DISCUSSION
One of the most critical environmental issues today is ground water contamination. About 70% of all the water available in our country is polluted. In India, more than 76% of the population who lives in several rural settlements of varying population are dependent on ground water as a source of drinking water (Kumar et al., 2007). Therefore the preservation, protection and management of the quality of life is dependent on the environmental components, apart from industries, agriculture and other economic parameters. The extensive use of pesticides in the present decade is also major culprits of environmental pollution. Apart from this, trace elements are often considered to be toxic to man, despite their active role as cofactor in many metabolic pathways. The trace elements are frequently encountered by human beings in several ways. Fluoride is one of such trace elements.

The present study was undertaken in order to evaluate ameliorative efficacy of melatonin and amla against toxic effect of sodium fluoride in some vital and endocrine organs of male Wistar rats (Rattus norvegicus) for duration of 60 days. The tissues selected for the study were liver, kidney (vital organs) and thyroid, adrenal (endocrine organs). Sodium fluoride (NaF) was administered orally at doses of 5 and 10 mg/kg body weight/day for two months. The doses used were based on the LD$_{50}$ value of fluoride (Pillai et al., 1987). Oral administration was preferred in view of water being the main source of fluoride among the human population in endemic areas.

Antioxidants melatonin and amla extract were fed with fluoride (High dose) and alone to the respective groups at doses of 10 mg/kg b.w and 20 mg/kg bw for 60 days to investigate ameliorative effect on fluoride toxicity. For comparison of ameliorative
effects of antioxidants, withdrawal studies for 30 and 60 days were also conducted and various indices and histology were done.

The various parameters studied at the end of respective treatments were body and organ weights. The concentration of glycogen and phosphorylase activity in the liver and levels of creatinine in kidney were carried out to investigate the alterations. Total protein levels, activities of alkaline phosphatase and acid phosphatase were also estimated during the course of the investigation. In addition the activities of some antioxidants indices viz., lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), levels of glutathione (GSH) and total ascorbic acid (TAA) were evaluated in vital organs like liver and kidney and in endocrine organs like thyroid and adrenal. The serums T3, T4, TSH levels from serum were studied to evaluate thyroid functions. Similarly, the tissue levels of fluoride were done in all the groups of animals in addition to histopathology of these organs.

GRAVIMETRIC STUDIES

The results revealed that doses of NaF (5 and 10 mg/kg) for a period of 60 days produced significant alterations in the body and organ weights which are good indices of overall growth pattern, particularly in long term study. Our findings are in accordance with other earlier reports (Sharma et al., 2007; Tiwari and Pande, 2011; Vasant and
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Narshimhacharya, 2012) who have attributed the reduction in body weights to reluctant food intake. It is possible that fluoride may have suppressed the hunger centers of central nervous system, resulting in increased food intake without enhancing energy assimilation, prompting a decline in body and organ weights (Vasant and Narshimhacharya, 2012), as found in our study.

Reports from our laboratory have revealed that NaF for a period of 7, 15 and 30 days did not produce any significant alteration in the gravimetric parameters, and it was effective from the 45th day onwards and the effects were more pronounced after 60 days treatment. (Chinoy and Patel, 1998). Another study from our laboratory on reproductive organs also supports this finding on gravimetric parameters (Rao and Bhatt, 2012). A similar decline in body weights occurred in rats and pups when the female mother rats were fed with fluoride chronic dose (200 ppm) for two generations (Basha and Sujitha, 2011). Khandara et al. (2011) also reported that a decline in the body weight in rabbits fed with 10 mg/kg NaF for seven months. The results of the present study further corroborate the above data as a significant reduction in the body and organ weights by fluoride treatment for 60 days depending on dose. The mechanism by which the growth rate is inhibited by fluoride could be due to alteration in food intake as well as reduction in protein synthesis. Gradual decline in the weight of liver, kidney, thyroid and adrenal were observed through our treatment. The low weight of these organs might be due to decline in protein levels along with reduced metabolic activity and hormonal status as observed in the present study.
Discussion

Mitigation of NaF caused decreased gravimetric parameters were observed with co-supplementation of melatonin and amla. Withdrawl study for 30 and 60 days after NaF ingestion showed partial recovery in gravimetric indices in the animals.

BIO CHEMICAL INDICES

The liver has an important place in toxicology. Virtually all substances absorbed from the gastrointestinal tract pass through the liver before entering the central circulation. The liver with its metabolic detoxicating function is extremely vulnerable to harmful substances. Kidney act as a natural filtration system in body. They filter out harmful chemicals and other body toxins, otherwise might find their way into bloodstream and cause blood poisoning. The glands of the endocrine system and the hormones they release influence almost every cell, organ, and function of an organism. The endocrine system is instrumental in regulating development, growth, tissue function, metabolism and reproductive processes.

In the present study, fluoride feeding induced liver, kidney, thyroid and adrenal functional alterations. The results revealed a significant dose dependent decrement in total protein levels in all these tissues after 60 days of NaF treatment. In support of our data Shashi (2003) reported decreased protein synthesis in vitro and in vivo by fluoride treatment. Shashi (2003) has also reported the level of proteins in adrenal gland exhibited a significant fall in the NaF treated rabbits. It is mainly due to impairment of the polypeptide chain initiation (Chinoy et al., 1997; Holland and Hongslo,
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1978; Hoerz and McCarty, 1971) and weak incorporation of aminoacids into proteins or possibly inhibition of DNA synthesis followed by a decrease in mRNA transcription, (Holland and Hongslo,, 1978 ; Zahvoronkov and Strochkova, 1981) by fluoride ions. Fluoride inhibits key enzymes of the glycolytic pathway and thus reduces energy metabolism and protein synthesis (Holland, 1979a,b). Shashi et al. (1992; 1994) also revealed a dose-dependent decline in protein levels in skeletal muscle and brain in experimental fluorosis in rabbits. It also inhibits amino acid uptake by cells and reduces protein synthesis. (Helgeland, 1976). Cenesiz et al (2005) reported decreased protein levels in Tuj sheep exposed to 13.8 mg F/L in their drinking water for 24 weeks. The results of the present study corroborate with the above data as significant decrement in the levels of total proteins in liver, kidney, adrenal and thyroid of rats treated with NaF for 60 days gradually. Queeq et al. (2002) also reported total serum protein levels were decreased in adult rats after oral treating with sodium fluoride at three doses, 10, 20, 30 mg/kg daily for 90 days. The overall decline in protein levels due to the treatment of NaF would affect the activities of various enzymes, reduce secretions in the cells and organs of fed rats.

The activity of the oxidative mitochondrial enzyme succinate dehydrogenase, showed a significant decline in liver, kidney, thyroid and adrenal tissue following the treatment of fluoride. Succinate dehydrogenase (SDH) catalyzes the oxidation of succinate to fumarate in the cycle of Krebs. This decrement in SDH activity affects the conversion of succinate to fumarate and may cause a block in Krebs cycle altering the oxidative energy metabolism of the tissue. Since SDH is a mitochondrial enzyme, any structural or functional alteration in mitochondria may influence its metabolic activity by
reducing net ATP production. Mathur (2012) also reported decreasing trend of ovarian SDH enzyme reactivity after 5 and 10 mg/kg doses of NaF in female Swiss albino mice. Earlier studies carried out in our laboratory (Chawla and Rao, 2012; Rao et al., 2009; Chinoy and Mehta, 1999, Patel et al., 1994,) had also reported a decrement in the activity of SDH in soft tissues of mice and rats by NaF treatment. The decrease in SDH might be similar to that of isocitrate dehydrogenase, another TCA cycle enzyme which leads to accumulation of citric acid. The reduction in activity of SDH in gastrocnemius muscle of fluoride-treated mice is reported by Chinoy et al. (2004 a, b). In agreement with our data Vani and Reddy (2000) and Pang et al. (1996) have also reported decrements in SDH levels of brain and muscle of NaF treated animals and the results have been correlated with alterations in the structure of mitochondria and skeletal muscle fibres (Meda, 2012, Shashi, 1989).

Adenosine triphosphatase (ATPase) is a hydrolytic enzyme involved in the conversion of ATP to ADP and release of energy. The ATPase activity in the present study resulted in a significant decline in these vital and endocrine organs of NaF treated animals. Miao et al. (2005) also reported that the activities of ATPase was reduced by F treatment in silkworm, Bombbyx mori L. Similarly reduced activity of ATPase in muscle, kidney and reproductive organs have been reported in a number of studies following fluoride ingestion in mice (Chinoy et al., 2005 a,b,c; Vani and Reddy, 2000; Chinoy, 1991a, b; 1992; 1995; 2003; Chinoy and Mehta, 1999; Chinoy and Sharma, 1998; Chinoy et al., 1991 a, b; 1993; Chinoy and Sequeira, 1989) A reduction in its activity by NaF treatment signifies inhibition of the electron transport system. The decrease in ATPase could also be related to the changes in Ca$^{2+}$ levels since it is necessary for activation of
these enzymes. All these factors would eventually lead to a liberation of less energy and
thus affect energy metabolism of vital organs as well as endocrine tissues by fluoride
treatment. The alteration in activities of adenosine triphosphatase (ATPase) after fluoride
treatment as in the present study was also reported by others in support of our data
(Lakshmi and Reddy, 2000, Shashi, 1989) Park et al. (1999) suggests that the energy
metabolism is disturbed probably due to structural and functional changes in tissues.
Partanen, (2002) reported decreased kidney ATPase activity in the animals treated with
NaF. Reduction in SDH and ATPase activities in these tissues hence resulted in loss of
energy status of NaF ingested groups.

A group of phosphatase enzymes are associated with numerous functions
at the cellular level, Acid and alkaline phosphatases are from this group of enzymes
associated with important functions of the body. Acid phosphatase (ACP) is a lysosomal
enzyme involved in many activities such as phagocytosis, dissolution of tissue
components, fat absorption in intestine, cellular differentiation and keratinization.
Alkaline phosphatase (ALP) is a membrane bound enzyme and is associated with the
transport of metabolites through the cellular membrane. The alkaline phosphatase is a
well known indicator of multiple toxicity cases, including those related to hepatic and
renal dysfunctions. In the present study both these enzymes decreased in a dose
dependent manner in liver, kidney, thyroid and adrenal gland in the low and high dosed
NaF treated animals. Reduction in phosphatases activities might be a consequence of
changes in the permeability of plasma membrane and lysosomal activity. This
observation is in agreement with the results reported by others (Fujii and Honda, 1972)
Similar results were also reported in a study of Vasant and Narsimhacharya (2012) who found out that activities of hepatic and renal phosphatase enzymes decreased in the groups of animals treated with 100 ppm sodium fluoride for 30 days. Sondhi et al. (1995) also reported decline in the phosphatase activity in the group of Swiss albino mice treated with 100 ppm of fluoride for 15 and 30 days. Significant reduction of acid and alkaline phosphatases in ovary and uterus and alteration in plasma ALPase concentration of female rats treated with 6 ppm fluoride for 15 and 30 days were reported by Sharma et al. (2007) and is directly related to the tissue damage. In agreement with our data, Zhan et al. (2006a,b) have reported decreased ALP activity in the kidney of pig treated with NaF. Sharma et al. (2007) have reported elevation in enzyme activity of acid phosphatase in serum traditionally used as a lysosomal marker enzyme (Araki et al., 1995). Orzechowska-Juzwenko and Orzechowski (1980) reported decreased activity of granulocytes alkaline phosphatase and increased count of leucocytes in peripheral blood during 4-week administration of water contaminated with sodium fluoride in doses of 20 mg/kg of body weight in people and animals from the area surrounding the factories, contaminated by fluorine compounds. Similar results of ACP reduction were also documented by Chen et al. (2005), who investigated that the acid phosphatase activity decreased drastically with the increased fluoride concentration in the food of silkworm. Partanen (2002) also reported inhibition of human renal acid phosphatases by nephrotoxic micromolecular concentration of fluoride. Inhibition of this lysosomal enzyme, as observed in the present investigation, may be due to the altered lysosomal activity since the toxicant acts on cellular components to inhibit its synthesis (Thaker et al., 1996). These changes are also contributory to loss of tissue metabolic, functional and
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structural integrity of these tissues by NaF treatment as was evident by biochemical and histological disturbance in these organs.

All these biochemical indicators altered in these tissues of fluoride ingested rats were correlated with the exerted histopathological changes and accumulation of fluoride in these tissues as described later in our discussion. Thus NaF induced decrement in total protein levels, SDH, ATPase, ACPase and ALPase were revived with administration of antioxidants like melatonin and amla. Withdrawal studies of NaF induced toxicity for 30 and 60 days showed partial or no much recovery in all these parameters of respected organs.

ANTIOXIDANT DEFENSE SYSTEM

Oxygen is a biradical and has a tendency to form toxic reactive oxygen species (ROS) or free radicals. The most common ROS include: the superoxide anion (O2-), the hydroxyl radical (OH ), singlet oxygen (1O2 ), and hydrogen peroxide (H2O2). Superoxide anions are formed when oxygen (O2) acquires an additional electron, leaving the molecule with only one unpaired electron. Antioxidants means "against oxidation." Antioxidants work to protect lipids from peroxidation by free radicals. These are manufactured within the body and can also be extracted from the food, which humans eat such as fruits, vegetables, seeds, nuts, meats, and oil. There are two lines of antioxidant defense within the cell. The first line, found in the fat-soluble cellular membrane consists of vitamin E, beta-carotene, and coenzyme Q. Of these, vitamin E is considered the most
potent chain breaking antioxidant within the membrane of the cell. Inside the cell, second line water soluble antioxidant scavengers are present. These include ascorbic acid, glutathione peroxidase, superoxide dismutase (SOD) and catalase.

The formation of lipid free radicals and lipid peroxides is considered an important feature of cell injury. The major fatty acids that undergo lipid peroxidation in the cell membranes are all polyunsaturated fatty acids (PUFAs). Abundance of free radicals could lead to uncontrolled chain reactions and lipid peroxidation (Marks et al., 1997). The data of current investigation revealed that fluoride feeding to rats induced lipid peroxidation in all soft tissues of rats after 60 days in a dose dependent manner. This increased peroxidation of lipids concomitantly increased TBARS levels losing cellular functions in these organs by fluoride intoxication. Fluoride induced lipid peroxidation has been reported in a number of animal models. Shivarajashankara et al. (2003) reported increased lipid peroxidation in the blood of young rats treated with 100 ppm of fluoride. Nabavi et al. (2012a) also reported increased lipid peroxidation in rat kidneys treated with 600 ppm fluoride. Similar results were reported by Hassan and Abdel-aziz, (2010), whose research data indicated that sodium fluoride (10.3mg/kg bw) administration induced oxidative stress as evidenced by elevated levels of lipid peroxidation in red blood cells, kidney, testis and brain tissues in rats. Inkielewicz-stepniak and Czarnowski, (2006;2010) reported the applied fluoride (25 mg F/L) caused an increase of nitric oxide levels (NO), intensified lipid peroxidation (LPO) and decreased total antioxidant status (TAS) in serum, brain, kidney and liver of rat. So an excess production of ROS by NaF may be also explained by its ability to produce alteration in mitochondrial function. As a consequence, an imbalance in antioxidant protective mechanisms takes place, leading to
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oxidative stress in the cell. Further Akdogan et al. (2004) documented increased TBARS levels by 21, 70 days NaF treatment to rabbits. In a report by Chlubek et al. (1999), the effect of increasing concentrations of NaF (25, 50, and 500 M) on lipid peroxidation in the mitochondrial fraction from human placenta was described. Güven and Kaya (2005) documented increased MDA levels in fluorotic sheep. Wang et al. (2004) also reported increased lipid peroxidation in human embryo hepatocytes exposed to 40 µg/mL and 80 µg/mL for 24 hours. Madhusudan et al. (2010) studied that when pregnant female rats were treated with 100ppm of fluoride for 21 days, pups of these females had enhanced levels of TBARS in discrete regions of central nervous systems. Reddy et al. (2003) also documented increased MDA levels in the blood of fluorotic patients. In our laboratory it is documented daily dosages of 5 and 10 mg NaF/kg bw administration orally to male rats for 60 days altered its antioxidant indices in the testis which was confirmed by increased lipid peroxidation (LPO) (Rao and Bhatt, 2012). In another study in our laboratory Chawla and Rao (2012) have reported enhanced levels of MDA levels in ovary by NaF administration to female rats. Basha and Sujitha (2011) have also reported increased lipid peroxidation in myocardial tissues by administration of chrome levels of fluoride in rats. Oncu et al. (2007) have reported increased lipid peroxidation levels in two generation of rats after exposed to long term fluoride in their drinking water. All these studies are in agreement with our observation that LPO levels remained increased gradually in all these tissues of treated rats.

The enzyme superoxide dismutase (SOD) catalyzes dismutation of superoxide radical leading to production of hydrogen peroxide which in turn is detoxified by the
enzyme, catalase (Rzeuski et al., 1998). Catalase is found mainly in the peroxisome and removes H2O2 and O2, reducing the hydrogen peroxide levels. It is an efficient inhibitor of LPO when hydrogen peroxide accumulates in a cell containing free ferrous ions. The enzyme, SOD is considered as a first line of defense against oxygen toxicity and central regulator of ROS levels by hydrogen peroxide, which in turn is detoxified by the enzyme catalase to molecular oxygen (Acharya et al., 2008). It also protects oxygen metabolizing cells against harmful effects of superoxide free radicals. Catalase is a heme protein, which catalyzes the reduction of hydrogen peroxides and protects the tissues against the destructive effects of highly reactive hydroxyl radicals. In the present investigation, NaF caused a decline in the activities of free radical scavenging enzymes viz., superoxide dismutase (SOD) and catalase in all tissue studied. In support of our data Reddy et al. (2003) documented, reduction in the activities of SOD and catalase following NaF treatment in rabbits. Inkielewicz et al. (2006) have reported that the activities of these enzymes were inhibited by NaF treatment in rats. Decreased activities of superoxide dismutase and catalase in liver, kidney, adrenal and thyroid gland of treated rats rendering the tissue susceptible to injury. Production of the superoxide anion (O2\textsuperscript{-}) is caused by an incomplete oxygen oxidation. These radicals are liable to react with several molecules, provoking their destabilization. The most important consequences are the denaturation of the proteins and peroxidation of membrane lipids, with an increased permeability of the cell membrane. Reports by Sun et al. (1998) have revealed decreased SOD and catalase activities in fluorotic mice in agreement with the results of the present study. These enzymes, SOD and CAT are considered primary enzymes since these are involved in direct elimination of ROS (Halliwell and Gutteridge, 1989). Reduction in the activities of
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SOD and catalase caused deleterious effects in tissue structure which could be due to accumulation of superoxide anions radicals and hydrogen peroxide. Nabavi et al. (2012a, b) reported decrement in the SOD and CAT in the rat blood erythrocytes and brain treated with NaF. Reddy et al. (2003) documented decrements in the levels of SOD and CAT in the blood of patients with skeletal fluorosis and in rabbits treated with 150ppm NaF for six months. Morales-González et al. (2010) reported 50 ppm of fluoride treatment to rats decreased levels of these enzymes in the rat erythrocyte membranes. It has been reported that acute or chronic intoxication with NaF in experimental animals, as well as in human who live in zones of fluorosis, produce oxidative stress in several organs, such as kidney, brain, liver and gonads. In such subjects, the clinical signs of fluorosis appear accompanied by alteration in the enzyme activities of SOD and CAT. The mechanism by which NaF induces oxidative stress in these organs is not known with certainty. Nevertheless, studies conducted in vitro show that NaF may interact with enzymes that contain a transition metal as part of their co-factors or in their active site as well as replaces hydroxyl ions.

Glutathione (GSH) comprises upto 90% of the non protein thiol content of mammalian cells. It acts as a nucleophilic ‘scavenger’ of many compounds and their metabolites via enzymatic and chemical mechanism, converting electrophilic centers to ether bonds. Total thiol (-SH) groups too play an important role in balancing the structure of protein and also the cellular equilibrium. In the present study there is a dose dependent decrement in the total – SH groups and GSH contents. 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. Glutathione conjugation helps in detoxification by binding electrophiles that could otherwise bind to proteins or nucleic acid, resulting in cellular damage and genetic mutation. Studies of Chouhan and Flora, (2008) also support these results. Reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) by glutathione peroxidase (GPx) by consuming H2O2 and again this oxidized glutathione is converted back to reduced glutathione (GSH) by glutathione reductase (GR) through conversion of NADPH to NADP. Alteration in the total –SH contents including GSH, probably upsets the intracellular equilibrium of free radicals and antioxidant enzymes, resulting into an oxidative stress of rat organs in our observation. Shuhua et al. (2012) also reported that NaF of concentration from 5 to 20 mg/L can stimulate BV-2 cells to change into activated microglia displaying upregulated OX-42 expression. GSH and GPx activities significantly decreased in fluoride-treated BV-2 cells as compared with control cells.

Ascorbic acid (AA) is known to be powerful reducing agent which acts as an antioxidant for detoxifying several toxic substances and helps in activating several enzymes (Kutsky, 1973). Reduction of ascorbic acid indicates its involvement in overcoming stress. NaF brought about a significant decline in total ascorbic acid levels in liver and kidney treated with low and high doses of fluoride indicating that fluoride treatment causes stress in the animals leading to rapid utilization of the vitamin. This suggests that the stored ascorbic acid is rapidly oxidized in the tissues under fluoride induced stress and converted it to its dehydroform which consequently showed an increase as obtained in this present study. According to Lewin (1976) maintenance of
proper levels of ascorbate is essential for preventing the conversion of adrenalin and noradrenalin to adrenochromes, which are highly toxic compounds acting as potent inhibitors in many enzymatic reactions. It also increases C-AMP levels by hindering the PDE activity in cell physiological changes. The administration of NaF to rats is reported to cause a marked decrease in the levels of TAA in male reproductive organs of male rats (Sharma et al., 2008a). Shivarajashankara et al. (2001) also found out decreased brain TAA levels treated with 30 and 100 ppm of NaF for 11 weeks. In support of our data Sharma et al. (2008a) reported decreased levels of ascorbic acid in the reproductive organs of rats treated with 5.8 ppm of fluoride for 60 days, these authors mentioned that ascorbic acid is reported to play an important role in general oxidation-reduction processes. Cui et al. (2003) also reported the same on the reproductive organs. Jenkins et al. (1970) reported coexistence of severe forms of fluorosis and ascorbic acid deficiency. Animal study reported in ICMR Bulletin, (1979) mentioned considerable reduction in cellular ascorbic acid content, indicating that fluoride ions interfered with vitamin synthesis pathway. It causes disturbance in the utilization and probably synthesis of ascorbic acid leading to changes in its metabolism that might also be influenced by the decreased GSH levels. 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). Tissue GSH is involved in the mechanism of detoxification by scavenging free radicals in rat liver (Liang et al., 1999, Satsangi and Dua, 2000) and in the conversion of dehydroascorbic acid into the reduced form of it (Rao et al., 2012). Thus, GSH and ascorbate in cellular functions are related, as noted in our present study.
Glutathione Peroxidase (GPx) is also an antiperoxidative enzyme, which is present in cytosol and mitochondrial matrix. GPx converts hydrogen peroxide to water along with the conversion of GSH to GSSG. Glutathione reductase (GR) is also a key enzyme to reduce oxidative stress by enhancing reduced levels of glutathione by reduction of GSSG to GSH simultaneously converting NADPH to NADP. In the present study GPx and GR are found to decrease during exposure to NaF. NaF might have inhibited the GPx and GR directly by impairing the functional groups and/or indirectly by rendering the supply of reduced glutathione and NADPH. Bharti and Shrivastava (2009) reported decrement in GR levels in pineal gland of buffalo fed with NaF. Similar result was also obtained by Basha and Madhusudan (2010), who have documented decreased GR levels in animals exposed with NaF during pre and post natal duration. Blaszczyk et al. (2008) reported decrement in the levels of GR in kidney of rats fed with NaF. Blaszczyk et al. (2009) in another study found decreased levels of GR in soft tissues and blood exposed to NaF. Another studies from our laboratory also reported decreased levels of GR and GPx in different tissues of rats exposed to NaF (Rao and Bhatt, 2012; Meda, 2012). Reddy et al. (2003) also documented reduced levels of GR in rabbits treated with 150 ppm of NaF for 6 months, and also in patients suffering with skeletal fluorosis.

Glutathione-s-transferase (GST) catalyses the conjugation of reduced glutathione via a sulfhydryl group to electrophilic centers on a wide variety of substrates. Its activity detoxifies endogenous compounds such as peroxidised lipids, as well as breakdown of xenobiotics. GST may also bind toxins and function as transport proteins, which gave rise to the early term for GSTs of “ligandin”. GST is considered to contribute to the biotransformation of xenobiotics conjugating to the compounds which are often
electrophilic and somewhat lipophilic in nature, with reduced glutathione to facilitate dissolution in the aqueous cellular and extracellular media, and then are removed from the body. This activity detoxifies endogenous compounds such as peroxidized lipids (Leaver and George, 1998) as well as breakdown of different xenobiotics. In our present investigation, GST activity was significantly increased in liver, kidney, thyroid and adrenal gland of treated animals, suggesting a high oxidative burden within the body of animals intoxicated with fluoride. This may be correlated with earlier report (Podder et al., 2008a, b) on significant increase in chromosomal aberrations of bone marrow cells in mice exposed to lower doses of F in comparison with higher dose. In the previous animal experiments, the authors (Shivarajashankara et al., 2001; Vani and Reddy, 2000) reported increased GST levels in the fluoride treated for shorter periods (14–120 days) to the animals. Thus reduced activities of GPx, GR as well as GSH levels followed by an increase in GST activity indicated the adverse effect of NaF on tissues to impose oxidative toxicity. Finding of Shivarajashankara et al (2003) demonstrated that NaF imposes a loss of antioxidant potential. An increase in ROS generation is also accompanied by a decline in the antioxidant enzymes. Agalakova and Gusev (2011), documented augmented oxidative stress leading to excessive generation of ROS, lipid peroxidation, decrease in the GSH/GSSH ratio, and alterations in activities of antioxidant enzymes, as well as inhibited glycolysis thus causing the depletion of cellular ATP and disturbances in cellular metabolism. Fluoride triggers the disruption of mitochondria outer membrane and release of cytochrome c into cytosol, which activates caspases-9 and -3 (intrinsic) apoptotic pathways. Extrinsic (death receptor) Fas/FasL-caspase-8 and -3 pathway was also described to be implicated in fluoride-induced
apoptosis. Fluoride decreases the ratio of antiapoptotic/proapoptotic Bcl-2 family proteins and upregulates the expression of p53 protein. Thus, anti stress indices expressed alteration which augmented altered functions of tissues by fluoride poisoning and is related to histological manifestations and higher fluoride burden in these tissues, as noted in this study.

These antioxidant parameters were comparable to controls when animals were treated with melatonin and amla along with high dose of fluoride. Withdrawal studies of high dose of NaF treated animals for 30 days and 60 days showed not much recovery was obtained.

Glycogen is a polymer of glucose. In the present study, treatment with fluoride resulted in a significant accumulation of glycogen but an inhibition of phosphorylase activity in the liver of rat treated with NaF. Underwood (1977) reviewed the effects of fluoride on carbohydrate metabolism. Fluoride is known to act as an inhibitor of glycolysis either by enolase mediated inhibition or decrease in the activity of isocitrate dehydrogenase. As a result of intoxication, animals were unable to utilize glycogen substrate, leading to its accumulation in liver. Chinoy et al. (2004a,b) reported enhanced glycogen levels in the liver gastrocnemious muscles of rat treated with 5 mg/kg for 30 days in correlation of our present data. A significant decline in phosphorylase activity was noticed in the silkworm, *Bombyx mori* l. treated with NaF (Miao et al., 2005). A significantly declined phosphorylase activity and increased glycogen levels in crabs treated with 30 ppm of fluoride for 15 days was also recorded. Similar enhancement in glycogen was reported in different soft tissues of rats and mice (Chinoy and Sequeira, 1989, Chinoy et al., 1993; 1994a,b,c; Patel et al., 1994). In support of these results,
Dousset et al. (1987) found a decrement in glycogen turnover and citrate accumulation in rats fed with NaF. The fluoride induced decline in the activity of glucose-6-phosphate dehydrogenase in rats would also affect the glycogen metabolism. These biochemical changes were correlative to histological changes of liver in the treated animals. Recently Vasant and Narsinhacharya (2012) also recorded fluoride effects on liver glycogen metabolism in light of our observation.

Cholesterol is a key molecule involved in the membrane fluidity and other physiological metabolism. Alteration in the level of cholesterol in various tissues and serum revealed structural and functional damage in liver and kidney. In the present study vital tissue cholesterol levels were enhanced gradually due to NaF intoxication to rats. Papierkowski et al. (1999) also reported increased serum cholesterol level in the animals treated with fluoride in rats with nephritic syndrome. To support our data this lipid alteration might changed membrane properties and these alterations led to structural changes as noted in liver histology with fluoride burden.

Creatinine is a key biomarker of kidney integrity and its concentration is an important measure of renal function. Any kind of change in the creatinine concentration in kidney, serum or urine is directly related to the structural and functional alteration in kidney. The data of present study revealed an increase in the levels of creatinine in the kidney of rat by NaF treatment. In support of our data Zhan et al. (2006) have reported elevated creatinine levels in young pigs treated with 100 and 250 ppm of fluoride for 50 days. Nabavi et al. (2012 b) have reported increased creatinine levels in kidney as well as in serum of rats treated with 600ppm fluoride for one week. Serum concentration of
creatinine is used as the first line investigation of glomerular function. This result is supported by structural changes in kidney and further correlated with fluoride burden.

The thyroid hormones, triiodothyronine (T₃) and thyroxine (T₄) are tyrosine-based hormones produced by the thyroid gland that are primarily responsible for regulation of metabolism. Iodine is necessary for the production of T₃ and T₄. These hormones increase the basal metabolic rate (BMR), affect protein synthesis, help regulate long bone growth (synergy with growth hormone) and neuronal maturation, and augment the body's sensitivity to catecholamines (such as adrenalin) by permissiveness. These hormones also regulate protein, fat, and carbohydrate metabolism, affecting how cells use energetic compounds and also stimulate vitamin metabolism. Numerous physiological and pathological stimuli influence thyroid hormone synthesis.

In our present study T₃, T₄ and TSH levels were estimated from serum and found to be decreased in a dose dependent manner in animals treated with different doses of NaF. Reduced levels of TSH might be due to indirect effect of fluoride acting on hypothalamic-pituitary axis and/or direct at receptor level at target site. These damaging effects, all of which occur with small concentrations of fluoride, have obvious and easily identifiable effects on thyroid status. In support of our data Basha and Sujitha (2011) has reported significant decrease in the serum-free thyroxine (FT4) and free triiodothyronine (FT3) levels in fluoride-treated group. These effects are further evidenced by loss of follicular structure, due to its replacement instead of iodine and decrement in thyroglobulin synthesis.
Zhan et al. (2006b) also found a significant decrease in free thyroid hormones (FT3 and FT4) in fluoride-treated rats which are in agreement with the results who reported decreased level of serum FT3 and FT4 in young pigs fed with 100, 250, and 400 mg fluoride/Kg diet. Trabelsi et al. (2001) also reported a significant reduction in the plasma free T4 level in 14-day-old mice whose mothers had been treated with 0.5 g NaF/L in drinking water. Wu et al. (2008) have suggested that the decreased levels of thyroid hormones might be due to an inhibition of the absorption of iodine through fluoride interaction, insufficient synthesis, and secreton of thyroglobulin and oxidized iodides from the thyroid gland owing to follicle injury after excessive intake of fluoride. Perhaps, changes in the thyroid hormone levels in the present study might be correlated with imbalanced oxidant/antioxidant system which further led to a reduction in learning memory ability and hypothyroid condition (Basha and Sujitha, 2011).

Adrenal cholesterol and ascorbate are indicators of its steroidogenesis of adrenal cortex. The involvement of ascorbate to increase C-AMP levels in steroidogenic process of gonads and adrenal is well known. Further, reduced ascorbate in adrenal is also related to a decline in GSH levels in it. It is known that GSH is involved in conversion of dehydroascorbate to reduced ascorbic acid in adrenal and gonads. In this connection, fluoride effects on gonadal steroidogenesis studies, Chawla and Rao (2012) achieved increments in ovarian ascorbate and cholesterol levels followed by included female hormones. Rao and Bhatt (2012) also noted abnormal Leyding cell androgen synthesis in fluoride treated rats in light of our data.

In the present study, the analysis of fluoride levels in liver, kidney, thyroid and adrenal gland of NaF treated rat revealed an enhancement, which indicates that, the
fluoride accumulation in these tissues and might affect their structure functions and metabolism. Idris and Wihardja (2008) have documented thyroid gland has a capacity to absorb and accumulate fluoride highest in soft tissues after aorta because the shape of fluoride is similar to iodine, which is essential element for thyroid gland and thyroid hormone like thyroxine that has four iodine atoms. So fluoride can impede active transport of iodine ions into the thyroid gland because both are included into the halogen group. Same reports were also documented by Chinoy and Mehta (1999) in serum, urine, testis, cauda epididymis, liver and kidney of treated mice and revealed a significant enhancement which reflected on fluoride accumulation in theses tissues and would affect their metabolism.

Susheela (1985) has reported a significant increase in the urine, skeletal muscle, liver and kidney F levels following NaF ingestion to rabbits and by Mathews et al. (1996) in fluorotic individuals of North Gujarat. Similarly, Chinoy and Patel (1998) found an enhancement in the levels of fluoride in the serum, uterus and ovary of mice. Chawla and Rao (2012) also noticed high accumulation of fluoride in ovary and brain of fluoride treated mice. Increased levels of fluoride in brain and reproductive tissues of rats were also documented and correlated with histopathological changes (Bhatt, 2012; Meda 2012). Likewise, fluoride burden in the tissues studied in our present investigation indicated that their structures and functions were affected and these pathological changes were supportive to biochemical lesions caused by fluoride treatment in this study.

Liver and kidney exhibited degenerative changes gradually with cellular atrophy. These changes were more prominent in 60 days treated animals treated with high
dose. Glomerular necrosis was also evident. Similarly results were also obtained by Chatopadhyay et al., (2011), Abdo et al., (2011) and also in our laboratory by Chawla et al., (2008) revealing their effects are correlated with biochemical changes in the vital organs. Fluoride accumulation also brought about thyroid follicular atrophy, loss of thyroglobulin production in degenerative follicles probably reflecting on reduced production of thyroid hormone. It is also known to by replace by iodine making it prone to its histopathological change. Altered Cortico steroids production by adrenal cortex also exhibited morphological changes induced/exerted by fluoride feeding. Medulla of adrenal also exhibited regression changes by fluoride ingestion to rats leading to atrophy of chromaffin cells by there reducing anti-stress activity. Thus, adrenal histopathology was related to oxidative stress as observed by reduced ascorbate and GSH levels and defense mechanism in this study.

All the biochemical profiles of vital and endocrine organs, histopathology and fluoride burden indicated a definite amelioration after co-supplementation of antioxidants to fluoride intoxicated rats in our study. However no recovery was obtained after withdrawal of treatment for 30 and 60 days.

AMELIORATION BY MELATONIN AND AMLA (EMBILLICA OFFICINALIS)

Melatonin is naturally-occurring in all living organisms (Lynch, 2004). It is a hormone that is produced by the pineal gland in the brain. Melatonin levels vary in 24 hour cycles and are controlled by our body clock. Normally its production is reduced by being in bright light. Levels increase at night. Hence it is often called ‘the hormones of darkness’
Some plants have small amounts of melatonin as well. Melatonin appears to be important in helping regulate the internal body clock's cycle of sleep and wakefulness. Other claims are made for it: it has anti-oxidant and free radical scavenging properties and some say it has anticancer and anti-ageing effects. Numerous reports document its beneficial and antioxidant effects in stress conditions. (Reiter, 2001; Tan et al., 2007)

In the present study, after administration of antioxidants like melatonin at the doses of 10 mg and 20 mg/animal/day respectively, body and organ weights did not reveal any significant differences compared to that of control. It can be attributed to free radical scavenging capacity of the antioxidants given along with fluoride feeding to animals. Amelioration of ATPase, SDH, phosphates enzymes and normal levels of proteins were observed in our study with melatonin treatment along with NaF. Melatonin is highly lipophilic and passes through plasma membrane and enters the mitochondrial compartment where it exerts potential antioxidative effect. Leon et al. (1954) also reported that melatonin increases the efficacy of the electron transport chain by limiting free radical formation and promotes ATP synthesis. Hence, melatonin preserves the integrity of the mitochondria and helps to maintain cell function and survival. Levels of phosphatases enzymes were also mitigated and were comparable to control by the melatonin supplementation. Studies from our laboratory have reported ameliorative role of melatonin on the NaF induced toxicity on blood, liver, ovary and testis in vivo and in vitro (Rao and Bhatt, 2012; Rao et al., 2011; Chawla and Rao, 2012; 2008, Rao and Tiwari, 2006). Shang et al. (2004) reported that melatonin can protect the mitochondria from the damage induced by ROS generation through its effective antioxidative potential.
in justification of our data. Melatonin sheltered the altered ATPase activities in PCB (Arocolor 1252) treated animals (Venkataraman et al., 2008) in support of our results. Oner et al. (2002) documented that melatonin prevented the alteration in the ATPase activities in rats after ethanol intoxication.

In the present study enzymatic and non-enzymatic antioxidants like GPx, GR, GST, SOD, Catalase, GSH, TAA, were mitigated in all the tissues of vital and endocrine in the animals supplemented with melatonin along with NaF feeding. In accordance to our data other reports revealed ameliorative effect of melatonin against ionizing radiation and drug toxicity (Reiter et al., 2001; Karbownik et al., 2001; Suryanarayan et al., 2007). Navara and Nelson, (2007) proposed the beneficial effects of pharmacological doses of melatonin due to its antioxidants properties. Reports from our laboratory documented restoration of all biochemical and antioxidative parameters with melatonin due to mercury and NaF induced toxicity in animals (Patel, 2010; Rao et al., 2010; Rao and Bhatt; 2012; Meda, 2012). Mercury and Aluminium induced oxidative stress and LPO levels were decreased by melatonin supplementation in liver, kidney and brain (Sener et al., 2003; Mahieu et al., 2009). It was documented that besides melatonin’s ability to scavenge ROS, it has been demonstrated to activate the antioxidative enzymes (Pal and Chatterjee, 2006). Melatonin supplementation also led to an increase in SOD activity in accordance with the finding of Antony et al. (2008) who reported that its administration induced an increase in the mRNA levels of dismutase. Similar results in modulation of renal and hepatic antioxidant defense system were reported by Bharti et al. (2011) with the treatment of melatonin. These beneficial effects of melatonin might be due to its
property as an electron donor. Reports also suggested that melatonin detoxifies numerous ROS including H2O2, singlet oxygen and also reactive nitrogen species. Sarabia et al. (2011) reported melatonin mitigated pesticide or time intervals, corroborating preventive role of melatonin.

Biochemical profiles and morphology of vital and endocrine organ tissue fluoride levels were mitigated significantly in the animals supplemented with melatonin along with the treatment of NaF. Melatonin is one of the most effective oxygen free radical scavengers. 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. AFMK alone is detectable in unicellular organism and metazoans. A single AFMK molecule can neutralize up to 10 ROS/ RNS since many of the products of the reaction/derivatives are themselves potent 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 (Tan et al., 2007) exerting the best protective role against toxicity imposed by the toxicant like fluoride in this investigation. It is also known to be better antioxidant than vitamins. The biochemical profiles, antioxidant indices, fluoride burden mitigation by melatonin in fluoride fed rats are well supported by restoration of normal histoarchitecture of endocrine and vital organs along with their normal hormonal and vital functions.

Amla fruit comes from *Emblica officinalis*, a tropical and sub-tropical medium sized tree that grows in arid areas. Its tonic qualities are very strong, lending it medicinal
value in the treatment of numerous diseases, including fever, cough, asthma, anemia, hemorrhage, liver dysfunctioning and alcoholism (Tasduq et al., 2005). The fruit is a very rich source of vitamin C. It was proposed that superior effect of the "vitamin C" component is actually the more stable and potent anti-oxidant effect of the tannins that appeared to be the vitamin.

Ameliorative effects of role of amla were reported on alcohol, ochratoxin and fluoride induced toxicity in animals (Chakrabarty and Verma, 2010; Reddy et al., 2007; Vasant and Narasimhacharya, 2012). Anil Kumar et al. (2004) have also reported protective effects of amla on oxidative stress and biochemical toxicity in rats challenged with dimethyl hydrazine. Amla extract obtained from Himalaya products, has various compounds like ascorbic acid, flavinoids, polyphenols, gallic acid, embillicanins (A and B), tannins etc. Chinoy and Patel (2001) have reported that treatment of ascorbic acid, a major content of amla extract, increased concentrations of Na+, K+, Ca+ and so possibly binds with F ions and helps in reducing its toxic effects. Vasant and Narasimhacharya (2012) suggested that the fruits of *E. officinalis* exhibit anti-hyperglycemic, anti-hyperlipemic, anti-peroxidative and antioxidant properties in fluoride exposed animals and therefore, fruit of *E. officinalis* are useful in regulating fluoride induced toxicity and improve the antioxidant status.

In the present study mitigative effects of amla was observed in body and organ weight of vital and endocrine organs. Energy metabolic parameters like SDH, ATPase and other biochemical parameters like total proteins, ALKPase, ACPase, were comparable to control after amla extract was supplemented to animals along with high
dose of fluoride. Corroborating our data, Chakraborty and Verma (2010) reported amelioration of amla on ochratoxin induced spermatotoxic effect. The levels of LPO and GST were reduced in vital as well as in endocrine organs of amla fed rats compared to NaF fed animals and results were comparable to controls. In antioxidant system, enzymatic parameters like SOD, CAT, GPx, GR and non enzymatic components like total –SH, GSH and TAA were mitigated in both the vital and endocrine organs with co-supplementation of amla along with NaF. Reddy et al. (2010) documented that amla fruit extract administration to alcohol treated rats also significantly increased plasma total proteins, the A/G ratio, and uric acid levels. Mir et al. (2007) reported recovery in abnormal histopathology of liver caused by CCL4 and TAA. Bhattacharya (2000a,b) reported antioxidative activity of tannoid principles of amla in chronic stress induced changes in rat brain. Antony et al. (2008) reported amla is very effective in inflammation and dyslipidemia. All these results support our data in regard to its amelioration of fluoride toxicity nearer to control levels.

This ameliorative effect of amla extract could be due to the possession of various components, ascorbate being the highest. All of these components have antioxidant properties that pool in the counteracting of fluoride toxicity in tissues. The herbal products are also novel agents for such studies, as there is less/no toxic even at higher doses. The hormonal status of thyroid, adrenal gland and vital organ functions along with their histological features were well mitigated along with reduced accumulation of fluoride, thus resuming their normal integrity of all tissue studied.

Withdrawal studies on animals for 30 and 60 days documented partial/or no recovery comparable to control group of animals. In accordance to our study Chinoy and
Patel (1998) have documented no or partial recovery in NaF fed animals with withdrawal treatment. Release of fluoride that was stored in the tissue during 2 month NaF exposure can account for this result, which released slowly and required probably more time for excretion. Further, reversibility might be longer, that is dependent on animal species, duration, response and other factors, revealing the antioxidants are better.

Thus, sodium fluoride has definite toxic effects on vital and endocrine organs with respect to their structure, metabolism and functions as noted by altered physiology of these tissues by fluoride intoxication. However, the fluoride induced effects could be effectively reversed by supplementation of melatonin and amla as noticed in this study. Melatonin is better antioxidant and mitigates numerous toxic effects exerted by various toxicants due to its free radicals scavenging cascade than other antioxidants like vitamins as suggested by Tan et al. (2007).