The present investigations were carried out to explore the in vivo effects of administration of sodium fluoride (NaF) on the structure and physiology of some male reproductive organs of adult male Wistar rats (*Rattus norvegicus*) weighing between 250-300 g. Sodium fluoride (NaF) was administered orally at two doses, low (5 mg/kg body weight) and high (10 mg/kg body weight) for 60 consequent days. The doses used were based on the LD50 value of fluoride, (Pillai et al., 1987; 1988). Ameliorative effects of melatonin (10 mg/kg body weight) and amla aqueous extract on fluoride induced toxicity (20 mg/kg body weight) were also studied. In different set of experiments, the sodium fluoride treatments were withdrawn after 30 days and 60 days in order to study the reversibility of the induced effects if any, upon cessation of treatment. Oral administration was selected, since drinking water is the major source of fluoride is found in foods, beverages and pharmaceutical as well as mineral water, etc.

Various tissues studied at the end of each treatment were body and organ weights of testis, cauda epididymis and vas deferens. To evaluate free radical induced cell injury by fluoride, the activities of some antioxidant enzymes, viz., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-s-transferase (GST) and levels of lipid peroxides, glutathione (GSH), total ascorbic acid (TAA) and total -SH were determined in the testis and cauda epididymis. To find out the effect of fluoride on
Protein metabolism, levels of total proteins were determined in these tissues. To study the effects of sodium fluoride on energy metabolism, activity of succinate dehydrogenase (SDH), acid phosphatase (ACPase), alkaline phosphatase (ALPase) in testis and cauda epididymis. Activity of Adenosine triphosphatase (ATPase) was investigated in all the organs. In addition, some specific parameters in testis viz., cholesterol, total lipids, activities of 3β and 17β hydroxysteroid dehydrogenases (3β and 17β HSDs) and serum testosterone levels were investigated to study the alterations in steroidogenesis. The tissue fluoride levels were also investigated in all tissues to know its tissue burden.

Similarly, sperm parameters of cauda epididymis and vas deferens viz., sperm motility, count, viability and morphology were studied in addition to fertility test. The androgen dependent parameter like sialic acid was also done in these tissues. Histology as well as histocytochemistry studies of testis, cauda epididymis and vas deferens were carried out during the course of the investigation in all respective groups.

GRAVIMETRIC STUDIES

In the present study, a significant reduction in body weights of toxicant treated animals was recorded in comparison to control. Besides weight loss, the animals also showed signs of intoxication like yellowish fur, a cachetic appearance and rigidity in the organs of the body. Similar results were reported in our laboratory and others (Chinoy, 1995; Banu Priya et al., 1997; Chinoy and Sharma, 1998 and Paul et al., 1998; Vani and Reddy, 2000; Chawla et al., 2008; Basha et al., 2011) in rats and
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mice fed with different concentrations of fluoride. Zhang et al. (2011) also observed that the surface of rat incisors fed with fluoride had chalky color change and cross striations could be seen on the enamel surface. Chawla et al., 2008; Rao et al., 2009; Chawla and Rao, 2012 and Rao and Bhatt, 2012 also have same trend in body weights of NaF treated rats. The weight of body depends on the food and fluid consumption, fluids retention, organ and skeleton weights, metabolism of protein, carbohydrates and lipids and excretory materials etc. The fluoride ions act as enzymatic poisons, interrupting protein synthesis, glycolysis, altering antioxidative pathways, and causing hematological abnormalities (Camargo, 2003; Kumar et al., 2007). These changes may influence the body weight. According to Chinoy et al., (1991a) changes in food and water intake lead to change in the body and organ weights, which are good indices of overall growth pattern, particularly in long term study.

The significant decline in the weights of testis, cauda epididymis and vas deferens was observed gradually in the present study like to that of whole body weights. From our laboratory, similar results were found by fluoride treatments of different doses and duration in male and female rodents (Chinoy and Sequeira, 1989a; Chinoy and Patel, 1996; 1998a; Patel and Chinoy, 1997; Chinoy and Sharma, 1998). Similarly, Ghosh et al. (2002) also observed a decline in weight of testis, prostate and seminal vesicle with sodium fluoride (20mg.kg.day) water ingestion for 29 days. Sodium fluoride (4.5, 9.0 ppm) exposure led to a decrease in testicular index in 75 days of treatment in rats (Pushpalatha et al., 2005). Its treatment further
brought about a significant reduction in the weight of testis, epididymis, and ventral prostate (Gupta et al., 2007) and Solanki et al. (2008) also obtained same results that reduction in testis, cauda epididymis and seminal vesicle weights in 270 days in fluoride water (5.8 ppm) treated rats.

Functional and structural integrity of reproductive tissues depends on circulating level of androgen and therefore, any small change in androgen level may result in reduction in the weight of reproductive organs (Chinoy et al., 1982). The reduction in the weight of testis after treatment possibly also related to loss of spermatozoa and spermatids, which make up a substantial proportion of testicular volume, because of disruption of spermatogenesis (Chatterjee et al., 1994; Mathur et al., 1995; Ma et al., 2008) by fluoride feeding. The reduced serum testosterone level in our study might be responsible for loss of weight of testis, cauda epididymis, vas deferens, seminal vesicle and coagulating glands as well as whole body weights in our study.

In present study, supplementation of melatonin (10 mg/kg body weight) and amla (20 mg/kg body weight) as antioxidants along with sodium fluoride for 60 days did not reveal any significant changes in body and organ weight as compared to control rats. This would be attributed to active detoxification of the toxicant and free scavenging ability of antioxidants.

Sodium fluoride treatment was withdrawn for 30 and 60 days to investigate reversibility of NaF effects. But, results revealed insignificant or no recovery in the body and organ weights. To support our data, Ekambaram and Paul (2002) had
reported that NaF treatment decreased food and water intake, reduced body-weight gain and no increases in body and organ weight after NaF withdrawal in support of our observation.

**FLUORIDE EFFECTS ON SOFT TISSUES**

In the current investigation, fluoride feeding to rats affected testis, cauda epididymis and vas deferens metabolism, structure and functions.

**A. OXIDATIVE STRESS AND ANTIOXIDANT PARAMETERS**

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. Examples include oxygen ions and peroxides. Reactive oxygen species (ROS) are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproducts of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during time of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically (Devasagayam et al., 2004). This may result in significant damage to cell structures. Cumulatively, this is known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. Free radicals are highly reactive species that have unpaired electrons, e.g. the hydroxy and superhydroxy radicals. In general, harmful effects of these species on the cell include damage of DNA, oxidations of polyunsaturated fatty acids in lipids (lipid peroxidation), oxidations of amino acids in proteins, and oxidatively inactivate specific enzymes by oxidation of co-factors (Brooker, 2011).
The most important consequences are nucleic acid destabilization, lipid peroxidation and change in permeability of the cell membrane (Subramaniam et al., 1994).

The fundamental biochemistry of these antioxidant enzymes is involved in the rapid conversion of superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$) in the presence of SOD in order to prevent the former from participating in the formation of highly pernicious hydroxyl radicals. The $H_2O_2$ generated in this manner is a powerful membrane permeates oxidant in its own right that has to be rapidly eliminated from the cell in order to prevent the induction of oxidative damage to lipids, proteins and DNA. Important enzymes involved in curbing these harmful effects include superoxide dismutase (SOD) which catalyses dismutation of superoxide radical leading to the formation of hydrogen peroxide which in turn is detoxified by the enzyme glutathione peroxidase (GPX) and catalase (CAT) (Rzeuski et al., 1998). Furthermore, activation of molecular oxygen is catalysed by small molecules (e.g. xenobiotics) and iron complexes. Exogenous (and endogenous) low-molecular weight molecules can catalyse the electron transfer to oxygen (leading to the formation of $O_2^{•−}$) from various reductases (e.g. NADPH reductase, NADH dehydrogenase, xanthine dehydrogenase, aldehyde oxidase). Such a redox cycle explains the toxic effects of several exogenous compounds (aromatic or nitroheterocyclic derivatives, iron complexes). These compounds can also generate ROS via direct activation of NADPH oxidase. $H_2O_2$, in conjunction with superoxide anion, which can damage cells by allowing the most reactive metabolites, hydroxyl radicals, to form. This formation occurs, via superoxide dismutase (SOD) and the Haber-Weiss reaction, the latter reaction being greatly accelerated by catalytic amounts of metal...
salts (iron or copper) (Halliwell and Gutteridge, 1989). In addition to $O_2^{•−}$, $H_2O_2$ and $OH^{•}$, the hydroperoxyl radical ($H_2O_2$) and the conjugated acid of superoxide anion play a key role in the initiation of the lipid peroxidation chain reaction in membrane lipids (Bielski et al., 1983; Alvarez and Storey, 1995).

Enhanced production of reactive oxygen species is stimulated under stress conditions such as chilling, desiccation, senescence and high light (Murage and Masuda, 1997; Mittler, 2002). All living cells possess both enzymatic and non-enzymatic mechanisms which can overcome oxygen toxicity. The biological damage is controlled in vivo by a wide spectrum of antioxidative properties of compounds such as ascorbate, glutathione, $\alpha$-tocopherol and antioxidant enzymes (Larson, 1988; Fernandez and Videia, 1996). The most abundant low molecular thiol is glutathione, which maintains protein thiol groups in the reduced state and also acts as a protective physiological antioxidant in biological systems (Barclay, 1988; Glass and Stark, 1997). Lipid peroxidation (LPO) is a free radical mediated process that has been implicated in a variety of disease states. LPO involves the formation and propagation of lipid radicals, the uptake of oxygen and a rearrangement of the double and unsaturated lipids, resulting in a variety of degraded products that eventually cause destruction of membrane and cytosolic lipids. Generation of free radicals, lipid peroxidation, and altered antioxidant defense systems are considered to play an important role in the toxic effects of fluoride (Zhi-Zhong et al., 1989; Shivarajashankara et al., 2001a).

Das et al. (2001) reported an increased peroxidation leading to changes in cellular metabolism of vital organs. It is known that free radicals and lipid
peroxidation may play an important role in the mechanism of fluorosis (Sun et al., 1996; Bian et al., 1997). Fluoride is known to stimulate respiratory burst and the production of superoxide radicals in neutrophils of humans, rabbits and guinea pigs. The high reactivity of superoxide radicals may lead to chemical modification of proteins, lipids, carbohydrates and nucleic acids in living cells (Rzeuski et al., 1998).

High fluoride concentrations are reported to inhibit superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase followed by increased lipid peroxidation and glutathione-s-transferase levels, in the testis and cauda epididymis thus rendering the tissue susceptible to injury (Chinoy and Patel, 1998a; Chinoy and Sharma, 1998).

In the present study, lipid peroxidation was assessed as a marker of oxidative stress of the lipids in the testis and cauda epididymis of sodium fluoride treated rats. The data revealed a significant increase in the lipid peroxidation levels in the tissues of treated rats. Fluoride induced an increase in lipid peroxidation has been reported in a number of animal models (Chinoy and Patel, 1998a; Chinoy and Mehta, 1999a; Shivarajashankara et al., 2001a; Chinoy and Shah, 2004a; Shivarajashankara and Shivashankara, 2012) and human beings (Shivarajashankara et al., 2001b). This increase in LPO by fluoride treatment may be a result of either overproduction or accumulation of ROS resulting from loss of antioxidases/antioxidants and it indicates damage to membrane lipids. Others (Oncu et al., 2007; Hassan and Abdel-Aziz, 2010) have also reported that sodium fluoride (10.3 mg/kg body weight) administration induced oxidative stress as evidenced by elevated levels of lipid peroxidation and nitric oxide in the testis. Basha and Madhusudhan (2010) and Rao and Bhatt (2012)
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also documented that lipid peroxidation levels were increased in testis of sodium fluoride treated rats.

Earlier also similar findings were noticed on lipid peroxidation in vital organs, endocrine glands and reproductive organs (Vyas, 2012). Das et al. (2005) had reported an increase in LPO levels in reproductive tissues, sperm pellet, and metabolic tissues like the liver and kidney. Wang et al. (2004) observed that, fluoride can cause lipid peroxidation, DNA damage, and apoptosis in human embryonic hepatocytes. Karaoz et al. (2004) had concluded that chronic fluorosis caused a marked destruction in kidney tissues of F1 and F2 rats by inducing lipid peroxidation. Elevated lipid peroxidation products (pro-oxidants) and diminished antioxidant defense enzymes suggest fluoride plays a significant role in exerting oxidative stress in the postmenopausal women living in an area of endemic fluorosis (Ravula et al., 2012). All these reports thus justified our observations.

The superoxide dismutase (SOD) is the enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide which in turn is detoxified by the enzyme catalase (Rzeuski et al., 1998). Thus, these are an important antioxidant defense markers in nearly all cells exposed to oxygen. Catalase is found mainly in the peroxisome and removes H2O2 produced during oxidation. It catalyses the conversion of H2O2 to H2O and O2, reducing the hydrogen peroxide levels. It is an efficient inhibitor of LPO when hydrogen peroxide accumulates in cells.

In the present study, sodium fluoride caused a decline in the activities of free radical scavenging enzymes viz. superoxide dismutase (SOD) and catalase in testis
and cauda epididymis of NaF treated rats. In support of our data Sarkar et al. (2006) documented a reduction in the activity of SOD and catalase following sodium fluoride treatment. Fluoride is known to inhibit SOD activity in the red blood cells of rats after chronic administration, for 4 months in different doses (Shivarajashankara et al., 2001a). Similar findings from our laboratory and others have been reported, where SOD, GPX and catalase activities in several tissues of mice exposed to high fluoride levels at different doses were affected (Chinoy and Sharma, 1998; Chinoy and Patel, 1998a; Vani and Reddy, 2000; Chinoy and Memon, 2001; Qin et al., 2001; Jhala et al., 2008; Chawla et al., 2008; Rao and Bhatt, 2012). Vasant and Narasimhacharya (2011) have reported a decline in these non-enzymatic and enzymatic components of defense system in hepatic and renal tissue by sodium fluoride ingestion. Yue-Hai et al. (2011) and Rao and Bhatt (2012) have reported activities of these enzymes were inhibited following sodium fluoride treatment in support of our data. The administration of sodium fluoride significantly increased the lipid peroxidation and decreased activities of antioxidant enzymes, viz., catalase and superoxide dismutase, in the regions of the heart was obtained (Basha and Sujitha, 2011). Nabavi et al. (2012) have reported the depletion of SOD and CAT enzyme activities after sodium fluoride treatment in rat brain in light of our study.

Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. Superoxide dismutase, glutathione peroxidase (GPX), glutathione-S-transferase (GST), glutathione reductase (GR), and catalase (CAT) are the most important antioxidant enzymes. The antioxidative enzymes, catalase and glutathione peroxidase serve to decompose hydrogen peroxide to water (Meister
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peroxides in rat testis after fluoride feeding. The studies carried out by Blaszczyk et al. (2010) confirmed the disadvantageous effect of NaF upon the antioxidative system in rats, where changes in activities of antioxidative enzymes such as SOD, CAT, GPX, GR, and GST were noticed. A dose of fluoride given to rats by Stawiarska-Pieta et al. (2012) for 35 days found altered levels of GPX, GR and GST activities in liver tissue. Similarly, decrements in these enzymatic and non-enzymatic components of defense system in hepatic and renal systems, as well as female tissues are reported by sodium fluoride intoxicated animals (Vasant and Narasimhacharya, 2011; Chawla and Rao, 2012). These data support our findings of testicular and caudal oxidative stress by fluoride poisoning.

Further Chattopadhyay et al. (2011) documented that selective low (15 mg sodium fluoride (NaF/L) and relatively high (150 mg NaF/L) doses of fluoride (F) treatment to Swiss albino mice through drinking water elicited changes in glutathione level (GSH), glutathione-s-transferase (GST) activity, malondialdehyde (MDA) production in both liver and kidney of rats. Albino rats administered 100-ppm sodium fluoride in their drinking water for four months, brought about changes in the activities of GPX and GST in the brain and liver (Shivarajashankara et al., 2001a). A significant increase was observed in the activity of the glutathione-s-transferase by NaF treatment in the rat testis (Rao and Bhatt, 2012). Thus reduced activities of the GPX and GR as well as GSH levels followed by increase in GST activity indicated the adverse effect of sodium fluoride on testis and cauda epididymal antioxidant system to impose toxicity. The low activities of the glutathione peroxidase (GPx), glutathione reductase (GR) and increased glutathione transferase (GST) enzyme levels were also
noted in rats by fluoride treatment (Stawiarska-Pieta et al., 2012) corroborating our data.

Glutathione (GSH) is a tripeptide with a gamma peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain. Glutathione (GSH) plays an important role mainly in detoxification (Levitt et al., 1962) and acts as a cofactor or a substrate of some enzymes. The oxidized form of glutathione (GSSG) is converted into the reduced form (GSH) by glutathione reductase (GR) (Foyer and Halliwell, 1976). It is also important for preventing the oxidation. Glutathione is the most abundant low molecular weight thiol containing compound in the cell. It is an antioxidant, preventing damage in important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella et al., 2003). Glutathione comprises up to 90% of the non protein thiol content of mammalian cells and performs a vital role in maintaining their metabolic and transport functions. It acts as a nucleophilic “scavenger” of many compounds and their metabolites via enzymatic and chemical mechanisms, converting electrophilic centers to ether bonds. Its depletion to about 20% to 30% of total glutathione levels impairs cell defenses against toxic action, which might lead to cell injury and death (Reed, 1990). Therefore, GSH is involved in the mechanism of detoxification of various xenobiotics (Meister and Anderson, 1983), inhibition of lipid peroxidation by scavenging free radicals (Li et al., 1999; Satsangi and Dua, 2000) as well as reducing dehydroascorbic acid to the reduced form. It is known that glutathione (GSH) could inhibit
peroxidation, scavenge free radicals and protects cell membranes (Patel, 1987). NaF has highly affinity for sulfahydryl (-SH) groups, inactivate numerous enzymatic reactions, amino acids, and sulfur-containing antioxidants (GSH), with a subsequent decreased oxidant defense and an increased oxidative stress. Total thiol (-SH) plays an important role in maintenance of the cellular equilibrium and protein structures.

In present study total –SH groups and glutathione levels significantly decreased in testis and cauda epididymis after 60 days sodium fluoride treatment. Therefore it indicates rapid oxidation and altered protein synthesis. The depleted glutathione by sodium fluoride strongly suggests that, like several exogenous compounds, fluoride might largely be dependent on glutathione for detoxification. From our laboratory also same data were reported on depletion of glutathione levels in sperm suspensions of rats and guinea pigs treated with fluoride (Chinoy et al., 1995; 1997a,b). Chawla and Rao (2012) and Rao and Bhatt (2012) also observed a depletion in glutathione levels in reproductive tissue of NaF treated rodents.

Ascorbic acid is one of the most powerful natural antioxidants. As an antioxidant, ascorbic acid scavenges neutrophilic oxidants, $\text{H}_2\text{O}_2$, and hydroxyl radicals (Elmadfa and Koenig, 1996). It is known to protect membranes from free radical damage (Barja, 1996; Perez et al., 2002). Vitamin C is an important biological active reactant that plays a dynamic role in several oxido-reduction reactions. The occurrence of ascorbic acid in the testis and semen of several animals including human beings is known. Distribution of ascorbic acid in various tissues and its physiological involvement in the overall metabolism in living system has been established. Depletion of ascorbic acid indicates its involvement in overcoming
stress. Total ascorbic acid concentration was decreased in testis of sodium fluoride treated rats in the present study. The fluoride causes disturbance in the utilization and probably synthesis of ascorbic acid leading to a change in its metabolism, which might be influenced by decrease in glutathione levels.

In support our data, a decrease in GSH but an increase in its oxidized form GSSG in blood of NaF exposed rats has been reported by Kaushik et al. (2001). A reduction in the levels of ascorbic acid was obtained in blood of rats administered with fluoride (100 ppm) treatment (Shivarajashankara et al., 2003). The administration of NaF to rats was reported to cause a marked decrease in the level of TAA in male reproductive organs of male rats (Sharma et al., 2008). Ascorbic acid mobilization was observed in adrenal following fluoride water (1.5, 3, 4.5 and 6 ppm) treatment to rats leading to a significant reduction in its levels as compared to control group (Sharma et al., 2004a). Ascorbic acid is considered to be the most important antioxidant of the plasma acts and as anti-stress factor. Its plays a significant role in the amelioration of fluoride induced toxicity (Verma and Sherlin, 2001; Wang et al., 2004; Pawlowska-Goral et al., 2004; Vasant and Narasimhacharya, 2012) in animals.

Hence from the present findings, it could be concluded that fluoride increased lipid peroxidation concomitant with alteration in the activities of related antioxidant enzymes viz., SOD, CAT, GPX, GR, GST and non-enzymatic antioxidants viz., GSH, TAA in testis and cauda epididymis of treated rats. Generation of free radicals, increased lipid peroxidation and altered antioxidant defense systems, all these indices might play an important role in manifesting toxic effects of fluoride.
indicating vulnerability of reproductive organs to free radical toxicity and was supported by histopathological observation and fluoride burden noted in these tissues of rats treated with fluoride.

Administration of melatonin and amla to fluoride fed rats, antioxidant system was unaffected and all indices were comparable even to control groups showing amelioration of these compounds, due to their protective effects. However, in withdrawal studies for 30 and 60 days, the recovery was insignificant.

B. OTHER BIOCHEMICAL PARAMETERS

ENERGY METABOLISM PARAMETERS

The results of the present study revealed a significant dose dependent reduction in total protein levels in reproductive tissues after 60 days of sodium fluoride feeding. Several studies from our laboratory by Chinoy and co-workers have obtained a decline in protein levels of various soft tissue and serum of rats, mice, rabbits and guinea pigs fed with sodium fluoride at different dose and durations (Chinoy, 1991a,b; Chinoy and Sequeira, 1989a; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Patel, 1999; Chinoy and Memon, 2001; Chinoy and Shah, 2004c,d; Chawla et al., 2008; Rao et al., 2009; Chawla and Rao, 2012). In chronic fluorosis, children also led to a sharp fall in proteins and albumin levels (Cenesiz et al., 2005). To support our data, Qujeq et al. (2002) reported that sodium fluoride at three doses, 10, 20 and 30 mg/kg daily for 90 days, the average total serum protein level of the rats in the treatment group decreased significantly as
compared with that in the controls. The level of proteins in adrenal gland also exhibited a significant decline in the NaF treated rabbits (Shashi, 2003).

In the reproductive system, the total protein contents run parallel to the growth of the reproductive organs and are androgen sensitive in males. The living organisms depend essentially on proteins, which directly or indirectly regulate the biochemical processes. The decrease may be due to blocking of the metabolism of amino acids, thereby preventing cells from synthesizing protein. In fact, studies have shown that fluoride inhibits protein synthesis (Chinoy et al., 1994a) and interferes with amino acid metabolism. Fluoride inhibits protein synthesis by weakening the beginning of the peptide chain and by preventing the production of peptide chains in ribosomes. It also inhibits amino acid uptake by cells and reduces protein synthesis (Helgeland, 1976). The reduction in protein contents of fluoride treated animals supports the view that fluoride inhibits oxidative deacarboxylation of branched chain amino acid and simultaneously promotes protein breakdown. Edwards et al. (1984) indicated that fluoride could also disrupt the hydrogen bonding of protein molecules. Hydrogen bonding is an important in the maintenance of tertiary structure of protein molecules. Such disruption would result in enzyme inhibition and therefore reduced protein concentration may be explained which helped to retard growth and weights of fluoride fed animals in this study.

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 these reproductive tissues of fluoride treated rats. The decline in its activity might affect the conversion of succinate to fumarate and may cause a block by fluoride in
the Krebs cycle, leading less synthesis of ATP molecules. Earlier studies carried out in our laboratory (Chinoy, 1991a,b; 1992; Chinoy and Sequeira, 1989a; Chinoy and Mehta, 1999a; Chinoy et al., 1991a,c; 1992a; 1993b; 1994a,b,c; Patel et al., 1994) had also reported a decline in the activity of SDH in reproductive and other organs of mice and rats by NaF treatment. In addition, SDH is a mitochondrial enzyme and its decreased activity indicates a possible alteration in mitochondrial structure and function as a result of fluoride ingestion in the tissue (Chinoy and Patel, 1998a; Chinoy and Sharma, 1999b). Fluoride treated male and female mice revealed structural disorganization of mitochondria and rupture of cristae through ultrastructural studies in liver, ovary and uterus (Chinoy and Patel, 1998b; Chinoy and Sharma, 2000; Chawla et al., 2008; Rao et al., 2009). Similar results were reported that SDH activity was significantly reduced in other tissue like gastrocnemius muscle and in brain of sodium fluoride-treated mice and rats compared to controls indicating halted ATP, necessary for their need (Vani and Reddy, 2000; Rao and Bhatt, 2012) in light of our data.

Adenosine triphosphatase (ATPase) is a hydrolytic enzyme and important to releasing energy by the conversion of adenosine triphosphate to adenosine diphosphate. It is involved in the process of spermatogenesis in testis by the breakdown of ATP and release of energy. The activity of the enzymes SDH and adenosine triphosphatase (ATPase) reflect on the state of oxidation and energy metabolism of a tissue. In the present study, the total ATPase activity showed a significant decline in all reproductive organs in treated rat. ATPases are a class of enzymes that catalyze the decomposition of adenosine triphosphate (ATP) into
From our laboratory and other researchers had obtained similar results regarding ACPase activity. Chinoy and Shah (2004b) and Chinoy et al. (2005a) were reported a marked decline in enzyme activity of acid phosphatase in kidney (5 mg/kg body weight) and in ventral prostate (10 mg/kg body weight) of NaF treated mice for 30 days. Similarly, Chawla et al. (2008) also elucidated that orally administered sodium fluoride (Naf 10 mg/kg body weight) for 30 days to adult female mice decreased enzyme activity in their liver and ovary. The decrease in acid phosphatase enzyme activity in testis and cauda epididymis in this study may be due to degradation of the testicular and caudal components, in turn disturbed the internal milieu of reproductive organs in this investigation.

Alkaline phosphatases (ALKPase) are a group of enzymes which hydrolyse phosphate at alkaline pH. Alkaline phosphatase has ubiquitous distribution in all tissues of the body especially in cell membrane where it is associated with the transport of metabolite across the membrane. It is highly sensitive to different heavy metals and its inhibition leads to disturbances to the cellular functions affecting membrane permeability (Thaker et al., 1996). ALKPase has been investigated especially in the germinal epithelium and in the peritubular tissue in the testis of the domestic fowl in addition to plasma membranes.

In present data, the alkaline phosphatase activity is lowered in the testis and cauda epididymis and vasal tissues of treated rats as compared to control groups. To support our observation Agrawal and Sharma (2008) reported fluoride (5.8 ppm) water exposure to rats for 60 and 120 days decreased serum acid and alkaline phosphatases enzyme activities. Miao et al. (2005) had observed a significant
reduction in alkaline phosphatase activity in the mid gut of silk worms. A significant decrease in alkaline phosphatase activity in serum after NaF treatment was suggested of hepatic dysfunction (Eraslan et al., 2007). Inhibition of these enzyme activities thus might be also due to alteration in lysosomal activity and membrane permeability respectively. The activity of alkaline phosphatase declined significantly in the kidney of sodium fluoride treated mice reported by Chinoy and Shah (2004c) and Rao et al., (2009) in support of our data.

Cholesterol, one of the primary cell constituents, is present in large amounts in nervous tissues and is a most important precursor in the synthesis of steroid hormones (Dorfman et al., 1963; Eik-Nes and Hall, 1962). The present study demonstrated a peak rise in total lipids and cholesterol in testis after sodium fluoride exposed rats. Androgens are synthesized from cholesterol. But an increase in its concentration in testis is an indicative of non-utilization by the testis leading to a fall in circulatory androgens in rats due to androgen suppressive effects of the test substance. Loss of circulating testosterone levels and steroidogenic enzymes (3β and 17β HSDs) in our study support anti-androgenic nature of the toxicant in support of earlier investigation (Chinoy, 1992; 1996; Narayana and Chinoy, 1994a; Chinoy and Mehta, 1999a; Chinoy et al., 2004a,b).

Our findings were supported by other researchers, who documented that the high cholesterol accumulation in testis following sodium fluoride treatment for 60 days to rats (Sharma et al., 2005, 2006b, 2008a; Solanki et al., 2008). The significantly reduced activities of 3β- and 17β-HSDs in mice testis after fluoride+aluminium...
treatment for 30 days correlated with an accumulation of cholesterol (Chinoy et al., 2005b), affecting on its interference in testicular metabolism and androgenesis.

Earlier studies (Narayana and Chinoy, 1994a) have also reported similar changes as well as a decline in circulating testosterone levels in rats and human populations in endemic areas of North Gujarat (Chinoy et al., 1992a). Chinoy and Mehta (1999b) reported a significant decline in the activities of 3β- and 17β-HSD in NaF treated mice affecting steroidogenesis corroborating with our investigation.

Sialic acid is a sialomucopolysaccharide which is essential for the maintenance of the structural integrity of sperm membranes, besides aiding in their maturation. It is secreted by epididymis in addition to other markers like carnitine, ascorbate, glycosidases and glycerylphosphoryl choline (GPC) for sperm maturation and fertilizability. The secretions of these components are controlled by androgens (Rajlakshmi, 1985). In the present study, the sialic acid levels were assessed and found to be reduced by fluoride in cauda epididymis and vas deferens of treated rats.

A slight change in circulating androgens might affect epididymal function affecting sperm maturation. In our study, fluoride feeding affected serum androgen levels, that probably affected epididymal, testis and vasal functions. It was further supported by loss of testicular steroidogenesis due to accumulation of cholesterol and lipid by inhibiting enzymes involved in steroidogenic process in our study. These effects were still evidenced by altered histological structure of these tissues in our study in addition to fluoride burden prevailed.
From our laboratory, Chinoy and Sequeira (1992) observed an inhibition of sialic acid concentration in testis of mice. Chinoy et al. (1994c) further reported decreased sialic acid concentration of caput and cauda epididymis with sodium fluoride exposure for 30 days. In support of our data, Nag et al. (1977) documented that during maturation process sperm physiology might be affected, due to low level of sialic acid after fluoride feeding, leading low fertility rate.

**SPERM PROFILE AND FERTILITY RATE**

Sperm quality is one of the important indices of male reproductive function. Changes in sperm quality induced by fluoride have been demonstrated *in vivo* and *in vitro* in many species, including rat, mouse, rabbit, guinea pig and human (Zhang et al., 2004). The low sperm motility obtained might be correlated with its reduced metabolic activity by fluoride in the present study. Any negative impact on motility would seriously affect fertilizing ability (Pathak et al., 2000). Similarly, Chinoy and Narayana (1994) revealed that human spermatozoa lost their motility *in vitro* in the presence of 250 mM NaF within 20 minutes incubation, while Schoff and Lardy (1987) demonstrated that bovine sperm treated with 30 mM fluoride became immobile within two minutes. Previous studies carried out by Chinoy and associates in our laboratory (Chinoy et al., 1991a,c; 1992b; 1994c; 1995; 1997b; Chinoy and Sequeira, 1992; Chinoy and Sharma, 2000; Narayana and Chinoy, 1994b) found similar effects on spermatozoa by NaF treatment in rodents.

Kumar et al. (2010, 2012) observed that significant decrease in sperm count, motility and foreword progressive motility in sodium fluoride fed male rabbits. The
decrease in sperm density could be correlated with testicular spermatogenic arrest following NaF ingestion in mice as obtained by others (Chinoy and Sequeira, 1989b; Kour and Singh, 1980). After NaF treatment the spermatozoa exhibited loss of acrosome integrity and deflagellation which resulted in an increase in the number of abnormal forms. It also caused head and tail abnormalities of epididymal spermatozoa in rat and rabbit spermatozoa (Narayana and Chinoy, 1994b; Chinoy et al., 1991a). The head to head agglutination process could be due to changes in sperm acrosomal or plasma membrane proteins causing stickiness and their clumping (Chinoy et al., 1991a). The above mentioned alterations in sperm might be the outcome of altered and hostile internal milieu of the cauda epididymis and vas deferens of NaF treated rats as the epididymal and vasal micro-environment is important for sperm maturation and maintaining them viable, motile and normal morphological state. These studies support our observation of loss of sperm count, motility, viability and morphology in this study. These induced sperm effects led to a loss of fertility of treated rats. Similarly, impaired fertility rate was reported after sodium fluoride treatment in mice, rats and rabbits (Chinoy, 1991a,b; Chinoy and Sequeira, 1992; Chinoy and Sharma, 1998; Chinoy et al., 1992b; Narayana and Chinoy, 1994b; Collins et al., 2001; Chinoy et al., 2006). Neelam et al. (1987) reported infertility among married men in highly endemic areas in India. Elbetieha et al. (2000) and Huang et al. (2008) observed reduced fertility rate with impairment in reproductive function of fluorotic mice. Fluoride can cause low sperm quality and diminished fertility (Ghose et al., 2002; Cui et al., 2003). Gupta et al. (2007) also documented that sodium fluoride administrated in drinking water of 2, 4, and 6 ppm
concentration for 6 months to male rats adversely affected their fertility and reproductive system to support our data.

**FLUORIDE LEVELS IN SOFT TISSUE**

The fluoride levels in testis, cauda epididymis and vas deferens of sodium fluoride treated rats indicated its accumulation in these organs, that adversely affected their structure, metabolism and functions in this study. Susheela (1985) has reported a significant increase in the urine, skeletal muscle, liver and kidney $F^-$ levels following NaF ingestion to rabbits. Inkielewicz and Krechniak (2003) have observed, the fluoride content in liver, kidney, brain, testis, and serum and was determined at the beginning of the experiment and after 2, 4, and 12 weeks of exposure. In all the tissues and organs (serum, brain, liver, kidney and testis) the fluoride content increased in a dose-dependent and a time-dependent manner.

Similarly, Chinoy and Patel (1998a) found an enhancement in the levels of fluoride in the serum, uterus and ovary of mice. The retained fluoride in the serum, thus would affect the general body metabolism probably by altering soft tissue functions. High fluoride levels at different doses of fluoride have been reported in several tissues of mice (Chinoy and Sharma, 1998; Chinoy and Patel, 1998a; Vani and Reddy, 2000; Chinoy and Memon, 2001; Sun et al., 2003). Our investigations are compared to that of above results, where enhancement in fluoride levels in the reproductive tissues was observed in sodium fluoride treated rats. Fluoride levels increased in blood and urine in individuals living in endemic areas of North Gujarat (Mathews et al., 1996). All the above results of fluoride burden in different tissues
are agreement to support our findings affecting their normal histology, spermatogenic arrest, atrophy of Leydig cell, tubular epithelium of epididymis and vas deferens as well as their histocytometric results.

Oxidative stress, alteration in energy metabolism and other biochemical indices, spermiogram and fertility rate and increased fluoride levels were correlated with histopathological and histocytometric changes noted in the tissues viz., testis, vas deferens and cauda epididymis of treated groups. These quaunta of changes were markedly ameliorated by supplementation of melatonin and amla to fluoride fed animals, as their values were comparable to control groups. However, withdrawal studies indicated marginal or no significant recovery in all tissue after 30 and 60 days showing more time to regain normal physiology after toxicant ingestion to rats.

MELATONIN AND AMLA (EMBLICA OFFICINALIS) AMELIORATION

The pineal gland is the main origin center of melatonin. Through secretion of melatonin, it also regulates the circadian rhythms, sleep wake cycle and also slows down the aging process. Melatonin is a hormone that helps regulate the onset of puberty and helps protect the body from cell damage caused by free radicals. It too maintains biological clock and also exerts a powerful antioxidant activity in mammals. It can easily cross blood–brain barrier and cell membranes (Hardeland, 2005; Reiter et al., 2010; Pohanka, 2011). This antioxidant is a direct scavenger of radical oxygen and nitrogen species including; OH, O₂⁻, and NO (Poeggeler et al., 1994; Arnao and Hernandez-Ruiz, 2006). Arnao and Hernandez-Ruiz (2006) reported
that melatonin works like with other antioxidants to improve the overall effectiveness from each antioxidant. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal or suicidal antioxidant (Tan et al., 2000; Pohanka, 2011).

Recent research indicates that the first metabolite of melatonin in the melatonin antioxidant pathway may be N(1)-acetyl-N(2)-formyl-5-methoxykynuramine or AFMK rather than the common, excreted 6-hydroxymelatonin sulfate. The AFMK alone is detectable in unicellular organisms. A single AFMK molecule neutralizes up to 10 reactive oxygen species/reactive nitrogen species (ROS/RNS), since many of these products of the reaction/derivatives (including melatonin) are themselves antioxidants. This capacity to absorb free radicals extends at least to the quaternary metabolites of melatonin, a process referred to as "the free radical scavenging cascade." This is not true of other, conventional antioxidants and is hence better one (Tan et al., 2007) comparatively.

In present study, these mitigative effects of melatonin was observed in body and reproductive organ (testis, cauda epididymis and vas deferens) weights following sodium fluoride treated rats for 60 days. Similar observations of mitigative role of melatonin in other tissues have been reported earlier (Chawla et al., 2008; Rao and Bhatt, 2012). The levels of LPO and GST were reduced in testis and cauda epididymis of NaF treated rats. In antioxidant system, enzymatic (SOD, CAT, GPX, GR) and non
enzymatic (total –SH, GSH, TAA) components were ameliorated to levels of controls in the reproductive tissues by melatonin co-supplementation in this study.

Energy metabolism indices like SDH and ATPase and other biochemical parameters viz., total proteins, acid phosphatase and alkaline phosphatase were mitigated and were compared near to control values in testis, vas deferens and cauda epididymis after co-administration of melatonin. Corroborating our data, other workers (Rao et al., 2009) reported that melatonin treatment along with NaF reduced the toxic effects of fluoride in reproductive and non reproductive tissues of human and rats as compared to the controls, confirming the antioxidative properties of melatonin. Reiter et al. (2007) documented that melatonin influences both the electron transport chain (ETC) and oxidative phosphorylation by increasing electron transport and ATP production in normal cells.

Bharti and Srivastava (2009; 2011) mentioned melatonin protected fluoride-induced oxidative stress in brain of rats through mechanisms involving enhancement of enzymatic and non-enzymatic antioxidant defense system, which strongly support our present data. They also suggested that melatonin can be useful in curing neurotoxicity induced by fluoride. Our results are in agreement with the work done by others in our laboratory, where melatonin ameliorates fluoride induced oxidative stress in mice and rats (Chawla and Rao, 2012; Rao and Bhatt, 2012).

The result also showed that melatonin administration to sodium fluoride treated rats manifested significant mitigation in the steroidogenic activity of testis followed by epididymal sperm and vasal biochemical profiles including fertility rates.
and hormone levels as compared to control animals. Accumulations of fluoride burden were also reduced after supplementation of antioxidant revealing that antioxidant is effective in elimination of fluoride directly or indirectly in the present study.

Reports from Sarabia et al. (2011), co-treated with melatonin plus the pesticide diazinon, morphological anomalies were not observed with both doses of the pesticide or time intervals, corroborating the preventive role of melatonin against damage induced by the pesticide possibly via melatonin receptor and extracellular signal–regulated kinase-mediated pathways and increase the fertility rate (Espino et al., 2011; Martin-Hidalgo et al., 2011). Casao et al. (2010) have also observed that the treatment accounted as increase in sperm motility and seems to increase both fertility and fecundity in ewes inseminated with semen from melatonin + fluoride treated rams during the oestrus. Chawla and Rao (2012) have reported that melatonin protects against fluoride induced histopathology of female mice. Melatonin has been found to ameliorate F-induced reproductive organ toxicity in male rats as reported by Rao and Bhatt (2012) to support our current investigation.

*Amla* (*Emblica officinalis*) is one of the most celebrated herbs in the Indian traditional medicine system. Although these fruits are known to contain high amounts of ascorbic acid (Vitamin C) (Tarwadi and Agte, 2007), the specific contents are disputed, and the overall antioxidant strength of amla may derive from its high density of ellagitannins such as emblicanin A (37%), emblicanin B (33%),
In present investigation, the protective effects of amla extract was observed in body and reproductive organs (Testis, cauda epididymis and vas deferens) weights following its supplementation with sodium fluoride to rats for 60 days. Vasant and Narasimhacharya (2012) have reported ameliorative role of amla against fluoride induced toxicity in rats. Lipid peroxidation (LPO) and glutathione-s-transferase (GST) levels were declined in testis and cauda epididymis of sodium fluoride treated rats. In this study, also other enzymatic (SOD, CAT, GPX, GR) and non enzymatic (total – SH, GSH, TAA) components of antioxidant system were mitigated in the reproductive tissues by amla co-supplementation.

Energy metabolic and metabolic parameters like SDH, ATPase, total proteins, and phosphatases were ameliorated with amla co-supplementation and were comparable to control values in testis, vas deferens and cauda epididymis. Vasant and Narasimhacharya (2012) reported that amla further revealed the levels of SDH, ATPase, total proteins and phosphatases reversed due to the antitoxic affect of amla against NaF toxicity in rats, which corroborate our findings. Amla fruit extract administration to alcohol-treated rats also significantly increased plasma total proteins, the A/G ratio (Albumin/Globulin ratio) and uric acid levels (Reddy et al., 2010). In study by Verma and Chakraborty (2008), administration of amla fruit aqueous extract (2 mg/animal/day) for 45 days along with ochratoxin caused significant amelioration in the ochratoxin-induced reduction in DNA, RNA and protein contents in the liver and kidney of mice. Amla co-supplementation during NaF treatment to rats evidenced significant amelioration in the steroidogenec
activity of testis followed by epididymal sperm and vasal profiles including fertility rate, and hormone levels as compared to control animals. Accumulation of fluoride burden were also reduced after supplementation of antioxidants revealing that antioxidant is effective in elimination of fluoride leading normal morphology of tissue in treated groups in the present study.

Chronic treatment of CCl₄ and TAA revealed abnormality in histopathology of liver which recovered by co-administration of amla (Fruit) (Mir et al., 2007). 50% hydroalcoholic fruit extract of *Emblica officinalis* have protective effect against antituberculosis drugs induced liver toxicity.

Out study also strongly suggest that both antioxidants of amla extract exerted protection against fluoride generated toxicity being slightly better by melatonin due to its free radical scavenging cascade phenomenon.

The data of upon withdrawal of treatment, insignificant or no recovery was obtained in most of the parameters studied. Chinoy and coworkers (Chinoy, 1992; 2002; Chinoy and Sequeira, 1989a,b; Chinoy and Patel, 1996; 1998a; 1999; Chinoy and Sharma, 1998; Chinoy and Mehta, 1999a,b; Chinoy and Memon, 2001; Patel and Chinoy, 1997; Chinoy et al., 1991b; 1994d; 1995; 1997a,b) have reported also partial or incomplete recovery in several biochemical indices in various organs after the withdrawal of NaF treatment for one or two months. Therefore toxic effects induced by fluoride were found to be random after termination of fluoride treatment inlight of our data. This might be due to difference in the animal species, the duration of exposure, varying tissue response provoked by administration of test chemicals, the dose used and duration of withdrawal, challenging antioxidant therapy is better.