AND FUNCTIONS OF OVARY, UTERUS AND FERTILITY RATE

ALL THE THREE ANTIDOTES TOGETHER MANIFESTED PRONOUNCED RECOVERY

(↓ = decrease; ↑ increase)