Mechanisms of Hepatotoxicity of Fluoride in Endemic Skeletal Fluorosis and Experimental Chronic Fluoride Toxicity

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Abstract
Dental and skeletal fluorosis are the well-known and much highlighted clinical entities of fluoride toxicity. However, fluoride exerts adverse effects even on the structure and functions of soft tissues such as brain, liver, pancreas, intestine, kidneys, red blood cells and thyroids. Animal studies have shown altered levels of serum liver function parameters, altered activities of liver tissue enzymes, disturbed liver cell architecture, oxidant stress and changes in antioxidants in liver. In humans with endemic skeletal fluorosis, liver function is found to be adversely affected. Fluoride exerts its effects by inhibition of metalloenzymes involved in vital metabolic pathways, induction of oxidative stress, inhibition of antioxidant enzymes, direct cytotoxic effect, modulation of metabolism by signal transduction via G Protein-coupled receptors, inhibition of synthesis of DNA, RNA and proteins, and by its interactions with other minerals. The animal experiments on chronic fluoride toxicity have reported varied findings which might be due to differences in dose, duration and mode of fluoride administration, animal species used, and organ-specific metabolic responses. Fluoride is a metabolic poison. Detailed research studies are required on mechanisms of fluoride toxicity and the antidotes to prevent or mitigate the clinical complications of fluorosis. There is a need for extensive studies on biomarkers of chronic fluoride intoxication.

Citation:

1. Introduction

Fluorine, 13th in the order of abundance of elements in earth’s crust, is highly electronegative and occurs ubiquitously as fluorides in nature (WHO, 1984). Fluoride is a compound of utmost concern to the human health. While minimum intake of fluoride is necessary to prevent dental caries, higher intakes for long time lead to detrimental effects on teeth, bones and soft tissues, the manifestations of which are together referred to as fluorosis (WHO, 1984; Susheela, 1999).

Fluorosis is a worldwide public health problem. In India, 17 out of 30 states, and more than 60 million people including 6 million children are affected by fluorosis (WHO, 1984; Susheela, 1999). The spectrum of clinical manifestations in fluorosis range from mottling of enamel to bone deformities and crippling. Dental fluorosis and skeletal fluorosis are the well-known and much highlighted clinical entities of chronic fluoride intoxication. However, fluoride exerts adverse effects even on soft tissues,
the non-skeletal fluorosis which is the focus of research in recent years (WHO, 1984; Susheela, 1999).

Liver is the largest organ of the body performing multiple functions. It plays a major role in metabolism of carbohydrates, lipids, proteins, minerals, vitamins and xenobiotics. Liver diseases have become one of the major causes of morbidity and mortality all over the globe (Dienstag and Isselbacher, 2005). As liver is the exclusive organ for metabolism of xenobiotics, and it is also a dumping site for toxic compounds and thus, many times a victim of their toxic manifestations. Exhaustive literature is available on soft tissue fluorosis in general and on hepatofluorosis in particular. Fluoride is known to be hepatotoxic. Not only the fluoride consumed through drinking water has hepatotoxic effects but also, organofluorides used as anaesthetics, antibiotics, antimalarial drugs, antidepressants, and other fluorides used in medicine have been shown to possess hepatotoxic actions (WHO, 1984).

However, this review focuses only on hepatotoxic effects of fluoride stemming from high levels of fluoride in drinking water in fluorosis-endemic areas, and chronic fluoride intoxication in experimental animals by administration of sodium fluoride through oral or subcutaneous routes.

2. Review of Literature

2.1 Fluoride Intake and Skeletal Fluorosis
The effects of fluorides on the health of man stem largely from dissolved fluorides present in ground and surface water (WHO, 1984). Owing to the universal presence of fluorides in the earth’s crust and influence of many hydrogeological and chemical factors, water samples contain varying amounts of fluoride, values as high as 2800 ppm have been reported (WHO, 1984). Water fluoride level of 0.5-1.0 ppm is considered optimal to offer beneficial effect and level above 1.0 ppm is shown to cause the manifestations of fluorosis (WHO, 1984). As per the recommendations of Food and Nutrition Board, National Research Council, Washington, the estimated safe and adequate intakes of fluoride are 0.1-1.0 mg/day for infants, 0.5-2.5 mg/day for children and adolescents, and 1.5-4.5 mg/day for adults. According to literature from WHO, the total daily intake of fluoride is 0.43-0.91 mg in areas with 0.4 ppm fluoride content in drinking water, and 1.0-5.5 mg in areas with more than 1.0 ppm fluoride in drinking water (WHO, 1984).

The authors carried out research work on chronic fluoride toxicity in Gulbarga of Karnataka state of India. This place is in the North interior of Karnataka, temperature here shoots up to 48 degree Celsius in summer. The area under study had borewells as the main source of ground water. The ground water fluoride levels are known to be higher in interior arid areas when compared to coastal areas (WHO, 1984). Gulbarga is in a belt of endemic fluorosis paralleling with areas of Andhra Pradesh where high prevalence of fluorosis is seen (Nawlakhe and Pramasivam, 1993; Susheela, 1999). The highest level of ground water fluoride was 13 ppm in the areas we studies in Gulbarga (Shivashankara et al., 2000). Children in the endemic fluorosis areas showed genu vulgam, genu varum, osteosclerosis of pelvic and femur bones, and osteoporosis of other bones. Few of the children were even crippled (Shivashankara et al., 2000). Similar observations were recorded by various authors in other endemic fluorosis areas (Krishnamachari, 1986; WHO, 1984).

2.2 Effects of Fluoride on Soft Tissues
Under normal circumstances soft tissues contain very little fluoride (<1.0 ppm). With prolonged exposure to fluoride, relatively large amounts of fluoride accumulate in tissues (Zhavoronkov, 1977). Fluoride affects almost all vital tissues of the body. Studies have reported Morphological and cellular architectural abnormalities, inflammatory changes, metabolic derangements and increased oxidative stress in experimental animals subjected to chronic fluoride intoxication (Michael et al., 1996; Monsour and Kruger, 1985; Vani and Reddy, 2000). In endemic skeletal fluorosis patients impaired functions of vital organs, paralysis, calcification of arteries, GIT lesions, metabolic derangements, and altered oxidant-antioxidant status leading to oxidative stress, have been reported (Monsour and Kruger, 1985; Shivarajashankara et al., 2003; Shashi and Thapar, 2008; Kour et al., 1981).

2.3 Fluoride Accumulation in Liver
Pharmacokinetic studies have shown the accumulation of fluoride to be slow but in greater amounts, after the absorption and that the accumulation continues for at least 3 hours (Geeraets et al., 1986). The highest reported fluoride concentration in the liver was 61 ppm in an endemic skeletal fluorosis patient with fatal radiculomyelopathy (Sauerbrunn et al., 1965).
2.4 Fluoride alters cellular architecture of liver
Assessment of the effects of chronic fluoride toxicity by Zhavoronkov has revealed marked liver damage referred to as fluorohepatopathy, which was characterized by decrease in the number of binuclear hepatocytes, destruction of mitochondria, and decrease in cytoplasm in animals (Zhavoronkov, 1977). Other researchers have echoed the above findings and also observed zonal necrosis, irregular shaped and pyknotic nuclei, disturbed arrangement of hepatic cords, mononuclear infiltration in portal triad areas, hepatic hyperplasia, and extensive vacuolization in hepatocytes in rats, mice, and guinea pigs (Chinoy et al., 1993; Kapoor et al., 1993; Babrowski et al., 2006; Kour et al., 1981; Shashi and Thapar, 2008).

2.5 Effect of Fluoride on Serum Liver Function Tests
Liver function is generally assessed by the assays of bilirubin, total proteins, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum. In Children and adolescents with endemic skeletal fluorosis, we observed reduced levels of total proteins and albumin, and increased levels of ALT, AST and ALP, in serum (Shivashankara et al., 2000). Significant elevations of cyclic AMP, ALP, AST and acid phosphatase (ACP), with positive correlations to water and serum fluoride levels, was seen in adult individuals suffering from endemic skeletal fluorosis (Chinoy et al., 1992; Shashi and Bharadwaj, 2011).

In rats, mice and rabbits subjected to long-term intake of high levels of fluoride via drinking water or by subcutaneous routes, impairment of liver function was observed. The observed biochemical changes in serum were, reduction in total proteins and albumin, and elevations of ALT and AST (Chinoy et al., 1993; Zhi-Zhong et al., 1989; Grucka-Mamczar et al., 1997; Chawla et al., 2008).

2.6 Effect of Fluoride on Liver Tissue Enzymes
Fluoride decreased or increased the levels of enzymes in liver tissue depending on the dose, duration, route of administration and animal species used. Rats administered 10 or 30 ppm fluoride in drinking water for 3 or 6 months showed increase in the activities of ALT and AST, and decrease in the activities of arginase, malate dehydrogenase and isocitrate dehydrogenase. Marked decrease in ALP, AST, ALT, acid phosphatase, ATPase, LDH and SDH in liver was observed in mice or rats which were administered various doses (10-200 ppm in drinking water or intraperitoneal doses) of fluoride for durations ranging from 1 to ten months (Chawla et al., 2008; Bogin et al., 1976; Singh and Kanwar, 1981). To substantiate the findings in above studies, *in vitro* studies have observed that fluoride is an inhibitor of those enzymes which require divalent metals as co factors. It has long been known that fluoride ions inhibit alcoholic fermentation and glycolysis. Warburg and Christian (1941) have shown that this is due to the inhibition of enolase, the enzyme of glycolytic pathway. It was suggested that the inhibition of enolase is due to the formation of a magnesium-fluoride-phosphate complex and studies have showed cooperativity of P$_i$ and F$^{-}$ binding and, more specifically, that F$^{-}$ is coordinated to the enzyme bound Mg$^{2+}$. Biotoxicity of fluoride ions results mainly from their inhibitory effect on the activity of many enzymes, mostly those concerned with ATP production and those which synthesize protein and DNA (Reiner et al., 1955; Haughen and Suttles, 1974; Sullivan, 1969; Goris et al., 1972; Tormanen, 2003). This is associated with high chemical activity of F ion and its affinity to Ca$^{2+}$ and Mg$^{2+}$, which catalyze a number of enzymatic reactions. Few such enzymes include enolase, ATPases, cholinesterases, arginase, ACP, SDH, esterases, isocitrate dehydrogenase, phosphatases and aconitase. Fluoride is proposed to be a competitive inhibitor of enzymes. In vitro and in vivo studies by Sullivan demonstrated significant inhibition of SDH activity by sodium fluoride by a mixed competitive and noncompetitive mechanism (Sullivan, 1969).

2.7 Fluoride and Hepatic Carbohydrate Metabolism
In vivo studies have reported modulation of hepatic carbohydrate metabolism by fluoride. Shearer and Suttie (1970) on administering fluoride (450 or 600 ppm, as NaF in diet for 3 days) to rats observed decreased levels of lactate and pyruvate, and increased level of citrate in liver. Fluoride ingestion was also shown to cause decreased activity of hepatic pyruvate kinase but no change in enolase. Chinoy et al. (1993; 2001) reported increased glycogen content of liver in rats subjected to chronic fluoride toxicity. Sodium fluoride (NaF) ingestion also caused an inhibition of glycogen phosphorylase in liver (Chinoy and Memon, 2001). In vitro studies on isolated rat hepatocytes incubated with NaF showed enhanced production of cyclic AMP, increased hepatic synthesis and release of glucose, and...
decreased formation of lactate and pyruvate (Shahed et al., 1979). Observations of above studies suggest that fluoride down regulates glycolysis and upregulates gluconeogenesis (Shearer and Suttie, 1970; Shahed et al., 1979). But, effect of fluoride on glycogen metabolism is still a subject of contradictions. Studies have revealed glycogen accumulation and decreased glycogen phosphorylase in liver (Chinoy et al., 1993; Chinoy and Memon, 2001); but its mechanism of action via increased production of cyclic AMP does not substantiate the phosphorylase-inhibiting action (Shahed et al., 1979).

2.8 Fluoride and Hepatic Lipid Metabolism
Chronic fluoride ingestion is shown to cause disturbances in lipid metabolism in the form of decrease (Singh et al., 1985), or increase (Saralakumari et al., 1988) in the concentrations of total lipids, triglycerides and cholesterol in the liver. Fluoride also caused a reduction in phospholipids and polyunsaturated fatty acids, and increase in saturated fatty acids (Wang et al., 2000). In experimental animals chronic intoxication with sodium fluoride caused elevation of serum triglycerides, total cholesterol, LDL cholesterol and reduction of HDL cholesterol in serum (Saralakumari et al., 2000). Children and adults in endemic skeletal fluorosis areas exhibited significantly increased triglyceride, but unaltered total cholesterol, HDL cholesterol and LDL cholesterol in serum (Shivashankara et al., 2000; Michael et al., 1996; Zhi-Zhong et al., 1989).

2.9. Fluoride and Protein Synthesis in Liver
Fluoride is shown to inhibit protein biosynthesis and reduce the protein content in liver. Studies on experimental animals have reported decreased levels of total proteins (Shashi, 2003; Grucka-Mamczar et al., 1997; Kathpalia and Susheela, 1978), acidic proteins (Shashi, 2003), and basic proteins (Shashi, 2003), and increased levels of free amino acids in liver (Shashi, 2003) on exposure to high-fluoride doses. In vitro studies have revealed that NaF affects the cellular protein biosynthesis by impairing peptide chain initiation (Vesco and Colombo, 1970). Incubation of He La cells with NaF resulted in inhibition of protein synthesis, disaggregation of polyribosomes, accumulation of 80 S ribosomes and decrease of free ribosomal subunits. After the removal of NaF the normal level of free ribosomal subunits was restored at the expense of a random dissociation of the ribosomes (Vesco and Colombo, 1970). The increase in free amino acids in the liver of rabbits chronically exposed to fluoride (5-50 mg NaF/kg/day, 15 weeks) further substantiated these findings (Shashi, 2003)

2.10. Fluoride inhibits DNA and RNA synthesis in liver
The key enzymes of DNA and RNA synthesis, DNA polymerase and RNA polymerase respectively, are metalloenzymes with zinc, a divalent cation. Hence, these are the potential targets for inhibition by fluoride. Fluoride is shown to inhibit the hepatic synthesis of DNA and RNA (Shashi, 2003; Holland, 1979), and an in vivo study has observed decreased hepatic contents of DNA and RNA in rabbits subjected to chronic fluoride ingestion (Shashi, 2003). Nucleoside triphosphatase, a Mg$^{2+}$-dependent enzyme is present in the liver nuclear envelope and is involved in nucleo-cytoplasmatic translocation of RNA. NaF inhibits this enzyme, which might be responsible for decrease in hepatic protein synthesis (Sidransky et al., 1982). Feeding 150 ppm fluoride (as NaF) for 4 weeks caused 44% DNA damage and increased apoptotic rate in liver tissue of rats (He and Chen, 2006).

2.11. Fluoride Interacts with Other Minerals
Liver tissue levels of trace elements also seem to be altered in chronic fluoride toxicity. Reduced levels of copper, manganese and zinc and elevated iron level in liver, were observed by Singh (1984) in rats receiving high levels of fluoride in drinking water (50, 100 and 200 ppm). Low levels of copper and zinc, and high levels of molybdenum in water and food contribute to the development of the genu valgum syndrome in fluorotic patients in South India (Shivashankara et al., 2000; Krishnamachanri, 1986). Fluoride is shown to inhibit the metalloenzymes requiring copper (Singh, 1984) and zinc (Vesco and Colombo, 1970). Fluoride is known to induce oxidative stress, and one of the mechanisms involved might be promotion of the activity of molybdenum-containing xanthine oxidase, a potential source of free radicals (Chinoy, 2003; Chlubek, 2003).

Hepatotoxic effects of fluoride are observed to be amplified in the presence of aluminium and arsenic. Aluminium and fluoride have close association in drinking water sources, environment and cooking utensils, where they form aluminofluoride complexes. Aluminium reinforces the fluoride’s stimulation of G protein coupled receptor-mediated signal transduction and increased adenylyl cyclase activity (Sternweis and Gilman, 1982). Combined treatment with NaF and Aluminium
chloride caused more significant decrease in protein content, SDH, cholinesterase and phosphorylase, and increase in glycogen content in liver, when compared to fluoride or aluminium treatment alone (Chinoy and Memon, 2001). Combined hepatotoxicity of fluoride and arsenic, which reinforce each other with respect to absorption, actions and toxic manifestations, has been reported (Nair et al., 2004).

2.12 Fluoride and Oxidative Stress in Liver
Oxidative stress is caused by increased generation of free radicals and depletions of antioxidants, and is implicated in the etiopathogenesis of many diseases and in the toxic actions of many compounds. Fluoride is known to induce oxidative stress. Because of its high electronegativity, F⁻ forms strong hydrogen bonds, especially with −OH and −NH moieties in biomolecules, and it has a potent ability to form stable complexes with polyvalent metal cations like Al³⁺, Fe²⁺, and Mg²⁺ (Chlubek, 2003; Chinoy, 2003).

Lawson and Wu (2003) by their in vitro study in earthworm proposed that fluoride binds to the active site copper on SOD, displacing water, thus acting as a competitive inhibitor. A decrease in SOD activity could be attributed to a direct action of fluoride on the enzyme rather than to increased generation of free radicals induced by fluoride intoxication. Fluoride stimulates respiratory burst and production of free radicals in neutrophils (Chlubek, 2003).

We observed increased levels of malondialdehyde (marker of lipid peroxidation), glutathione peroxidase (GSHPx) and vitamin C, and reduced levels of glutathione (GSH), uric acid and superoxide dismutase (SOD) in the blood of children with endemic skeletal fluorosis (Shivarajashankara et al., 2001a). Our studies also showed increased malondialdehyde, GSHPx, and glutathione S-transferase (GST), and decreased levels of GSH, total glutathione and ratio of GSH to GSSG, in liver, brain and blood of rats exposed to 30 or 100 ppm fluoride in drinking water for 3 months (Shivarajashankara et al., 2001b; Shivashankara et al., 2002; Shivarajashankara et al., 2003; Shivaraajashankara et al., 2002). The effects of fluoride on oxidative stress in liver, brain and blood were more pronounced when the rats were exposed to fluoride from prenatal days and earlier stages of life than the exposure at the adult stage of life. Various other studies have reported increased lipid peroxidation and altered antioxidant status of liver in experimental fluoride toxicity in animals (Inkiewicz-Stepniak and Czarnowski, 2010 Chawla et al., 2008).

While elevated MDA is a universal finding in the animal studies of oxidative stress in fluorosis, varied observations have been made on the changes in antioxidants in liver. Elevations in antioxidants in liver might be attributed to an adaptive response and protective mechanism of the liver tissue to fluoride-induced oxidative stress. Decreased levels of antioxidants in liver could be attributed to their depletion on combating reactive oxygen species generated in chronic fluoride toxicity.

2.13 Effect of Fluoride on Hepatic Detoxifying Enzymes
Fluoride activates the enzymes of xenobiotic metabolism in liver. In vitro study by Dierckx (1998) observed that sodium fluoride activated the phase I ethoxyresorufin-O-deethylase (to 240%) and pentoxysorufin-O-depentyllase (to 156%), and the phase II glutathione transferase (to 120%) in rat hepatoma-derived Fa32 cells. A time and concentration-dependent activation was observed, and maximum activation occurred at a concentration of 1.2 mM NaF. Our in vivo studies have shown significant elevation of GST in liver, brain and RBC, in chronic fluoride intoxication (30, 100 ppm fluoride) in young (during prenatal life and first ten weeks of postnatal life) and adult rats (one month old till 4 months); the GST activity in liver increased by about 140 % in liver (Shivarajashankara et al., 2001b; Shivashankara et al., 2002; Shivarajashankara et al., 2003).

2.14 Antidotes to Fluoride-induced Hepatotoxicity
Antioxidant vitamins C and E (Chinoy et al., 1993; Nair et al., 2004), methionine (Blaszczyk et al., 2010), melatonin (Chawla et al., 2008), caffeine (Inkiewicz-Stepniak and Czarnowski, 2010) and calcium (Chinoy and Memon, 2001; Nair et al., 2004) were effective as antidotes in mitigating hepatotoxicity from fluoride. These antidotes, when administered simultaneously with sodium fluoride, prevented/reversed histological abnormalities, changes in liver enzymes, and oxidative stress in experimental animals. Tamarindus indica and Moreinga oleifera plant extracts were tried successfully in amelioration of hepatotoxicity of fluoride in rabbits (Ranjan et al., 2009).
Conclusion
Fluoride has detrimental effects on liver. It can be considered as a hepatotoxic agent and a metabolic poison. Fluoride exerts its effects by inhibition of metalloenzymes involved in vital metabolic pathways, induction of oxidative stress, inhibition of antioxidant enzymes, direct cytotoxic effect, modulation of metabolism by signal transduction via G Protein-coupled receptors, inhibition of synthesis of DNA, RNA and proteins, and by its interactions with other minerals. The animal experiments on chronic fluoride toxicity have reported varied findings which might be due to differences in dose, duration and mode of fluoride administration, animal species used, and organ-specific metabolic responses. Though there is a consensus with respect to impaired liver function in endemic fluorosis, many hydrogeological and nutritional factors decide the extent to which liver function is impaired. There are contradictions regarding the mechanisms proposed for fluoride toxicity. Future studies need to explore the molecular mechanisms of fluoride toxicity and the beneficial effects of antidotes in amelioration of adverse effects of chronic fluoride intoxication. In India, fluorosis is highly prevalent and the clinicians need to consider soft tissue fluorosis as an important clinical entity to be examined while dealing with cases of skeletal fluorosis.

Highlights of the Article
- Fluoride has detrimental effects not only on the skeletal system but, also on the non-skeletal tissues such as liver
- Fluoride induces oxidative stress
- Fluoride inhibits many enzymes, and is a metabolic poison
- Soft tissue fluorosis could be an integral clinical component of endemic fluorosis

Competing Interests
The authors declare no conflicting interests

Authors Contributions
SSAR and SYM both have made substantial intellectual contributions to the paper. SSAR made the design of the review article and extensive literature survey. SYM made the initial draft and SSAR did the critical evaluation of the draft. Both the authors approved the final draft.

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