CHAPTER 11

Review of Literature
Fluorine, one of three halogens known to have a biological function, does not occur in the elemental state because it is highly reactive electronegative non-metal with an oxidation potential greater than ozone (Greenwood et al., 1994; Graw-Hill, 1992). This element was isolated in 1886 by Nobel Laureate Henri Moissan and it combines directly with most elements and indirectly with a few to form fluorides. Fluorides are ubiquitous in nature and are present in rocks, soil, water, plants, foods and even air. The geological survey of India reported that topaz, apatite, rock phosphate nodules and phosphorite are wide spread in the earth’s crust in India and contain high percentages of fluoride. As a result of rich mineral content and high rainfall, fluoride leaches out and contaminates the water and the soil affecting mainly teeth, bones and joints (WHO technical report series, 1994).

2.1 Sources of fluoride:

Water accounts for the major source of fluoride intake in an adult, because most foods are low in fluoride except for marine fish, clams, lobster, crab, shrimp, and some teas (Taves, 1983; Kumpulainen et al., 1977). Preparation of food in the home with fluoride-containing water also increases fluoride intake.

The major influence on total fluoride intake, however, is probably the fluoride content of drinking water. To calculate total fluoride intake, sources other than water, such as milk, infant formulas, beverages, food, and other fluoride products, must also be considered. For an adult male residing in fluoridated community, estimates of daily fluoride intake from food and beverages range from 1 to 3 mg/day. This range is reduced to ≤ 1.0 mg/day in a non-fluoridated area (Nopakun et al., 1990).

Most foods have fluoride concentrations less than 0.5 ppm (Taves, 1983). The recommended concentration range is 0.7-1.2 ppm, depending on the average regional temperature. The lower levels are recommended for warmer regions where water intake tends to be higher (Galagan et al., 1957). Beverages include soft drinks, fruit juices and drinks, tea, and gatorade as well as several others consumed less frequently. Beverages fluoride concentration reflects those in the water used for preparation. In general, they range from 0.1 to about 1.4 ppm (Clovis et al., 1988, Pang et al., 1992). Toothpastes, the most frequently used dental products; contain fluoride at 1000-1500 ppm either as sodium fluoride or disodium
monofluorophosphate (Whitford, 1994). The halo effect occurs when food and beverages are produced with fluoridated water and then shipped for consumption in communities that do not have fluoridated water. Of particular importance in this regard are soft drinks and juices (Whitford, 1994). There can be significant environmental pollution with fluoride that comes from unprotected mines, industrial emission, coal burning fertilizers and pesticides (W.H.O, 1994). Water fluoride level of <1 ppm is said to be in safe limits. Toxic levels of fluoride present in water sources in AP are indicated in Map1.

2.1.1 Recommended levels of water fluoride:

The recommended fluoride content in water is 1.0 ppm (WHO Technical Report Series, 1994). This varies with temperature as water requirement increases in hot climates. Intake of fluoride recommended for tropical and temperate climate is 0.5 and 1 ppm respectively.

2.1.2 Recommended levels of fluoride intake per day:

The fluoride contents from all the sources determine the human intake of fluoride. In the majority of endemic areas around the world, the main contribution is from water and significant amounts come from foods. Recommended levels of fluoride intake are presented in table: 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Recommended intake of Fluoride per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3 yrs</td>
<td>0.50-1.5 mg</td>
</tr>
<tr>
<td>3-5 yrs</td>
<td>Maximum of 2.5 mg</td>
</tr>
<tr>
<td>6-17 yrs</td>
<td>Maximum of 2.5 mg</td>
</tr>
<tr>
<td>Adults</td>
<td>1.5-4.0 mg</td>
</tr>
</tbody>
</table>

Map 1: Map showing toxic level of fluoride present in water sources in AP.
2.2 Fluoride Metabolism:

2.2.1 Absorption:

Ingested fluoride can be absorbed from both the stomach and intestinal mucosa. In the stomach, where low pH favors formation of highly diffusible hydrogen fluoride (pKa = 3.4), absorption is very rapid (Whitford et al., 1984). Conditions of high gastric acidity therefore favor fluoride absorption whereas alkalinity decreases fluoride absorption (Whitford, 1996). Fluoride absorption from the small intestine occurs by non-pH dependent diffusion (Nopakun et al., 1989). Under conditions where fluoride is ingested as apart of a total diet, most of the ingested fluoride is likely to be absorbed from the small intestine (Ophaug, 1990) that indicated in fig 1.

Flow chart 1: Metabolism of fluoride in human body

When fluoride is ingested in the fasting state, its absorption is 100%. The presence of food reduces efficiency of fluoride absorption to 50-80% (Parkins et al., 1966). The mechanism by which fluoride is absorbed is by passive diffusion by way of membrane channels (Parkins et al., 1966). The possibility that fluoride might be absorbed by an active process (Stookey et al., 1964) was strongly challenged by the observation that fluoride transfer across the intestinal membrane was unaffected by metabolic inhibitors such as sodium cyanide, sodium iodoacetate, and 2,4-dinitrophenol (Ekstrand, 1978).
2.2.2 Transport and Tissue Uptake:

At usual fluoride intake and rate of absorption, plasma fluoride concentration ranges from 10-20 μg/L or 0.5 -1.0 μM (Forbes, 1990). Ionic fluoride in the plasma is not protein-bound (Whitford, 1996).

Fluoride is rapidly removed from the plasma by mineralized tissue in exchange with other anions such as hydroxyl ion, citrate, and carbonate in contrast to soft tissue, which does not accumulated fluoride. In an adult, over 95% of total body fluoride is found in bones and teeth. Total body fluoride has been estimated to be about 2.6 grams, which is second only to the trace element iron (Nielsen, 1996).

The most efficient uptake of fluoride into bones and teeth occurs during the period of rapid development. Thus mature bone takes up fluoride considerably slower than newly forming bone. Higher concentrations of fluoride are also found in surface layers of mineral structure than in deeper layers, and fluoride released during bone remodeling is largely redeposited (Guo et al., 1988). Fluoride is deposited to a greater extent in trabecular bone compared to compact bone (Rao et al., 1995). For teeth, maximum fluoride deposition occurs during childhood (Maheswarai et al., 1981) and unlike bone it is not subject to resorption associated with the remodeling process. As in bone, the deposition of fluoride into teeth follows a gradient. Thus the outer layers of surface enamel may contain 3000μg F-/gm in contrast to 100μg F-/gm at the dentin-enamel junction (Maheswarai et al., 1981). The concentration of fluoride in the outer enamel layer can also be influenced post-eruptively by oral fluoride exposure by way of surface demineralization-remineralization events.

Neuman and Neuman (1958) proposed that the uptake of fluoride from the extracellular fluid by bone occurs by a three-stage process: (1) by ionic exchange in the hydration shell of the crystallite; (2) by exchange with an ion into vacant spaces deeper with the crystallite; and (3) by migration of surface ions into vacant spaces deeper within the crystallite (Neuman et al., 1958). The major variable, which affects the rate of fluoride uptake by bone, is age or the stage of skeletal development. The crystallites of younger bone are smaller, more numerous and loosely organized. They are heavily hydrated, and therefore, they offer a much larger surface area for the uptake of fluoride than does more mature bone. In the elderly, it
is likely that a net loss of fluoride from bone occurs due to the increased rate of bone resorption relative to that of bone accretion.

Substitution of the fluoride ion into the hydroxyapatite crystal structure for the hydroxyl and carbonate ions (fluorapatite) promotes crystal formation which is associated with increased apatite crystal size, reduced crystal distortion, and reduced solubility of the apatite crystal (Eanes, 1983).

2.2.3 Excretion:

Normally, about 90% of total the excretion occurs by way of the urine (Hodge et al., 1970). Loss of fluoride in perspiration is considered to be negligible except under unusual circumstances (Schiffl et al., 1982; Spac et al., 1985). Renal clearance of fluoride is linear with glomerular filtration rate and about 60% of filtered fluoride is reabsorbed (Spac et al., 1985; Ekstrand et al., 1978). The rate of fluoride clearance is lower in children than adults, which allows for greater fluoride retention at a time when bone and teeth are rapidly developing. Cessation of bone growth is associated with an increase in the amount of fluoride excreted in the urine.

Fluoride reabsorption has been shown to be inversely related to kidney tubular fluid pH (Taves, 1968). Acidosis would therefore increase fluoride retention by way of the formation of highly diffusible hydrogen fluoride, whereas alkalosis would increase urinary fluoride excretion by decreasing fluoride reabsorption. A urinary fluoride level > 0.1 ppm is said to be diagnostic of fluorosis. Fecal fluoride is usually considered to be that which was not absorbed.

2.3 Biochemical functions of fluoride:

2.3.1 Mineralized tissue:

The major crystalline salt of mature bone and teeth is predominately composed of calcium and phosphorus in the form of hydroxyapatite with the chemical formula \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). Addition forms of calcium and phosphorus, such as \( \text{CaHPO}_4\cdot2\text{H}_2\text{O} \) (calciumBiphosphate) and \( \text{Ca}_3(\text{PO}_4)_2\cdot3\text{H}_2\text{O} \) (Calcium Phosphate), represent a fraction of salts called the amorphous phase that can be readily mobilized (Eanes, 1983). In bone, the amorphous fraction may be as much as 20-30% of the total salts.
Substitution of the fluoride ion into the hydroxyapatite crystals in place of hydroxyl and carbonate ions (fluoroapatite) promotes crystal formation, which is associated with increased apatite crystal size, reduced crystal distortion, and reduced solubility (Okazaki, 1992). The unique function of fluoride is attributed to high degree of reactivity of fluoride ion coupled with its ionic radius (Greenwood et al., 1984).

The chemical structure of fluoroapatite $\text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 \cdot \text{F}_{2x}$ indicates that various degrees of fluoridation of the mineral matrix denoted by “x” (Simonen et al., 1985).

Fluoride distribution in bone and teeth is not homogeneous. The degree of fluoridation of the hydroxyapatite crystal is inversely related to the solubility of the crystal. The significance of the substitution of fluoride into the hydroxyapatite crystal is much more pronounced in teeth than in bone.

2.4 Biomarkers of fluoride exposure:

Knowledge of fluoride availability during pre-eruptive period of tooth formation allows assessment of the potential for later development of fluorosis. Knowledge of its availability post-eruptively, provides a guide to the potential level of protection from caries. Fluoride biomarkers may also serve to assess the impact of water fluoridation on bone quality and other physiological conditions (WHO technical report series, 1994).

2.4.1 Historic markers: Bone and Teeth:

The body’s burden of fluoride is best reflected in the calcified tissues, though enamel is not the tissue of choice because most of its fluoride was taken up during tooth formation. After tooth eruption, exposure to widely fluctuating concentrations of fluoride in the oral cavity significantly affects fluoride levels in the surface layers of enamel, where the highest concentrations of fluoride are found. Bone fluoride concentrations are much better indicators of fluoride exposure and body burden, though fluoride is not uniformly distributed throughout the bone. For example, cancellous bone has higher fluoride concentrations than does cortical bone (WHO technical report series, 1994).
2.4.2 Contemporary markers: Urine, Plasma, and Saliva:

The values of urinary, plasma and salivary fluoride obtained are not a direct measure of fluoride accumulation in the body, although they are indicative of the body burden because of an incompletely defined relationship between fluoride concentrations in bone and in the extracellular fluids. These fluids include urine, plasma and ductal saliva. Ductal salivary fluoride is related to the concentration in plasma by a factor of about 0.8. Samples taken from fasting subjects have most value because the fluoride concentrations in these two fluids are influenced significantly by intake during recent hours. Urinary fluoride excretions are also related to those of plasma, but they are more variable than those of ductal saliva because of variations in urinary flow and pH (WHO technical report series, 1994).

2.4.3 Recent markers: Nails and Hair:

The concentrations of fluoride in nails and hair appear to be proportional to intake over longer periods of time. Nails grow at about 0.1 mm/day so the average level of fluoride intake over a 1-3 week period can be estimated. Fluoride in hair could be used to estimate intake over longer periods (W.H.O technical report series, 1994).

2.5 Toxic effects of fluoride:

Invitro studies have shown that fluoride is essential to initiate the deposition of calcium phosphate in "matrix vesicles" and thus it facilitates the nucleation process prior to bone mineralization. Fluoride plays an essential role in the formation of enamel in teeth. Some amount of fluoride is considered to be essential for the metabolism of bone and teeth: large doses of fluoride are known to be toxic. Toxic effects are of two types (Anasuya Das, 1998).

1. The acute fluoride intoxication and
2. The chronic fluoride intoxication

2.5.1 Acute Toxicity: This is a result of a single massive dose of fluoride. The signs and symptoms of acute fluoride toxicity are nausea, vomiting, diarrhea, abdominal pain, excessive salivation and lacrimation, pulmonary disturbances,
cardiac insufficiency and weakness, convulsions, sensory disturbances, paralysis and coma (Kathy, R).

2.5.2 Chronic Toxicity: The only known adverse effect associated with the chronic ingestion of relatively low levels of fluoride (1-2 mg/L in the drinking water) is dental fluorosis, which is hyper mineralization of enamel resulting from excess fluoride reaching teeth during the developmental stages. Chronic ingestion of relatively high levels of fluoride can cause skeletal fluorosis.

The following are the factors, which could influence the severity of fluorosis (Teotia et al., 1994). 1) Fluoride concentration in the drinking water; 2) daily intake of fluoride; 3) duration of fluoride exposure; 4) continuity of residence in the endemic area; 5) fluctuations in the fluoride intake; 6) age at the time of fluoride ingestion; 7) nutritional status, particularly the dietary intakes of calcium and vitamin D; 8) physical hard work in a hot environment.

Fluorosis is of two types:
1. Industrial fluorosis
2. Endemic fluorosis

1. Industrial fluorosis:

It is observed in factory workers chronically exposed to fluoride gas emanating from industries based on fluorine containing ores. Eg. cryolite industries. Chronic exposure to fluoride in aluminum works may cause industrial fluorosis, which is characterized by increased mineral content of the bone tissue. Decreased bone mineral density was found in aluminum workers compared to the control group particularly in age group of 40-44 and 50-54 years (Czerwinski et al., 1977).

2. Endemic fluorosis:

It is a result of consumption of water and foods containing high concentrations of naturally occurring fluoride over prolonged periods. In both these types of fluorosis, dental and skeletal tissues are the primary targets and show most significant changes. The most prominent features of endemic fluorosis are dental and skeletal abnormalities, widely known as dental fluorosis and skeletal fluorosis respectively.
2.6 Biomarkers of fluoride exposure:

Epidemiological studies by Dean and colleagues in the 1930s clearly demonstrated the relationship between dental fluorosis in humans and the level of fluoride in water supplies (Dean, 1934). These and other studies have shown that in a population there is a direct relationship between the degree of fluorosis and the plasma and bone fluoride levels on the one hand, and the concentration of fluoride in drinking water on the other hand. These studies suggested that fluorosis could be used as a biomarker for the level of fluoride exposure though dental fluorosis is a reflection of fluoride exposure only during the time of enamel formation (W.H.O 1994).

2.6.1 Dental fluorosis:

Dental fluorosis is an aesthetic and social problem besides being a health problem. Teeth exhibit the first sign of chronic fluoride toxicity. Ameloblast (the enamel forming cells) is the most sensitive cell to fluoride toxicity. Enamel exhibits the first clinical changes in chronic fluoride toxicity. These dental changes help in the diagnosis of fluorosis. The characteristic feature of dental fluorosis is dental mottling. In this condition, the structure of the enamel is affected during formative stages resulting in hypoplasia known as mottled enamel. Minute abnormal white flecks, yellow flecks characterize the mottling or brown spots or striations scattered irregularly over the tooth surface (fig 1). The discoloration may be in spots; streaks are invariably horizontal in orientation, as during development new layers of the matrix are added horizontally.

The outermost covering of the tooth is the hard structure in the body, with inorganic compounds, mainly with calcium salts. Enamel protects the tooth besides giving colour and luster. Normally healthy dental enamel is semi-transparent, smooth and milky white in appearance. However, appearance of white opaque patches on the enamel may be indications of the initial phases of dental fluorosis. In extreme cases of fluorosis, not only does the entire dental enamel turn opaque white to brown, but also the teeth tend to break off easily and even their shape may begin to be affected.
In dental fluorosis the discoloration will be away from the gums and on the enamel surfaces and it can never be removed, as it is an integral part of tooth matrix. Calcium rich constituents of teeth, viz., enamel and dentin have strong affinity for fluoride during the formation of teeth. Fluoride combines with calcium during the mineralisation of teeth forming calcium fluoroapatite crystals. Enamel matrix is laid down in incremental lines before and after birth. Hence, dental fluorosis is invariably seen as horizontal lines or bands on the surface of teeth and never as vertical bands. It may also appear as spots.

Teeth commonly affected are 1) central incisors 2) lateral incisors and 3) molars of the permanent dentition. Fluorosis affects both the inner and outer surfaces of teeth. Teeth affected by fluorosis being poorly calcified (hypomineralized) loose enamel under the normal masticatory stress. Enamel has no regenerative capacity. Once it is lost, it is lost forever. The dentin is then exposed. Cavities formed in dentin spread much faster and involves the pulp easily, leading to loss of teeth. The teeth once affected by dental fluorosis cannot be reverted to
normal. But the disclosed teeth can be masked by bleaching and or by other methods.

Based on the severity of changes, attempts have been made to classify the degree of dental fluorosis into different grades (fig.2)

2.6.1.1 Classification of dental fluorosis (WHO, 1997):

0 Normal. The enamel surface is smooth, glossy and usually a pale creamy-white colour

1 Questionable. The enamel shows slight aberrations from the translucency of normal enamel, which may range from a few white flecks to occasional spots.

2 Very mildly. Small, opaque of the enamel areas scattered irregularly over the tooth but involving less than 25% of the label tooth surface.

3 Mild. The white opaque of the enamel of the teeth is more extensive than for code 2, but covers less than 50% of the tooth surface.

4 Moderate. The enamel surfaces of the teeth show marked wear and brown stain is frequently a disfiguring feature.

5 Severe. The enamel surfaces are badly affected and hypoplasia is so marked that the general form of the tooth may be affected. There are pitted or worn areas and brown stains are wide spread; the teeth often have a corroded appearance.

6 Excluded (e.g. a crowned tooth).

7 Not recorded.
Fig 2: Pictures of different grades of dental fluorosis
2.6.2 Skeletal Fluorosis:

This is associated with a daily intake of fluoride > 1 ppm over prolonged periods. Endemic skeletal fluorosis was first reported from the Nellore district (which was then under Madras presidency) of the southern part of India in 1937. It is a para metabolic bone disease. Fluoride is a cumulative toxin, which can alter accretion and resorption of bone tissues. It also affects homeostasis of bone mineral metabolism. The total quantity of ingested fluoride is the single most important factor, which determines the clinical course of the disease.

Skeletal fluorosis is not easily recognizable until the disease has developed to an advanced stage. Excessive quantities of fluoride when deposited in the skeleton are more in cancellous bone compared to cortical bone. Changes in the bone will then be revealed through radiographs. Maximum ill effects of fluoride are detected in the neck, spine, knee, pelvic and shoulder joints. It also affects joints of the hands and feet. The usual complaints of the patients, viz., pain in the neck, back, joints and rigidity begin in regions where cancellous bones predominate. With increased severity of skeletal fluorosis, pain is associated with rigidity and restricted movement of cervical and lumber spine, knee and pelvic joints as well as shoulder joints.

The various skeletal changes in endemic fluorosis are best described as follows:

2.6.2.1 Gross changes in the skeleton:

Skeletal changes involving overall increase in bone mass, 2-3 times the normal is a characteristic feature of fluorosis (Weatherell and Weidmann 1959; Singh et al 1962; Reddy et al 1969). The changes will be first noticed in the vertebral column and pelvis and thereafter in the rib cage and limb bones. The bones become whitish and occasionally mottled like the teeth.

A clear indication of chronic fluorosis is the calcification and ossification of ligaments and interosseous fasciae occurring along with periosteal new bone formation and development of exostoses on long bones and osteophytes in the spine. It is in the muscular attachments and tendinous insertions that new bone formation occurs, as a result of which there is a thickening of the cortex and narrowing of the medullary cavity. The effect on the vertebral column is seen in roughening of pedicles, laminae, spinous and transverse processes. The osteophytes projecting into the spinal canal and intervertebral foraminae may press upon the cord and spinal
roots and thus account for the radiculomyelopathic features in chronic fluorosis. The spine is converted into a single rigid bone as a result of ossification of spinal ligaments and fusion of the adjacent bony structures. The bones of pelvis exhibit changes essentially similar to those found in the spine. The skull is rarely involved, although there may be thickening of the calvaria and a roughening of the outlines of the foramen magnum.

2.6.2.2 Histopathology:

Among the effects of high fluoride ingestion may be the calcification of tendons, ligaments and occasionally, muscles as well as the stimulation of osteoblastic activity. Some bones are more prone to exostosis formation than the long bones.

The extensive production of new bone in the fluorotic skeleton is usually accompanied by increased bone resorption.

Skeletal fluorosis has been linked to a number of bone diseases; the dense radiographic picture of the skeleton has resulted in comparison with osteosclerosis; the presence of broad osteoid seams has suggested osteomalacia; the way in which the bone formation may proceed side by side with bone destruction is reminiscent of Paget's disease; and the often extensive resorption points to osteoporosis. Certainly, fluorotic bones can exhibit signs common to each of these conditions, but a unique distinction is the presence of high levels of fluoride in the bone (Jolly et al., 1969).

Histopathological changes of endemic fluorosis occur only at higher levels of intake than 1-4 ppm.

2.6.2.2.i Chemical composition of fluorotic bones: The deposition of fluoride in bone takes place mainly by two mechanisms. In the first, fluoride exchanges with the hydroxyl ion on the surface of existing crystals. In the second, new bone is formed by osteoblastic and osteoclastic activity. It is probable that there are initially changes in the chemical composition and deposition of bone salts in the organic matrix, possibly mediated by altered enzyme reactions.

2.6.2.2.ii Deformities and crippling fluorosis: This advanced stage of fluoride intoxication results from the continuous exposure of an individual to 20-80 mg fluoride ion daily over a period of 10-20 years. Such heavy exposure is associated with a level of at least 10ppm in the drinking water supply. The commonest deformities are kyphosis, flexion deformity of the hips, flexion deformity of the
knees and the fixation of the chest in the position of inspiration due to calcification of cartilage. The advanced picture of crippling fluorosis is strikingly uniform. The quadriplegic patient bent with kyphosis and with markedly restricted movements of his spine, with contractures of hips and knees, provides a grim picture of the result of excessive fluoride intake (Jolly et al., 1969).

2.6.2.3 Clinical features:

Stage 1: This is a relatively asymptomatic stage. It usually encountered in young adults whose only complaints are vague pain in the small joints of hands and feet, knee and spinal joints.

Stage 2: In this stage, obvious stiffness of the spine with limitations in movement is observed.

Stage 3: By this stage the development of kyphosis is obvious. The subjects experience difficulty in walking and suffer from the following problems. Stiffness and limitation of the movements of various joints especially inability to bend downwards, backwards and neck rigidity. This loss of movements is because of the calcification of intervertebral ligaments, which renders the vertebral column rigid. This abnormal calcification also results in narrowing of intervertebral foramen and spinal canal and compression of vertebral disc (Anasuya Das, 1998). Manifestation of fluoride toxicity in skeletal fluorosis is presented in fig 3 (Susheela et al., 1993).

![Diagram showing stages of skeletal fluorosis]

**Normal healthy individual**
A. Can bend body and touch the floor/feet
C. Can touch chest with chin
E. Can Stretch hands, fold arms and touch Back of head

**Fluoride toxicity manifestation**
B. Unable to bend without folding knees
D. Unable to bend neck-touching chest with chin not possible
F. Unable to stretch hands fold arms and touch back of head
The stage at which skeletal fluorosis becomes crippling usually occurs between 30 and 50 years of age in the endemic regions. Newcomers to a hyperendemic region may sometimes develop symptoms of skeletal involvement within 4 years of their arrival (Siddiqui, 1955). Men suffer more than women from severe effects of the disease presumably because their work is usually more strenuous than that of women (Siddiqui, 1955; Jolly et al., 1968). The factors which govern the development of skeletal fluorosis are (a) the prevalence of high levels of fluoride intake, (b) continual exposure to fluoride, (c) strenuous manual labour, (d) poor nutrition and (e) impaired renal function due to disease (Pandit et al., 1940; Daver, 1945). In regions with very high fluoride content the disease may affect younger age groups including children. The longer the exposure to fluoride the higher will be its incidence. Epidemiological observations revealed that nutritional status might influence chronic fluoride toxicity. The incident of fluorosis is higher in tropical and subtropical countries probably on account of higher drinking water consumption. It is observed that in adults, exposed to high fluoride ingestion, the hydroxyl bonds of the hydroxyapatite material in bone are partly replaced by fluoride. It is surmised that in order to immobilize fluorine from the circulating fluoride phase in the body (blood and cellular fluids), the body’s defense mechanism fixes excess fluorides into hydroxyapatite material of the bone by replacement of OH- by F- (Teotia and Teotia, 1992) irreversibly as long as exposure persists. In the process, the rate of synthesis of bone formation or osteosclerosis, a basic symptom of subjects suffering from skeletal fluorosis.
Fig 4: Pictures of different grades of skeletal fluorosis
2.6.2.5 Radiological Features:

The most significant effect of chronic fluoride ingestion is seen in the skeletal system. Radiology offers the best method of diagnostic confirmation of the condition. Commensurate with structural change, there is functional change in fluorotic bone. Abnormal bone also has abnormal density.

Radiological features in fluorosis include four types of changes I) osteosclerosis ii) osteoporosis, iii) osteomalacia and iv) features suggestive of hyperparathyroidism. These changes are seen in varying combinations in different regions of the skeletal system. There is inter-individual variation in the severity and extent of these changes. The single most important indicator of osteosclerosis is calcification of interosseous membrane “of the forearm (Krishnamachari, 1986). Shortt et al (1937) in their description of the disease mentioned that all patients of fluorosis have evidence of osteosclerosis, inner osseous membrane calcification and calcification of muscular attachments.

In patients of endemic genu valgum, Krishnamachari et al described the occurrence, in addition to osteosclerosis, of lines of growth arrest, subperiosteal resorption of phalanges, cystic expansion of short long bones and expansion of lower ends of long bones (Krishnamachari, 1973). Teotia et al (1976), described that in addition to the features of osteosclerosis mentioned above which occur in 55 percent of cases, unusual radiological features are also seen. These features include thinning of the cortex, sub-periosteal phalangeal resorption, dense and white epiphyses and metaphyses, alveolar resorption and lines of growth arrest. Simultaneous occurrence of osteosclerosis and osteomalacia is common in fluorosis patients of the tropical countries. Osteosclerosis is the predominant lesion in fluorosis patients who have adequate dietary intake of calcium while osteomalacia picture predominates in patients who, in addition to being exposed to fluoride, are also on marginal or sub- optimal dietary intake of calcium.

2.6.2.6 Bone Mineral Density:

The density of new bone is increased. This property may be helpful in screening subjects from endemic fluorotic areas, for detecting early signs of skeletal fluorosis. However, since this bone also exhibits areas of irregular mineralisation of
the bone matrix, density measurements alone may yield unreliable information. Higher bone mineral density level was seen in early postmenopausal women with endemic skeletal fluorosis. BMD measurement is a tool in the diagnosis and management of fluorosis (Yildiz et al., 2003). Postmenopausal BMD values in both endemic fluorosis and control were significantly less than premenopausal BMD values. Although the differences were less prominent in women with endemic fluorosis, menopause is still the major determinant of BMD in the spine and femur (Yildiz et al 2003).

2.6.3 Genu valgum:

A variant of the severe form of skeletal fluorosis, called “Genu valgum” (or knock-knee syndrome), has been reported from various parts of the world. Young and adolescent people are affected with this syndrome with greater prevalence among boys. These subjects exhibit not only signs of dental and skeletal fluorosis, but also extensive osteoporosis of the limb bones. The syndrome was observed among people whose staple diet was sorghum (Jowar). Low calcium status predisposes to the condition (Krishnamachari et al., 1976).

2.7 Fluoride effects on cells involved in bone metabolism:

Bone is a metabolically an active tissue that undergoes continuous remodeling by two counteracting processes, namely bone formation and bone resorption (Markus J Siebel, 2005). Two key cell types are responsible for bone formation and bone resorption, the osteoblast and osteoclast, respectively. Osteoprogenitor cells give rise to osteoblasts. Osteoprogenitor cells are a self-renewing population of cells that are committed to the osteoblast lineage. They originate from mesenchymal stem cells. Osteoblasts contain a single nucleus, line bone surfaces, possess active secretory machinery for matrix proteins, and produce very large amounts of type I collagen. Because they also produce and respond to factors that control bone formation as well as bone resorption, they play a critical role in the regulating skeletal mass. Osteoclasts are giant, multinucleated phagocyte cells that have the capability to erode mineralized bone matrix. They are derived from cells in the monocyte/macrophage lineage. Their characteristic ultrastructural
features allow them to resorb bone efficiently by creating an extracellular lysosome where proteolytic enzymes, reactive oxygen species, and large numbers of protons are secreted. Osteoclastogenesis is controlled by local (Cytokines, growth factors) as well as systemic regulators (e.g., PTH, Vitamin D and other steroid hormones).

2.7.1 Fluoride effects on Osteoblasts:

The single clearest effect of fluoride on the skeleton is its stimulation of osteoblast proliferation.

The mechanism, by which fluoride increases osteoblast number, has been reviewed by Libanati et al (1996). Farely et al (1983) found that a fluoride concentration of only 10 µM could increase osteoblast proliferation and alkaline phosphatase activity. Because this fluoride concentration exceeds usual serum fluoride concentration at nutritional levels of fluoride intake, it is not possible to say with certainty that this mitogenic effect of fluoride is significant to every day changes in cell activity.

A scheme to explain what is presently known about the mitogenic effect of fluoride is binding of a growth factor to a cell surface receptor which activates tyrosyl protein kinase activity, which in turn activates a series of phosphorylation reactions leading to a stimulation of DNA synthesis and osteoblast proliferation. Fluoride stimulates phosphorylation by either an inhibition of phosphotyrosyl protein phosphatases or by increasing the activity of phosphotyrosyl kinase or by a combination of both processes. Observations that support this proposed mechanism include: 1) the mitogenic effect of fluoride on cells of skeletal origin requires a bone cell growth promoter such as the insulin-like growth factor I (IGF-1) (Farley et al., 1996); 2) osteoblastic phosphotyrosyl protein phosphatase (PTPP) is inhibited by fluoride in the micromolar range (Lau et al., 1989) which would prevent dephosphorylation of mitogenic signalling proteins; 3) tyrosyl phosphorylation of signalling proteins such as MAP kinase (mitogen-activated protein) is required to control cell proliferation (Pouyssegur et al., 1992); 4) phosphotyrosyl protein phosphatases are cellular regulators of tyrosyl phosphorylation (Gomez, 1991), and
5) tyrosine kinase activity of osteoblast-like cells has been reported to be stimulated by fluoride (Burgener et al., 1995).

Fig 5. A Mechanism for the Mitogenic Effect of Fluoride on Bone cells

Most reports document increased osteoblast number; however, some investigators have documented a complex and paradoxical effect of fluoride in patients with skeletal fluorosis. Boivin et al. (1989) reported that, in biopsy of bone cores taken from 29 patients with skeletal fluorosis, there is an apparent increase in the production of osteoblasts with a concomitant increase in a toxic effect of fluoride at the cell level. They provided data to indicate that chronic exposure to fluoride in both endemic and industrially exposed subjects led to an increase in bone volume, an increase in cortical width, and an increase in porosity. However, there was no reduction in cortical bone mass. Osteoid parameters (unmineralized type-1 collagen) were also significantly increased in fluorotic patients. Interestingly, the fluorotic group had more osteoblasts than the control group, with a very high proportion of quiescent, flattened osteoblasts, but the mineral apposition rate was significantly decreased. It appeared as though the increased numbers of quiescent, cells were in a prolonged inactive period. Thus, the conclusion drawn by these investigators was that fluoride exposure increased the birth rate of new osteoblasts, but at high
concentrations there was an independent toxic effect on the cells that blocked the full manifestation for the increase in skeletal mass. The addition of these effects represents, however, a marked increase of bone formation at the organ level (Bovin et al., 1989).

2.7.2 Fluoride effects on Osteoclasts:

The effects of fluoride on osteoclast activity, and by extension the rate of bone resorption, are less well defined than its effects on osteoblasts. Most reports studying the effect of fluoride on osteoclast function indicate an inhibition. In fact, the effect might be mediated through G-protein-coupled pathways as in the osteoblasts. Moonga et al. (1993) showed that fluoride, in the form of AlF₄⁻ resulted in a marked concentration-dependent inhibition of bone resorption. In association with this inhibition, they found a marked increase in the secretion of tartrate-resistant acid phosphatase (TRAP). TRAP presumably originated from the osteoclast; however, its function as a secreted enzyme is not known.

The effects of fluoride on bone resorption and osteoclast function in vivo present a complex picture. Some well-controlled animal studies document a decrease in osteoclast (as well as odontoclast) activity. In these studies, rodents and rabbits were exposed to doses of fluoride ranging from clinically relevant to high. Time courses ranged from days to weeks, and the findings indicated a statistically significant decrease in the number and activity of resorbing cells (Faccini, 1967; Lindskog et al., 1989; Kameyama et al., 1994). Other studies documented little or no statistically significant effect of fluoride on osteoclast activity (Marie and Hott, 1986; Huang, 1987). Yet other work that utilized skeletal turnover and remodeling showed an increase in resorption after fluoride therapy (Kragstrup et al., 1984; Snow and Anderson, 1986). These studies based their conclusions on the initiation of basic multicellular units (BMUs) and extent of remodeling surface. In the field of skeletal research, it has been accepted that adult bone remodels itself through the generation of BMUs. This unit is a temporal description of remodeling starting with osteoclastic bone resorption and progressing through a coupled stimulation of bone formation. All BMU activity, thus, is initiated with the action of an osteoclast. An increase in remodeling surface also implies an increase in BMUs. Snow and Anderson (1986) and Kragstrup et al. (1984) demonstrated an increase in resorption
under the influence of fluoride by measuring BMU numbers and remodeling surface, respectively. Because these data were derived from intact in vivo animal models, the investigators could not conclude that the effects of fluoride on osteoclastic bone resorption were direct. Unfortunately, there were not enough histological or biochemical marker data in this report to determine whether the fluoride effect was direct or indirect.

2.8 Biochemical changes in fluorosis:

2.8.1 Blood fluoride:

Fluoride is rapidly absorbed from stomach and the proximal part of intestine and is taken by blood to various tissues in a short time following ingestion. Ionic fluoride is measured in biological fluids using fluoride ion-specific electrode. In normal persons only trace amounts of ionic fluoride are detected. In established cases of skeletal fluorosis, ionic fluoride in serum had been shown to be significantly elevated. Serum fluoride does not always indicate the chronic nature of fluoride toxicity (Anasuya Das, 1998). The plasma fluoride accounts for three-fourths of the total amounts of fluoride found in whole blood; cells account for the rest. Fluoride in plasma exists in free ionic and bound forms, the latter bound to the serum albumin forming about 85% of the total amount of fluoride in plasma (Taves, 1968). Plasma fluoride in normal individuals in non-fluoridated areas ranges from 0.14-0.19 ppm and is higher in fluorotic patients (Singer and Armstrong, 1969). However, on abnormally high intake of fluoride through water and foods, the regulatory mechanism may be disturbed leading to elevated levels of fluoride in plasma (Anasuya Das, 1998).

2.8.2 Serum Calcium, Phosphorus and Alkaline Phosphatase:

In skeletal fluorosis, metabolism of bone related minerals are altered to a varied extent. These metabolic alterations are reflected to some extent in serum levels of the minerals concerned. Fluoride ion replaces hydroxyl ions of hydroxyl apatite in the bone to form fluoroapatite. It is a large crystal which being relatively more stable may not allow an easy exchange of calcium between the bone and blood. Such an abnormality may lead to decrease in serum calcium levels (Havivi et al., 1966; Zipkin et al., 1963). Serum alkaline phosphatase (ALP) activity is an indicator of biochemical abnormality of bone metabolism in fluorosis. Significantly
elevated levels (2-5 folds) of ALP are seen in fluorosis. This is related to degree of bone mineralization (Anasuya Pas, 1998). Alkaline phosphatase is often found to be elevated in fluorotic cases, which may be due to increased turnover rather than any specific effect of fluoride on the enzyme (Rosenquist 1974). A significant increase in serum ALP activity has been considered as an indicator of biochemical abnormality of bone mineral homeostasis in fluorosis. Studies reported that fluoride is a potent enzyme poison. It injures all the cells in bone (osteoblast and osteocytes). The cell initiates a repair response and results in increased serum alkaline phosphatase (SAP) production in both cell populations. The repair response in osteoblasts results in increased proliferation, matrix production and SAP production. When the repair process in osteoblasts fails, the osteoblasts undergoes either apoptosis or necrosis and is replaced by proliferation of osteoprogenitor cells. These new osteoblasts will be injured in turn, and increased repair and cell death would be repeated. This activation of a repair response in osteoblasts would contribute to increase SAP (Farely JR et al., 1983; Marie PJ et al; 1986; Tomkinson et al; 1997; Noble BS et al., 1997).

2.8.3 Hormonal Changes:

Abnormal calcification of dental and skeletal tissues, as well as ligaments is a basic feature of fluorosis. Therefore, efforts have been made to study the circulating levels of hormones, which are directly or indirectly involved in calcium and bone metabolism, namely vitamin D, calcitonin, parathyroid and thyroid hormones (Anasuya 1998).

2.8.3.1 Vitamin D:

Vitamin D is primarily responsible for maintaining blood calcium and phosphorus levels, and assisting in calcium absorption, which builds and maintains strong bones. It is necessary for the calcification of bone. In the absence of vitamin D, Ca absorption from the gut is diminished. Cartilage cells of matrix and the osteoid matrix are not calcified when fluoride intoxication occurs in a vitamin D deficient individuals the clinical expression of the disease varies. Defective ossification causes typical deformities in bone such as knock knees, bowing of legs, spinal curvature and also malformations of the chest and pelvis (Chatterjee, 1985). Vitamin D status of individuals modifies the expression of skeletal fluorosis.
Circulating levels of vitamin D metabolites 25 (OH) D3 have been reported to be normal in fluorosis (Teotia et al., 1978).

2.8.3.2 Calcitonin:

Calcitonin acts to lower blood calcium and phosphate concentrations, primarily or exclusively by inhibiting osteoclastic (bone resorption) activity. Calcitonin does not play a major role in calcium homeostasis in humans, and its primary importance seems to be to protect against excessive bone resorption (Bringhurst et al., 2002; Goodman, 2003). At high concentrations, calcitonin can also increase urinary excretion of calcium and phosphate, but these effects in humans are small and not physiologically important for lowering blood calcium (Goodman, 2003). Some investigators reported increased levels of calcitonin (Teotia et al., 1978) in the plasma of fluorotic subjects, while some others have found its concentration below the measurable range (Krishnamachari et al., 1976).

2.8.3.3 Parathyroid hormone:

The primary effect of parathyroid hormone is to increase calcium concentration and decrease phosphate concentration in blood (Bringhurst et al., 2002; Goodman, 2003). The major mechanisms by which this effect occurs include the mobilization of calcium phosphate from the bone matrix, primarily from increased osteoclastic activity; in the kidney, increased reabsorption of calcium, decreased reabsorption of phosphate, and increased activation of vitamin D; and increased intestinal absorption of calcium (Bringhurst et al., 2002; Goodman, 2003). Parathyroid hormone is also important for skeletal homeostasis (bone remodeling). Regulation of parathyroid hormone secretion is inversely related to the concentration of ionized calcium (Bringhurst et al., 2002; Goodman, 2003).

Increased levels of parathyroid hormone are seen in fluorosis. This increase in PTH correlated well with excess fluoride ingestion, which may be responsible for maintaining serum calcium levels and may have a role in toxic manifestations of fluorosis (Gupta et al., 2001).
Fluoride ingestion → Increased blood fluoride → Sequestration of calcium → Lowering of serum calcium (hypocalcemia) → Increased PTH (Sec hyper parathyroidism) → Increase in immature osteoblast → Bone resorption → Failure of mineralization → Decreased bone calcification → Restoration of serum calcium

Flow chart 2: Showing a possible mechanism of secondary hyperparathyroidism due to high fluoride ingestion (Ref: Gupta et al., 2001).

2.8.3.4 Thyroid hormones:

Thyroid hormones are essential for normal bone growth, but in excess can lead to bone fractures (Ammann et al., 1998). Production of thyroid hormones is regulated by a negative feedback mechanism, i.e., when the pituitary gland senses a drop in FT3 levels in circulation, it releases more TSH to stimulate the thyroid gland which in turn accelerates the production of the thyroid hormone T4, now considered a "pro-hormone". The major source of circulating T3 is from peripheral deiodination of T4 and not from thyroid secretion (Visser, 2004). Osteoblasts mediate the thyroid hormone stimulation of osteoclastic resorption. However, a wider range of thyroid hormone concentrations enhance the production of cytokines that promotes the formation of osteoclasts (Ammann et al., 1998). Barat (1998) reported normal levels of thyroid stimulating hormone (TSH) and triiodothyronine (T3) but elevated thyroxine (T4) in fluorotic subjects.

2.8.4 Urinary fluoride:

Fluoride is eliminated almost exclusively via the renal route. A healthy kidney is essential for body's handling of fluoride. In a normal person not more than 0.1 to 0.5 mg of fluoride is excreted in 24 hours time. Fluoride excretion depends on
several factors important of which are (1) total daily consumption of the element, (2) degree of renal efficiency (3) age (4) physiological state, (5) interaction of fluoride with other factors such as binders. Hodge et al (1970) in their review considered that urinary fluoride is the best indicator of intake level of the element. Many investigators observed a good correlation between intake and urinary loss of fluoride. In endemic fluorosis, urinary fluoride varies between 1.2 and 10.0 mg per 24 hours.

2.8.5 Bone Metabolism:

Bone is the target organ in fluoride intoxication. The structure of bone makes it possible for this organ to actively interact with fluoride ion. New bone formation with irregular mineralization has been the hallmark of fluorosis. Teotia et al (1976) observed irregular distribution of osteoid tissue among calcified trabeculae of the bone. It has been generally believed that several factors influence the type of bone changes seen in fluoride intoxication. The nature of fluoride exposure, nutritional status, hormonal responses, age and perhaps the sex of the person in human instance, the type of bone, the dose and duration of fluoride exposure, dietary habits in man are some of the variables which influence the outcome.

Fluorotic bone shows increased density. This is due to an increased deposition of bone mass per unit volume of the bone. Information quoted in literature tends to suggest that in spite of the increased thickness of the bone in this condition; physical attributes of the bone in terms of its capacity to withstand strain are not satisfactory. A tool to predict an early stage of skeletal fluorosis is to measure bone density. Analysis of bone biopsy samples for fluoride is a good laboratory procedure to confirm fluoride intoxication in man either due to hydric fluorosis or due to industrial exposure. Fluoride content of bone has been shown to correlate well with intake.

Fluoride ion has great affinity for calcium ion. Biological interactions between these two elements are important factors in determining the clinical course and metabolic course, which the disease is likely to follow. Their interactions are influenced by several factors including the relative intakes of the two elements, hormonal levels and urinary loss of the minerals. Calcium-Fluoride interactions can be studied in two ways i) metabolic balance studies involving cold calcium and ii) Calcium turnover studies using radioactive calcium. Srikantia and Siddiqui (1965)
carried out cold calcium balance studies in six adult patients with proven skeletal lesions due to fluorosis and in four matched controls. They observed a significantly higher retention of calcium in fluorosis patients. Narasingarao et al (1968) confirmed the earlier observations cited above.

Calcium turnover studies in experimental fluorosis by Srrangareddy and Narasinga Rao (1971) observed high fluoride when fed on a low calcium diet caused increased retention of calcium. Srikanta and Siddiqui also reported that patients suffering from fluorosis retained significantly higher amounts of calcium than do normal subjects.

Griffiths et al (1975) demonstrated that dietary calcium plays an important role in determining the type of bone lesions, which would emerge during the process of experimental induction of fluorosis in monkeys. Low calcium diet predisposed to the development of osteomalacia in fluoride toxicity. Increased metabolic turnover of the bone, impaired bone collagen synthesis and increased avidity for calcium are features in fluoride toxicity.

The basic effect of excess fluoride on bone is the causation of a high bone turnover state, which can also be induced to a milder extent by low calcium diet itself. Therefore, the formation of high bone turnover state is the pathogenic basis for low dietary calcium intake to exacerbate the severity of skeletal fluorosis (Li et al., 1997). Fluoride toxicity also exaggerates the metabolic effects of calcium deficiency on bone (Teotia et al., 1997). Increased parathyroid hormone levels are responsible for maintaining serum calcium levels in fluorosis (Gupta et al., 2001).

2.8.6 Collagen metabolism:

Fluoride is a toxic element, which is known to interfere with metabolism of many tissues, bone being the major target tissue. Secondary hyperparathyroidism, which occurs in fluorosis, causes breakdown of bone collagen to varying extent. Hydroxyproline, is produced in large amounts whenever there is collagen breakdown. Normally, hydroxyproline is excreted in urine in amounts not exceeding 30 mg per day. Urinary hydroxyproline is better correlated to bone resorption (Weiss et al., 1969). In osteosclerotic type of skeletal fluorosis characterized by increased new bone formation, urinary total hydroxyproline is not altered (Anasuya et al., 1974; Rao et al., 1978). In contrast, in endemic genu valgum, a manifestation of fluoride toxicity associated with secondary hyperparathyroidism, total
hydroxyproline in urine is significantly increased (Krishnamachari et al., 1976). It was hypothesized that in skeletal fluorosis, bone collagen breakdown products are reutilized for the purpose of new bone formation (Anasuya et al., 1974). Increase in urinary hydroxyproline in osteoporotic form of fluorosis is perhaps due to increased rate of bone resorption that occurs in this clinical condition, in addition to increased bone formation rate (Anasuya et al., 1974).

2.9 Biochemical markers of bone metabolism:

The structure, cyclical metabolism and hormonal regulation of bone are reflected by markers of formation, resorption and/or turnover. Bone turnover can theoretically be assessed by comparing the amount of substances that are released during resorption with the amount of substances associated with formation. Biochemical markers of bone formation and resorption provide a new and potentially important clinical tool for the assessment and monitoring of bone metabolism (Christenson, 1997).

2.9.1 Bone formation markers: - Bone formation markers indicate osteoblast activity; bone specific alkaline phosphatase and the N-terminal and C-terminal extension peptides of procollagen reflect formation of organic matrix in bone.

A number of biochemical markers of bone turnover/metabolism which reflect the activity of osteoblasts (bone formation) and osteoclasts (bone resorption) (Siebel, 2005) have been described in the table 2 below.

| Table 2: Biochemical markers of Bone formation and Bone resorption |
|--------------------------|--------------------------|
| **Bone Formation Markers- serum** | **Bone Resorption Markers - Plasma/Serum** |
| Osteocalcin (Bone Gla-Protein) | Tartrate-resistant acid phosphatase |
| Total and bone specific alkaline phosphatase. | Free pyridinoline and deoxypyridinoline |
| Procollagen I carboxy (PICP) and N-terminal (PINP) extension peptides | Type I collagen N and C-telopeptide break down products |
| | Urine |
| | Pyridinoline and deoxypyridinoline |
| | Type I collagen N and C-telopeptide break breakdown |
| | Hydroxyproline |
| | Hydroxylysine glycosides |
2.9.1.1 Alkaline phosphatase (ALP):

Alkaline phosphatase is a glycoprotein dimer of molecular weight 140,000 daltons. Osteoblasts are rich in alkaline phosphatase. Alkaline phosphatase is also found in liver, placenta, kidney and intestine, all of which may contribute to the total amount of ALP found in blood. The liver, renal and bone specific isoenzymes are coded by the same gene; therefore, differences between these isoenzymes are due to post translational modification. The precise function of the enzyme is yet unknown, but it obviously plays an important role in the mineralization of newly formed bone (Siebel, 2005). Increased levels of this enzyme have been found in fluorotic subjects (Misra et al., 1992, Raghamulu et al., 1997). The bone specific alkaline phosphatase (BAP) isoenzyme predominates during childhood and adolescence (Christenson, 1997). BAP is much more effective than total ALP in assessing bone formation because of its higher specificity (Siebel, 2005).

2.9.1.2 Osteocalcin:

Osteocalcin, the most abundant non-collagen protein of bone matrix is synthesized from osteoblasts. It is a small protein of 49 amino acids that is rich in glutamic acid (GLA). It is also known as bone GLA protein. Although osteocalcin binds both free calcium and hydroxyapatite, its physiological role is unknown. In the process of matrix synthesis, some osteocalcin is released and circulates in blood with a short half-life; subsequently it is cleared by kidney (Christenson, 1997). Osteocalcin in serum or plasma can be measured by immun assay. Increased levels of osteocalcin have been found in fluorotic subjects (Srivastava et al., 1989).

2.9.1.3 Procollagen I-extension peptides:

Type I collagen is synthesized by osteoblasts as a large precursor protein termed as procollagen-I. The carboxy terminal and amino terminal ends of procollagen-I undergo extracellular cleavage and yield two relatively large extension peptides termed PICP and PINP. PICP is a globular protein and has a MW of 115 kDa, is stabilized by disulphide bonds. PINP is an elongated protein and has a MW of only 70 kDa, is rich in proline and hydroxyl proline. Both the peptides are cleared by liver endothelial cells and are currently measured by immunoassays (Christenson, 1997; Siebel, 2005).
2.9.2 Bone resorption markers: Except for tartrate-resistant acid phosphatase, the majority of bone resorption markers are degradation products of bone collagen.

2.9.2.1 Tartrate resistant acid phosphatase (TRAP):

Tartrate-resistant acid phosphatase (TRAP) has been used for several years as a marker enzyme of bone resorbing osteoclasts. There are five isoenzymes of acid phosphatase in blood, the major sources of which are bone, prostate, platelets, erythrocytes and spleen. Only one (bone isozyme) is tartrate-resistant (TRAP) and is abundant (Christenson, 1997). This enzyme, also named as type 5 acid phosphatase, is secreted into the circulation by osteoclasts and it has been suggested that serum TRAP could be a useful marker of bone resorption. In human blood, tartrate resistant acid phosphatase (TRAP) exists in two different glycosylated forms referred to TRAP-5a and TRAP-5b. TRAP 5a is derived from macrophages and TRAP 5b from bone-resorbing osteoclasts. The only structural difference between these two forms is that TRAP 5a contains sialic acid, which is not found in TRAP 5b. Resorbing osteoclasts secrete TRAP-5b into blood circulation as an enzymatically active form that is rapidly inactivated, degraded into fragments and finally removed from the circulation through liver or the kidneys. Recent evidence suggests that serum TRAP-5b activity is a specific and sensitive marker of bone resorption. It is the only marker known whose serum levels reflect specifically the bone resorption rate, as it is released into circulation exclusively from osteoclasts. More recently, specific immunoassays for TRAP-5b have been described and results indicate that this marker may be useful to assess osteoclasts activity (Seibel, 2005). Increased levels of this enzyme have been found in rabbits receiving fluoride (Turner et al., 1997).

2.9.2.2 Collagen breakdown products:

In Type I collagen, amino terminal and carboxy terminal ends are each linked to a helical portion of a nearby molecule by a pyridinium cross-links. The amino- and carboxy-non helical ends are termed as N-telopeptides (NTX) and C-telopeptides (CTX), respectively. Degradation by osteoclasts during the resorption process releases NTX and CTX fragments of various sizes, still attached to helical portions of a nearby molecule by a pyridinium cross links, into circulation for metabolism or excretion in urine. With additional degradation in the liver and
kidney the fragments are finally broken down to their constituent modified and unmodified amino acids, and the pyridiniums, pyridinoline (PYR) and deoxypyridinoline (D-PYR) (Christenson, 1997).

2.9.2.2.i Hydroxy proline (HP):

Hydroxyproline is a modified amino acid that is the product of posttranslational hydroxylation of integral proline residues of type I collagen. 90% of the HP liberated during the degradation of bone collagen is primarily metabolized in the liver and subsequently; it is excreted in the urine. The measurement of urinary hydroxyproline is usually affected by dietary intake of gelatin. So, HP measurement is generally performed after hydrolysis to convert all peptides and polypeptide sources of the modified amino acid to the free form. Colorimetric and high performance liquid chromatographic (HPLC) methods are available for precise measurement (Christenson, 1997). Increased levels of urinary hydroxyproline have been found in fluorotic subjects (Anasuya et al., 1974; Weiss et al., 1969).

2.9.2.2.ii Pyridinoline (PYR) and Deoxypyridinoline (DPYR) cross-links:

The pyridinium cross-links pyridinoline (PYR) and deoxypyridinoline (DPYR) are trifunctional cross-links formed during the extra cellular maturation of fibrillar collagens.

Pyridinoline is found in cartilage, bone, ligaments and vessels whereas deoxypyridinoline is almost exclusively found in bone. Pyridinoline and deoxypyridinoline cross-links are released from bone in a ratio of approximately 3:1. Hydroxylysine is essential for the formation of covalent pyridinium cross-links (PYR and DPYR). PYR is derived from 3 hydroxylysine residues whereas DPYR is derived from one lysine and 2 hydroxylysine. About 60% of the pyridinium cross-links released during bone resorption are bound to a protein and the remaining 40% is free. In addition, the urinary excretion of pyridinum crosslinks is independent of dietary source since neither PYR nor DPYR are taken up from food. HPLC and immunosay are the two methods that have been widely used for measuring PYR and D-PYR in urine before or after hydrolysis. Although the pyridinium cross-links are not entirely bone-specific, they appear to be superior markers of bone resorption compared with hydroxyproline (Christenson, 1997; Siebel, 2005). Ando et al. (1998)
reported that high concentrations of fluoride in food directly stimulate bone resorption and cause excretion of higher levels of urinary deoxypridinoline.

2.9.2.iii Cross-linked telopeptides:

When type I collagen is degraded by osteoclasts during the resorption process, amino- and carboxy terminal fragments of collagen are released with cross links attached. These fragments with attached crosslinks are called telopeptides. N-telopeptides (NTX) and C-telopeptides (CTX) are highly bone specific because osteoclasts are not active in the degradation of other type I collagen containing tissues. Because the vast majority of these fragments are relatively small, they readily pass through the glomerulus into the urine. Specific immunoassays for NTX and CTX in serum and urine are available. Telopeptides are currently viewed as the best indices for assessing bone resorption (Christenson, 1997). Topuz et al (2006) reported that the urinary CTX levels were markedly increased in fluorotic subjects.

2.9.3 Advantages and disadvantages of bone markers:

A number of biochemical markers of bone metabolism/tumover have been described and these reflect the activity of osteoblasts (bone formation) or osteoclasts (bone resorption). These markers have the following advantages for the measurement of bone turnover: (1) they are noninvasive; (2) can be repeated on many occasions; (4) and reflect bone cell activity in the entire skeleton. The disadvantages are: (1) they do not provide information about the work of individual cells; (2) they do not reflect the process of mineralization; and (3) their levels may be affected by the rate of clearance.

2.10 Oxidative stress and Antioxidants:

Oxidative stress has been defined as the inability of the organ or cell to defend itself against the oxygen derived species, resulting in oxidative injury (Halliwell and Gutteridge, 1985).

Oxidative stress occurs when there is an imbalance in the generation and removal of radical species within an organism. A radical is any molecule that contains one or more unpaired electrons. The majority of the radicals involves with oxygen are referred to as reactive oxygen species (ROS) (Seis, 1991). The term reactive oxygen species (ROS) is now generally preferred because of single oxygen,
hydrogen peroxide, hypochlorous acid, peroxide, hydroperoxide and epoxide metabolites of endogenous lipids. Radicals in toxicology refer to those that exist in a free and uncombined state, which is able to interact with various tissue components. Once free radicals interact with tissues, many changes can occur. These changes can create a disturbance in the pro-oxidant-antioxidant balance in favour of the former (Seis, 1985) leading to potential damage.

2.10.1 Lipid peroxidation:

Lipid peroxidation is generally defined as an oxidative deterioration of polyunsaturated lipids and is known to occur in all biological membranes (Halliwell et al, 1992). Lipid peroxidation mainly occurs in 3 stages. Poly unsaturated fatty acids (PUFA) side chain reacts with a reactive free radical to form a lipid radical, which later reacts with molecular oxygen forming a peroxyl lipid radical. This forms the initiation stage. In the propagation stage, the peroxyl radical reacts with another PUFA side chain of the lipid moiety to yield a lipid hydroperoxide and lipid radical there by increasing the number of radicals. Finally the lipid peroxidation can be terminated either by reactions between the radicals or by the chain breaking antioxidants like vitamin E (Waldeck & Stocker, 1996).

**Initiation**

\[ \text{LH} + R^* \rightarrow L^* + RH \]

\( \text{R = Initiator, O}_2, \text{OH}^* \text{ etc) } \)

**Propagation**

\[ L^* + O_2 \rightarrow LOO^* \]

\[ LOO^* + L \text{H} \rightarrow LOOH + L^* \]

**Reinitiation**

\[ \text{LOOH} + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} \text{ OH} + \text{LO}^* \rightarrow \text{LOH} \]

\[ \text{LOOH} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} \text{H}^* + \text{LOO}^* \rightarrow \text{LOOH} \]

**Termination**

\[ \text{LOO}^* / L^* / \text{IH} \rightarrow \text{LOOH} / \text{LH} / \text{LO} + \text{I}^* \]

\[ \text{LOO}^* + \text{LOO}^* \rightarrow L^* = \text{O} + \text{LOH} + O_2 \]

\[ \text{LOO}^* + \text{I}^* \rightarrow \text{LOOH} - \text{L} \]

\( \text{IH = Antioxidant} \)
Lipid peroxidation can be initiated either non-enzymatically through metal ion centered reactions (non-enzymatic lipid peroxidation) or through enzymatic insertion of \(O_2\) by the enzymes like cyclooxygenases and lipoxygenases (enzymatic lipid peroxidation) (Sarala, 1997). Extensive lipid peroxidation in biological membranes causes impairment of membrane function, decreased fluidity, inactivation of membrane bound receptors and enzymes, cross linking with sulphhydryl groups of enzymes etc, (Halliwell and Gutteridge, 1990). Thus uncontrolled lipid peroxidation mediates a variety of degenerative diseases leading to cell death.

2.10.2 Antioxidants:

Any substance when present at low concentration compared to those of an oxidizable substrate, significantly delaying or preventing oxidation of substrate is called an antioxidant (Halliwell, 1995). Antioxidants, in reality, are specific reducing agents promoting the reduction of oxidized products.

According to the mode of action, antioxidants may be classified as free radical scavenger/terminator, chelator of metal ions, capable of catalyzing lipid peroxidation. Antioxidants which have a vital role in the prevention of oxidative damaging effects of free radicals fall into two classes: (1) preventive antioxidants which reduce the rate of chain reaction and (2) chain breaking antioxidants which interfere with chain propagation. The former decompose peroxides or sequester metal ions to reduce the generation of free radicals whereas the latter scavenge free radicals to inhibit the radical attack and/or break the chain oxidation. The preventive antioxidants include catalase and other peroxidase that react with ROOH, and chelators of metal ions such as DTPA (diethylene triamine pentaacetaate) and EDTA (ethylenediamine tetraacteate). Chain-breaking antioxidants are often phenols or aromatic amines. In vivo, the principal chain-breaking antioxidants are superoxide dismutase (SOD) which acts in the aqueous phase to trap superoxide free radicals (\(O_2^-\)); perhaps urate and vitamin E, which act in the lipid phase to trap \(ROO^\cdot\) radicals. Ascorbic acid, thiols (GSH), tocopherol, uric acid, \(\beta\)-carotene, bilirubin and ubiquinol were known to serve as chain-breaking antioxidants (Halliwell, 1990). Naturally there is a dynamic balance between the amount of free radicals generated in the body and antioxidants quench and/or scavenge them and protect the body against their deleterious effects (Finkel and Holbrook, 2000; Nose, 2000).
A few important antioxidants are mentioned here. Catalase and Glutathione-S-transferase.

2.10.2.1 Catalase:

Catalase is one of the most important antioxidant enzymes, which reduces the toxic hydrogen peroxide into water and protects the cell from possible oxidative damage (Krinsky et al., 1992). Catalase acts on hydrogen peroxide generated either through metabolism of endogenous substances or exogenous compounds. Since it removes reactive hydrogen peroxide from cell, it is important in detoxification mechanisms. Catalase plays a vital role in removing peroxyl radicals and has been able to protect cells from toxic injury. Catalase is present predominantly in peroxisomes in liver and kidney. Decreased levels of this enzyme have been found in fluorotic subjects (Vijayabhaskar et al., 2007; Shanthakumari et al., 2006).

2.10.2.2 Glutathione-S-Transferase (GSTs):

Glutathione-S-Transferases are a group of multifunctional proteins involved in the detoxification of a wide spectrum of compounds. GSTs are known to play a central role in biotransformation of a variety of xenobiotics including oxidants. The well known catalytic functions of GSTs are: conjugation, peroxide reduction and isomerisation. GSTs are the drug metabolizing and detoxifying enzymes involved in the intracellular transport and metabolism of steroids and hormones (Jakoby and Kenn, 1977). GSTs catalyse the reactions between the SH group of GSH and are potential alkylation agents, neutralizing their electrophilic sites and rendering them more water soluble (Sen and Hannmen, 1994). GSTs play a prominent role in antioxidant defense mechanism by former two catalytic functions, conjugations and peroxide reductions (Board, et al 1991). The peroxidase activity of GST is termed as non-selenium glutathione peroxidase (Non-Se-GSHPX), which is expressed by the Ya containing isozyme of GST (Burgess et al, 1987). The GSTs of class Mu are known to be induced by the active oxygen radicals (Murata et al, 1990). Decreased levels of this enzyme have been found in fluorotic subjects (Vijayabhaskar et al., 2007; Shanthakumari et al., 2006).
2.11 Prevalence of Fluorosis in India:

Fluorosis is a crippling and painful disease caused by intake of fluoride. Fluorosis can affect young and old. Fluorosis is an endemic disease prevalent in 17 states. In 1960 only four states of India were identified for endemic fluorosis - namely Andhra Pradesh, Punjab, Tamil Nadu and Uttar Pradesh. In 1980 the problem was also identified in Delhi, Gujarat, Haryana, Karnataka, Madhya Pradesh, and Rajasthan. Today there is clear epidemiological and clinical evidence that the problem exists in India. About 25 million people from these areas were found to be seriously affected with endemic fluorosis and about 40 million are at risk (ITRC, 1995).

Depending on the seriousness of problem, India states have been categorized into 3 groups (Map 2)

1. 70-100% districts are affected in Andhra Pradesh, Gujarat and Rajasthan
2. 40-70% districts are affected in Bihar, Delhi, Jharkhand, Karnataka, Maharashtra, Madhya Pradesh, Orissa, Tamil Nadu and Uttar Pradesh.
3. 10-40% districts are affected in Assam, Jammu and Kashmir, Kerala, Chattisgarh and West Bengal (Susheela, 2003).

In Andhra pradesh, the fluorosis affected areas are Nalgonda, Ranga Reddy and Mahaboobnagar in Telangana region; Prakasam, Guntur and Krishna in coastal region and Anantapur, Kurnool and Cuddapah in Rayalaseema region (Lingeswara Rao, 2003). Rajgopal and Tobin (1991) reported that fluoride content increased with the depth of water source. The concentration of fluoride in drinking water is said to vary from one geographical region to another. According to Sangh et al. (1996) the fluoride content of drinking water varied from 0.5 to 25.0 ppm in different parts of India.

The fluoride content in drinking water samples varied from 1.6 to 20.6 ppm in different areas of Andhra Pradesh (Ahuja, 2001). In the Telangana region, water fluoride level was reported to be 20.6 ppm in Nalgonda district, in Medak district the fluoride level was 3.0 ppm, in Mehboobnagar district the fluoride level was 6.4 ppm, in Warangal district the fluoride was 5.8 ppm, in Kareemnagar district the fluoride level was 4.9 ppm, in Hyderabad district the fluoride level was 4.8 ppm, in Nizamabad district the fluoride level was 3.0 ppm, in Adilabad district the fluoride level was 2.8 ppm.
In Coastal region, water fluoride level was 12.0 ppm in Prakasam district, in Visakhapatnam district the fluoride levels above 5 ppm, in Guntur district the fluoride level was 10.0 ppm, in Nellore district the fluoride level was 8.0 ppm, in Srikakulam district the fluoride level was 3 ppm, in East Godavari district the fluoride level was 1.6 ppm.

In Rayalaseema region, the reported water fluoride level was 10.1 ppm in Anantapur district, 9.6 ppm in Kurnool district, 4.0 ppm in Cuddapah district and 3.0 ppm in Chittoor district. Based on research studies drinking water fluoride levels in different endemic areas are presented in table 3.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Area</th>
<th>Fluoride levels (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chakma et al., (2000)</td>
<td>Tilapalpuni village, Hirapur village, Mandla (Dt)</td>
<td>9.22 - 10.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 - 13.5</td>
</tr>
<tr>
<td>Sharma, (2003)</td>
<td>Jammu and Kashmir, Himachal Pradesh, Rajasthan, Bihar, West Bengal, Chattisgarh, Orissa</td>
<td>0.17 - 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 - 6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 - 6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>An average 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 - 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.2 - 13.2</td>
</tr>
<tr>
<td>Shivasankara et al., (2000)</td>
<td>Gulbarga (Dt), Karnataka</td>
<td>0.6 - 13.4</td>
</tr>
<tr>
<td>Moudgil et al., (1986)</td>
<td>Faridabad town, Haryana</td>
<td>7.3 - 29.0</td>
</tr>
<tr>
<td>Susheela et al., (1996)</td>
<td>Palam and mega city of Delhi</td>
<td>1.2 - 2.5</td>
</tr>
<tr>
<td>Choubisa et al., (1997)</td>
<td>Dungarpur (Dt), Rajasthan</td>
<td>0.5 - 10.8</td>
</tr>
<tr>
<td>Susheela et al., (1993)</td>
<td>Faridabad (Dt), Haryana</td>
<td>0.25 - 8.00</td>
</tr>
<tr>
<td>Saralakumari and Ramakrishna Rao (1993)</td>
<td>RallaAnanthapuram, Andhra Pradesh</td>
<td>7.2 - 10.70</td>
</tr>
<tr>
<td>Misra et al., (1992)</td>
<td>Lucknow</td>
<td>0.55 - 12.0</td>
</tr>
</tbody>
</table>
Map 2: Map showing Endemic states of Fluorosis in India
2.12 Prevalence of Fluorosis studies

2.12.1 Dental Fluorosis: Based on research studies the prevalence of dental fluorosis in different endemic areas is presented in table 4.

Table 4: Prevalence of Dental Fluorosis in Different Endemic Areas

<table>
<thead>
<tr>
<th>Authors</th>
<th>Area</th>
<th>Age group (yrs)</th>
<th>N</th>
<th>Dental fluorosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chakma et al., (2000)</td>
<td>Tilapalpuni village</td>
<td>20 - 20</td>
<td>84</td>
<td>51.1</td>
</tr>
<tr>
<td></td>
<td>Hirapur village, Mandla (Dt)</td>
<td></td>
<td>36</td>
<td>22.2</td>
</tr>
<tr>
<td>Choubisa et al., (1997)</td>
<td>Dungarpur (Dt), Rajasthan</td>
<td>16 yrs</td>
<td>1224</td>
<td>62.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 yrs</td>
<td>1364</td>
<td>69.7</td>
</tr>
<tr>
<td>Misra et al., (1992)</td>
<td>Lucknow</td>
<td>3-12</td>
<td>69</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 - 90</td>
<td>78</td>
<td>96.1</td>
</tr>
<tr>
<td>Múxta agarwal and Purva johri (1998)</td>
<td>Banasthali village, Rajasthan</td>
<td>2 - 18</td>
<td>198</td>
<td>100</td>
</tr>
<tr>
<td>Pushpa Bharathi and Meera Rao (2003)</td>
<td>Dharwada (Dt), Karnataka</td>
<td>7 - 9</td>
<td>224</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 - 12</td>
<td>32</td>
<td>43.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 - 15</td>
<td>133</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 - 80</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Saralakumari and Ramakrishna Rao (1993)</td>
<td>Ralia Ananthapuram, Ananthapuram (Dt), Andhra Pradesh</td>
<td>20 - 20</td>
<td>162</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>156</td>
<td>92.3</td>
<td></td>
</tr>
<tr>
<td>Shivasankara et al., (2000)</td>
<td>Gulbarga (Dt), Karnataka</td>
<td>3 - 10</td>
<td>46</td>
<td>89</td>
</tr>
<tr>
<td>Susheela et al., (1993)</td>
<td>Faridabad (Dt), Haryana</td>
<td>16</td>
<td>1953</td>
<td>58</td>
</tr>
</tbody>
</table>
2.12.2 Skeletal fluorosis: Based on research studies prevalence of skeletal fluorosis in different endemic areas is presented in table 5.

Table 5: Prevalence of Skeletal fluorosis in Different Endemic Areas

<table>
<thead>
<tr>
<th>Authors</th>
<th>Area</th>
<th>Age group(yrs)</th>
<th>N</th>
<th>Skeletal fluorosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chakma et al., (2000)</td>
<td>Tilapalpani village</td>
<td>&gt;20</td>
<td>142</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>Hirapur village, Mandla (Dt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choubisa et al., (1997)</td>
<td>Dungarpur (Dt), Rajasthan</td>
<td>21 – 60</td>
<td>1955</td>
<td>27.8</td>
</tr>
<tr>
<td>Misra et al., (1992)</td>
<td>Lucknow</td>
<td>13 – 90</td>
<td>78</td>
<td>46.1</td>
</tr>
<tr>
<td>Saralakumari and Ramakrishna Rao (1993)</td>
<td>Ralla Ananthapuram, Andhra Pradesh.</td>
<td>&gt; 20</td>
<td>129</td>
<td>76.3</td>
</tr>
<tr>
<td>Shivasankara et al., (2000)</td>
<td>Gulbarga (Dt), Karnataka</td>
<td>3-10</td>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>Susheela et al. (1993)</td>
<td>Faridabad (Dt), Haryana</td>
<td>&gt; 16</td>
<td>1953</td>
<td>27</td>
</tr>
</tbody>
</table>

2.13 Fluoride Status studies:

Susheela et al. (1996) reported serum fluoride levels, which ranged from 0.05 to 0.63 ppm and urinary fluoride levels, which ranged from 0.30 to 24.1 ppm in subjects residing in fluorotic areas of Delhi. Pushpa Bharathi and Meera Rao (2003) reported the mean fluoride intake of adults, which ranged from 6.72 to 15.4 ppm per day in Dharwada district of Karnataka where fluoride levels ranged from 4.0 to 10.5 ppm. Simiarily Baby Devaki and Ramalakshmi (2001) also reported the mean fluoride intake range of 4.8 to 6.3 ppm, the mean serum fluoride levels, which ranged from 0.1 to 0.9 ppm and urinary fluoride levels ranged from 1.91 to 2.66 ppm for fluorotic adults in Raphadu mandal of Ananthapur district where the water
fluoride levels ranged from 1.3 to 3.3 ppm. Yadav et al. (2003) reported the mean urinary fluoride levels of 1.58 ppm in Bahadurgarh, 1.48 ppm in Beri, 1.50 ppm in Jhajjar, 1.51 in Matanhall and 1.56 ppm in Sahalaws for children affected with dental fluorosis residing in Jaipur district of Haryana. Gupta et al. (2001) reported the serum fluoride, urinary fluoride and fluoride intake levels of fluorotic children (n = 50 from each area) residing in four endemic fluorotic areas of Rajasthan. Water containing 2.4, 4.6, 5.6 and 13.5 ppm of fluoride. All children were in an age group of 6 to 12 years. Serum fluoride, urinary fluoride and fluoride intake levels were 0.79 ppm, 9.45 ppm and 7.35 ppm for 50 fluorotic children residing in Ramasagar Ki Dhani; 1.10 ppm, 15.90 ppm and 11.97 ppm for 50 fluorotic children residing in Rampura; 0.10 ppm, 17.78 ppm and 14.45 ppm for 50 fluorotic children residing in Shivadaspura and 1.07 ppm, 14.56 ppm and 32.56 ppm for 50 fluorotic children residing in Rajpurai respectively.

2.14 Bone Mineral Status Studies as related to fluorosis:

Bone mineral studies revealed that the fluorosis-affected subjects had normal levels of calcium and phosphorus. Alkaline phosphatase levels were significantly elevated in fluorosis subjects. Misra et al. (1992) reported, the mean serum calcium, phosphorus and alkaline phosphatase levels of 9.39 mg/dl, 4.1 mg/dl and 229.70 U/L respectively in adults residing in endemic area of Lucknow. Raghuramulu et al. (1997) also reported the mean serum calcium, phosphorus and alkaline phosphatase levels of 9.6 mg/dl, 5.6 mg/dl and 7.1 Bodensky Unit respectively in fluorotic adults and the mean serum calcium, phosphorus and alkaline phosphatase levels of 10.6 mg/dl, 4.8 mg/dl and 5.5 Bodensky Unit respectively in non-fluorotic adults residing in non-endemic area of Hyderabad. Srikantia and Siddique (1965) reported the serum calcium, phosphorus and alkaline phosphatase levels were 10.05 ± 0.16 mg/dl, 3.57 ± 0.25 mg/dl and 16.9 ± 2.72 BU for fluorotic adults (n = 31) residing in endemic villages of Nalgonda district. Shivashankara et al. (2000) reported the serum calcium; phosphorus and alkaline phosphatase levels were 9.4 ± 1.29 mg/dl, 4.95 ± 0.55 mg/dl and 447.67±111.5 IU/L for 3-10 yrs age group of fluorotic children (n=46) residing in endemic area of Kheru Nayak thanda of Gulbarga district. Chakma et al. (2000) reported the serum calcium; phosphorus and alkaline phosphatase levels were 9.5 ± 0.5 mg/dl, 3.4 ± 0.9 mg/dl and 16.6 ± 6.4 King Armstrong Units for children and adolescents (below age 20) in Tilalpani village.
and Hirapur village of Mandal district. Gupta et al. (2001) evaluated the serum calcium level was 9.23 mg/dl for fluorotic children residing in Ramasagar Ki Dhani; 10.75 mg/dl for fluorotic children residing in Ramapura; 9.68 mg/dl for fluorotic children residing in Shivadaspura and 10.39 mg/dl for fluorotic children residing in Rajpurai respectively.

2.15 Ca-turnover studies:

An alteration in bone metabolism is a basic feature of fluorosis. Calcium plays an important role in bone metabolism. National Institute of Nutrition conducted studies on turnover of calcium in fluorosis (Narasingarao et al., 1968; Narasingarao et al., 1977). In calcium turnover studies carried out on monkeys, experimental fluorosis was induced by normal diets as well as diets containing low amounts of vitamin C and calcium, which aggravated the conditions (Sriranga reddy and Srikanthia, 1971). Ca$^{45}$ kinetics showed that the cumulative retention of radioactive calcium was high in experimental animals as compared to control group. These data clearly indicated that the level of dietary calcium influences fluoride toxicity (Sriranga reddy and Narasingarao, 1977). Radioactive studies with calcium$^{45}$ in skeletal fluorosis subjects indicated that there is increased absorption and lowered urinary excretion (Narasingarao et al., 1968). Calcium turnover studies using Ca$^{47}$ in subjects with genuvalgum had higher rate of total body calcium turnover as compared to fluorosis subjects (Narasingarao et al., 1977).

2.16 Hormonal Status Studies as related to fluorosis:

Hormonal status studies revealed that the fluorosis-affected subjects had normal levels of 25(OH) vitamin D levels but elevated PTH levels.

Srivastava et al. (1989) reported serum parathyroid hormone levels, which were significantly elevated and normal serum 25(OH) vitamin D levels in fluorotics. Raghuramulu et al. (1997) reported the mean serum 25(OH) vitamin D3 levels of 38.3 ± 3.86 ng/dl for skeletal fluorosis subjects, 47.9 ± 24.56 ng/dl for age match subjects without bone deformity in endemic area and 73.9 ± 20.67 ng/dl for controls from non-endemic area. Sivakumar et al. (1976) reported that the concentration of parathyroid hormone was considerably higher in subjects with fluorosis i.e., 2674 pg/ml as compared to normal i.e., 1240 pg/ml, but this level was 5390 pg/ml higher in genuvalgum subjects. Gupta et al. (2001) reported the serum parathyroid hormone
level of 31.64 pmol/l for fluorotic children residing in Ramasagar Ki Dhani; 40.98 pmol/l for fluorotic children residing in Rampura; 75.07 pmol/l for fluorotic children residing in Shivadaspura and 125.10 pmol/l for fluorotic children residing in Rajpurai respectively. Barat (1998) reported the mean thyroid stimulating hormone, triiodothyronine and thyroxine levels of 2.56 ± 0.36 µU/ml, 1.53 ± 0.076 µg/ml and 9.16 ± 0.63 µg/dl respectively for fluorotic adults and the mean thyroid stimulating hormone, triiodothyronine and thyroxine levels of 2.56 ± 0.36 µU/ml, 1.53 ± 0.076 µg/ml and 9.16 ± 0.63 µg/dl respectively for non-fluorotic adults residing in non-endemic area of North Gujarat.

2.17 Oxidative and Antioxidant enzyme status studies as related to fluorosis:

Oxidative and Antioxidant enzyme status studies revealed that the fluorosis-affected subjects had increased levels of malondialdehyde and decreased levels of catalase and glutathione-s-transferase. Vijayabhaskar et al (2007) reported the mean serum malondialdehyde, catalase and glutathione-S-transferase levels of 3.49 ± 0.19 nmol/ml, 64.96 ± 3.82 KU/gm Hb, 47.07 ± 2.56 IU/L respectively for fluorotic adults residing in endemic area of Nalgonda district. Shanthakumari et al (2006) reported the mean serum malondialdehyde, catalase and glutathione-s-transferase levels of 270 nmol/mL RBC, 0.72 ± 0.08 U*10^-4/mL RBC and 1.16 ± 0.21 U/mL RBC respectively for fluorotic adults residing in endemic area of Alangayam subdivision of Vellore district. Saralakumari and Ramakrishna (1991) reported the mean serum malondialdehyde levels of 447.01 ± 26.84 µ moles of MDA formed/mg protein for fluorotic adults residing in endemic area of Ralla Ananthapuram of Ananthapur district. Shivarajashankara et al.(2001) reported the mean serum malondialdehyde levels of 256.0 ± 9.04 nanomoles/gm Hb for fluorotic children residing in endemic area of Kheru Nayak Thanda of Gulbarga district.

2.18 Criteria for Recruiting the Study Population:

The following biochemical markers have been chosen for selecting the study population (fluorotic and non-fluorotic adults) for the present investigation.

1. Primarily, serum and urinary fluoride levels were estimated to assess fluoride status in both study and control groups.
2. Liver function tests such as bilirubin, SGOT and SGPT were performed to exclude abnormal subjects from the study population.

3. Serum and urine levels of creatinine were estimated to evaluate renal status. The subjects with renal disorders were excluded from the study population.

4. Serum calcium, phosphorus and alkaline phosphatase levels were estimated to evaluate bone mineral status in study population.

5. Calcitropic hormone levels such as those of parathyroid hormone and 25-hydroxy vitamin-D3 were evaluated to assess hypo- and hyperparathyroidism and also to know the influence of these hormones on calcium homeostasis and on bone metabolism in study population.

6. Levels of thyroid hormones such as thyroid stimulating hormone, triiodothyronine and thyroxine were estimated to evaluate thyroid status in study population.

7. Serum malondialdehyde levels, catalase and glutathione-s-transferase activities were estimated to evaluate oxidative stress and antioxidant enzyme status in both study population.

2.19 **Selection of Biochemical Markers of Bone Metabolism**

The degree of bone metabolism/turnover (bone formation and resorption) is assessed by evaluating bone formation and bone resorption markers.

1. In the present study, Bone specific alkaline phosphatase (BAP) has been selected as a marker of bone formation because it is produced in extremely high amounts during the bone cycle’s formation phase and is, therefore, an excellent indicator of bone formation activity.

2. Serum TRAP-5b was chosen as bone resorption marker, since it is the only serum resorption marker to assess osteoclastic activity.

3. Urinary bone resorption marker such as urinary C-terminal telopeptide of type-1 collagen levels is selected in this study to evaluate the degree of collagen degradation.