DOES VITAMIN D DEFICIENCY AND RENAL DYSFUNCTION PLAY A ROLE IN THE PATHOGENESIS OF FLUOROTOXIC METABOLIC BONE DISEASE (FMBD)?

Thesis submitted to

The Tamil Nadu Dr. M.G.R. Medical University

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To fulfil the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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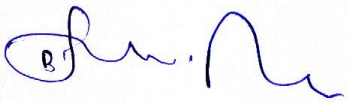
July, 2016

01st July, 2016

Vellore

DECLARATION

I hereby declare that the thesis entitled "**Does Vitamin D deficiency and renal dysfunction play a role in the pathogenesis of Fluorotoxic Metabolic Bone Disease (FMBD)?"** is based on the results of the work carried out by me for the degree of **DOCTOR OF PHILOSOPHY** under the supervision of **Dr. R. Selvakumar**, Department of Clinical Biochemistry, Christian Medical College, Vellore. This work has not formed the basis of any associateship, fellowship, degree or diploma of any other University.



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CERTIFICATE

This is to certify that Mr. Joseph Dian Bondu has completed his requirements for the submission of his thesis entitled "**Does Vitamin D deficiency and renal dysfunction play a role in the pathogenesis of Fluorotoxic Metabolic Bone Disease (FMBD)?"** for the degree of **DOCTOR OF PHILOSOPHY** under my supervision. This thesis is a bonafide record of the research work carried out by the candidate during the period of study under my guidance and is not formed the basis of any associateship, fellowship, degree or diploma of any other University. This thesis represents independent research carried out by the candidate, under my supervision.

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The project titled " *Role of vitamin D deficiency in the pathogenesis of fluorotoxic metabolic bone disease (FMBD).*" has been reviewed by the Research Committee of the Christian Medical College which considered its objective, study design and budget. This study has been approved for conduct at the Christian Medical College, Vellore under the direction of Dr. R. Selvakumar, Former, Professor, and Mr. Joseph Dian Bondu, Lecturer, Department of Clinical Biochemistry.

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This is to certify that the animal studies done by **Joseph Dian Bondu**, as part of his Ph.D. thesis titled **"Role of vitamin D deficiency in the pathogenesis of fluorotoxic metabolic bone disease (FMBD)"** was approved by the Institutional Animal Ethics Committee of Christian Medical College, Vellore on 8/6/2011. (IAEC approval Number 12/2011)

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CERTIFICATE

This is to certify that Mr. Joseph Dian Bondu has completed his requirements for the submission of his thesis entitled "**Does Vitamin D deficiency and renal dysfunction play a role in the pathogenesis of Fluorotoxic Metabolic Bone Disease (FMBD)?"** for the degree of **DOCTOR OF PHILOSOPHY** under my supervision and has used the anti-plagiarism software to prove the authenticity of this thesis.

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CONTENTS

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BALP	Bone Alkaline phosphatase
BMC	Bone Mineral Content
BMD	Bone Mineral Density
CLU	Clusterin
CRF	Chronic Renal Failure
DEXA	Dual Energy X-ray absorptiometry
EDTA	Ethylene Diamine Tetracetic acid
ELISA	Ensyme linked Immonosorbent assay
FHA	Fluoridated Hydroxyapatite
FMBD	Fluorotoxic Metabolic Bone Disease
GFR	Glomerular Filtration Rate
HA	Hydroxyapatite
HF	Hydrogen Fluoride
IC	Ion Chromatography
ISE	Ion Selective Electrode
IGF-1	Insulin like growth Factor-1
IGF-2	Insulin like growth Factor-2
KIM	Kidney injury marker
25-OHD$_3$	25- hydroxyl Vitamin D_3
1,25 (OH)$_2$ D$_3$	1,25-dihydroxy Vitamin D_3

OPN	Osteopontin
ppm	Parts Per million
PTH	Parathyroid hormone
RANKL	Receptor activator of Nuclear factor kappa B ligand
TGF-β	Transforming Growth factor beta
UVB	Ultraviolet B rays
VDR	Vitamin D receptor

INTRODUCTION

DOES VITAMIN D DEFICIENCY AND RENAL DYSFUNCTION PLAY A ROLE IN THE PATHOGENESIS OF FLUOROTOXIC METABOLIC BONE DISEASE (FMBD)?

1. INTRODUCTION

Fluorosis is a major public health problem, and it is endemic in about 24 countries worldwide which have recorded high levels of Fluoride in drinking water. The geographical belt of endemic Fluorosis includes Japan, Iraq, Iran, Afghanistan and the countries extending from Turkey to China (1) (2). About 100 million people are known to be affected by Fluorosis (1) and India is one of the countries which lie in the affected geographical belt. The magnitude of the problem is alarming, as about 62 million people in India, which includes 6 million children, have developed Fluorosis, due to the consumption drinking water with high levels of fluoride. The states which are worst affected are Rajasthan, Bihar, Jharkhand, Punjab, Chhattisgarh, Maharashtra, Haryana, Andhra Pradesh, Karnataka and Tamil Nadu (3). Thus the researcher has aimed to study the prevalence of high ground water fluoride levels in the various villages in the district of Vellore, Tamil Nadu .

The toxic manifestations of Fluorosis are Dental Fluorosis, Skeletal Fluorosis and Genu Valgum which is found among children (4). Dental fluorosis occurs when there is excess Fluoride ingested over the years of tooth calcification (especially during the first 7 years of life) (5).The early signs may be “mottling” of dental enamel (6), loss of the shiny appearance and chalky white patches on the teeth. These white patches then turn yellow and sometimes even brown or black (7). Skeletal Fluorosis is associated with intake occurring all through life of water with a fluoride content of 3.0 to 6.0 ppm or more (6). The is deposition of fluoride in the skeleton may lead to disabilities like musculoskeletal dysfunction, arthritis, ankylosis of spine with radiculopathy , osteoscleros**i**s with ligament calcifications as well as peripheral

neuropathy (8). Genu valgum is the bowing of lower limbs occurring with the bone disorders of osteomalacia, osteosclerosis and osteopenia (9). This has been reported from some districts of Andhra Pradesh, Rajasthan, Bihar, Karnataka and Tamil Nadu. Studies have shown that it is children with a higher intake of fluoride that exhibit this syndrome (10) (11).

The World Health Organization (WHO) has prescribed the acceptable levels of Fluoride to be <1.5ppm in drinking water (12), while the Indian standard of permissible drinking water levels is less than 1 ppm(12).But some states in India, have markedly high levels of fluoride. Water fluoride levels of more than 1.5 ppm have been recorded in about 17 states in India (13). The literature states that eventhough high levels of Fluoride consumption can lead to dental and skeletal fluorosis an optimal amount of consumption is essential for adequate bone mineral deposition.

Fluorosis is also known as Fluorotoxic Metabolic Bone Disease (FMBD). Teotia studied the prevalence of disorders of calcium and bone metabolism among the Indian population from 1963 to 2005. Among the 4, 11,744 subjects found to have these disorders, 52% had nutritional bone diseases, 43% had endemic Fluorosis and 5% had metabolic bone disease (14). Therefore studying the causes, related factors and consequences of this public health problem is essential.

As stated earlier, Fluoride is known to cause skeletal fluorosis and has a role to play in bone metabolism, as it affects the mitotic activity of the osteoblasts in bone. (15). It has also been found that fluoridated hydroxyapatite crystals increase cell attachment of osteoblasts, influences the propagation and delineation of osteosarcoma cells in SAOS- rat. The fluoride anion may alter the crystalline structure of the bone tissue as it has been observed to replace hydroxyl in the hydroxyapatite crystals. (16).

Studies have shown conclusive results of the viability of osteoblasts being affected by the accumulation of Fluoride in the bone. Although Fluroapatite crystals have a larger size than hydroxyapaptite crystals, they offer less surface exchange, are less soluble and more stable while being less responsive to the actions of the parathyroid hormone (17).

Severity of Fluoride toxicity may be influenced by the following factors:

- Consumed water fluoride content
- Daily ingestion of fluoride
- Continuation and the length of fluoride exposure
- Intake of Vitamin D and calcium
- Age, sex, occupation
- Composition of food in relation to content of Magnesium, Calcium, Phosphate and aluminium ions.
- Status of bone remodelling

The function of Vitamin D in remodelling of bone is already eastablished and it has been found to alter the calcium deposition, matrix ossification, and osteoblastic activity. (18). Deficiency of Vitamin D, a common nutritional deficiency in India, may predispose a person to develop FMBD, as studies suggest that low levels of Vitamin D may lead to bone deformities (19) (20). The researcher has extensively studied the area of the synergistic effect of Vitamin D deficiency in the development of FMBD.

Renal damage is known to occur in Fluorosis as increased blood fluoride levels have shown to be associated with impaired renal function (21).A few case reports have shown that fluorosis causes changes in the glomerular filtration rate and severe tubular damage (22) (23). Some of the studies have suggested a vicious cycle where in fluoride induced renal damage leads to increase in the blood fluoride levels, since the kidneys normally excretes Fluoride,

which results in further damage to the kidneys. But this area has still to be researched extensively.

Though the effects of fluorosis have been shown to be debilitating but the researcher has found a dearth of literature studying the effects of fluorosis and the associated factors. This public health problems calls for immediate action owing to the severity of disabilities caused and its preventable nature.

AIMS AND OBJECTIVES

2. AIMS OF THE STUDY

- To establish the Ion Chromatography method for the estimation of Fluoride in drinking water
- To study the role of fluoride in renal tubular damage and to study the role of vitamin D deficiency in the pathogenesis of Fluorotoxic metabolic bone disease (FMBD).

OBJECTIVES

1. To validate the method of Ion Chromatography for estimation of Fluoride levels in drinking water in comparison to the established ISE methodology.
2. To assess the Fluoride levels in the drinking water of the villages in Vellore district using Ion Chromatography.
3. To standardize a rat model of Vitamin D deficiency and assess the changes in bone density and changes in specific biochemical parameters after 4 months on a Vitamin D deficient diet.
4. To study the changes in bone morphology and metabolism induced by varying levels of fluoride intake in the rat model of Vitamin D deficiency.
5. To study the effects of fluoride intake in drinking water on the renal tubular function in the rat model of Vitamin D deficiency.

HYPOTHESES

H1: Concomitant Vitamin D deficiency and/or hypocalcemia may exacerbate Fluorotoxic metabolic bone disease (FMBD).

H2: Ingestion of high Fluoride in drinking water leads to renal tubular damage.

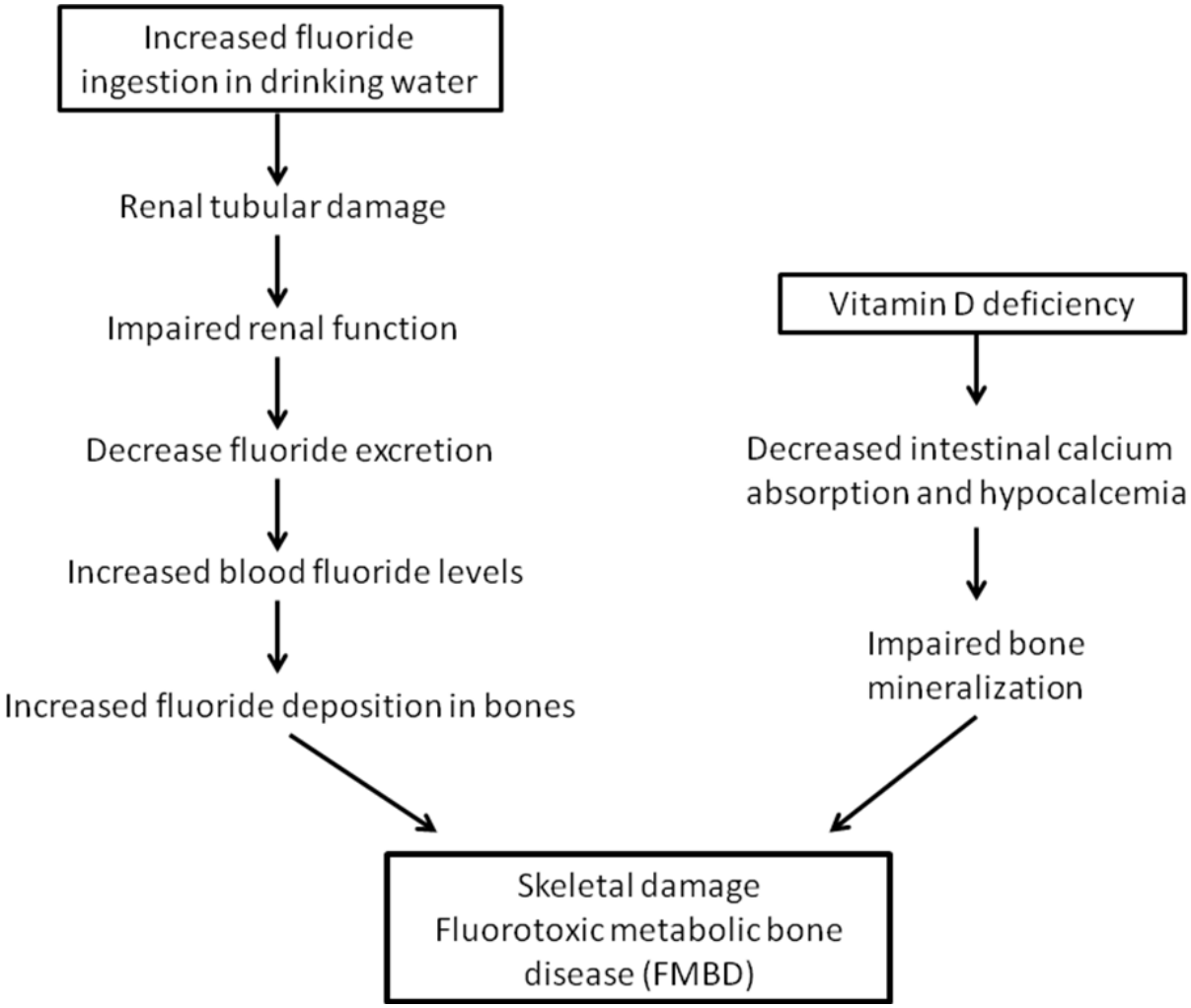


Figure 1: Schematic representation of these Hypotheses.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

Overview

Fluoride is an essential nutrient ion which arises from fluorine. This element exists in the form of a gas and is abundantly present (17^{th} most abundant element) in the earth's crust, but does not exist in its elemental state in nature. Fluorine coexists with other compounds like fluoride compounds which are the mineral constituents of rock (24). Fluoride is one among the 14 elements which are physiologically essential for normal mineralization of bones and formation of dental enamels (25). About 96% of the Fluoride in the human body is found in the teeth and bones.

In nature, fluoride exists in the ionic forms of fluorspar and rock phosphate. As it is abundant and universal in the earth's crust, all water sources contain varying concentrations of fluoride. Therefore, the major source of fluoride for humans is drinking water (26). High content fluoride ground water has been found in the geographical belts extending from Syria through Jordan to Kenya and from Turkey to China. India is situated in the fluoride belt which extends from Afghanistan, Iraq, Iran, Turkey, Japan and China (27). It is interesting to note that out of the 85 million tons of fluoride deposited in the earth's crust, 12 million tons are found in India (28). This amounts to 14% of the world's total. This throws light on the widespread and alarming problem of fluoride contamination of groundwater in India.

Fluorosis is an important public health problem in 24 countries which have a high content of fluoride in their drinking water and which lie in the geographical belt from Turkey to China (29). About 100 million people are said to be affected by Fluorosis (1). High Fluoride concentrations in ground water have also been measured in the USA, Africa, Asia as well as in India (27). Endemic fluorosis was identified as being prevalent in India since 1937,

affecting about 17 of its 28 constituent states. The states in which fluorosis is endemic have recorded drinking water levels more than 1.5 mg/L. The World Health Organization (WHO) and the Bureau of Indian standards have set the permissible maximum limit of fluoride in drinking water to be 1.5mg/L and 1mg/L respectively (6). The magnitude of the problem is alarming, as about 62 million people in India, which includes 6 million children, have developed Fluorosis, due to the consumption drinking water with high levels of fluoride. The states most seriously affected by fluorosis are Rajasthan, Bihar, Jharkhand, Punjab, Chhattisgarh, Maharashtra, Haryana, Andhra Pradesh, Karnataka and Tamil Nadu (3) **(Figure3.1).**

Intake of high amounts of Fluoride can be toxic, but on the contrary, studies have proved that daily fluoride supplementation with lower optimal amounts; enhance bone mineral deposition and acts as mitogenic stimulus to osteoblastic activity (30). Dental fluorosis (affecting enamel of teeth), skeletal (affecting bones) and non-skeletal fluorosis (affecting soft tissues) may result from long term consumption of high fluoride containing drinking water (4). Dental, renal and skeletal damage due to fluorosis are irreversible, but are preventable by appropriate and timely intervention. Although people consume drinking water with fluoride levels exceeding the safe limits set by WHO, yet not all that are exposed to exceeding levels of fluoride develop fluorosis. It is, therefore, clear that there are other factors involved in the pathogenesis of fluorosis in addition to excessive intake of fluoride containing drinking water (31).

Fluoride levels in drinking water – the Indian scenario

There have been a few studies, conducted in some of the states in North India that have assessed the fluoride levels of drinking water. The Phulera Tehsil, district of Jaipur – in Rajasthan had drinking water fluoride concentrations of 1.2- 18 ppm (32). Drinking water in the Dausa district of Rajasthan was found to have a fluoride level of 14.7ppm (33). Of the 40 villages studied in the Ladnu block of Nagaur district of Rajasthan, 31 were found to have groundwater Fluoride concentrations above 1.5 ppm. The groundwater Fluoride levels ranged from 0.5 ppm (recorded at Hudas) to 7.1ppm (recorded at Roja) (34). Drinking water in Gaya region of Bihar had measured fluoride values from 0.2 – 14.4 ppm (35), while in Durg district of Chhattisgarh the levels ranged from 0.3- 7.8 ppm (36). It was interesting to note that out of 238 villages studied in Palamau district of Jharkhand, majority had elevated fluoride concentrations which were capable of causing major health concerns. Among the villages assessed, in the Palamau district, one drinking water source had recorded fluoride level as high as 12ppm (37). Levels of Fluoride in groundwater at Yavatamal district in Maharashtra had been found to be ranging from 0.2 to 5.0ppm. (38)

In South India the scenario is no different. Studies done in 2 districts of Andhra Pradesh found the fluoride concentration of ground water to be from 0.1 – 8.8 ppm in the Nalgonda district (39) and from 0.78- 6.1ppm in the Anantapur district (40). Bore well water in the Nalgonda district was found to have fluoride levels ranging between 0.5- 5 ppm (41), and in the ground and surface water of Mysore at Karnataka levels ranged from 0.2 – 3 ppm.(42).

In the state of Tamil Nadu, many studies have assessed Fluoride levels in various districts. In Nilakotti, Dindigul district fluoride levels were found to range from 0.6-5.64 ppm (43).In the East costal area the drinking water had between 0.02- 1.54 ppm of fluoride (44). Ground

water in Tiruchendur, Tuticorin district had fluoride levels of between 0.6-1.3ppm (45).Yet another study done at Tuticorin had found levels ranging from 0.9- 4.3 ppm (46). Tiruchirappali east and west taluk recorded fluoride levels of 0.2- 2.06 ppm (47). Another study done at SIPCOT zone at Kudikadu, Cuddalore (dist.) had found the fluoride levels to be ranging from 1.1-2.1ppm (48). Palacode (dist.) and Dharmapuri(dist.) had levels of 1.4- 4.4 ppm (49) and 0.89- 2.1 ppm (50) respectively. 9 blocks of Kanyakumari (dist.) had found levels of fluoride to be between 1.5- 1.7 ppm (51).

In Vellore, a study done at the Walaja block in Palar river basin found fluoride levels to be between 0.6- 0.8ppm (52) whereas the ground water at Poongulm had levels of 0.9-3.86 ppm (53). At Sowedakuppm it was found to be 0.97-3.05ppm whereas in Panchayats of Thirupattur block and Marimanikuppam the levels were between1.04- 3.24ppm and Narasingapuram 2.3 – 4.59ppm Panchayats of Alangayam block in Vellore dist (53).

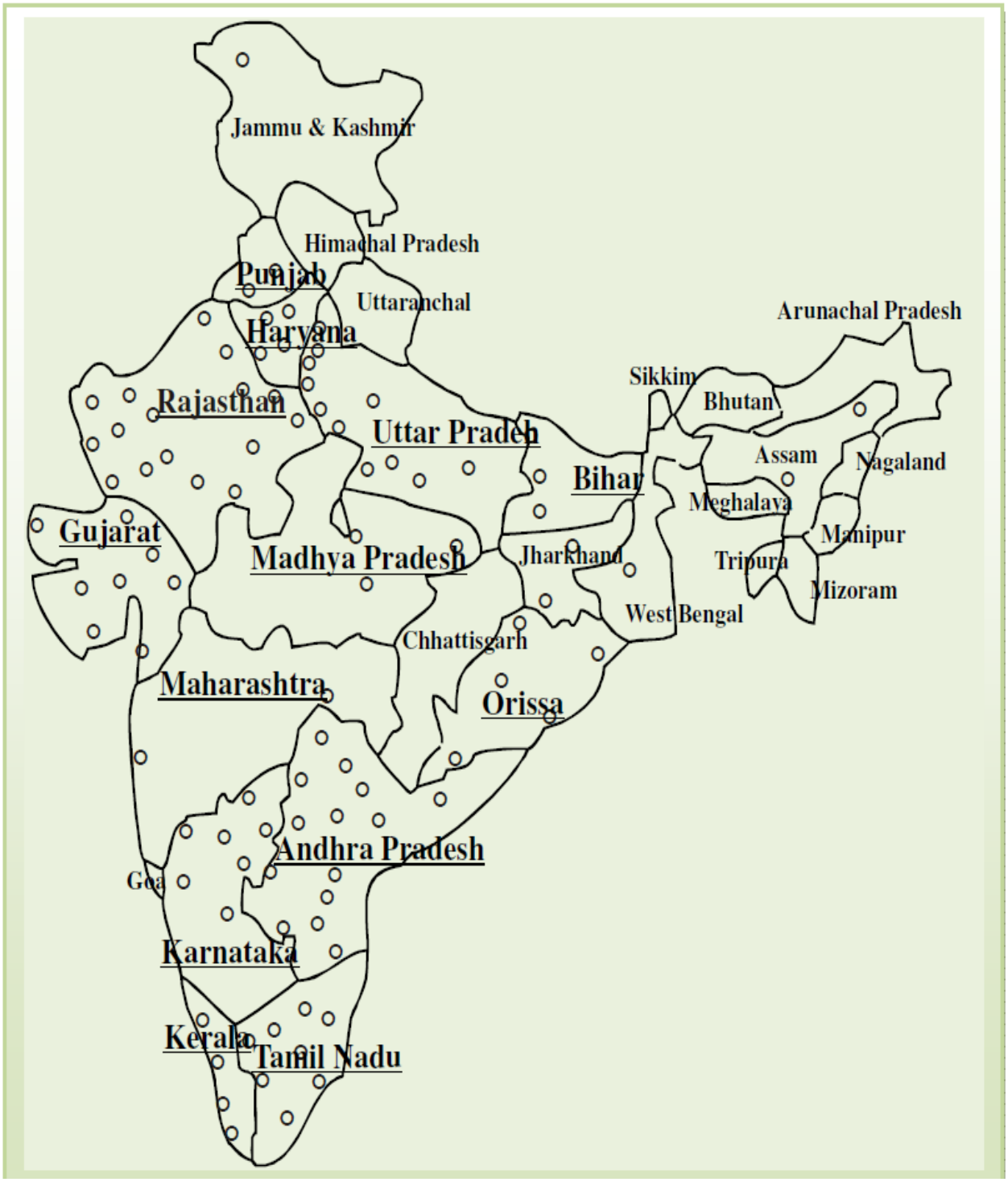


Figure 3.1: The occurrence of Fluorosis in various states underlined and districts (circles) in India. (Source: National survey performed by Teotia, 1963- 2003).

Physiology and Metabolism of Fluoride

The gastric assimilation and dispersion in the body as well as the renal excretion of fluoride are all pH dependent. When the pH is above 3.4, fluoride in the ionic form exists in the lesser proportion when compared to the undissociated hydrogen fluoride (HF) (more than 50% of Fluoride) (54). Fluoride is able to cross the cell membrane into the cells through the lipid bilayer, in the form of undissociated HF rather than in the ionic form. This is owing to the pH concentration gradient (55).

Intestinal Absorption

Water soluble fluoride in the form of sodium fluoride, silicofluoride, or sodium mono-fluorophosphate, are more readily absorbed than the less-soluble fluoride forms viz. calcium fluoride, magnesium fluoride and aluminium fluoride. However before absorption in the lower intestine sodium monofluorophosphate may require dephosphorylation (56).

Fluoride absorption (20-25%) occurs as passive diffusion in both the small intestine and the stomach. The low pH environment in the stomach enables better absorption of undissociated HF. The fluoride which is not assimilated in the stomach is then taken up in the upper portion of small intestine in the ionic form (55). The bioavailability of fluoride in various fluoride salts may be altered by 40% owing to the varied preparations (e.g. coating) (57). Other variable factors in the absorption of fluoride are the presence of cations like magnesium, aluminium and Phosphorous anions, which decrease the absorption of Fluoride. Calcium may also have an inhibitory action on fluoride absorption (58) (59) Fluoride absorption from high carbohydrate foods like rice which is inclusive or exclusive of calcium along with meat protein was associated with delayed assimilation and decreased peak of plasma

concentrations (60). Fluoride absorption from milk and milk based infant feeds can be as low as 25% due to the presence of Calcium (61). It is worthy to note that fluoride absorption can be hindered by presence of calcium from foodstuffs but not from calcium supplements (58) (59).

Fluoride in water, where it is naturally present or added as fluorosilic acid, is absorbed in proportion to the concentration present and the time required to reach the maximum plasma levels is about 0.7-0.9 hours. This is independent of the water hardness and the calcium concentration in the water but there it was observed that there were large between subject variations (62).

Fluoride assimilation is affected by various factors and a variation in absorption efficiency of fluoride from different foods exists. But out of the total fluoride ingested, only about 80-90% is absorbed (56).

Transport in Blood

Following ingestion of a single dose, the plasma fluoride concentration will peak in about 0.7-0.9 hours irrespective of the dose or the form of the fluoride ingested (62). After that the decline in the plasma levels occurs due to the uptake of the fluoride into calcified tissue (Bone) and also through excretion in the kidneys. The serum levels may return to baseline within 3 – 11 hours. The ionic form of fluoride does not bind to plasma proteins or other serum compounds and is ultrafiltrable by the kidney but its concentration in the body cannot be homeostatically controlled. The concentration of ionic fluoride is twice as high in the red cells as the undissociated form. The levels may be assessed by potentiometry or Ion Chromatography. Non-ionic form mostly consists of fat-soluble fluorocompounds that can

also be analysed by the above mentioned methods following ashing. The biological significance of the non-ionic form fluoride is still unknown (63).

Distribution to tissues

Until a steady state is established, fluoride is absorbed and rapidly dispersed by circulation both in the intracellular and extracellular fluid, which are not under homeostatic control (64). The soft tissue may contain about 1% of the absorbed fluoride and the ratio with plasma levels may be ranging from 0.4 to 0.9 among rats (65). The kidney, adipose tissue, brain and pineal gland may have varied levels of fluoride. The kidney may accumulate higher levels of fluoride, but the blood-brain-barrier is impermeable to ionic form of fluoride (56). The net influx of the fluoride ions into and exit off the cells may be controlled by a few factors which alter the pH gradient i.e. diet, drugs, physical activity levels, altitude of dwelling, or the period of an illness (55). Acidotic states may lead to higher plasma levels as there may be reduction in renal excretion. The major quantity of fluoride (40%) is retained in the calcified tissues such as the bones and teeth where it may be reversibly but tightly bound (55). In children, below 7 years of age, fluoride may be higher (by about 55%) in calcified tissues (66). Remobilization of fluoride from bone is caused by interstitial ion exchange or by remodelling and resorption of bone (55).

Elimination

Absorbed fluoride in the plasma is primarily excreted by the kidneys (45% in children and 60% in adults) (66). But in younger children and infants the amount of fluoride excreted may be only about 10-20% as the bones have a higher capacity to store fluoride (67). The renal

glomeruli filter the ionic form and are also responsible for partial reabsorption by the renal tubules owing to the pH concentration gradient. The renal clearance has been found to be 30-50 ml/ min in adults but this is reduced in the case of renal dysfunction (68) (69). The amount of fluoride in the body may be assessed by analysis of blood, bone and by urine concentrations.

Bone physiology

Bones are part of the living tissue found in our body. They possess blood vessels and are made up of cells that are capable of repair and proliferation. There are about 300 soft bones in a newborn which later fuse and become 206 hard bones in adulthood (71).

Major functions of the bone are:

- Structural support and protection of the vital organs
- Contains marrow which produces the blood cells
- Acts as a store house for minerals like calcium

There are two types of tissues that are present in bone namely:

1. Rigid outer layer known as the cortical bone which is thick and strong.
2. Inner spongy layer is known as the trabecular (cancellous) bone that is lighter and less thick in comparison to the compact bone.

Constituents of bone:

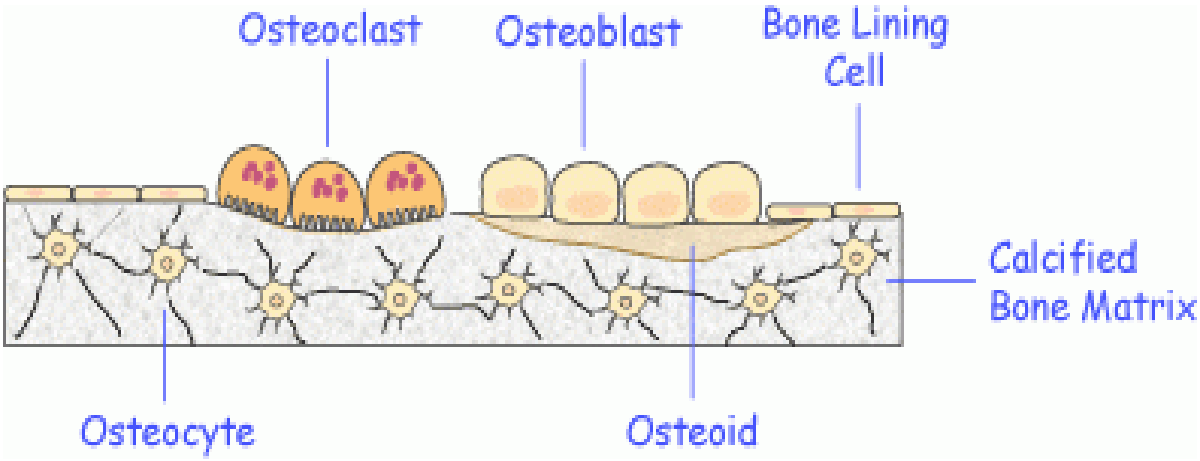


Figure 3.2: Structure and constituents of bone (70).

The bone consists of osteoblasts and osteocytes, which are the cells of bone formation. They also contain bone resorption cells called osteoclasts. The matrix consists of collagenous and non-collagenous proteins (osteoid) as well as inorganic salt deposits in the matrix **(Fig. 3.2)**

The bone cells are involved in bone formation, resorption and remodelling.

- **Osteoblasts**: The osteoblasts are accountable for the synthesis of bone matrix and further mineralization. They are derived from mesenchymal stem cells and line surfaces of the bone which are not undergoing remodelling.
- **Osteocytes:** The osteocytes are the cells situated deep in the bone matrix of the calcified bone and are included in the newly formed osteoid. The osteocytes which are

present deep in the bone tissue are in close contact with other osteocytes, osteoblasts and the bone lining cells, all through a complex system of cell processes called "canaliculi". These cells are considered to react to any change in physical force over the bone and they may pass signals to the cells lining the bone surface to initiate bone resorption.

- **Osteoclasts:** The osteoclasts are larger cells derived from the hematopoietic origin and are multinucleated. Their main function is bone resorption, and they are attached to the surface of bone where active resorption takes place.

Bone matrix:

The osteoid comprises almost 94% of type I collagen and the non-collagenous proteins. The hardening of bone occurs when the osteoid matrix contains the mineral salts which are the crystalline complexes of the phosphorus and calcium (hydroxyapatite). The calcified bone consists of the 25% of organic matter, 70% of inorganic minerals (hydroxyapatite) and only 5% of water.

Bone growth and development

During life, bone grows both longitudinally and circumferentially. The longitudinal growth of the bones takes place at the growth plates and the proliferation of the cartilage occurs at the epiphyseal and the metaphyseal areas of long bones. The process of bone formation is known as "osteogenesis". This consists mainly of two processes:

i. The replacement of the connective tissue membranes with bone tissue, results in formation of flat bones. This is known as intramembranous ossification.

ii. The Endochondral ossification involves the bone tissue replacing the hyaline cartilage resulting in the formation of long bones. These bones may continue to grow through childhood and the adolescent age and increase in length and circumference.

Bone modelling:

When bone formation and resorption occur on separate surfaces it is known as 'bone modelling' which may occur from childhood through the adulthood. Bone shape changes in response to the physical forces and the physiological factors which leads to the gradual adjustments occurring within the bone. Biochemical influences may influence the independent actions of bone addition and removal by the osteoblasts and osteoclasts. It has been observed that during bone modelling bone resorption and bone formation do not occur simultaneously. Bone modelling occurs less frequently than remodelling in adults. Bone modelling may be influenced by pathological influences of hypoparathyroidism, renal osteodystrophy or in treatment with anabolic agents (71).

Bone remodelling:

Bone remodelling occurs by replacement of the old bone tissues with the new bone tissue. This in turn renews the bone strength and mineral homeostasis of bone. This process occurs continuously, from birth to death, to prevent microdamage by resorbing the old bone and forming new bone (72). This occurs mainly to maintain the bone mass via osteoblasts and the osteoclasts. Bone formation and bone resorption occurs in the following phases:

- **Activation:** in this phase pro-osteoclasts are influenced by the cytokines and growth factors to become mature active osteoclasts. The pro-osteoclasts bind to the bone matrix through the interaction of the integerin receptors and the RGD contain peptides in the bone matrix proteins (arginine, asparagine and glycine)

- **Resorption**: the old mineral matrix is digested. This phase may take about 2-4 weeks during the remodelling cycle.

- **Reversal:** resorption ceases in this phase and bone formation commences through the presence of the monocytes and osteocytes present at the bone formation sites. This area has still to be researched extensively but it is known that the osteoclasts resorb the bone and new bone formation may be influenced by the combined action of the bone matrix derived factors (TGF-β, IGF-1, and IGF-2), bone morphogenetic proteins and fibroblast growth factor.

  TGF-β is released from the bone matrix, and by inhibiting the release of receptor activator of NF-κB ligand (RANKL) from osteoblasts, it reduces the bone resorption. This presence of TGF-β may be indicated by the increased serum osteocalcin and bone specific alkaline phosphatase

- **Formation:** Osteoblasts act to synthesize the new bone tissue. This may take about 4 to 6 months to complete. New collagenous matrix is formed by the osteoblasts, which also regulate the mineralization of the bone by releasing calcium and phosphate concentrated by bound matrix vesicles. When the bone formation is over, about 50-

70% of the osteoblasts undergo apoptosis and the remainder are converted into osteocytes.

- **Quiescence:** In this phase osteoblasts lining the bone surface remain dormant although they may still regulate the influx and efflux of the mineral ions into and out of the extracellular fluid.

Bone remodelling regulates the addition and loss of bone mineral density which affects bone strength directly. The ratio of the receptor activation of the NF-kB ligand (RANKL increasing bone resorption) to the osteoprotegerin (give rise to osteoblasts) may regulate this cyclic process.This process has been found to be influenced by the presence of the PTH, Calcitonin, colony stimulating factor, (CSF) and 1, 25-dihydroxyvitamin D.

Fluoride and its effect on bone metabolism

Fluoride anion is thought to replace hydroxyl group in hydroxyapatite crystals which may cause alteration in the crystalline structure of bone (73). In a study performed among rats, it has been found that in the presence of fuoridated hydroxyapatite crystals there is an increase in osteoblast cell attachment, its proliferation and differentiation in the osteosarcoma cells of these animals (16). Fluoride has been found to stimulate bone formation at the cellular as well as at the tissue level (30, 74). This results in positive outcomes among osteoporotic patients, where there may be a positive balance in the bone remodelling cycle and better trabecular connectivity (75). It has also been demonstrated that fluoride causes positive calcium balance among osteoporotic patients. In contrast, excessive fluoride affects bone mineralization causing excessive osteoid formation and incomplete mineralization (76). Rats exposed to varying levels of Fluoride were studied, and it was observed that serum ALP and Bone ALP levels (osteoblast surface marker and bone formation marker) were increased in the group exposed to high (150 ppm) levels of fluoride in drinking water in comparison to control group rats which were only treated with low fluoride containing drinking water (77). Similar findings were found among human subjects in the same study. This study strongly suggested that the viability of osteoblasts is affected by the accumulation of Fluoride in bone. This may be because of role of fluoride in promoting osteoblast mitosis. This is suggestive that the accumulation of Fluoride in bone may promote osteoblast mitosis, and thus accelerate bone turnover.

Serum Osteocalcin, is a reliable indicator of changes in bone metabolism, which is released into the blood during bone formation. Animal experiments have shown that when rats were exposed to low doses of Fluoride there were no significant changes in bone osteloblast activity. However after they were treated with high (150 ppm) doses of Fluoride for a period

of 90 days, there was a considerable increase in the osteocalcin levels suggesting an increase in the osteoblastic activity. In contrast, levels of serum osteocalcin among rats treated with very high levels of Fluoride (400 ppm) over a period of 30 and 90 days had decreased significantly (77). This may be explained as the inhibitory effect of high doses of fluoride on the bone osteoblasts.

Another group was studied for the role of fluoride on the osteoblastic cell activity by using Fluoridated hydroxyapatite (FHA) discs (0 - 0.0577mol F-/mol apatite) to culture the osteosarcoma cells of the SAOS-3 rats. Their cell proliferation, attachment, differentiation and morphology were studied. Pure Hydroxyapatite (HA) discs were used as control in the study. It was observed that there was more cell attachment, proliferation and higher alkaline phosphatase activity present among the cells cultured on the FHA discs when compared to the HA discs. Thus, suggesting that fluoride ions have significant effect on osteoblastic cell activity (16). The in vivo effect of Fluoride on osteocalcin levels has also been studied in7 healthy male humans, following the administration of sodium fluoride for 3 weeks the fasting serum calcium, phosphorus ALP, 25-OHD, PTH and osteocalcin were assessed. These biochemical parameters did not show any change but, the osteocalcin levels were found to be higher preceding the administration of sodium fluoride (78,79) . This study suggested that administration of fluoride increases levels and osteocalcin and stimulates osteoblastic activity, thus accelerating bone turnover.

Fluoride and its effect on Bone mineral density

There has been no clear evidence of the role of fluoridation and its effect on BMD. The BMD of 24 young women from Regina, Canada, consuming drinking water with fluoride levels 0.1 ppm was lower than 33 women from Saskatoon who were consuming drinking water containing higher levels of fluoride (1.0 ppm) (80).Thus reiterating that, water fluoridation does have a positive impact on the BMD (axial spine) of young women. The effect of elevated levels of fluoride in drinking water on the BMD was studied in more detail, using dualphoton absorptiometry of the lumbar vertebrae in 23 subjects consuming large amounts of mineral water with high levels of fluoride (8.5 ppm). For over a period of 5 years the subjects consumed about 0.75 litres every day. It was interesting to note that the BMD was higher in these subjects than in the control group. Thus, it may be inferred that BMD and levels of fluoride in drinking water are positively correlated. (81).

Among naturally fluoridated drinking water areas and neighbouring areas of Taiwan, 248 women above the age of 40 years were assessed for BMD. After assessing the lumbar spine, it could be concluded that the women living in areas where drinking water contains low levels of fluoride (<0.6 ppm) had slightly decreased bone densities when compared to those ingesting drinking water with fluoride levels of >0.6 ppm (82).

A population based study, assessed BMD of the neck of femur and spine among 3,222 perimenopausal women. Fluoridated water (1.0- 1.2 ppm) was consumed among 969 women for a period of 10 years and were compared to the women (n=2,253) consuming lower fluoride containing drinking water (<0.3 ppm). There was a significant higher BMD among the women consuming higher levels of fluoride in drinking water than the women consuming lower levels of fluoride. Considering other factors like age, weight, physical activity, menopausal status and dietary intake of calcium, it was found that there may have been a

slight increase in the axial BMD in women consuming higher levels of fluoride in drinking water (83).

While assessing human vertebral bone for the strength, porosity, and mineralization in relation to fluoride content, another study (84) found that increased mass of bone was correlating with increased levels of fluoride. A radiographical examination of the vertebrae of 80 male subjects was done to examine the consequences of increased fluoride on the bone. With rising levels of fluoride, the bones were less porous and less mineral per unit of bone was present which were suggestive of Osteomalacia like changes, as opposed to the changes observed in Osteoporosis. There was no apparent change in the bone strength with higher fluoride levels. Some other-studies (84) have suggested on the contrary that with increased mineralization, caused by fluoride, there may be a greater occurrence of brittle bone disease which may predispose to fractures. Thereby, fluoridation is believed to increase the bones mass by improving the mineralization but may not influence the bone strength. Thus, predisposing individuals with higher exposure to fluoride, to a greater risk of developing fractures.

Effect of Fluoride on the Kidneys

Blood fluoride levels are principally determined by the rate of excretion of fluoride in urine, viz. renal function (85). It has been shown that blood fluoride levels increase significantly in the impaired renal functioning (86). However, a review of the current literature shows that the relationship between fluorosis and renal function has not been clearly evaluated and published studies are few. A few case reports have shown that chronic fluoride intoxication affects renal function. In a study among 40- 60 year old residents of areas endemic for fluororis at Southern Algeria, where the water fluoride content was around 4.5 ppm, the subjects were shown to be prone to renal dysfunction manifested as impaired tubular function with a slight decrease in glomerular filtration rate (GFR) (87). In another study, patients with fluorotoxic metabolic bone disease due to chronic fluoride intoxication showed severe renal tubular impairement and reduced GFR (8). Autopsy findings in one of these patients showed atrophy of the tubules and loss of nephrons with resultatnt glomerular changes.

Children who drink water containing fluoride more than 2 ppm were found to have increased levels of N-acetyl-β-D-glucosaminidase (NAG) and glutathione –S- transferase (GST) in urine. Both of these enzymes are markers of renal tubular damage, (88). A case study has also suggested a probable link between ingestion of fluoride-rich mineral water (8.5 ppm of fluoride) over long periods and the development of end stage renal disease (89). Fluoride-induced renal dysfunction can result in increased blood fluoride levels, which in turn can lead to further renal damage, thus, setting up a vicious cycle. However, this hypothesis has not been fully investigated.

A study done in an endemic fluorotic area of the Punjab in 1963 had found that 28 out of 409 cases had blood urea levels ranging from 15- 50 mg/100ml with an average of 33mg/100ml (90). Six patients had a urea clearance test out of which five were found to have decreased

urea clearance signifying renal dysfunction. An increase in the ratio of urinary phosphate concentration to serum levels was observed in renal insufficiency. In an average of 67 cases studied, significant aminoaciduria was found to be present in 4 cases. Existence of aminoaciduria along with mild increase in blood urea, impaired urea clearance, and a high phosphorous ratio imply mild disturbances of the renal function.

The role of calcium and sodium in presence of high levels of fluoride and its effect on kidneys was assessed. Dose dependent calcium and sodium in drinking water in conjunction with high fluoride has been found to be the major reason for chronic renal failure (CRF) in the tropical regions of Sri Lanka (91). The urea-Creatinine-Fluoride clearances among 25 fluorotic patients and on 10 healthy non-fluorotic subjects, were found to be low than the controls. Overall these studies seem to indicate long duration of exposure to toxic levels of fluoride intoxication leads to glomerular dysfunction humans (92).

At the Medical College, Patiala in Punjab, 25 cases of fluorosis (radiologically confirmed) were studied for kidney function. Tests showed a significant reduction of creatinine clearance and structural abnormalities in some of the patient's kidneys was also described. However no significant renal tubular changes were demonstrated by either water loading or water deprivation test (93).

Role of Fluoride in kidney damage- Animal experiments

One study in 2013, used male rats, recently weaned, to assess the risk of kidney injury following ingestion of 15ppm and 50 ppm of fluoride through drinking water over 40 days. This study used early and sensitive biomarkers of renal tubular damage and found a considerable increase in urinary beta 2 microglobulin (dose-dependent) and cystatin C while

there was increase Osteopontin (OPN), clusterin (Clu) and kidney injury molecule (Kim-1), clusterin (Clu), osteopontin (OPN) observed. There were also an increased mRNA expression levels of above mentioned tubular markers. The effect of fluoride on renal tubular structure and histopathologically confirmed tubular damage could be appreciated (94).

In an experiment in 2002, Fluoride doses of 5mg, 10mg, 20mg and 50mg/kg body weight per day were injected into young albino rabbits for 15 weeks and they were then sacrificed. At a higher dose (20mg and 50 mg) cytoarhitechture of the kidney exhibited an increasing amount of cloudy swelling, tissue necrosis with tubular epithelial degeneration, extensive vascularisation in renal tubules, hypertrophy, atrophy of glomeruli, exudation, interstitial edema and interstitial nephritis (95) Another group used young pigs and randomly divided them into three groups which received same basal diet but were additionally supplemented with 0mg, 100mg, 250mg of Fluoride for 50 days. Severe renal histological changes were observed under light microscopy Atrophic glomeruli, glomerular capsule dilatation, tubule dilatation and severe tubular leakage was also seen with an increase in serum urea nitrogen and creatinine (96).

In a study done among mice in 1980, 100 adult male albino mice were separated into three groups. Firstly, group A were fed 10ppm of Fluoride, Group B with 500ppm and Group C with 1000 ppm for period of three months. Cloudy swelling of renal tubular cells was most consistently observed in the kidneys of mice. Glomerular atrophy, tubular cell necrosis and areas of interstitial infiltration of round cells were found among the high dose group B and C (97).

In the 1960's, a study evaluated Wistar rats fed with 50 ppm and 100 ppm of Fluoride in drinking water. After 6 months, 2 of the 12 rats which received 100 ppm fluoride showed significant renal tubular dilatation at the cortico-medullary region of kidneys (98).

The question which remains is whether chronic excessive fluoride intake causes renal damage or whether the systemic fluorosis was caused by impaired renal function. As the researcher has found only a few studies regarding the role of Fluoride in causing tubular damage, this present study is designed to throw light on this very important question.

Vitamin D – its metabolism and functions

Vitamin D is a fat-soluble Vitamin (99) which has two forms namely Vitamin D_2 (Ergocalciferol) and Vitamin D_3 (Cholicalciferol**) (Fig 3.3).** Vitamin D_2 is end product of the UV light radiation of the ergosterol (steroid mostly found in fungi) (100). 7-dehydrocholesterol in the skin absorbs the ultraviolet B radiation from the sunlight during exposure and is converted to form Previtamin D_3. Due to its unstable nature, Previtamin D_3 gets rapidly converted to Vitamin D_3. It is then expelled out of the skin cells into the extracellular space where it is captured by the Vitamin D-binding protein into the dermal capillary bed (101) (102). The efficiency of synthesis of Vitamin D depends on the amount of UVB photons penetrating the epidermis, but melanin pigmentation (103) and the use of sunscreen lotions both absorb the UVB photons efficiently. Thus, resulting in decline in production of Vitamin D_3 by almost 90% (104). Even with excess exposure to sunlight there is no chance for Vitamin D intoxication as any excess Vitamin D_3 produced is destroyed (105).

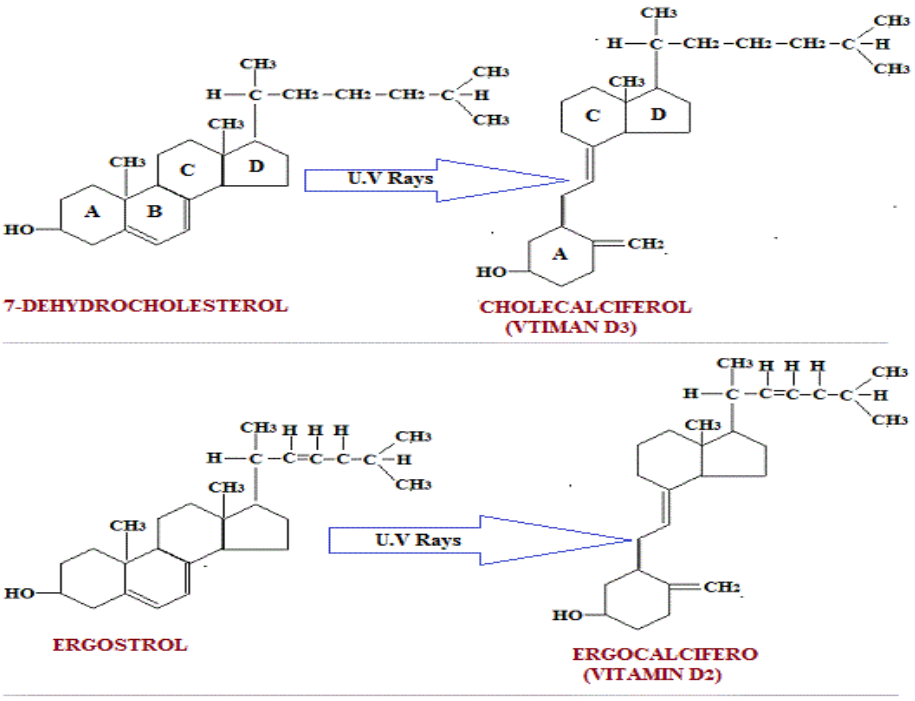


***Figure 3.3*:** Biochemical structures of Vitamin D_2 and Vitamin D_3

The food sources of Vitamin D_2 and Vitamin D_3 are fortified foods (dairy products, orange juice and soya milk) and fish (100). Vitamin D_2 and D_3 are present in the chylomicrons and are assimilated into lymphatic system. There they then bind to Vitamin D binding protein (DBP) and lipoproteins after entering into the plasma (106) (107). The Vitamin D is then released from the DBP into liver where it undergoes hydroxylation through the action of enzyme Vitamin D-25-hydroxylase and becomes 25-OHD. This is the form found commonly circulating in the body where its measurement is used to assess the individuals Vitamin D status. Its measurement may also be useful in diagnosis of secondary hyperparathyroidism, rickets and osteomalacia. The half-life of 25-OHD is 2 weeks. The complex of 25(OH) D bound to DBP then binds to megalinin at the plasma membrane of renal tubule cell and is then move into the cell (106) (107)

Once inside the renal tubular cell, 25(OH) D is acted upon by the enzyme 25-hydroxyvitaminD-1α-hydroxylase to form 1, 25-OHD. This biologically active form is responsible for maintaining homeostasis of calcium and phosphorus. This is accomplished when 1, 25-OHD interacts with its receptor, Vitamin D receptor (VDR) in the small intestines. The complex between the 1, 25-$(OH)_2$ D_3 and VDR then combines with the retinoic acid X receptor. In turn, this complex then binds to the Vitamin D responsive element (VDRE) for the epithelial calcium channel. The expression of the calcium channel allows more calcium to enter the cell. In the cell, the Vitamin D dependent calcium-binding protein, (calbindin 9K), may assist in relocation of calcium into the blood. $1,25(OH)_2D_3$ also enhances the phosphorous absorption from the small intestine (108) **(Fig. 3.4)**

Though the main effect of Vitamin D is the deposition of minerals in the osteoid of the bone this direct effect cannot be differentiated from the indirect effects. In some studies it has been shown that the increase in both the cortical and the trabecular bone coincided with the over expression of VDR in the mature cells of osteoblastic lineage(109).This animal model used osteocalcin based promoters which were expressed in the later stages of osteoblasts and osteocytes as well as the hypertrophic chondrocytes. It is indeed interesting to observe that osteoblast over expression resulted in an proliferation in bone formation and a decline in bone resorption. But the above mentioned osteoblastic cells had less capacity to activate functional osteoclasts. Other studies (110) have also found that in vivo, there were fewer osteoblasts and lesser mineral apposition. Which allows us to conclude that, atleast in the later stages of osteoblastic development, VDR does show positive effects of increased bone formation and reduction in bone resorption.

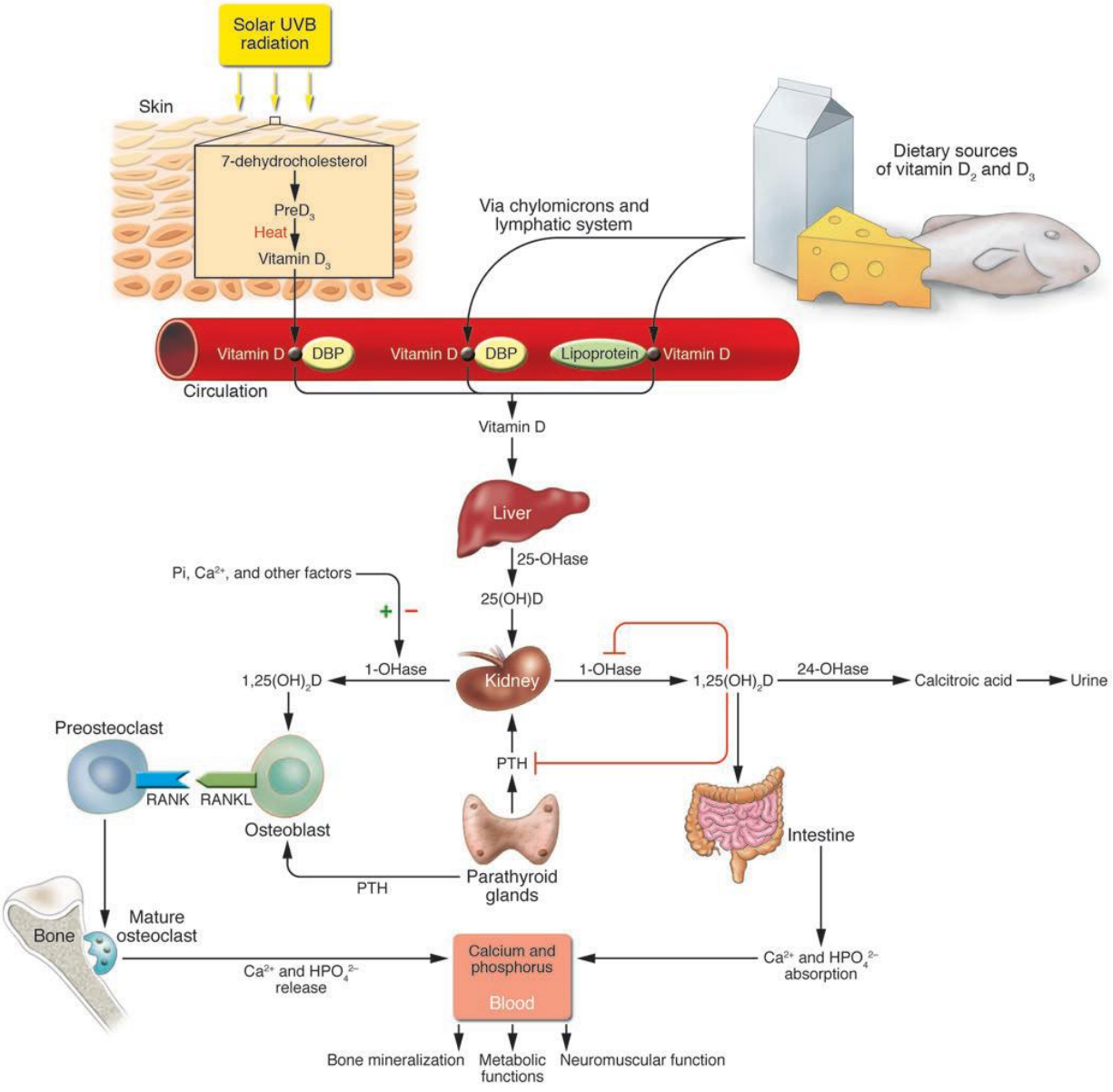


Figure 3.4: Action of Vitamin D in the maintenance of calcium and phosphorus homeostasis (Source: Holick, 2007)

As earlier stated, Vitamin D plays a vital role in calcium homeostasis. Its interaction with VDR at the osteoblasts induces plasma membrane protein receptor activator of NF-kB ligand (RANKL). Preosteoclasts become mature osteoclasts when the RANKL binds with RANK on the plasma membranes of preosteoclasts. Hydrochloric acid is released from the mature osteoclasts as collagenases act upon the bone by dissolving it and allowing calcium and phosphorous to be released into the blood circulation (111).

Thus Vitamin D has an important function in maintenance of serum calcium and phosphorus levels. In doing so it also supports bone mineralization, neuromuscular transmission and many metabolic functions. Vitamin D deficiency may cause Rickets and also prevents the adequate absorption of calcium and phosphorous. Only about 10-15% of dietary calcium and 50-60% of dietary phosphorous is absorbed in a Vitamin D deficient state. The calcium levels slowly decrease in the body. In response, parathyroid hormone (PTH) is released from the parathyroid gland. PTH then acts by increasing calcium reabsorption in both the proximal and distal convoluted tubules. In a similar manner to PTH, 1,25(OH)2D also increases the expression of RANKL on the osteoclasts thus improving production of osteoclasts, and mobilizing the stored calcium from the bone (112).

Vitamin D deficiency

Among the Indian population, the main source of Vitamin D is through exposure to the UVB light from the sun. Some of the risk factors in developing Vitamin D deficiency are listed below:

- Lack of exposure to sun due to lifestyle changes
- Darker skin colour
- High levels of pollution
- Overcrowded residences
- Not consuming Vitamin D containing foods

A study was carried out among 5137 apparently healthy schoolchildren (aged 10–18 years) in urban New Delhi, of whom 3089 (1079 boys, 2010 girls) were from the lower socio-economic status group (LSES group), and 2048 (968 boys, 1080 girls) were from the Upper socio-economic group (USES group). Hypovitamosis D occurred more among females (41.6%) than in males (27.4%). Hypovitaminosis D was seen in majority (92.6%) of the LSES group (severe: 11.2%; moderate: 39.5%; and mild: 42.1%) when compared to the USES group (84.9%) (severe: 4.9%; moderate: 25.5%; and mild: 57.6%) (113). Thus, concluding that there is a high occurence of clinical and biochemical hypovitaminosis D even among healthy school-going children.

Vitamin D deficiency among the healthy children ranging from age 3 months to 12 years from the upper socio-economic background attending the OPD of private Paediatric hospital was studied at Chandigargh (114). This study was conducted among 338 children (188 boys and 150 girls) and 40.2% were found to have Vitamin D deficiency, 25.4% were Vitamin D insufficient whereas only 34.2% had sufficient levels of Vitamin D. This study also found

that clinical signs of Vitamin D deficiency were observed among only 8.53% and the mean Vitamin D level was 16 ng/ml.

A cross-sectional study conducted in Northern India (115) found that adolescent girls from rural low socio-economic background had 88.6% incidence of Vitamin D deficiency among 121 adolescent girls. It is also known that seasonal variations significantly influenced the Vitamin D levels among the subjects.

A study to assess the prevalence of Vitamin D deficiency among the school-children in South India was conducted in the paediatric outpatient department (OPD) in a tertiary care teaching hospital situated in sub urban metropolitan area of Chennai over a period of nine months. The occurence of Vitamin D deficiency was 37.4% whereas Vitamin D insufficiency was seen in 24.8% of the subjects. The nutritional status of the children was considered where it was found that 34.3% of children were underweight, 58.3% had normal nutrition and 7.4% were found to be overweight. The clinical vitamin D deficiency was present only among 3% children. Female children had a greater risk of vitamin D deficiency and more so among the adolescents. Obese children and those who belonged to the upper socio-economic status were found to have a higher incidence of Vitamin D deficiency (116).

Another cross-sectional study done to study Vitamin D deficiency in the north-west population, had recruited 150 healthy male and female subjects. A fasting sample was collected to assess the levels of 25-hydroxy vitamin D (25(OH) D) levels. The overall prevalence was 90% .Of those with Vitamin D insufficiency, 96 (94.12%) were urban subjects but only 39 (81.25%) were rural subjects (117).

57 subjects from the rural area of North India were assessed for the presence of 25 OHD deficiency even after abundant exposure to sunlight. Mean values for 25OHD ranged from 36.4+/- 22.5 nmol/L. Males had higher values than females and 70% were Vitamin D deficient (118).

At Delhi, a study had assessed Vitamin D status among the 50 years and above population (119). A total of 1346 healthy subjects were assessed and then divided into two groups. One had individuals from age of 50 to 65 years and the second had individuals above 65 years. Vitamin D deficiency was found in 50% of the population studied.

Among south Indian population, a study aimed at studying serum calcium and Vitamin D deficiency among healthy south Indians. They assessed 191 rural and 125 urban subjects. Fifteen per cent wereVitamin D deficient, 54% Vitamin D insufficient with only31% having normal Vitamin D values (120).

Studies among the postmenopausal women (n=164) of South India examined parameters including their daily dietary calcium intake, phytate to calcium ratio and other bone mineral parameters. About 82% of the subjects had varying low levels of 25-OHD (121).

Vitamin D deficiency and its effect on bone mineral density

Vitamin D hypovitaminosis has been shown to alter calcium metabolism, matrix ossification, bone remodelling and oteoblastic activity. Thereby, affecting the bone mineral density (122).

A literature review of the Vitamin D status and bone mineral density among the Chinese population (123) found 293 studies of which 11 research studies were selected. A wide range of serum 25-OHD levels were found and the mean serum Vitamin D was found to be in the range of 29-82 nmol/L. There was a relationship between the 25-OHD concentrations and BMD but these results were not conclusive for the middle aged and elder population in China.

In another study at done South India, (124) among women of reproductive age (n=55) and post-menopausal women (n=136), they studied the 25-OHD status and BMD. The other biochemical assessments done were serum calcium, phosphorous, albumin, alkaline phosphatase, creatinine, 25-OHD and intact PTH. After studying the women over a period of 1 year, it was found that 76% of them were 25OHD deficient (<20ng/ml), 70% were 25OHD insufficient (20-30ng/ml) and 7% had replete levels of 25OHD (>30ng/ml). On assessing their BMD, it was found that in comparison to women of reproductive age group the postmenopausal women had lower BMD at the hip trochanter, forearm, lumbar spine antero-posterior and lateral aspects. The majority of the osteoporotic changes were noted among the postmenopausal women at different sites. There was no association` between the biochemical indices and the BMD but it was noted that Vitamin D deficiency coexists lower BMD in women. In these subjects preventive measures such as Vitamin D and calcium supplementation may be required.

Lower levels of 25(OHD) (coexisting with secondary hyperparathyroidism and increased bone turnover) have been associated with incidence of osteoporosis. On the contrary, adequate levels of Vitamin D have been shown to prevent fractures due to osteoporosis. A

study done among 400 osteoporotic patients(125) revealed that the Bone Mineral Density (BMD),which is the gold standard technique to investigate osteoporosis, was lower among the patients with coexisting 25-OHD insufficiency and deficiency when compared to patients having normal levels of 25-OHD. In addition there was a positive correlation between BMD and 25-OHD and a negative correlation with the parathyroid hormone level, which were statistically significant. This study proposed effective management of low bone mass involving treatment of hypovitaminosis D.

Further research has been carried out which reinstates the relation between Vitamin D, parathyroid hormone and BMD. A study done in Syria (126), assessed 25-OHD and PTH levels among 156 healthy adults aged between 18-53 years. DEXA scan of the lumbar spine and hipwas done to derive the BMD. Almost all the participants had 25-OHD levels <30ng/ml and 89.1% had values less than 20 ng/ml. It was found that secondary hyperparathyroidism was more prevalent among the subjects with decreased levels of 25-OHD than the subjects with increased levels of 25-OHD. The BMD was found to be lower among Syrian subjects when compared to Caucasian from Europe and North America.

In northern India, 5137 school going children were assessed from all strata of society ranging from the age 10 yrs to 18 yrs. (127). After assessment it was found that almost 10.8% school-going children are vitamin D deficient and there was a significant relationship between the socio-economic status and the vitamin D deficiency. The other biochemical parameters assessed were the serum calcium, inorganic phosphorus, alkaline phosphatase and immunoreactive PTH measured among 760 randomly selected children. The BMD of the forearm and the calcaneum were assessed among 555 children using a DEXA scan. The children from the upper socio-economic status had a higher mean 25-OHD value of 13.7± 0.4 ng/ml but the children from the lower socio-economic status had lower concentration with mean 10.4±0.4ng/ml. Very low (<9ng/ml) concentrations were observed among 35.7% of the

children. It was also found that the boys had higher levels of 25OHD when compared to girls. It is interesting to note that there was a negative correlation existing between the serum PTH and serum 25-OHD.

Reviewing the relation between 25OHD, PTH and BMD among the Indian population in Northern India, 1829 adolescents and 1346 adults (50years and above) were assessed. About 30-40% had moderate and severe Vitamin D deficiency. It could be concluded that lower BMD was found among individuals with decreased PTH levels but there is ambiguity about the role of Vitamin D in the same (128).

A cohort study done among Indian population was carried out to find the correlation of Vitamin D, BMD and PTH among adults with low BMD (129). A total of 102 patients among which 38 were male and 64 were female, with mean age of 62.5±6.4 years were assessed. 58 patients were diagnosed with Osteoporosis whereas 44 patients had osteopenia. The mean serum 25-OHD was found to be 21.3±0.5ng/ml and the mean iPTH was found to be 53.1±22.3 pg/ml. Vitamin D deficiency was confirmed among 84.3% of patients with serum 25-OHD levels below 30ng/ml. Though the iPTH levels, body mass index, gender and age were found to be significant predictors of BMD, yet no significant association was established between 25OHD level and BMD. A negative correlation exists between Serum iPTH and BMD at the lumbar spine and hip. This reiterates the important role of Vitamin D and PTH in the bone metabolism.

Yet another study assessing 110 Indo-Asian patients at a general rheumatology clinic, (130) had found that 77 of the patients were Vitamin D deficient and 33 had associated secondary hyperparathyroidism. DEXA scan was performed to assess the BMD at the femoral neck, lumbar spine, distal radius and radius. It was concluded that decreased bone mineral density was significantly associated with Hypovitaminosis D which is complicated with secondary hyperparathyroidism.

Fluorotoxic Metabolic Bone Disease and Vitamin D deficiency

There is a dynamic exchange of fluoride between the blood and the bone. The rate of incorporation of fluoride into the hydroxyapatite crystals of bones depends on the circulating fluoride levels (85). Toxic manifestations of excessive fluoride ingestion on the bone are collectively called as fluorotoxic metabolic bone disease (FMBD). There are two types of FMBD (131). One is an endemic variety that affects the elderly, and is characterized by new bone formation, musculoskeletal dysfunction, arthritis, peripheral neuropathy, ankylosis of the spine with radiculopathy and osteosclerosis with ligament calcification (28). The other affecting children predominantly, causing bone deformities like genu valgum and bowing. Radiologically, the children's bones show a mixture of osteomalacia, osteosclerosis and osteopenia (132) (10).

Vitamin D deficiency is very common in India and it has been suggested that dietary vitamin D deficiency may render individuals more susceptible to FMBD (133). In a study in Delhi 24 patients with FMBD were assessed and osteo-renal syndrome (71%) found to have a Vitamin D deficiency (8).

A case-control study to assess for deformities of bone among young children caused by Vitamin D deficiency and Fluorosis was done at Bihar (11). This study examined children from two villages between ages of 1.5 – 14 years. Their mean serum fluoride concentration was 7.9 ± 4.1ppm while that among the control group was 0.6±0.3 ppm. The urinary fluoride levels among the children with bone deformities were found to be 20 ± 17.4ppm and in those without deformity it was 15.5 ± 10.6 ppm. In the control group the urinary fluoride levels ranged from 2.4 ± 0.8 ppm. Vitamin D levels were lower in both groups, i.e. those with deformity (14 ± 8.1ng/ml) as well as those without deformity 22 ± 5.1ng/ml compared to control group values of 43 ± 28ng/ml.

In children, other factors than drinking water fluoride levels, have been found to affect the occurrence and severity of FMBD. The increased incidence of this syndrome among children belonging to the lower socioeconomic strata is suggestive of an important role of under-nutrition on fluoride-induced toxicity (134). It has been shown that the toxic effects of fluoride were severe and more complex among children with concomitant calcium or Vitamin D deficiency (17).

Calcium deficient children in areas where fluorosis is endemic showed evidence of secondary hyperparathyroidism, bone resorption and increased fluoride deposition in bones. It is suggested that fluoride exacerbates the clinical manifestations of rickets. Similarly, marginal calcium deficiency may also exacerbate the syndrome of FMBD in children living in areas where fluoride content in drinking water is high (135).

Among adults, a study showed that one out five patients with fluorosis had low serum calcium and subnormal levels of both 25-OHD3 and 1,25diOHD3 concentrations (37). The course of Fluorosis has also been found to be more severe and complex in patients with severe calcium and Vitamin D deficiency (20). In fact, observational studies have also shown that calcium and Vitamin D supplementation can reverse dental and clinical fluorosis in children (136).However, the role of Vitamin D deficiency in the pathogenesis of this condition is not yet known.

Therefore it may be concluded that the level of daily fluoride intake, duration of stay in an endemic fluorotic area, level of calcium and Vitamin D intake and the nutritional status of an individual, can all influence in the clinical course of Fluorosis.

4. SCOPE AND PLAN OF WORK

Scope

- This study will help establish IC as a reliable method in analysis of water fluoride levels in the various health care settings.
- This study will help in measurement of fluoride levels in drinking water to ensure availability of safe drinking water for human consumption and help in prevention of FMBD
- This study will help to understand in detail, the role of Vitamin D deficiency in the pathogenesis of FMBD
- This study will also help to study the effect of excessive intake of fluoride on the bone and kidneys with co-existing Vitamin D deficiency.
- This study will assist in discovery of novel methods in treatment of FMBD and help prevention of disabilities caused by the same.

Work Plan

- Standardization of the IC method – December, 2012 to June, 2013
- Collection of water samples from the villages in and around Vellore district- June, 2013- February, 2015
- Comparison of IC with ISE method- June, 2015 to July, 2015
- Selection of the Sprague-Dawley rats for study.- 2014
- Developing Vitamin D deficiency in the rat model- June, 2014- October, 2014
- Administration of fluoridated drinking water to the control and Vitamin D deficient rats. –October, 2014

- Assessing the whole body DEXA scan and the biochemical parameters- February, 2015to October, 2015
- Submission of Synopsis- April, 2016
- Thesis submission – July, 2016

MATERIALS AND METHODS

5. MATERIALS AND METHODS

I. Fluoride estimation in drinking water

- **Fluoride estimation in drinking water**

Water samples were collected from 165 randomly selected villages in Vellore district around Ranipet, Arcot, Ambur, Vaniyambadi, Gudiyatham, Alangayam, Jolarpet, Katpadi and Kaniyambadi over a period of 1 year and fluoride levels were assessed by using Ion Chromatography.

Ion Chromatography

Principle: This method separates molecules through ion exchange depending on their respective charges **(Fig. 5.1)**

Method: As the water sample is inserted into a stream of carbonate-bicarbonate eluent, it then passes through a number of ion exchangers. The fluoride ions are separated depending on their relative affinities at the strongly basic anion exchanger (guard and separator columns). After separation of fluoride ions, they are then passed through the hollow fabrication exchanger membrane (fiber suppressor) or micromembrane suppressor which has a strong acidic solution (regenerant solution) passing through continuously. In the micromembrane suppressor the fluoride ions are converted to the highly conductive acid while the carbonate-bicarbonate eluent is converted to weakly conductive carbonic acid. The separated anions are only measured in the acid form using conductivity **(Fig 5.2).** Retention time is compared to standards for identification and quantification is measured by the peak height or peak area (137).

***Figure 5.1*:** Ion Chromatography (Metrohm)

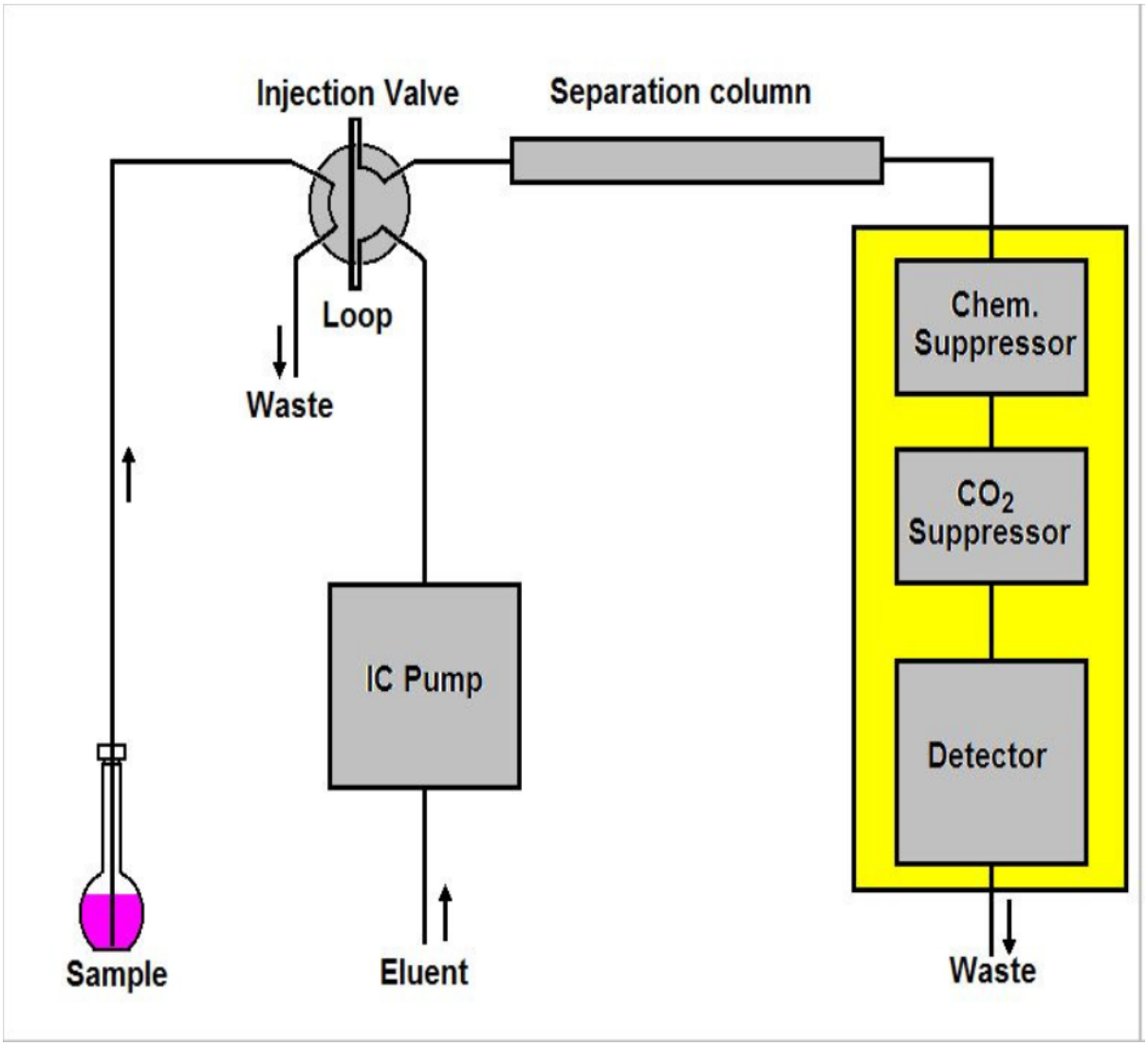


Figure 5.2: Schematic representation of sample processing using Ion Chromatography.

The fluoride levels in drinking water were assessed by using high performance liquid chromatography (HPLC) Ion Chromatography, with conductivity detection after chemical suppression (138).(Metrohm, AG, Switzerland)

Settings used in Ion Chromatography

- Columns used:
  i. Analytical column- Polyvinyl alcohol with quaternary ammonium groups.
  ii. Guard column- Polyvinyl alcohol with quaternary ammonium groups.
- Eluent- 3.2 mmol/L Sodium Carbonate, 1mmol/L Sodium Hydrogen Carbonate
- Suppressor- 50 mmol/L Sulphuric acid
- Recording time- 27 minutes
- Flow rate- 0.7 ml/minute
- Injection volume- 20 microlitre
- P max- 15 Mpa
- Colum temperature- room temperature
- Detector- Conductivity detector
- Stock anion standard solution- 10 mg/L(10 ppm) from FlukaSigma

The large particles (>0.45 microns) in the water samples as well as the large particles (>0.20 microns) in the reagent solutions must be filtered prior to processing as they may result in damage to the instruments columns and the flow systems.

II. Developing Vitamin D deficiency in rat model.

- **Animals :**

Experiments were done on male Sprague-Dawley rats weighing approximately 200gms. Approval was obtained from the Institutional Review Board (IRB), Institutional Animal Ethics committee and the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India

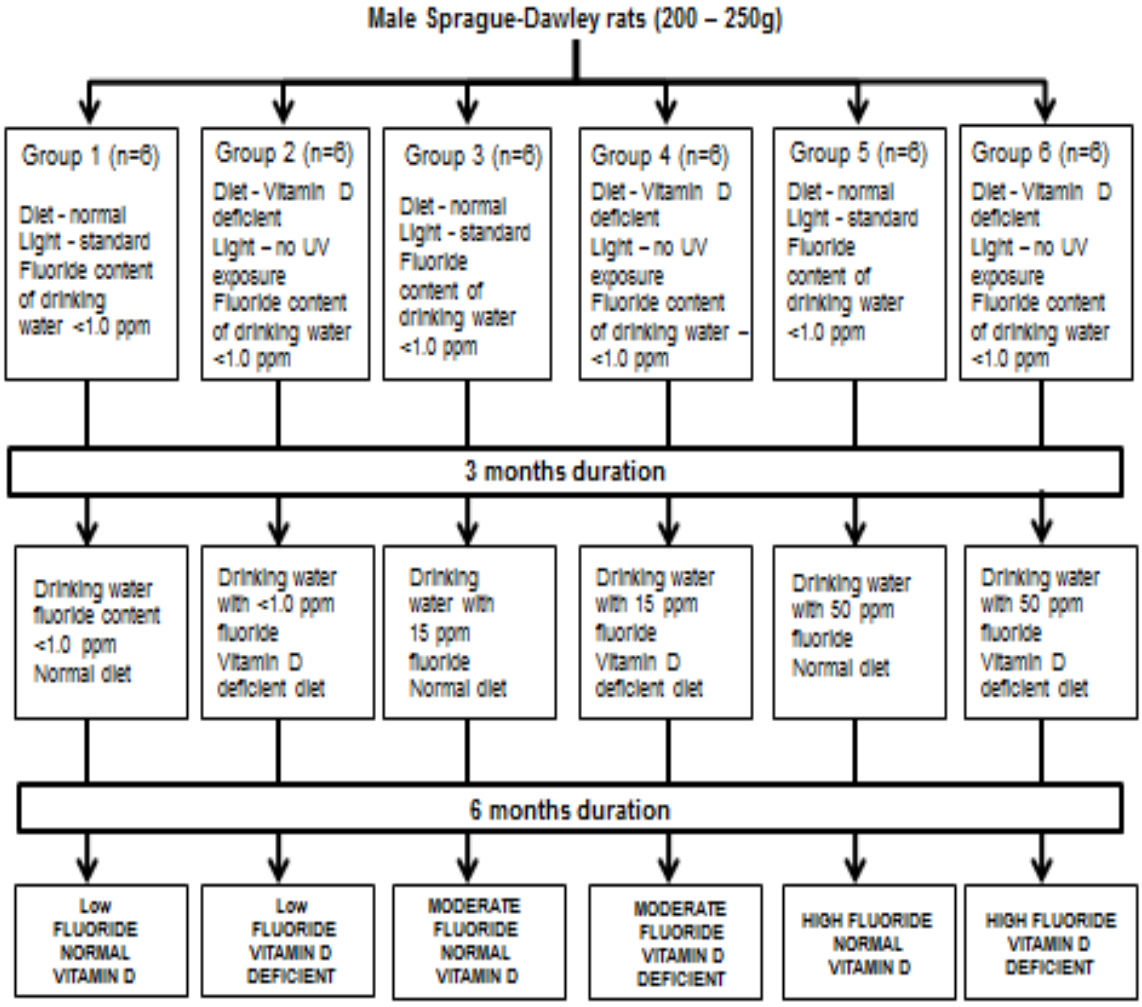


Figure 5.3: The basic design of the study.

- **Induction of vitamin D deficiency in rats:**

The male Sprague-Dawley rats were divided into 6 groups (as shown above) with 6 rats in each group. Rats in group 2, 4 and 6 received a diet deficient in vitamin D [obtained from National Institute of Nutrition (NIN), Hyderabad]. They were housed in rooms in the animal house where the only source of lighting was provided by incandescent bulbs that do not emit UV radiation in the spectrum required for conversion of 7-dehydrocholesterol to cholecalciferol (wavelength of 290–315 nm) in the skin. The animals were exposed to a 12 hour light-dark cycle per day. Animals in group 1, 3 and 5 were fed with a control diet that contained 400 IU of vitamin D per kg of diet (also obtained from NIN, Hyderabad). They were housed under standard lighting conditions. All animals received drinking water that contain normal level fluoride (<1.0 ppm).

- **Assessment of the rat model of Vitamin D deficiency:**

After 3-4 months, the rats that were maintained on vitamin D deficient diet were expected to become vitamin D deficient (139).This was confirmed by estimation of 25OHD and calcium levels in blood at the time point.

In addition, at this time, serum levels of phosphorus, creatinine, ALP and albumin were estimated in blood samples obtained from the retro orbital venous plexus sinus of the rats. For blood collection, standardized micro-hematocrit capillary tubes which were heaprinized were used. The rats were bled after administering general anesthesia using Xylazine at 10mg/kg and Ketamine at 75 mg/kg which were injected intraperitonealy. Whole body animal bone mineral density was estimated using DEXA scan after inducing general anaesthesia as in the above mentioned procedure. All biochemical assays were carried out using commercially

available kits in a Hitachi Roche cobas c702 and E170 automated electrochemiluminescence (ECLIA) modular system. Details of all kits used are given below.

III. Administration of various concentrations of fluoride in drinking water and analysing its effect on bone and kidney in Vitamin D deficient and control group rats.

- **Fluoridation of water and administration of fluoridated water**

The control group and experimental group of rats were administered water with drinking water of levels <1.0ppm. Water was assessed prior to administration using IC. The control group and deficient group rats were administered drinking water with varied levels of fluoride. After dissolving 34.5mg sodium fluoride (molecular weight = 41.99 g/mol) in 500 ml deionised water followed by adding it upto 1000 ml using deionised water to achieve fluoride concentration of 15ppm. 114 mg of sodium fluoride was used to achieve a fluoride water concentration of 50 ppm using the same procedure. The drinking water was then administered to the rats of the control and the deficient group of rats using feeding bottles which are generally used to feed rats.

The experimental and control group rats exposed to low (<1.0ppm) fluoride were caged in 2 separate cages, likewise for the rats exposed to the moderate (15ppm) and high fluoride (50ppm) containing drinking water.

- **Assessing effect of Fluoride on bone metabolism and on kidneys**:

After the initial 3- 4 months of pre-conditioning, the control group rats continued to be maintained on a standard diet while the experimental group rats were kept on the same Vitamin D deficient diet. Rats in groups 1 and 2 continued to receive drinking water which contained normal (<1ppm) concentration of fluoride. Rats in groups 3 and 4 received 15 ppm fluoride in drinking water and rats in groups 5 and 6 received 50 ppm fluoride in drinking water. This treatment was continued for 6 – 7 months.

Ionized plasma fluoride levels tend to be lower in rats than in humans. It has been shown that drinking water containing 15 and 50 ppm of fluoride produces plasma levels of ionized fluoride in rats equivalent to humans consuming 3 and 10 ppm of fluoride in drinking water (140) (141).Before sacrificing the rats, urine samples were obtained whereas blood samples were obtained after sacrificing at the end of this period.

To study the effect of fluoride on bone, the following tests were performed in rat serum: Vitamin D, parathyroid hormone (PTH) Osteocalcin, and C- terminal telopeptide. In addition, alkaline phosphatase, calcium, phosphorus, creatinine, albumin and fluoride were also estimated in rat serum. Details of all kits used are given below.

Bone densitometry evaluation on the animals was done at 6-7 months after exposure to fluoride, this was performed using DEXA scan to assess the whole body mineral density under general anaesthesia. After 6-7 months exposure to fluoride the animals were sacrificed and the femur and vertebrae were retrieved. The rat bone morphology was examined using light microscopy (142). The bone content of fluoride, calcium and phosphorus were estimated after ashing the bone (143) (144). Details of the kits used are given below. After treating the rats with fluoride for 6-7 months the serum creatinine (a marker of the glomerular filtration

rate) was estimated to assess glomerular function. Urine levels of cystatin C [low molecular weight (LMW) protein markers of proximal tubular reabsorptive capacity] were also measured, as well as urine fluoride levels. Details of all kits used are given below. Light microscopy was carried out on rat kidney sections to assess morphological damage in the kidney.

Measurement of the Biochemical parameters

(a) Blood withdrawal:

At the end of the3-4 months of pre-conditioning, 500- 1000 µL of blood was collected from all the rats under general anaesthesia. Blood withdrawal was done from the orbital venous plexus sinus which was punctured and blood was collected using Standard heparinized micro-hematocritglass capillary tube. At the end of the 6-7 months fluoride exposure, blood was withdrawn using a cardiac puncture after the rats were sacrificed.

(b) Urine collection:

For collection of 24-hour urine samples, the rats were housed in metabolic cages and the urine was collected in sterile containers.

(c) **Quantitative determination of 25-hydroxy vitamin D_3 in serum:**

This was done using Electrochemiluminescence immunoassay (ECLIA) competitive principle supplied by Roche Diagnostics, Germany. Total vitamin D was processed on e170 modular analyser.

There are 3 steps for incubation:

- Step 1-treatment with pre-treatment reagent releases vitamin D from vitamin D binding protein
- Step 2- a complex is formed between ruthenylated vitamin D binding protein and 25 hydroxy vitamin D which was released during pre-treatment of the sample.
- Step 3- after addition of 25-hydroxyvitamin D labelled with biotin,and streptavidin-coated microparticles, the free sites of the ruthenylated vitamin D binding protein become occupied with vitamin D labelled with biotin to form a complex.

This complex becomes bound to the solid phase via interaction of biotin and streptavidin. After incubation the reaction mixture is placed into measuring cell and iron particles are magnetically separated and unbound particles are removed using the Procell solution. Application of a voltage induces a chemiluminescent emission measured by a photomultiplier. The counts per second detected +by the system are inversely proportional to the 25 OH-vitamin D concentrations in the specimen. The results were calculated by the e 170 software and reported in ng/ml.

(d) **Measurement of serum calcium:**

Serum calcium were assayed by a colorimetric end point method using commercially available kit Cobas Gen 2. V.4.0 Roche Diagnostics, Germany. Calcium forms a purple coloured complex with 5- nitro 5- methyl –BAPTA in an alkaline medium. The complex reacts in a second step with EDTA. The intensity of the colour measured at 340 nm is proportional to the serum calcium. This method measures total calcium (ionized +chelated+ bound)forms in the serum (ref). The test is run on the Roche cobas c 702 analyser.

(e) **Measurement of serum phosphorus:**

Serum phosphate was assayed by a commercially available kit of Cobas , Roche Diagnostics, Germany. The test is run on the Roche c 702 analyser and the method is UV end point. Serum phosphorous present in the sample reacts with Ammonium molybdate and Sulphuric acid to form the unreduced form of phosphomolybdate complex and its absorbance is measured at 340nm (145).

(f) **Measurement of Alkaline phosphatase (ALP)*:***

Serum ALP was assayed by a commercially available kit Cobas , Roche Diagnostics, Germany. The test is run on the Roche c702 analyser.

P-nitrophenyl phosphate is formed in the presence of magnesium and zinc ions and is cleaved by alkaline phosphatase into phosphate and p-nitrophenol. The rate of formation

of paranitrophenol is measured colorimetrically and is proportion to the ALP activity in the sample(146).

(g) Measurement of Albumin:

Serum albumin was assayed by using a commercially available kit of Cobas Roche Diagnostics, Germany. Colorimetric end point bromocresol green method was used. Bromocresol green (BCG) binds with the albumin to form a complex (colour changes from yellow to blue green) at pH of 4.2 and photometric measurement of absorbance is performed (148). The test is run on the Roche Cobas c702 analyser.

(h) **Measurement of fluoride:**

Serum and urine fluoride was assayed using an ion selective electrode (Thermo Scientific Orion 4 star PH /ISE bench top PH meter) with a fuoride ISE electrode.
To perform the analysis, first take a non-glass beaker and place in it measured equal amounts of the sample and TISAB buffer. Mix the solution and clean the electrode by rinsing with deionized water. Dry it and then place it into the beaker containing the sample. Continue stirring the solution and look for a stable reading. The concentration of the sample is displayed on the meter. Calibration solutions used are fluoride standards provided by Thermo Scientific Orion 4 star PH /ISE bench top PH meter. Concentrations of the calibration solutions are 1 ppm and 10 ppm.

(i) **Measurement of Osteocalcin:**

Serum osteocalcin was assayed using a commercially available double antibody sandwich ELISA. My Biosource kit San Diego, USA was used.

The microplate was mounted with the pre-coated osteocalcin specific antibody. The controls and samples are pipetted into the wells and any osteocalcin which is binds to the immobilized antibody. Osteocalcin specific antibody (biotin conjugated antibody) is added to the wells after removing the unbound substances. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash, in order to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells. The colour develops in proportion to the amount of osteocalcin bound in the initial step. The colour remains and the intensity of the colour is measured.

(j) **Measurement of intact parathyroid hormone (PTH):**

Serum intact PTH was assayed using double antibody rat sandwich ELISA obtained from Immutopics Inc., USA.

(k) **Measurement of C terminal telopeptide of type I collagen :**

Serum C terminal telopeptidewas using a commercially available double antibody rat sandwich ELISA obtained from MY Biosource kit San Diego, USA .

Calibrators controls and rats serum were added into micro plate, which has been pre-coated with antibody specific for CTX-I , and wereallowed to interact.. After incubation there was a wash step to remove removing any unbound substances, then a biotin-

conjugated antibody specific for CTX-I was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate is added to the wells and colour develops in proportion to the amount of CTX-I present in the sample. The colour development is stopped and the intensity of the colour is measured.

Assessing the renal tubular damage

(i) Measurement of creatinine:

Serum creatinine was assayed using a commercially available kit Cobas Creatinine, Roche Diagnostics, Germany, rate blanked and compensated method.

Without the deproteinization of serum creatinine or creatinine in urine, Colorimetric kinetic jaffe alkaline picrate forms a coloured complex in alkaline medium. The rate of absorbane is depicted by change of the coloured complex which is proportional to the creatinine concentration in serum or in urine. The kinetic assay starts approximately the second to third minutes after picrate addition. First, the fast reacting non creatinine chromogens predominate and in the third minute slow reacting non-creatinine chromogens predominate (147). The test is run on the Roche cobas c702 analyser.

(ii) Measurement of Cystatin C:

Urine Cystatin C was assayed using double antibody rat sandwich ELISA obtained from MYBiosource kit San Diego, USA.

The Cystatin C present in the samples reactys with the anti-Systatin C antibodies which are present on the walls of the polystyrene microtitre wells. After this washing is carried

out to ensure the removal of the unbound proteins. The anti-Cystatin C antibodies conjugated with the horseradish peroxidise (HRP) are then added which then form complexes with the previously bound Cystatin C.. A second washing step is undertaken, following which the chromogenic substrate, 3,3', 5,5'- tetramethylbenzidine (TMB) (buffer) is added to assay the enzyme bound to the immunosorbent. The Cystatin C sample is tested at absorbance of 450nm and measured by the quantity of enzyme bound. The quantity of Cystatin C is then compared to the standard curve constructed using controls and corrected for sample dilution .

Assessing changes in Bone mineral density and Bone fluoride content among the control and Vitamin D deficient rats

Measurement of bone mineral density:

Whole body BMD of rats was measured under anesthesia using whole body DEXA scan.

Measurement of bone fluoride content:

Specimens of rat bone were obtained as described earlier. The researcher used the vertebrae to assess the bone fluoride content. Bone was dried at 105 °C for 12hours and then weighed. The specimens were then transferred into crucibles and pyrolyzed in an oven at 600 °C for 12 hrs. Cooled samples were then placed into polyethylene measuring vessels after washing the crucibles with 3% Hydrochloric acid then diluting in 5ml water. Then 1.5ml of the sample added with equal amount of TISAB buffer (pH 5.2) was added for measurement of fluoride concentration. The fluoride content was measured using an

ISE (as explained above) and expressed as a percentage of the dissolved bone ash weight (143)(144) and bone calcium and phosphorous was measured on the ash solution, using colorimetric end point method commercially available kit methods (Cobas, Roche).

Assessing the histopathological changes in Bone of rats

Histopathological studies:

The histopathological studies were carried out in the Department of Pathology .Samples of kidney tissue were fixed in 10% buffered formalin and sections were stained with hematoxylin and eosin for light microscopic examination. Bone vertebral tissue was also fixed in buffered formalin and decalcified bone sections were stained with hematoxylin and eosin, and also silver stained for light microscopic examination (142).

Statistical Analysis:

Data was screened for outliers and extreme values using Box-Cox plot and histogram (for shape of the distribution). Descriptive statistics was used to report the clinical characteristics and data was expressed as mean± Standard deviation. Mann-Whitney U test was used between group (experimental and control) and risk variables. Descriptive statistics were used. The Analysis of Variance (ANOVA) was used for determining statistically significant differences between the various treatment groups. A p value of less than 0.05 was considered statistically significant. The Statistical Package for Social Scientists (SPSS) version 17.0 was used for all statistical analysis.

RESULTS AND ANALYSIS

RESULTS

Objective 1-Validation of the method of Ion Chromatography for estimation of Fluoride levels in drinking water in comparison to the established ISE methodology.

Ion Chromatography

The settings are as follows:

- Fluoride column **Retention time** was- 3.2 minutes
- **Limit of detection** (LOD)- 0.027 ppm (equivalent to mg/L)

LOD may be expressed as:

$$LOD = \frac{3.3\ \sigma}{S}$$

Where σ = the standard deviation of the response

S= the slope of the calibration curve

The estimate of σ is based on standard deviation of blank run 10 x consecutively

- **Limit of quantification** (LOQ)- 0.083 ppm

The quantification limit (QL) may be expressed as:

$$QL = \frac{10\ \sigma}{S}$$

Where σ = the standard deviation of the response

S= the slope of the calibration curve

The estimate of σ based on standard deviation of blank run10 x consecutively

- **Linearity** of the Calibration graph was determined to be from 0.1 to 100 ppm

- **The Correlation coefficient** (R^2) was 0.999 and the relative standard deviation was 2.4% with Slope= 0.266and intercept = -0.016.

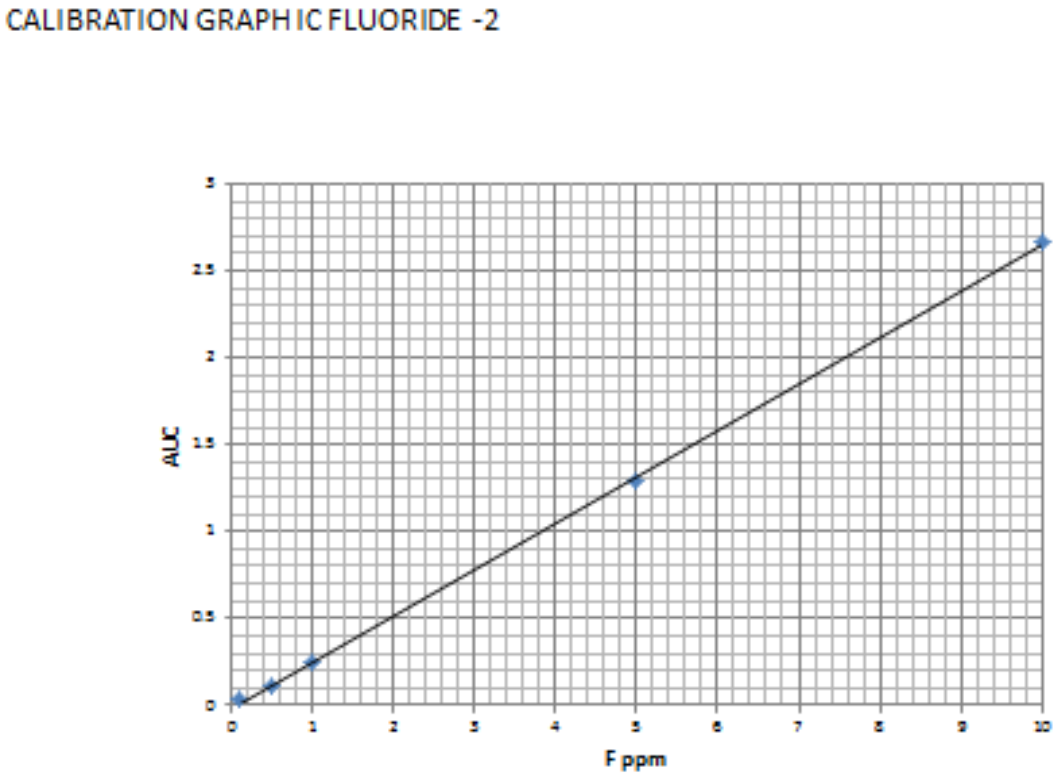


Figure 6.1: Calibration graph of fluoride

- **Precision**

Within run precision was determined using fluoride standard of 0.5, 1.0, 2.0, 5 and 10 ppm, where the researcher assessed the results after running each sample 10 times consecutively within a day.

The following results were obtained:

Table 1: Within- Run

Sample No.	**Mean**	**SD**	**CV**
1	0.53	0.019	3.60%
2	1.06	0.064	5.98%
3	2.03	0.077	3.80%
4	5	0.1	2.10%
5	10.5	0.2	2%

- **Linearity**

Samples were assayed under various dilutions using the deionised water in which the fluoride levels were undetectable using Ion Chromatography (Refer the linearity table below for representative data)

Table 2: Linearity table

Sample No.	**Dilution**	**Observed**	**Expected**	**%O/E**
1	8 in 8	2.6		
	4 in 8	1.38	1.3	106
	2 in 8	0.78	0.65	120
	1 in 8	0.49	0.325	150
2	8 in 8	5		
	4 in 8	2.57	2.5	100
	2 in 8	1.36	1.25	109
	1 in 8	0.77	0.625	123
3	8 in 8	11.6		
	4 in 8	5.56	5.58	95.8
	2in 8	2.6	2.9	90
	1in 8	1.4	1.45	96
4	8 in 8	32.3		
	4 in 8	14.7	16.15	91
	2in 8	7.55	8.05	94
	1 in 8	3.67	4.02	93

The values within ±10% or ± 0.2ppm are acceptable. The Linearity was acceptable in all the dilutions of samples up to 4 in 8 (0.5), up to 2 in 8 (0.25) in 3 samples, and 1 in 8 (0.125) in 2 samples.

Method comparison

49 paired samples drinking water samples with a fluoride concentration range of 0.5 to 10.2 were measured by using Ion Selective Electrode (ISE) and the Ion Chromatography (IC) method from Metrohm. The graph below shows the comparison of IC results on the Y axis and the ISE results on the X axis.

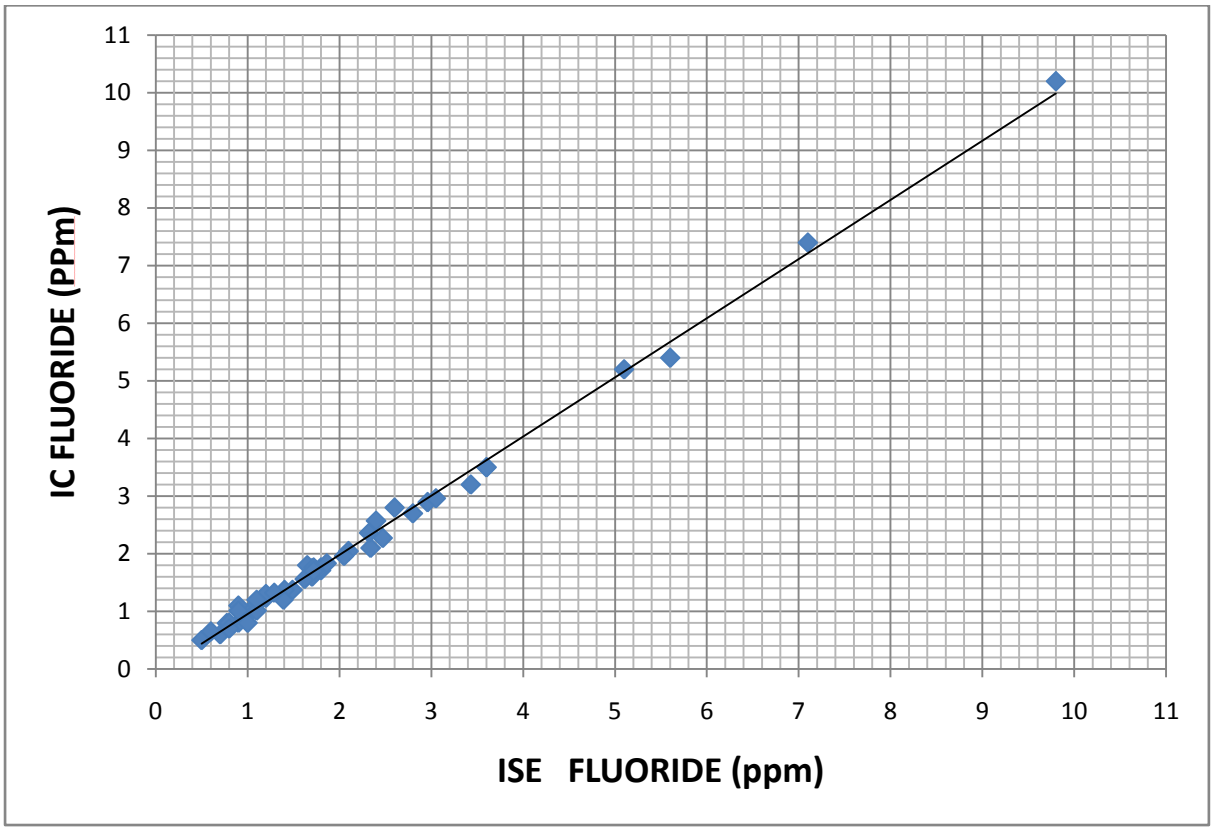


***Figure 6.2*: Comparison of the IC and ISE method. Linear regression (R^2) is = 0.998, the slope=1.02 and the intercept= -0.076**

The Bland Altman plot showed no bias between the ISE and Ion Chromatography methods **(Fig 6.3)**

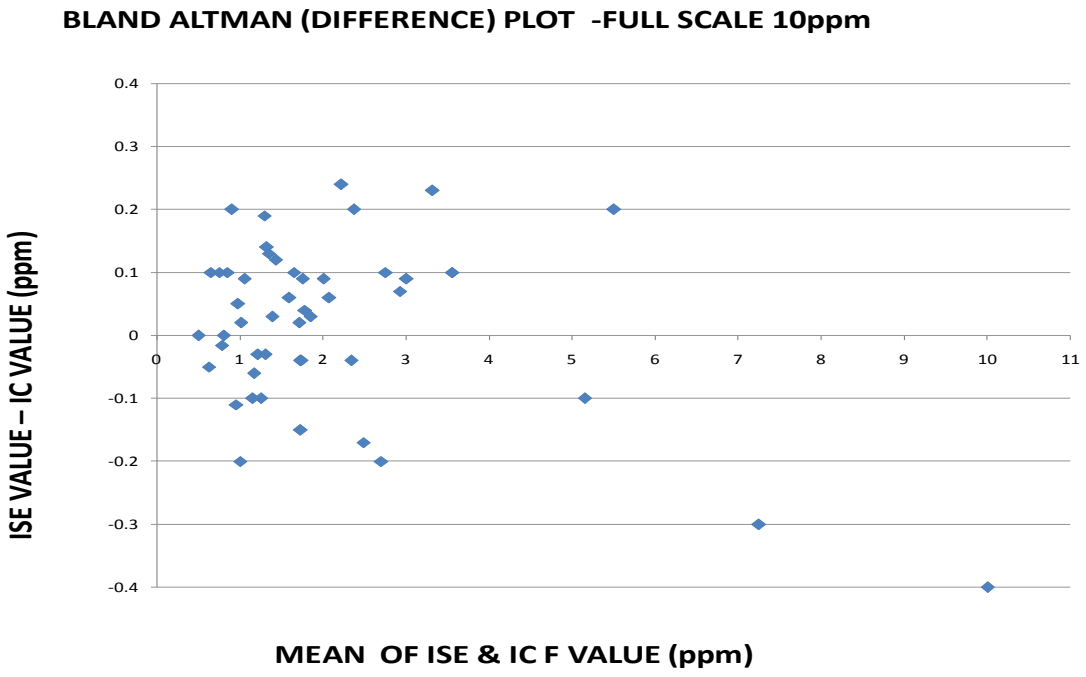


***Figure 6.3*:** The comparison of IC and ISE methodology using Bland Altman plot

Objective 2- To assess the Fluoride levels in the drinking water of the villages in Vellore district using Ion Chromatography.

Table 3: The ground water fluoride levels in the villages in and around Vellore district.(n=165)

Sr. No.	Name of Villages	Fluoride level in drinking water (ppm)	Sr. No.	Name of Villages	Fluoride level in drinking water (ppm)
1	Agravaram	1.3	84	Melpal	0.3
2	Agravarammalai	0.79	85	Melvisharam	1.1
3	Alan Kuppam	0.51	86	Methur	0.4
4	Alangayam	0.66	87	Mettukulam	0.98
5	Alangayam 2	0.8	88	MGR nagar	2.06
6	Alinjikuppam	0.5	89	Mislafah	0.5
7	Alrajvattam	2.36	90	Mittapalli	0.54
8	Ambur	0.7	91	MLA vattam	2.06
9	Ambur 2	0.7	92	Mookanur	1.44
10	Amburtuthipet	0.89	93	Nagathopu	0.24
11	Amman nagarkarimangalam	1.34	94	Narasingapuram	0.67
12	Ammanankoilvattam	1.1	95	Natrampalli	1.6
13	Andivattam	1.12	96	Natrampalli 2	1.23
14	Anna Nagar	0.6	97	Nayankuppam	0.3
15	Anna Nagar 2	0.8	98	Nellikuppam	1
16	Arcot	0.49	99	Nimayapattu	0.5
17	Arumparuthi	0.99	100	Oddapatti	2.81
18	AvviKam	0.7	101	Oosanpatti	0.5
19	Ayyganoor	1.1	102	Pachal	1.76
20	Bagayam	0.67	103	PalaimMinnur	0.6
21	Bairavanvattam	1.3	104	Palangkuppam	2.2
22	Balur	0.73	105	Pallikuppam	1.1
23	ButtuValayam	0.3	106	Palliteru	0.76
24	ChekkuMedu	0.6	107	Palnangkuppam	1.01
25	ChengiKuppam	0.96	108	Pattamalvattam	1.01

26	Chinnakamiyampattu	1.3	109	Peranampet	0.3
27	Chinnakonapattu	1.2	110	Periyakammiyampattu	3.56
28	Chinnamookanur	2.8	111	Periyamookanur	3.2
29	Chinnamookanur 2	2.2	112	Perumalkoilvattam	2.06
30	Chinnamookanurchapani	2.36	113	Perumalvattam	2.04
31	Chinnamookanurparisan	2.5	114	Pilliyar	0.34
32	Chinnamookanurpujoli	1.58	115	Pilliyar 2	0.34
33	Chinnamookanurrathanavel	1.73	116	Ponnai river	0.2
34	Chinnamookanursattapuri	2.2	117	Ponneri	1.21
35	Chinnavengayapalli	2.57	118	Poorigamanimitta	1.94
36	Dekanvattamjagalapuram	1.84	119	Poosariyur	0.8
37	Ekambaranallore	0.78	120	Prakasapuram	0.94
38	Ellapalli	1.2	121	Priyakammiyampattu	1.37
39	Ganapathi Nagar	0.2	122	Pudupattai	2.94
40	Gandhi nagar	2.27	123	Pudupettai 2	1.8
41	Girisamudram	0.9	124	Pudur	0.89
42	Goundavanur	1.23	125	Pudur 2	0.7
43	Gudiyatham	0.3	126	Pugalaikaranvattam	1.24
44	Gudiyatham 2	0.3	127	Pullaneiri	2.8
45	Indira nagar	0.9	128	PuthuiMedu	0.48
46	Jabupallam	0.5	129	Puthur	0.7
47	Jagiri	2.01	130	Raja Nagar	0.9
48	Jandakaranvattam	1.26	131	Rani pet	0.69
49	Jeevan Nagar	0.4	132	Salainagar	2.9
50	K. bandharapalli	1.2	133	Samayakaranoor	2.26
51	Kakathoku	0.5	134	Sambedi	0.5
52	Kalaraimedu	1.39	135	Sangagowndarvattam	1.6
53	Kalavai	0.78	136	SantroKuppam	0.44
54	Kalpudor	0.86	137	Sathuna	1.56
55	Kamalapuram	1	138	Senguttai	0.78
56	Kambiyampattanvattam	1.49	139	Serkadu	0.72
57	Kanigapuram	0.68	140	Sevoor	1.1
58	Kannadipalayam	0.8	141	Sivarampettai	0.5
59	Karthigaypuram	0.7	142	Solur	0.95
60	Karupanur	2.37	143	Somalapuram	0.4
61	Kasam	0.69	144	Somanaikkanpatti	1.66
62	Kaspa	0.52	145	Soornampettai	0.48
63	Kateriammankoil	1.14	146	T V Balaramanvattum	0.95
64	Katpadi	0.56	147	T verapalii	1.35

65	Katteri	1.2	148	Thiriyalam	3.1
66	Kilvisaram	0.65	149	Thiriyalam 2	1.56
67	Kona pattu	1.76	150	Thonnaiyanoor	1.58
68	Kona pattu 2	1.14	151	Thoplagunda	1.71
69	Konampatti	0.7	152	Thorapadi	0.88
70	KonduMossapill	0.5	153	Thzhaikaranvattam	1.34
71	Konerivattam	1.6	154	TV duraisamynagar	1.74
72	Kothur	1.3	155	Umarabad	0.2
73	Kothur	2.8	156	Unna Mangalam	0.9
74	KRS vattam	3.54	157	Vada Cheri	0.5
75	KRS vattam 2	3.2	158	Vallalar	0.99
76	Kugaiyanallore	0.76	159	Vaniyambadi	0.6
77	Kuntimariamimanvattam	1.17	160	Vasigam	0.5
78	Kuppusamyvattam	1.74	161	Vellakuttur	0.64
79	LakshmanPuram	0.7	162	Vepullampatti	0.85
80	Lalapet	0.97	163	Walajaanaikut	0.8
81	Lorry shed	1.83	164	Walajapudupet	0.59
82	Maniyakarvattam	1.4	165	Yettagal	0.28
83	Maruham	0.34			

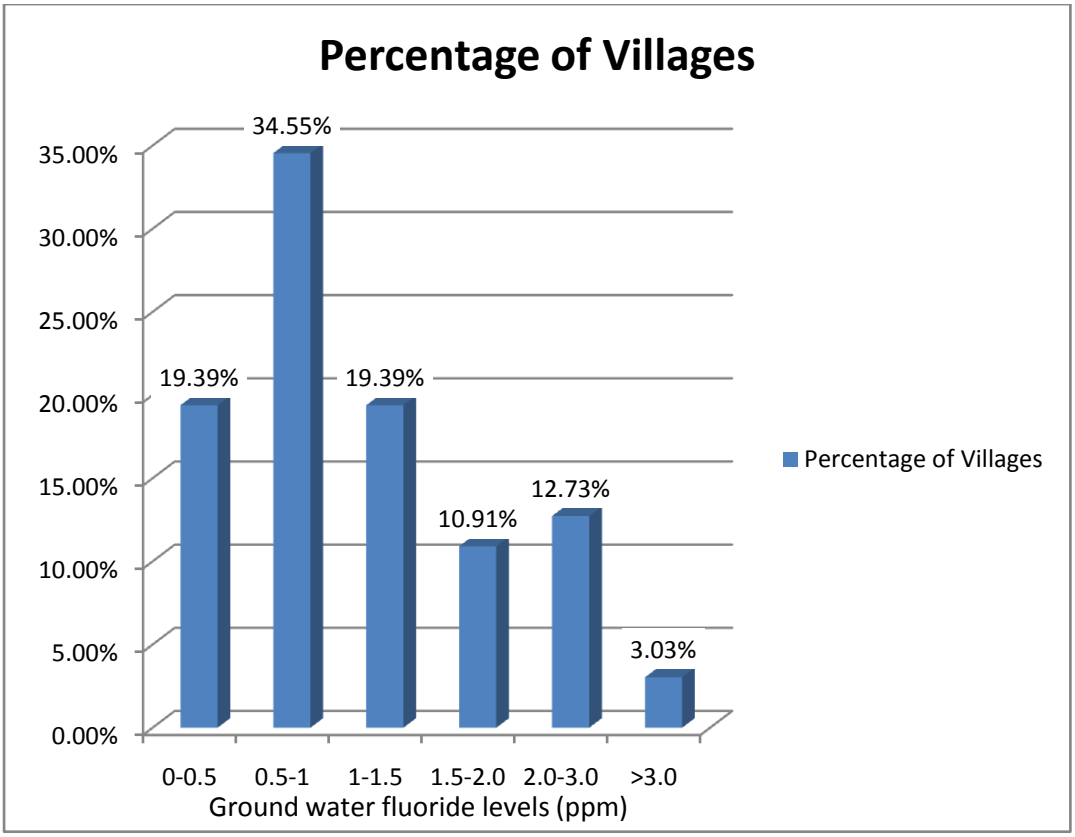


Figure 6.4: **Percentage of villages and the drinking water fluoride levels (n=165).**

The above given table lists the water fluoride levels from the villages in the district of Vellore. 165 villages were assessed and the findings showed that 32 (19.4%) had levels ranging from 1-1.5ppm, 18 (10.9%) of the villages had levels ranging from 1.5-2 ppm and about 21 (12.7%) had levels ranging from 2.0- 3.0ppm. Five (3.0%) of the villages had recorded levels > 3.0ppm. This signifies that 76 (46.06%) of the villages had levels above the recommended Indian standard (> 1.00 ppm).Of these most (44.42%) of the villages belong to the taluk of Jolarpet.

Objective 3- Standardized a rat model of vitamin D deficiency and assessed the changes in bone density and changes in specific biochemical parameters after 4 months on a Vitamin D deficient diet

Changes in Biochemical parameters

After 3-4 months of feeding the rats a diet with and without Vitamin D, and prior to exposure of any group to fluoride, the experimental group rats which were fed with the Vitamin D deficient diet, had developed Vitamin D deficiency. The levels of Vitamin D of the rats in the control and experimental group serum showed a marked difference and the values were 27± 6ng/ml (mean ± SD) and 4.5± 2ng/ml respectively. The control group had serum albumin levels of 3.3 ± 0.5g/dl, calcium levels of 10.1± 0.6mg/dl and alkaline phosphatase 52 ± 8 U/L levels whereas the experimental group had serum albumin levels of 3.2±0.5g/dl, calcium levels of 10.0±0.8mg/dl and alkaline phosphatase was 55 ± 8 U/L. Calcium, albumin and alkaline phosphatase levels in both groups were found to be nearly the same.

Changes in Bone Mineral Density (BMD), Bone Mineral Content (BMC) and fat mass after 4 months on a Vitamin D deficient diet.

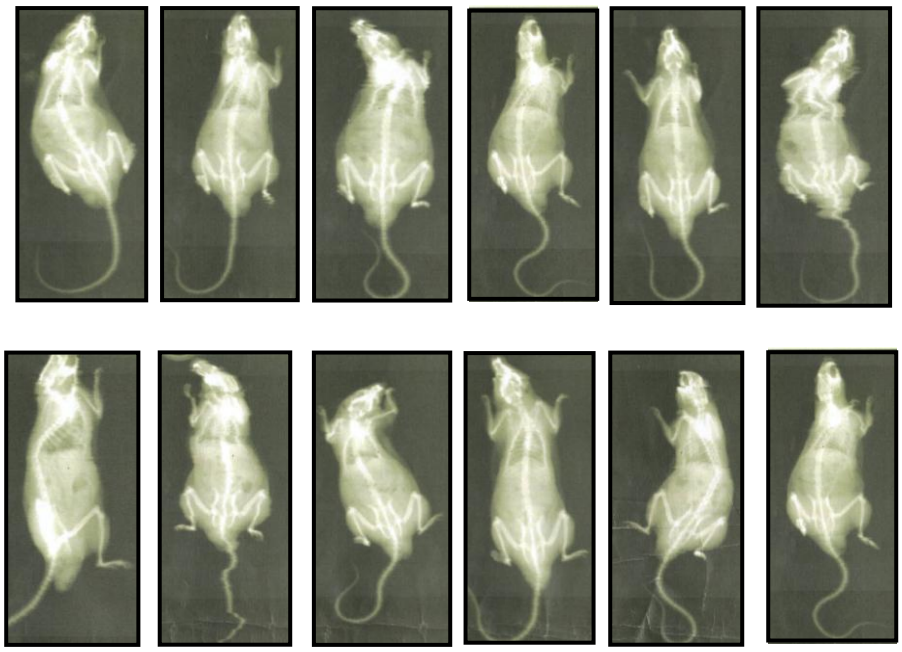
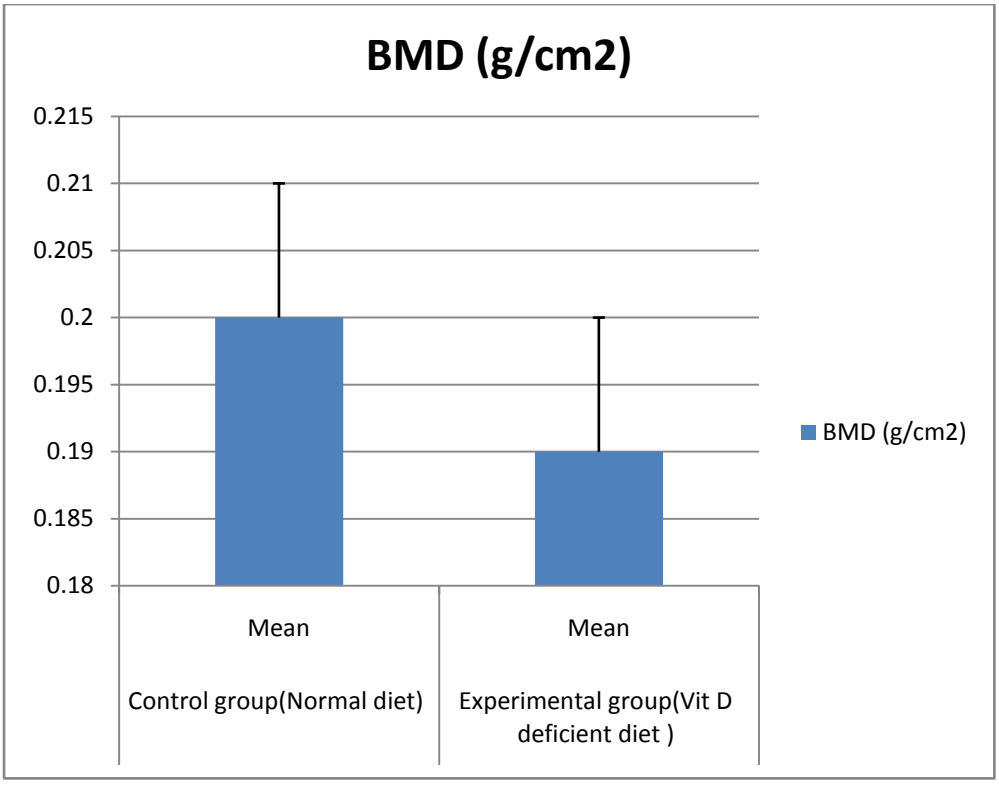


Figure 6.5 : The DEXA scan images of rats after 4 months on a Vitamin D deficient diet. The first row of rats (n=6) represents the Vitamin D deficient rats and the second row of rats (n=6) belong to the control group.

Table 4: The DEXA scan results prior to exposure to fluoride (n=18)

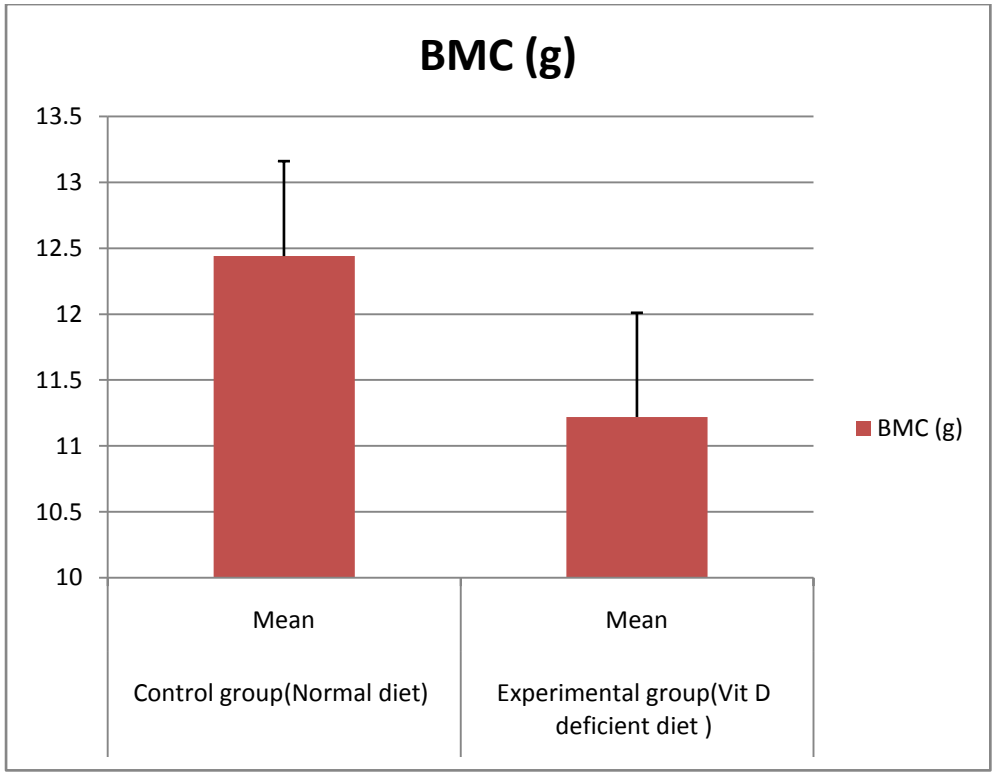
Test	Control group (Normal diet) n= 18		Experimental group (Vitamin D deficient diet) n=18		P value
	Mean	**SD**	**Mean**	**SD**	
BMC (g)	12.44	0.72	11.22	0.79	0.03*
BMD (g/cm2)	0.20	0.01	0.19	0.01	0.04*
Fat mass (g)	64.38	14.77	84.68	20.12	0.03*
% of fat	15.5	3.07	20.48	3.52	0.01**
Total mass (g)	371	21.11	377.75	18.49	0.29

p<0.05 , **p<0.01



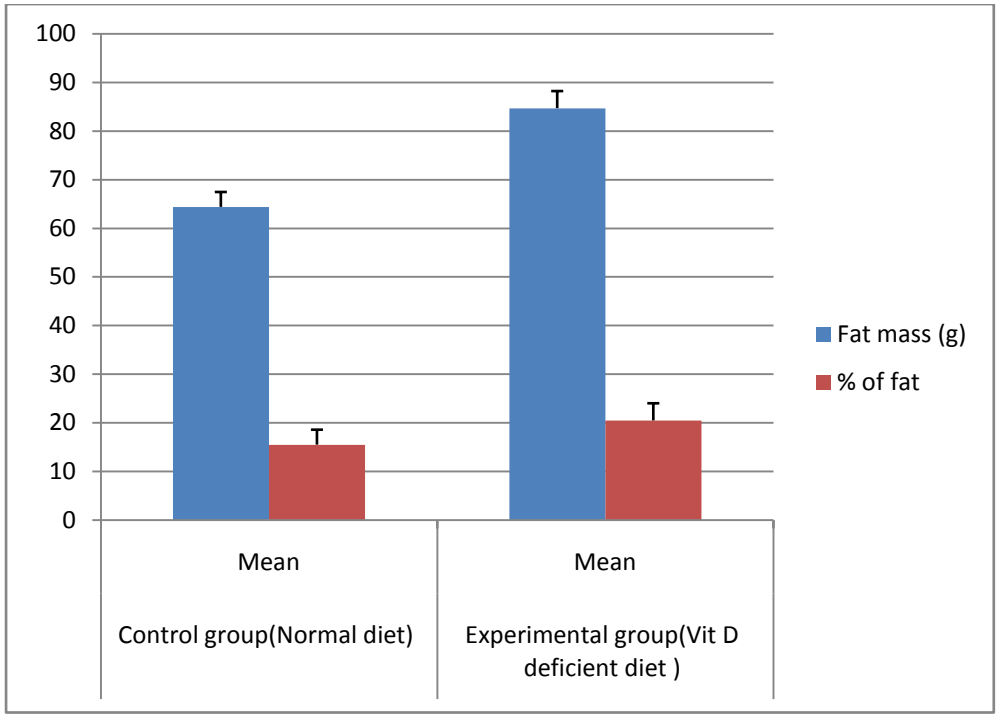
(p<0.05)

Figure 6.6: Comparison of Bone Mineral density (BMD) between the control and Vitamin D deficient rats (n= 18).



(p<0.05)

Figure 6.7: Comparison of Bone Mineral Content (BMC) between the control and Vitamin D deficient rats (n= 18)



Fat mass p<0.05) and fat percentage (p<0.01)

Figure 6.8: Comparison of fat mass, percentage and total mass of rats from the control and experimental groups (n=18)

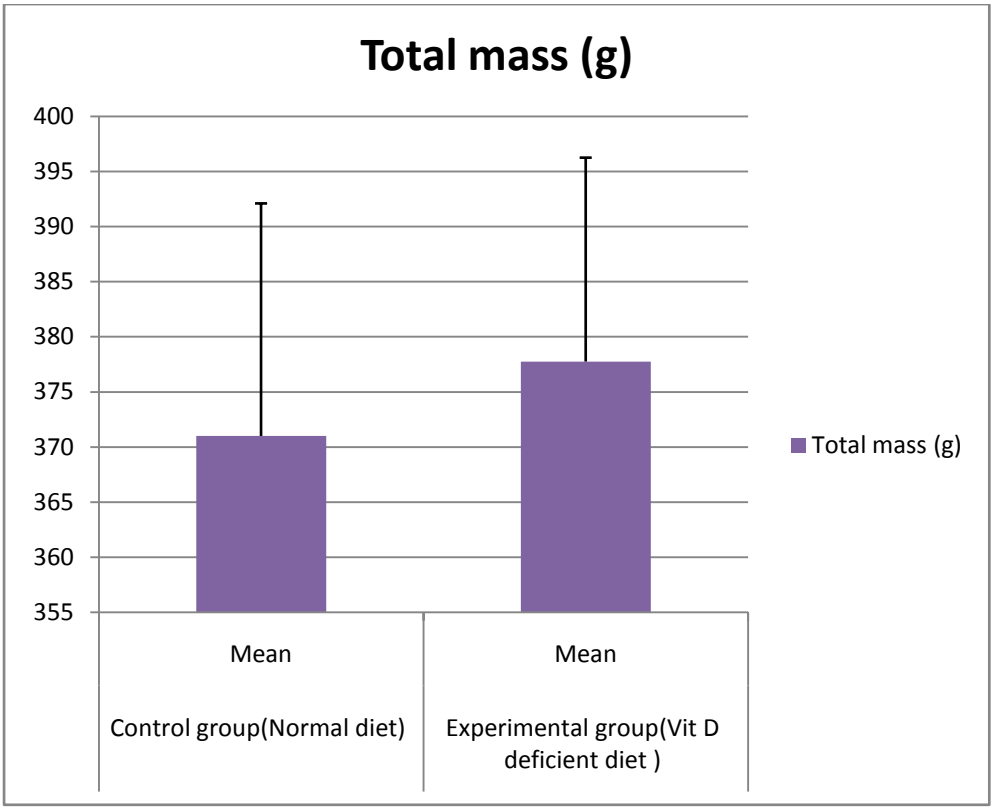


Figure 6.9: Comparison of Total mass between the control and Vitamin D deficient rats (n= 18)

The above given figures depict the DEXA scan results of the experimental and control group rats prior to the exposure to fluoride. The whole body BMC in both the groups were compared and it was found that the control group rats had a higher bone mineral content than the experimental group. This increase in BMC was found to be statistically significant ($p<0.05$). Also the bone mineral density of the rats in the control group was found to be significantly higher than the Vitamin D deficient group of rats ($p<0.05$). The researcher found it interesting to note that there was a significant increase in the fat mass and percentage of fat. The Vitamin D deficient rats had a significantly higher mean fat mass ($p<0.05$) and fat percentage ($p<0.01$) when compared to the control group rats. The total mass of the rats in the vitamin D deficient group was found to higher when compared to the control group. But this increase was not statistically significant.

Objective 4: Changes in bone morphology and metabolism induced by varying levels of fluoride intake in the rat model of vitamin D deficiency.

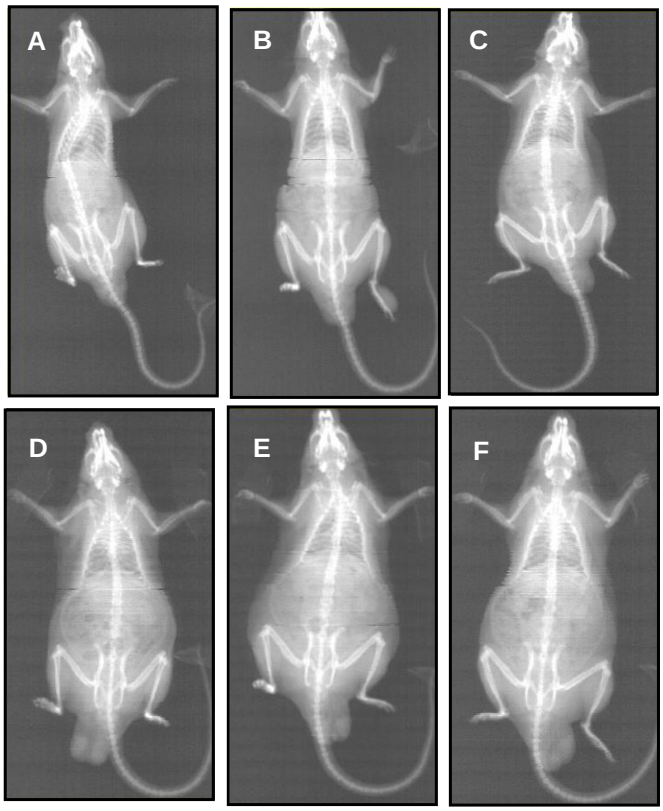


Fig : A, B and C - Control ,
D, E and F - Deficient

Figure 6.10 : The DEXA scan images of the rats from the control and Vitamin D deficient groups exposed to low levels of fluoride (<1.0ppm).

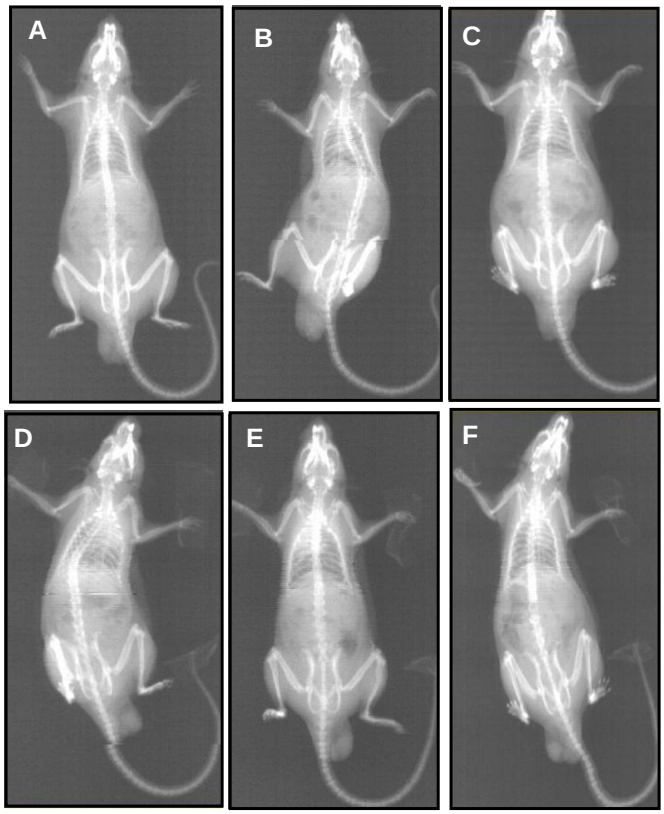


Fig : A, B and C - Control Medium ,
D, E and F - Deficient Medium

Figure 6.11: The DEXA scan images of the rats from the control and Vitamin D deficient groups exposed to moderate levels of fluoride (15ppm).

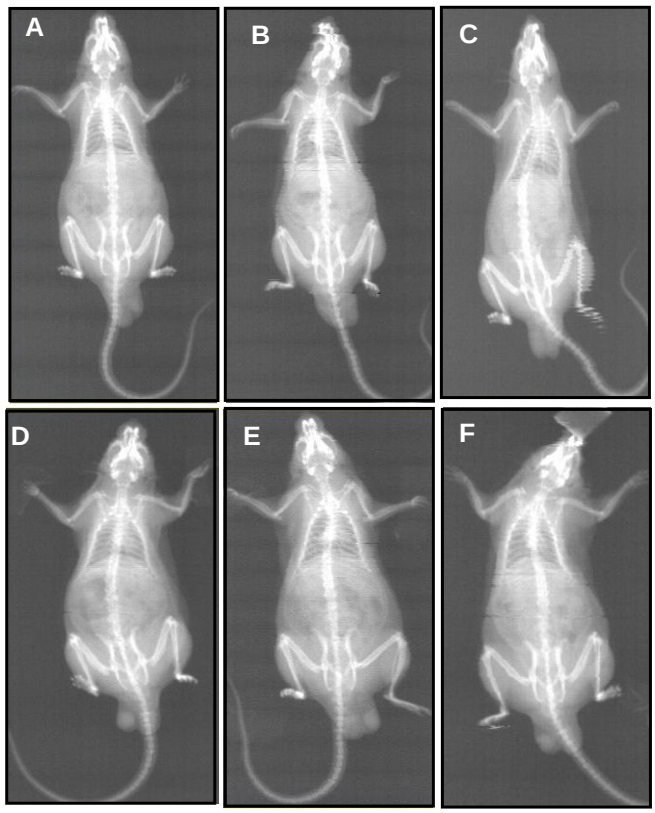


Fig : A, B and C - Control High ,
D, E and F - Deficient High

Figure 6.12: The DEXA scan images of the rats from the control and Vitamin D deficient groups exposed to high levels of fluoride (50ppm).

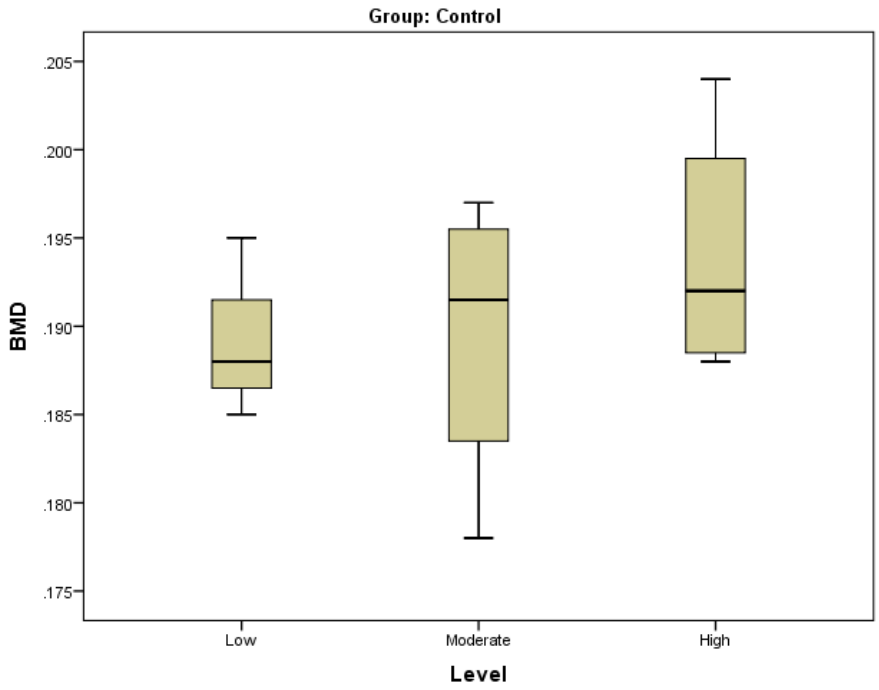
The above given figure represent the DEXA scan images of the rats belonging to the control and the Vitamin D deficient groups exposed to the various levels of fluoride in drinking water.

Table 5: The DEXA scan results after exposure to fluoride (n=18)

Group	Statistical significance	**BMC (g)**	**BMD (g/cm2)**	**Fat mass (g)**	**% of fat**	**Total mass (g)**
Within the groups	Chi-square	0.725	1.426	3.736	5.031	1.622
	P value	0.696	0.49	0.154	0.081	0.444
Between groups	Low (P value)	0.077	0.034*	0.034*	0.034*	0.032*
	Moderate	1	0.052	0.68	0.216	0.41
	High	o.522	0.011*	0.136	0.38	0.522

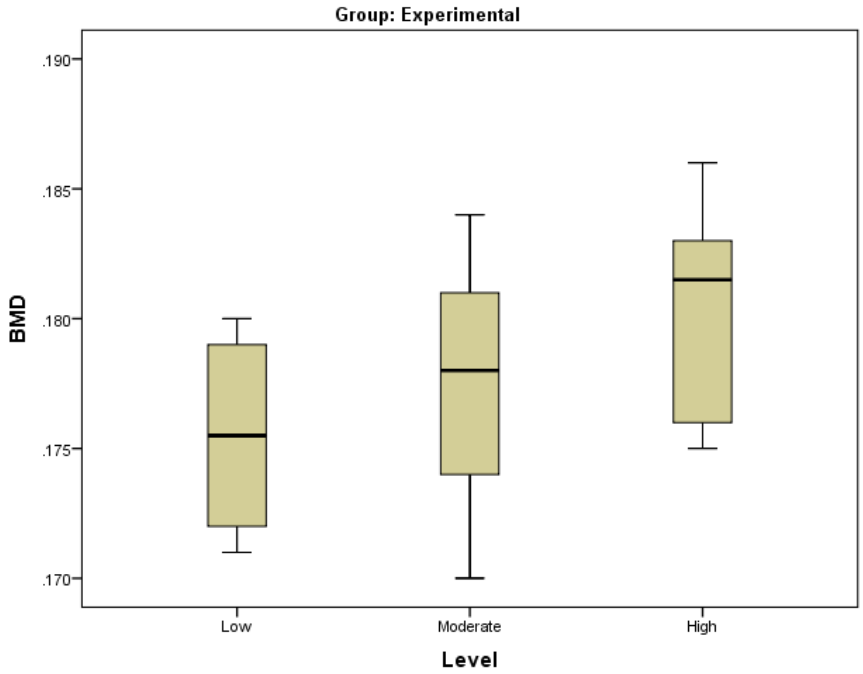
<u>Changes in Bone morphology using DEXA</u>

Bone Mineral Density



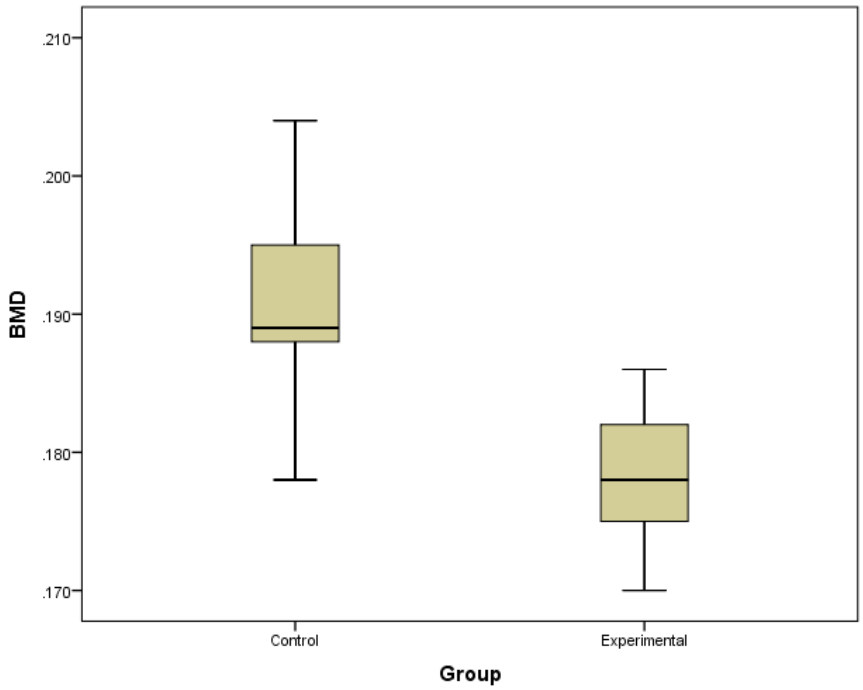
p value <0.05 (low fluoride), p value<0.01 (high fluoride)

Figure 6.13: Comparison of median and interquartile range of BMD of rats within the control group exposed to various levels of Fluoride (n=14)



p <0.05 (low fluoride), p<0.01 (high fluoride)

Figure 6.14: Comparison of median and interquartile of Bone Mineral Density (BMD) of rats within the experimental group exposed to various levels of Fluoride (n=16)

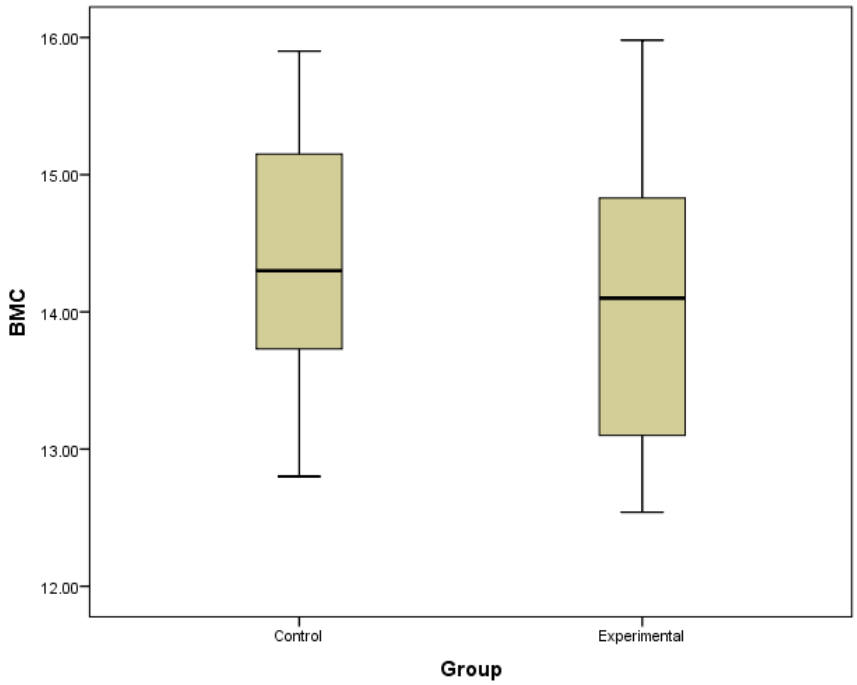


(P<0.05)

Figure 6.15: Comparison of median and interquartile Bone BMD of rats between the control group and experimental group exposed to various levels of Fluoride.

The figures given above describe the BMD. The BMD of the rats in control group (normal Vitamin D levels) treated with low levels of fluoride was less than (0.189±0.005 g/cm^2) the rats treated with moderate (0.19±0.008 g/cm^2) and high levels (0.194±0.007g/cm^2) of fluoride. A similar trend was observed in the experimental group, where rats treated with low fluoride levels had a lower BMD (0.176± 0.004g/cm^2) than the rats treated with moderate (0.177±0.007 g/cm^2) and high levels (0.181±0.04g/cm^2) of fluoride. Also all the data combined for the rats in the control group showed a higher BMD(0.191±0.007 g/cm^2) than that of all the data combined from the experimental Vitamin D deficient group(0.178±0.005 g/cm^2). There was statistical significance ($p<0.05$) found in between the groups of rats treated with low and high levels of fluoride among the experimental and control group rats.

Bone Mineral Content

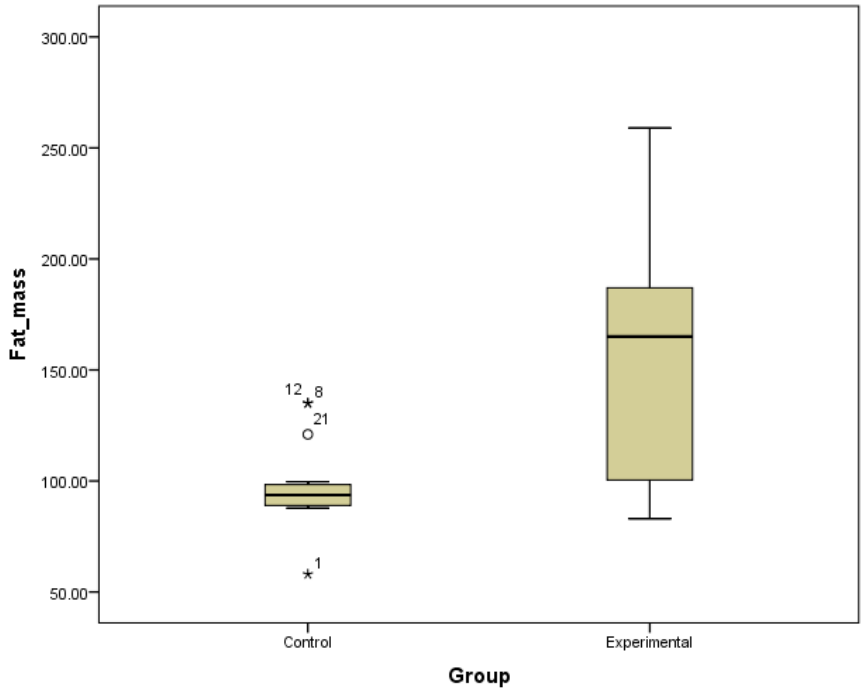


(P=0.70)

Figure 6.16: Comparison of median and interquartile of Bone Mineral Content (BMC) of rats between the control group and experimental group exposed to various levels of Fluoride.

The above figure describes the results of the whole body DEXA scan depicting the bone mineral content (BMC). The BMC of the control group rats treated with low fluoride levels (13.713±0.175 g) was lesser than the BMC of the rats with moderate (14.55 ±1.239g) and high levels (14.225±0.64g) of fluoride.In the experimental group of Vitamin D deficient rats it was observed that rats treated with low levels of fluoride had slightly higher BMC (14.55 ± 0.54 g) than the rats treated with moderate (14.02 ± 1.69g) and high levels (13.86 ± 1.032 g) of fluoride. The overall mean of the control group (14.29 ± 0.99 g) is slightly higher than the experimental group had lower (14.11 ±1.05 g) BMC. There was no statistical significance (p= 0.07) found in between the groups of rats treated with low and high levels of fluoride.

Fat Mass

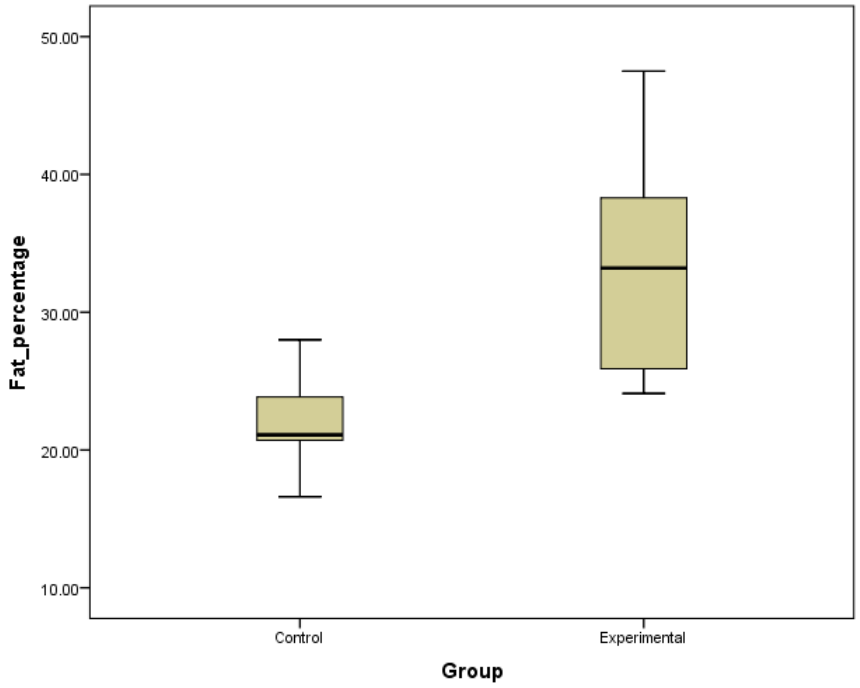


(P=0.15)

Figure 6.17: Comparison of median and interquartile of Fat Mass of rats between the control group and experimental group exposed to various levels of Fluoride.

The fat mass of the rats has been depicted in the above figure. These results also had shown interesting findings. Compared to the control group (97.67±19.57 g), the experimental group of Vitamin D deficient rats had had shown a significant increase (154.85±57.51g) in fat mass.

Fat Percentage

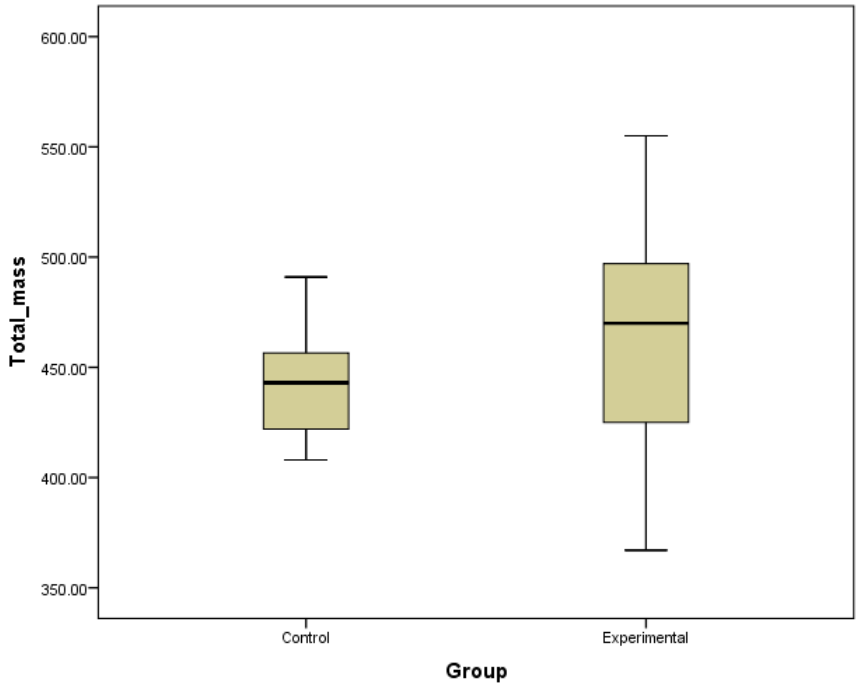


(P=0.08)

Figure 6.18: Comparison of median and interquartile of Fat percentage of rats between the control group and experimental group exposed to various levels of Fluoride.

The above figure describes the percentage of fat among the rats both in the experimental and the control groups. Rats from the experimental groups had shown significant increase (33.1±7.7) in the percentage of fat as well, when compared to the control group (22.5±3.34)

Total Mass



(P=0.44)

Figure 6.19: Comparison of median and interquartile of Total mass of rats between the control group and experimental group exposed to various levels of Fluoride.

The above figure describes the total mass of the rats between the groups. The total mass of the experimental group was found to be higher (467.47 ±56.39 g) than that of the control group (442.53 ± 26.54 g).

Biochemical Parameters

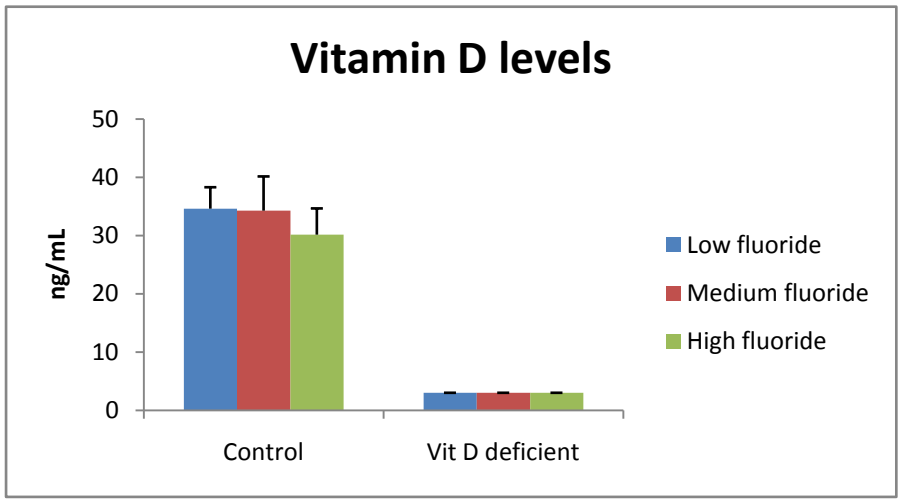


Figure 6.20: Comparison of serum Vitamin D levels of rats between the control group and experimental group exposed to various levels of Fluoride.

A number of biochemical parameters were assessed in control and experimental animals. The control group rats treated with low levels, moderate and high levels of Fluoride had Vitamin D levels of 34.6±3.68 ng/ml, 34.26±5.87 ng/ml and 30.14±4.50 ng/ml respectively. It was observed that the Vitamin D levels of the experimental group in comparison with control group were undetectable (<3ng/ml). Though there were no significant changes observed within the groups but these changes were highly significant ($p<0.001$) between the control and the Vitamin D deficient group of rats.

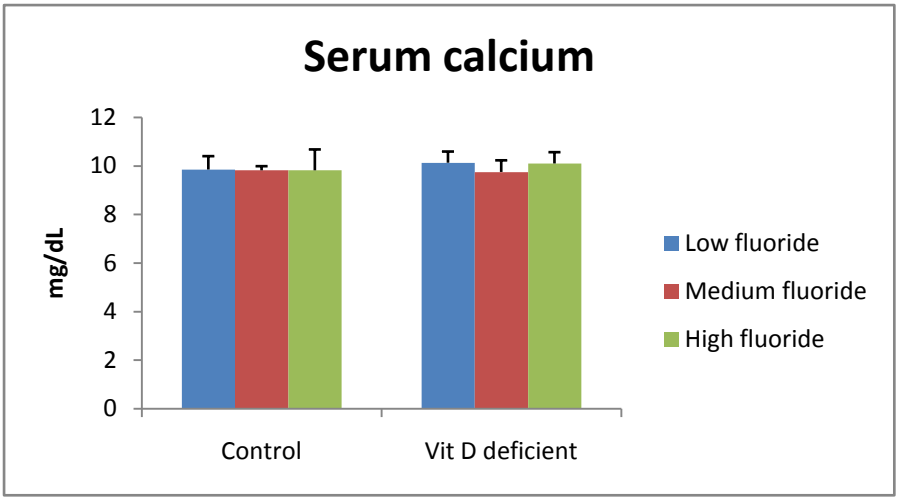


Figure 6.21: Comparison of serum calcium levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The above diagram represents the serum calcium levels among the control and experimental group rats. The control group rats treated with low, moderate and high levels of Fluoride had almost the same levels of serum calcium (9.85±0.55 mg/dl, 9.82±0.17mg/dl and 9.82±0.55mg/dl respectively). There were no significant changes observed in the serum calcium values after being treated with low, moderate and high levels of fluoride, among the Vitamin D deficient rats too (10.13±0.46mg/dl, 9.75±0.48mg/dl, 10.1 ±0.47 mg/dl). There were no significant changes noted even between the Vitamin D deficient and control group of rats.

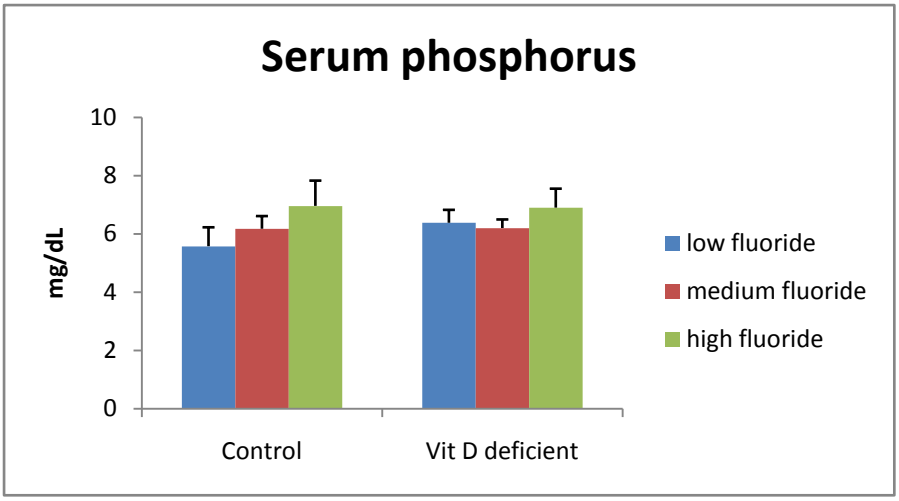


Figure 6.22: Comparison of serum Phosphorus levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The above diagram shows how the serum phosphorus levels of the rats in both groups. The rats in the control group, exposed to high levels of Fluoride, had significantly higher levels (6.96±0.87 mg/dl) of phosphorus when compared to the rats treated with moderate (6.18±0.44 mg/dl) and low levels (5.58±0.65mg/dl) of fluoride ($p<0.05$). Similarly the Vitamin D deficient rats with high levels of fluoride had higher levels (6.9±0.65 mg/dl) of serum phosphorus than the group of rats treated with moderate (6.2±0.29 mg/dl) and low levels (6.38±0.44 mg/dl) of Fluoride. The levels of phosphorus are seen to be increasing with exposure to the increasing levels of fluoride both in the control group and Vitamin D deficient group of rats. But no significant change was observed between the Vitamin D deficient and control group rats.

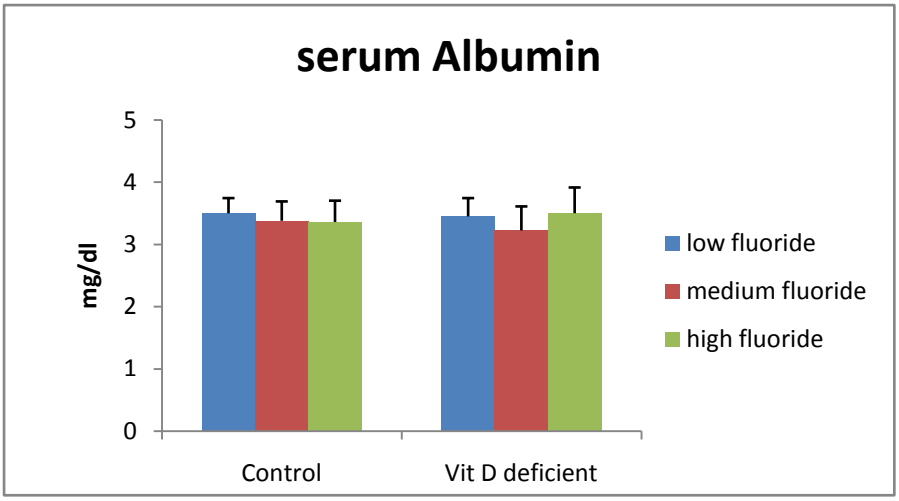


Figure 6.23: Comparison of serum Albumin of rats between the control group and experimental group exposed to various levels of Fluoride.

The above diagram compares the serum albumin levels of the rats in the control and experimental group. The control group rats treated with low, moderate and high levels of fluoride had recorded serum albumin levels of 3.5±0.25 mg/dl, 3.38 ±0.31mg/dl and 3.36±0.34mg/dl respectively. The control group rats had levels of serum albumin of 3.45 ± 0.29 mg/dl among those treated with low levels of Fluoride, 3.23±0.39 mg/dl among the rats treated with moderate levels of fluoride and 3.50±0.41 mg/dl among the rats treated with high levels of fluoride. There was no statistical significance found between or within the control or Vitamin D deficient groups of rats.

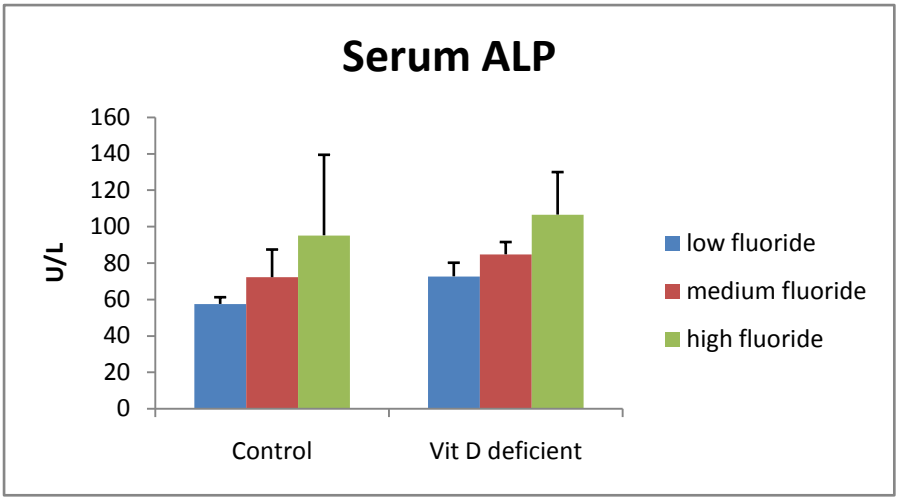


Figure 6.24: Comparison of serum Alkaline Phosphatase levels of rats between the control group and experimental group exposed to various levels of Fluoride.

Alkaline phosphatase was found to be increasing as the concentration of fluoride increases both in control and experimental group. Also, when compared to the control group, where the mean values of ALP in rats treated with low, moderate and high concentration of fluoride were 59U/L, 73U/L and 94U/L respectively; the experimental group had higher mean ALP values of rats treated with low, moderate and high concentration of fluoride 72U/L, 84U/L and 106U/L respectively. This change was found to be statistically significant ($p<0.01$). Though the ALP values are higher among the Vitamin D deficient group when compared to the control group, but this change was not found to be statistically significant.

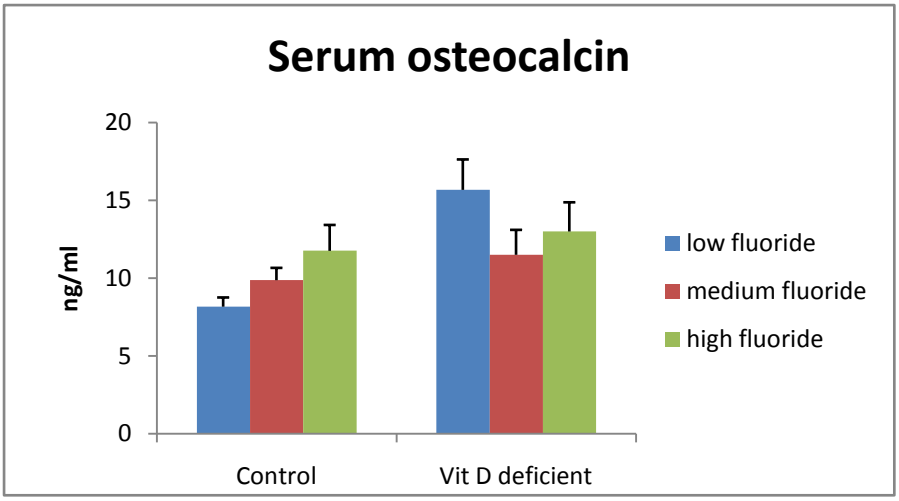


Figure 6.25: Comparison of serum Osteocalcin levels of rats between the control group and experimental group exposed to various levels of Fluoride.

In the control group the Osteocalcin (marker of bone formation) levels of rats treated with high levels of fluoride was found to be significantly higher (11.8±1.7 ng/ml) than the rats treated with moderate (9.9±0.79 ng/ml) and low levels of fluoride (8.3±0.69 ng/ml) ($p<0.01$).It could be appreciated that the levels of Osteocalcin increased with increasing levels of fluoride but this trend was not seen among the Vitamin D deficient group. The experimental group (Vitamin D deficient rats) treated with low, moderate and high fluoride levels had osteocalcin levels of 15.7±1.9 ng/ml, 11.5±1.4 ng/ml and 13.0±1.7 ng/ml respectively ($p<0.05$). This finding was found to be statistically significant. The osteocalcin levels of the Vitamin D deficient rats were found to be significantly higher than the rats of control group. ($p<0.001$)

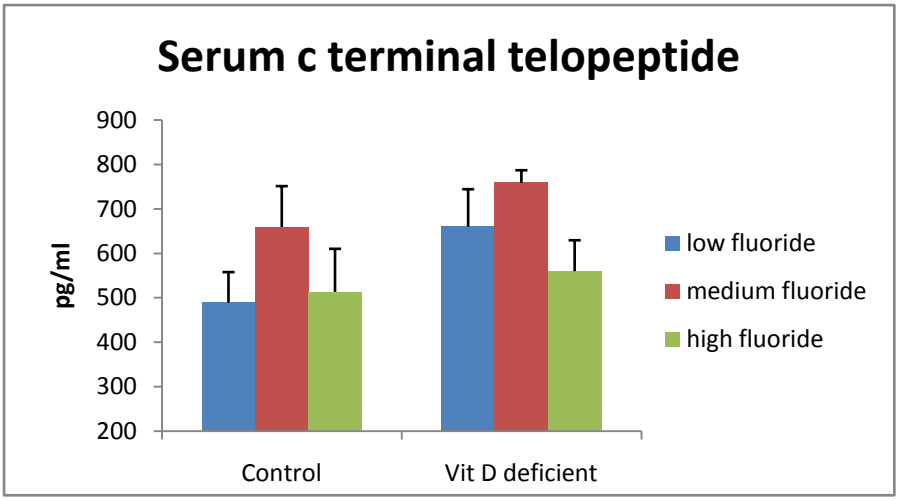


Figure 6.26: Comparison of serum C terminal telopeptide levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The above figure describes the levels of C terminal telopeptide (bone resorption marker) between the control and Vitamin D deficient rats. It was observed that the control group rats treated with low, moderate and high levels of Fluoride had values of 489±68.72 pg/ml, 659.25±91.96 pg/ml, 513.2±96.94 pg/ml respectively. These changes were found to be statistically significant ($p<0.05$) The C terminal telopeptide levels in the experimental group rats treated with low levels (660.17±84.09 pg/ml) and moderate levels (758.75±28.18 pg/ml) of Fluoride was significantly higher than the rats treated with high levels (559.8±69.62 pg/ml)($p<0.01$) of Fluoride. The rats from both the control and Vitamin D deficient group, treated with moderate levels of fluoride, were found to have a higher serum C terminal peptide when compared to the rats treated with low and high levels of fluoride. The levels of C terminal telopepetide were found to significantly higher among the rats from the Vitamin D deficient group when compared to the rats in the control group ($p<0.05$).

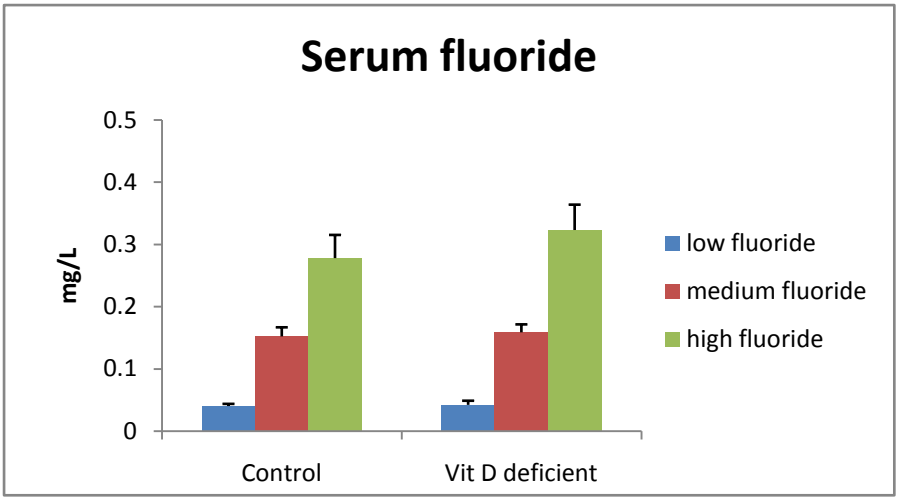


Figure 6.27: Comparison of serum fluoride levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The above given figure describes the levels of serum Fluoride in the control treated with high levels of Fluoride were significantly higher (0.28±0.04 mg/L) than the rats treated with moderate (0.15±0.02mg/L) and low levels (0.04±0.004mg/L) of Fluoride ($p<0.01$). The experimental group of Vitamin D deficient rats had also shown almost the same trend where in the rats treated with high levels of Fluoride had significantly higher levels (0.32±0.04mg/L) of serum Fluoride when compared to rats treated with moderate (0.16±0.01mg/L) and low levels (0.04±0.006mg/L) of Fluoride were found. These changes in the Vitamin D deficient group of rats was found to be highly significant ($p<0.001$). In both groups the serum Fluoride level had increased with increasing concentration of fluoride in the water (from<1ppm, up to50 ppm) but there were no changes found between the serum fluoride levels of rats from the Vitamin D deficient and the control group.

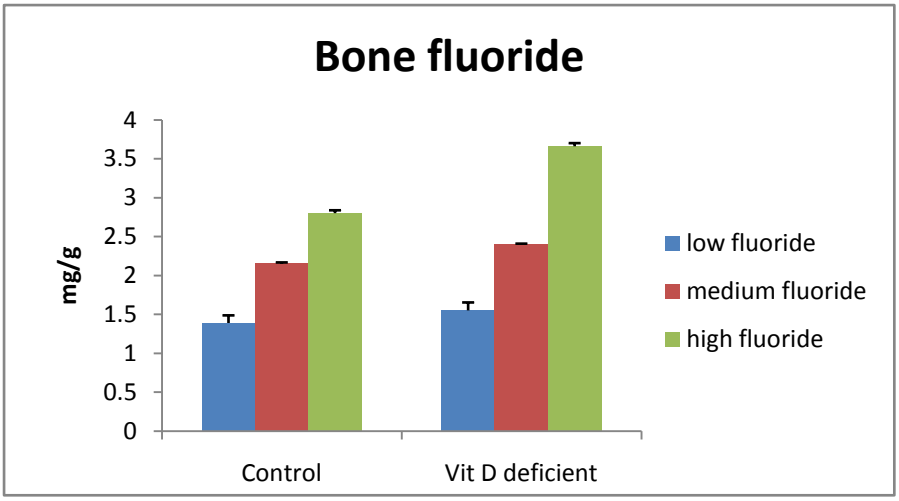


Figure 6.28: Comparison of bone fluoride levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The bone mineral content of fluoride, calcium and phosphorous were also assessed. The bone fluoride levels in rats from Control following the fluoride exposure i.e.< 1, 15 and 50 ppm were 1.46±0.1 mg/gm, 2.2±0.09 mg/gm, 2.8±0.1 mg/gm respectively. It was observed that with increasing fluoride concentration in drinking water, there was a significant increase in the bone fluoride levels as well ($p<0.01$). In the experimental group following the fluoride exposure i.e. < 1, 15 and 50ppm the levels of bone fluoride were 1.56±0.1 mg/gm, 2.4±0.05 mg/ gm, 3.7±0.23 mg/gm respectively. This increase within the Vitamin D deficient group of rats was also found to be highly significant ($p<0.001$). Even though the bone fluoride levels were higher in the Vitamin D deficient group than the control group rats, but these changes were not found to be statistically significant.

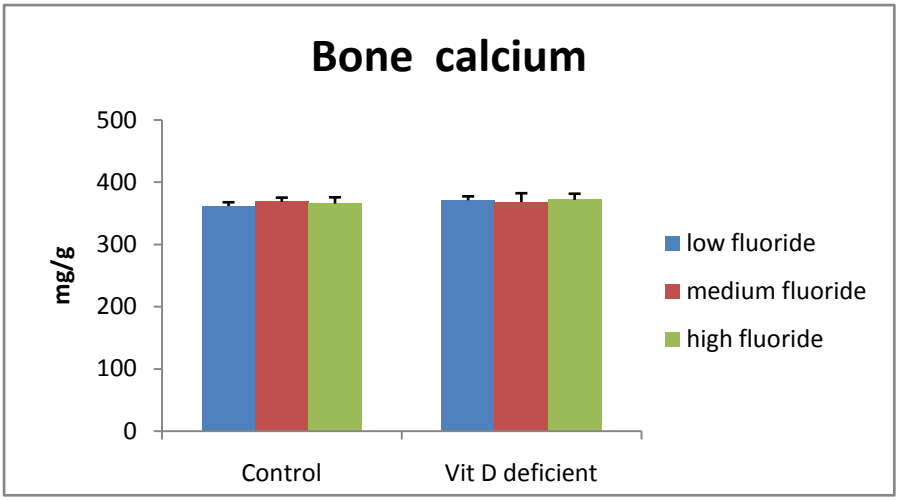


Figure 6.29: Comparison of bone calcium levels of rats between the control group and experimental group exposed to various levels of Fluoride.

The bone calcium levels, depicted in the figure above, of the experimental group rats treated with low levels of fluoride was 360±10mg/ gm wt, moderate levels of fluoride was 368±10.9 mg/ gm wt and high levels of fluoride was 371±9.8 mg/ gm wt. Whereas in the experimental group rats treated with low levels of fluoride had bone calcium levels of 371±10.9mg/gm wt, with moderate levels of fluoride was 370± 10.2 mg/gm wt and with high levels of fluoride was 372±11 mg/ gm wt. There was no significant increase in the bone calcium levels within and between the Vitamin D deficient and control group rats.

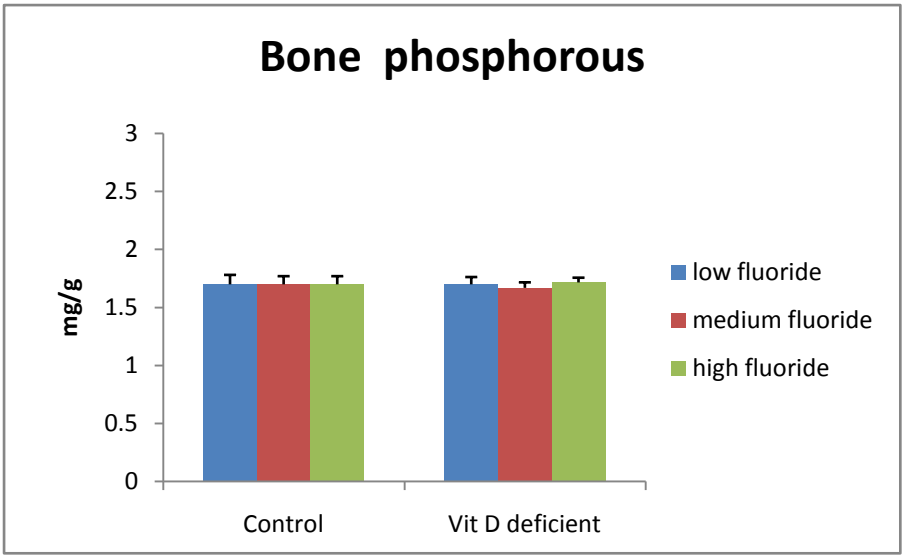


Figure 6.30: Comparison of bone phosphorous levels of rats between the control group and experimental group exposed to various levels of Fluoride.

There were no changes in the bone phosphorous levels as well. The levels in the control group 1, group 2 and group 3 rats were 1.7±0.05 mg/ gm, 1.7±0.01 mg/gm and 1.7±0.05mg/gm respectively. Whereas, in the experimental group 4, group 5 and group 6 the bone phosphorous levels were 1.71±0.05 mg/gm, 1.69±0.05mg/gm and 1.72±0.05mg/ gm respectively. There were no significant changes observed among the vitamin D deficient and control group rats or even between the groups.

Histopathological Findings

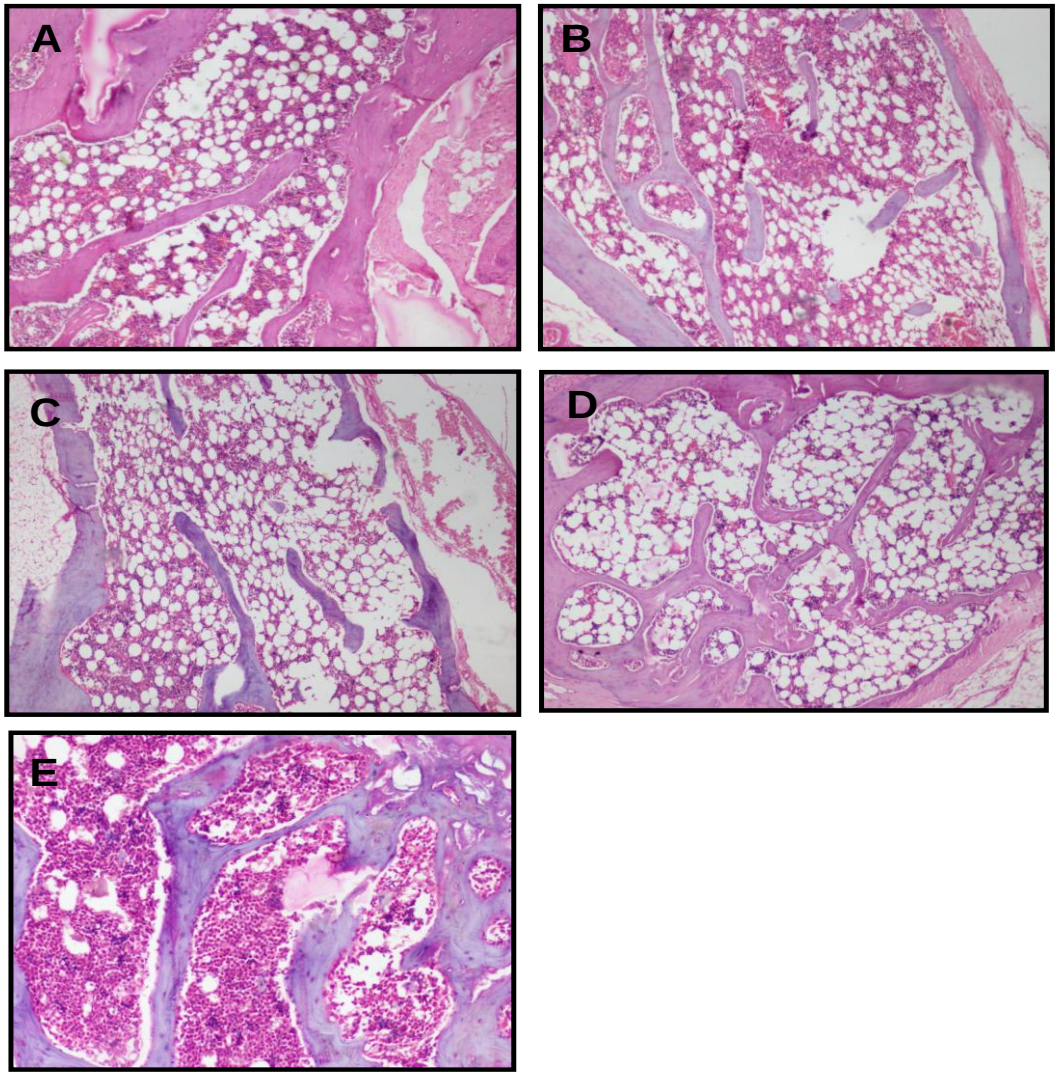


Figure 6.31: Figures A to E represent light microscopy images of sections of normal bone of rats from the control and experimental group exposed to various levels of Fluoride

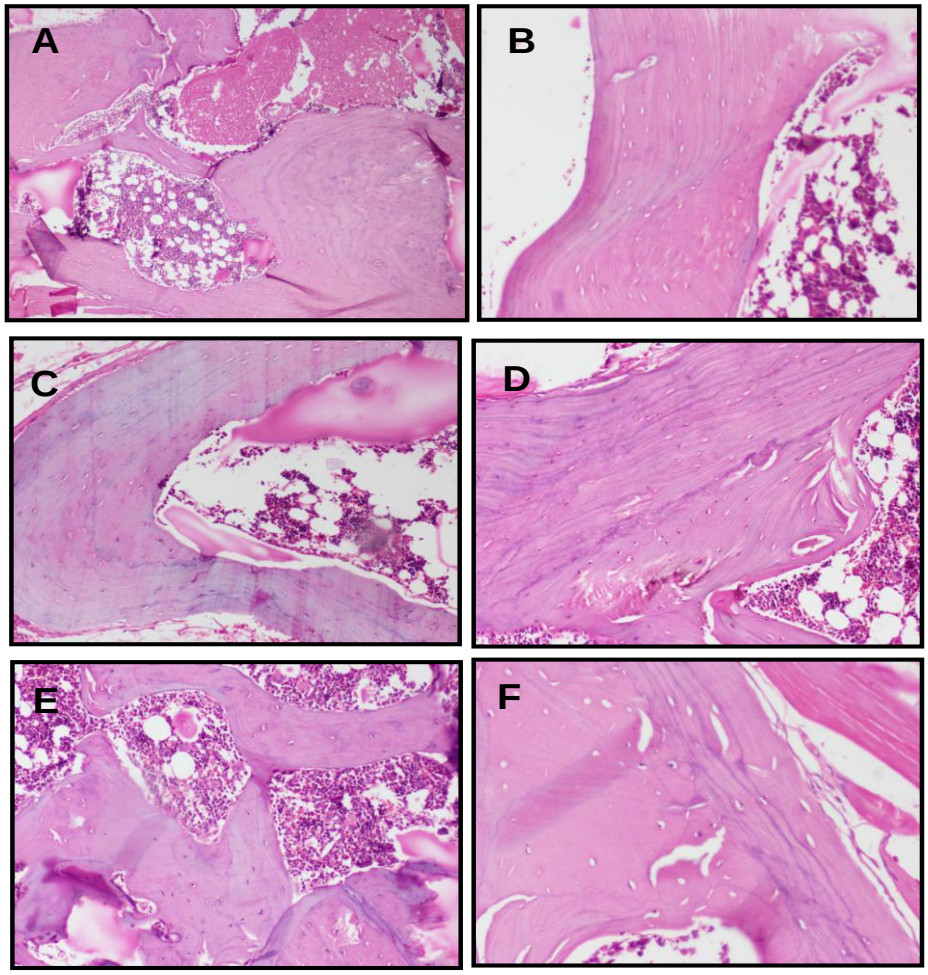


Figure 6.32: Figures A to F represent light microscopy images of sections of bone of rats from the control and experimental group exposed to high levels of Fluoride. The sections show mild thickening of bone with increased osteoid.

Bone histopathologic examination revealed normal bone in both control and Vitamin D deficient group rats treated with low and moderate levels of fluoride. **(Fig. 6.31)** But in the Vitamin D deficient rats treated (n=5) with high fluoride intake of 50ppm, examination revealed mild thickening of bone with increased osteoid. Two of the rats from the control group treated with high levels of fluoride, had revealed mild thickening and increased osteoid in the bone **(Fig. 6.32)**

Objective 5: Effects of fluoride intake in drinking water on the renal tubular function in the rat model of Vitamin D deficiency.

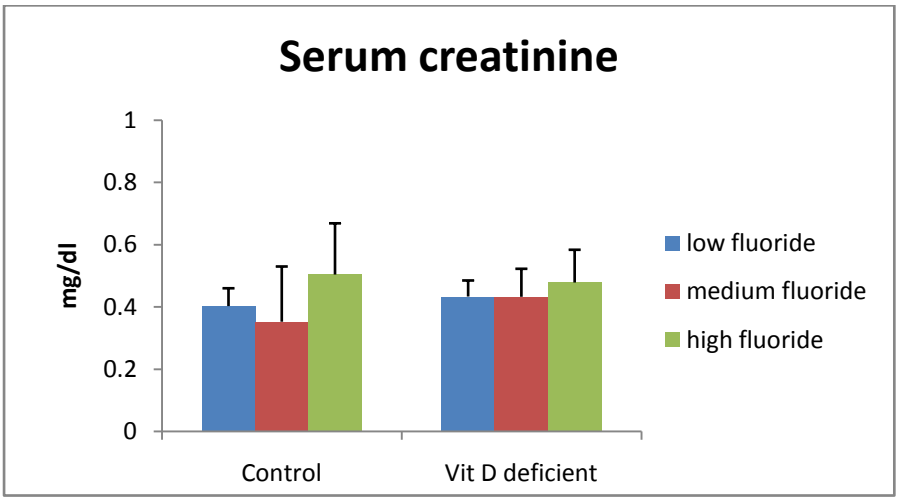


Figure 6.33: **Comparison of serum Creatinine levels of rats between the control group and experimental group exposed to various levels of Fluoride**

The study began with a total of 36 rats and it was observed that the serum creatinine (marker of renal function) of the rats in the control group treated with low levels of fluoride was lower (0.39 ± 0.12mg/dl) than the rats treated with moderate (0.43±0.04mg/dl) and high levels (0.5±0.17mg/dl) of fluoride. One of the rats from the control group treated with high levels of fluoride had serum creatinine level of 0.79 mg/dl. Likewise in the experimental group of Vitamin D deficient rats treated with low levels of fluoride had lower levels (0.43 ±0.05mg/dl) of serum Creatinine when compared to that of moderate(0.43±0.09mg/dl) and high levels (0.48 mg/dl±0.11mg/dl) of fluoride. One rat of the experimental group, treated with high levels of fluoride, had serum creatinine levels of 0.65 mg/ dl.

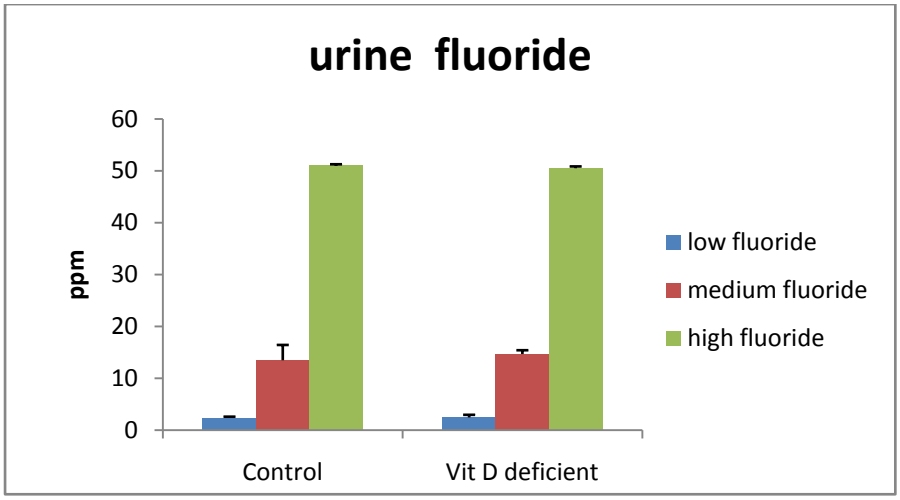


Figure 6.34: Comparison of urine fluoride levels of rats between the control group and experimental group exposed to various levels of Fluoride.

Urine fluoride levels were found to be increasing with the increasing intake of fluoride through drinking water. The urine fluoride levels among control group of rats exposed to high levels of fluoride was found to be higher (51±5.6 ppm) when compared with rats exposed to moderate (13.5±2.95ppm) and low levels (2.3±0.25ppm) of fluoride. Similarly, Vitamin D deficient rats exposed to high levels of fluoride had higher (49±6.0ppm) urine fluoride levels when compared to the rats exposed to moderate (14.7±0.73ppm) and low levels (2.45±0.5ppm) of fluoride in drinking water.

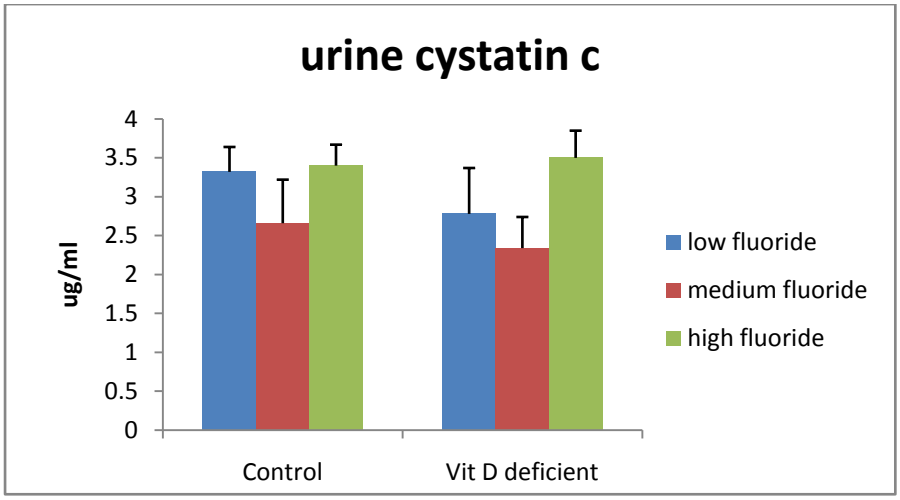


Figure 6.35: Comparison of urine Cystatin C levels of rats between the control group and experimental group exposed to various levels of Fluoride

Urine tubular marker Cystatin C was found to be lower (2.66 ±0.56ug/ml) among the rats exposed to moderate levels of fluoride when compared to the low (3.32± 0.32 ug/ml) and high fluoride (3.3± 0.27 ug/ml) exposure among the control group rats. The Vitamin D rats exposed to moderate levels of fluoride were also found to have lower (2.34 ± 0.4 ug/ml)levels of Cystatin C when compared to the rats exposed to low (2.78±0.59 ug/ml) and high levels (3.5±0.35 ug/ml) of fluoride.

Histopathological Examination of renal tissue

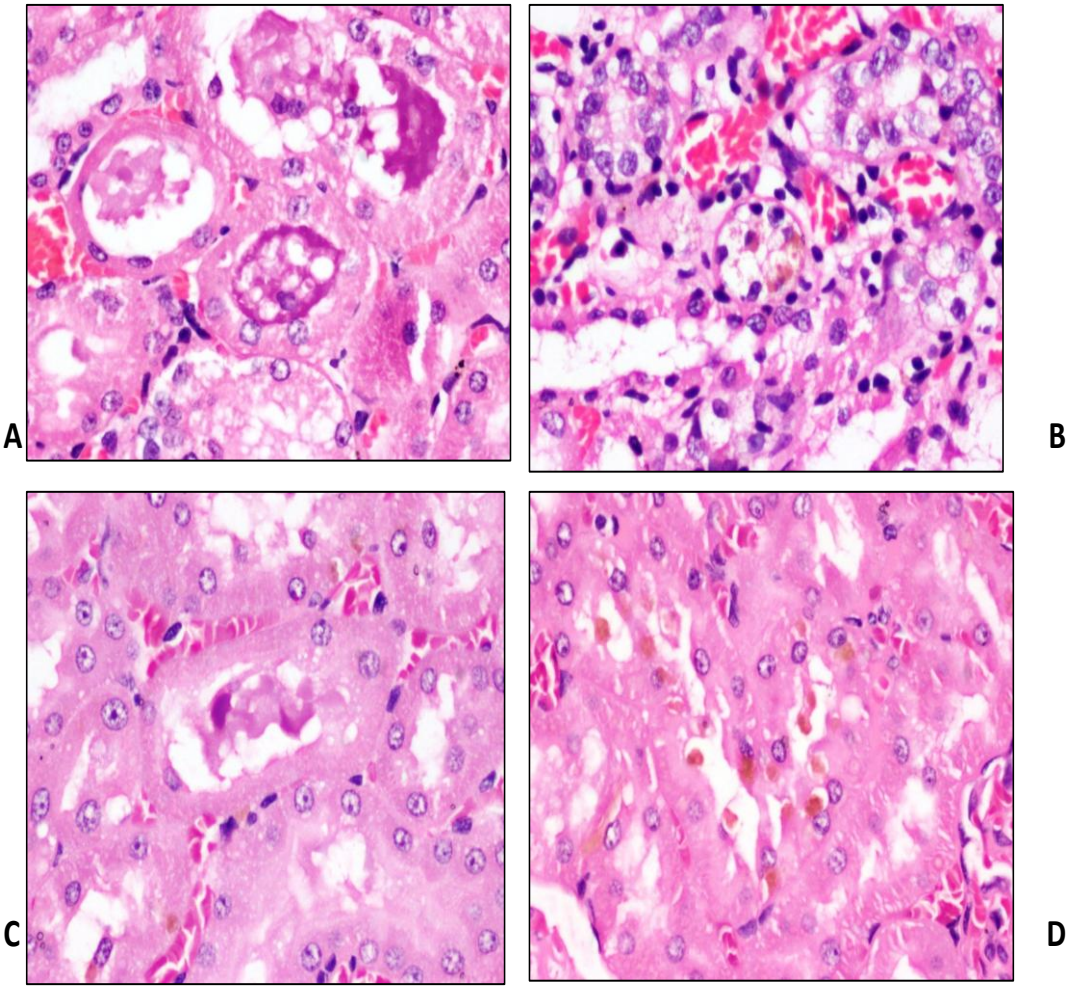


Figure 6.35: Figures(A) and (B) represents light microscopy sections of renal tissues showing acute tubular necrosis. Figures (C) and (D) represents the light micropy sections of the normal renal tissue

After sacrificing the 14 control group and 16 experimental group rats a light microscopic examination of the renal tissue was carried out. It was found that out of the rats treated with high levels of fluoride (50 ppm), each from the control and the Vitamin D deficient group, showed sparse necrotic debris in occasional renal tubules **[Fig 6.35 (A) (B)].**

DISCUSSION

DISCUSSION

The first objective was to validate the method of Ion Chromatography (IC) for estimation of Fluoride levels in drinking water in comparison to the established ISE methodology.

Drinking water is primarily used for human consumption but also is used for other household purposes. Drinking water is considered to be safe when it poses no danger to human health. Water analysis is the vital part of the chemical analysis of environmental samples. The development of the newer methods of water analysis and improving the existing methods is the major task at hand for an analytical chemist.

A variety of methods have been used for the chemical analysis of fluoride levels in drinking water which includes colorimetry, titrimetry, ion selective electrode (ISE) and amperometric titrations. However many of the methods are not specific and suffer from interference, limited sensitivity and are often difficult to automate. The new technology of IC which has replaced many wet chemical methods in water analysis was used in the present study to assess the levels of fluoride in drinking water (149). The standardization of this method showed a good within run precision, linearity and coefficient of variance. The Limit of Detection (LOD) in our analysis was found to be 0.027ppm but another study had found it to be 0.01ppm (150) and the Metrohm application note states it to be 0.05 ppm (151). This shows that the IC methodLOD correlates well. Limit of quantification (LOQ),in the IC method, was found to be 0.083 ppm, while other studies have stated it to be 0.125ppm (150). The present study is the first to compare drinking water fluoride levels using IC with conductivity detection after chemical suppression and the existing ISE method.

In comparison with the established method of ISE, using linear regression, the IC method showed a correlation coefficient $R^2 = 0.998$ with slope of 0.266 and an intercept of 0.016. IC is a novel method of assessing water fluoride levels but has its own drawbacks. For serum analysis, the fluoride has to be extracted prior to analysis and also chloride has to be removed from the sample. For urine analysis as well, chloride and other cations need to be removed from the urine sample prior to analysis as especially chloride was found to be interfering with the assay (152). The IC method requires sample extraction before analysis which makes it more costly, and time consuming. There was no change in the fluoride concentration following dilution but the interference of Chloride cannot be overcome by this process. These drawbacks make IC more tedious to perform than the ISE method which is much easier.

The second objective was to assess the Fluoride levels in the drinking water of the villages in Vellore district using Ion Chromatography.

In the present study, the researcher has assessed water fluoride levels from 165 villages in the Vellore district. The study findings showed that 19.39% of the villages had levels ranging from 1- 1.5ppm, 10.91% of the villages had levels ranging from 1.5-2 ppm and about 12.73% of the villages had water fluoride levels of 2.0-3.0 ppm. It was interesting to note that about 5 (3.03%) of the villages had water fluoride levels of >3 ppm. Out of the villages assessed most (44.42%) of these villages belonged to the Jolarper taluk and all of these villages had moderate to high (0.86- 3.56ppm) water fluoride levels. Among 165 villages, 46.06% of the villages had recorded water fluoride levels above the recommended Indian standards (<1.00ppm)

Earlier studies have also noted higher ground water levels in 4 panchayats of the Alangayam and Thirupattur blocks of Vellore districts. The ground water fluoride levels were ranging from 0.43- 4.59 ppm (53). Another study done at the Walaja block in Palar river basin had recorded water fluoride levels ranging from 0.6- 0.8 ppm (52).

It could therefore be inferred that people living in Jolarpet Taluk of Vellore district consume ground water with moderate to high levels of fluoride and therefore may be predisposed to dental and skeletal fluorosis

The third objective was to standardize a rat model of vitamin D deficiency and to assess the changes in BMD and changes in specific biochemical parameters after 4 months on a Vitamin D deficient diet (before exposure to fluoride).

The rats in the experimental group developed Vitamin D deficiency after 3- 4 months of administering the Vitamin D deficient diet as compared to the control group who were given a normal diet. This is consistent with the literature that also states that after 10 weeks of a Vitamin D deficient diet there were undetectable serum levels of 1, 25 dihydroxyvitaminD3 in the rats.(139)

It is known that Vitamin D stimulates the assimilation of calcium from the intestine in humans and stimulates mature osteoblasts in the mineralization of bone while making the calcium ion available for this purpose (18).Vitamin D is also known to facilitate mineral deposition in the bone matrix (122). On the other hand, Vitamin D deficiency is known to reduce the bone mineral density in humans (153). Thus Vitamin D plays a vital role in bone metabolism.

There has been ongoing research on finding new strategies of detecting even minute changes in the BMD with high accuracy and greater feasibility. DEXA has been found to be a sensitive, non-invasive and precise method of measurement of BMD in animals as well. One study assessed the BMD in the rats at the lumbar, proximal tail and tibial bones. It was observed that before and after 4 weeks of performing an ovariectomy in the rats, there was a significant decline in the spine BMD compared to the rats in the control group. The accuracy of measurement by DEXA was found to be good, precise and feasible to detect even the slightest change in BMD over short-periods of time (154).

In the present study, the whole body DEXA scans were done for the first time among rats. They were performed on rats from the experimental group which showed a significant decrease in BMD ($p<0.05$) and BMC ($p<0.05$) compared to rats in the control group. The literature states BMD was lower among the individuals with lower 25-OHD levels when compared to individuals with normal levels of 25-OHD. In south India, women from both the reproductive and the post-menopausal age groups were assessed for Vitamin D deficiency and BMD. It was observed that women from both the groups had Vitamin D deficiency coexisting with a low BMD(124). Another study was conducted in India, to assess the association between the Vitamin D, PTH and its effect on BMD. In 1829 adolescents and 1346 persons aged 50 years and above, Vitamin D deficiency again was found to be coexisting with low BMD. Less than half the subjects with Vitamin D deficiency had increased serum PTH and their BMD was decreased (128).

In the present study, there was a significant increase in the fat mass ($p<0.05$) and percentage of fat ($p<0.01$) among the experimental group (Vitamin D deficient) rats. Adipose tissue is known to regulate and be regulated by Vitamin D. The literature states that the increased intracellular calcium has a blunting effect on the lipolytic response to catecholamines by activation of the enzyme phosphodiesterase-3B (also known to mediate the antilipolytic

response of insulin) and by compromising efficiency of the insulin-stimulated glucose uptake. (155). It is been demonstrated that increased levels of PTH increase calcium in the adipocytes. As PTH is known to increase in the state of Vitamin D deficiency, it can be hypothesized that Vitamin D deficiency increases fat mass in individuals.

The biochemical parameters of serum calcium, albumin and alkaline phosphatase were found to be nearly the same in both groups of rats in accordance with another study (139).Vitamin D is necessary for the regulation of serum calcium, phosphate and alkaline phosphatase. However rats fed with a Vitamin D deficient diet for 10 weeks did not show any changes in the serum calcium. A study done among 1,210 subjects between 20-69 years old found 79.6%of the subjects to be Vitamin D deficient. Their serum calcium, phosphorous, and alkaline phosphatase levels were found to be unchanged when compared to the control group(156).Thus it is concluded that serum calcium, alkaline phosphatase and phosphate may not show changes in mild Vitamin D deficiency.

In conclusion, Vitamin D deficiency plays a major role in bone formation and in maintaining bone mineral content and density. There appear to be no changes in the biochemical parameters of calcium, albumin and alkaline phosphatase in the early stages of Vitamin D deficiency in this rat model.

The fourth objective was to study the changes in bone morphology and metabolism induced by varying levels of fluoride intake in the rat model of vitamin D deficiency.

The whole body DEXA scan revealed that the BMD of the control groups and the Vitamin D deficient groups appeared to increase with the increasing levels of fluoride in drinking water. In addition, the BMD of the rats belonging to the control group was higher than that of the experimental group (Vitamin D deficient). Interestingly there was a significant ($p<0.05$) change in the BMD between both the groups of rats treated with low, moderate and high levels of fluoride. In the animal model it was found that Wistar rats on being fed a diet rich in sodium fluoride and calcium had shown an increase in BMD (157). In humans as well, it was observed that women consuming water with lower fluoride levels (<0.6 ppm) had slightly decreased bone densities when compared to the women in areas with higher drinking water fluoride levels (>0.6 ppm) (82) . Earlier studies done in humans, have found an increase in BMD of the axial bone in women and an increase in the vertebral bone density among those consuming drinking water which is high in fluoride (>8.5 ppm) (81). Some studies have assessed the effect of fluoridation of water on the BMD of young women consuming lower levels of fluoride (<0.1ppm) in drinking water and comparing the BMD with the women consuming higher (>1.0ppm) levels of fluoride in drinking water. It was found that women who were consuming higher levels of fluoride were found to have higher BMD (80). To study the effect of the contributing factors of age, sex and menopausal status, perimenopausal women consuming higher levels of fluoride 1.0 to 1.2 ppm in drinking water were found to have a higher BMD in the axial bone when compared to women consuming lower levels of fluoride in drinking water (<0.3ppm) (83). Another study assessed the relationship between increased fluoride intake and BMD among male subjects. Radiological examination of the vertebral bone revealed that with increasing levels of fluoride, the bones were less porous and

had less mineral per unit of bone, suggesting Osteomalacia like changes (84) Thus reiterating that fluoridation of drinking water improves the mineralization of bone but may not affect the bone strength. This may predispose individuals exposed to excessive fluoride to higher risks of fractures.

The researcher has shown that the rats from the Vitamin D deficient group had a lower BMD than the control group rats. After exposure to high levels of fluoride, moderate in both the Vitamin D deficient and control group rats there was an increase in BMD observed. It has been demonstrated that there are other factors such as age, calcium intake, physical activity which may also influence BMD positively (84).The researcher has now shown that even in Vitamin D deficiency fluoride can cause an increase in BMD.

The BMC of the control group rats, treated with low levels of fluoride, was less than those treated with moderate and high levels of fluoride. There were changes in the BMC of the rats in the Vitamin D deficient group where in the BMC decreased with increasing levels of fluoride in drinking water. . However, the overall control group rats had a higher BMC than the Vitamin D deficient rats. Therefore, it may be inferred that fluoride content in drinking water may influence the BMC. These findings correlate to a study done among women with osteoporosis. It was observed that following daily treatment with high levels of sodium fluoride (30mg) and 1 gm of calcium per day for about a period of 3 years, there was an increase in the BMC in the spine of these women (158).

The present study showed that the rats belonging to the Vitamin D deficient group had a significantly higher fat mass and percentage of fat when compared to the control group. Vitamin D deficiency is known to be common cause of obesity in humans. The total mass of the rats was also higher in the Vitamin D deficient group when compared to the control group rats. This signifies fat mass, percentage and total mass are influenced in the presence of Vitamin D deficiency.

Biochemical estimations showed that Vitamin D levels of the rats in experimental group were undetectable and that the rat serum fluoride levels increase with increasing levels of fluoride concentration in drinking water. Serum ALP was found to be increasing with increasing concentrations of fluoride in both the groups. However there was no significant change in the serum calcium, phosphorous and albumin between groups. Serum Alkaline phosphatase levels were higher in the Vitamin D deficient rats when compared to the control group rats. These findings correlate to a study assessed the serum and bone ALP levels in rats exposed to varied levels of fluoride. It was found that rats exposed to high (150ppm) levels of fluoride had higher serum ALP levels when compared to the group exposed to low levels of fluoride in drinking water (77).The morphology of the bone was studied using pure hydroxyapatite discs, where it was found that there was increased cell attachment, proliferation and higher alkaline phosphatase activity among the cells cultured on these discs (16). This signifies that increased concentrations of fluoride increase osteoblastic cell activity.

Serum Osteocalcin is a sensitive specific marker for bone formation and a reliable indicator of changes in bone metabolism (159).Serum Osteocalcin levels were measured and were found to be increasing with increasing concentration of fluoride in the control group. But in contrast, among the low Fluoride, Vitamin D deficient rats, the serum level of osteocalcin was almost double that of their controls. This value fell and then rose slightly again in the moderate to high exposure groups. In each of the experimental groups serum osteocalcin was higher than in their respective control group. The study findings correlate to a study which had assessed the serum calcium, alkaline phosphatase, 25-OHD, PTH hormone and osteocalcin, before and after exposure to sodium fluoride for a period of 3 weeks among healthy male human subjects (78). It could be observed that the osteocalcin levels were higher after exposure to fluoride than the values prior to the exposure of fluoride. Another study found that on exposing the rats for 90 days to 150ppm doses of fluoride, there was a

significant increase in the osteocalcin levels which in turn signifies that fluoride influences the osteoblastic activity (77).The researcher has also shown this but found that Vitamin D deficiency complicates the changes observed.

C terminal telopeptide is a bone resorption marker and represents the bone osteoclast activity. Higher serum levels of C terminal telopeptide therefore indicate more osteoclastic activity (159). In the present study the researcher found that among the control group rats exposed to moderate levels of fluoride the C terminal telopeptide was elevated and a decrease in the levels was observed on exposing the rats to high levels of fluoride. Similar trend was observed among the Vitamin D deficient rats. Literature states that for past two decades, fluoride is known to inhibit the activity of the osteoclasts (160) (161). Present study findings were correlated with study done to assess the effect of fluoride on the osteoclasts. It was found that the activity of osteoclasts decreased with increasing concentration of fluoride exposure. In the present study, the researcher found that the higher concentration of fluoride has an inhibitory effect on activity of osteoclasts when compared to the moderate levels of fluoride.

The serum fluoride and bone fluoride content in both the groups of rats was found to be increasing with increasing levels of fluoride consumed in drinking water, but the Vitamin D deficient rats had higher bone levels of fluoride when compared to the control group rats. Though the Vitamin D deficient rats in each group had higher levels of fluoride than their controls but a statistically significant increase could not be demonstrated.

The bone calcium content and phosphorous levels were almost the same in both the control and Vitamin D deficient rats and did not show any significant change with increasing levels of fluoride in drinking water.

The histopathological examination had shown normal bone in the control and Vitamin D deficient groups exposed to low and moderate levels of fluoride. But five of the rats from the

Vitamin D deficient group exposed to high levels of fluoride had thickening of bone with increased osteoid. Two of the rats from the control group exposed to high levels of fluoride, as well had mild thickening and increased osteoid. This clearly demonstrates along with changes in BMD, BMC, Osteocalcin and C-terminal peptide that vitamin D deficiency makes the rats (5 versus 2) more susceptible to bone damage when exposed to high levels of fluoride (50ppm) in their drinking water. The Fluoride is causing a flux in bone remodelling. It gets incorporated in the bone and affects both construction and breakdown of bone.

In the Literature it is said that fluoride ions affect osteoblastic cell activity, possibly by promoting osteoblast mitosis and stimulating growth of cells of the osteoblast lineage, thereby enhancing bone osteoblast numbers and accelerating bone turnover (16, 77). This is evidenced by increase in levels of Alkaline phosphatase and Osteocalcin. This correlates with the present study findings where, it was found that with increasing fluoride concentration in water, the osteocalcin, ALP and bone fluoride content also increases both in the control and the Vitamin D deficient group. It is interesting to note that the ALP, bone fluoride content and Osteocalcin was found to be higher the Vitamin D deficient rats, with respect to the control group rats, as the fluoride concentration increased. Also, the bone resorption marker, C terminal telopeptide levels indicated that fluoride has an inhibitory action on the activity of osteoclasts with high levels of fluoride. Higher concentrations of fluoride were found to cause greater pathological changesi n the bone of Vitamin D deficient rats as well. Thus it may be inferred that fluoride affects the osteoblast activity and fluoride deposition in bone while Vitamin D deficiency may accentuate the effect of fluoride on the bone. This may lead to the causation of fluorotoxic metabolic bone disease.

The fifth objective was to study the effects of fluoride intake in drinking water on the renal tubular function in the rat model.

The researcher observed that the serum creatinine levels of the rats from the control and the experimental group (Vitamin D deficient) had not shown any significant changes when treated with low and moderate levels of fluoride. The Vitamin D deficient rats did not have a higher incidence of renal glomerular impairement after exposure to fluoride, as expressed as a rise in serum creatinine. Subtle changes in renal tubular function are indicated by increases in urinary cystatin C excretion. The researcher found that the urine tubular marker increased in both the control and Vitamin D deficient group of rats on exposure to high levels of fluoride but this was not statistically significant. The changes observed are thus inconclusive.

Male Wistar rats were treated with 15ppm and 50 ppm of fluoride in drinking water for 40 days. This study used early and sensitive biomarkers of renal tubular damage and found a considerable dose dependent rise in urinary beta 2 microglobulin and cystatin C with an increase in Kim-1, Clu and OPN (94). In the present study the researcher found that, on pathological examination of the renal tissue, two rats, one each from the control and the experimental group had shown signs of tubular damage on pathological examination and an elevated serum creatinine levels (0.79mg/dl and 0.65mg/dl respectively) after exposure to high levels of fluoride. Hence, it may be concluded that there is a selected incidence of renal tubular damage occurring with increased fluoride intake in drinking water.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

- Through this study, an Ion Chromatography method has been validated and has been found to have a good correlation with the well-established ISE method. Ion Chromatography is therefore a reliable method in assessment of fluoride levels in the drinking water, but may have a few drawbacks. IC method is known to be expensive and more tedious as it requires chloride and other cations to be extracted from serum and urine samples prior to analysis, which makes the ISE method much easier to perform.
- The IC method was used to analyse the ground water and drinking water levels of 165 villages in and around the Vellore district, Tamil Nadu. About 46.06% of the villages assessed had water fluoride levels recorded above the Indian standards (<1.00ppm); 19.39% of the villages had water fluoride levels ranging from 1-1.5ppm; 10.91% had levels ranging from 1.5-2 ppm and 12.73% had water fluoride levels ranging from 2.0-3.0 ppm.The Jolarpet taluk of Vellore district has been found to have high levels (0.86-3.56 ppm) of fluoride in drinking water, which makes the people residing in this areaat high risk to develop dental and skeletal fluorosis.
- Vitamin D deficiency was produced in an experimental group of rats. To assess the changes occurring in bone,in these animals,–full body DEXA scan, was usedfor the first time to assess BMDin control and Vitamin D deficient rats. There was a significant decrease in BMD ($p<0.05$) and BMC ($p<0.05$) among the vitamin D deficient rats. Thus, Vitamin D deficiency has been shown to play a vital role in bone formation and in maintaining BMC and BMD. It was interesting to note that there was a significant increase in the fat mass ($p<0.05$) and fat percentage ($p<0.01$) among the Vitamin D deficient rats. It may be hypothesised that Vitamin D deficiency can lead

to an increase in fat mass in individuals. The biochemical parameters of calcium, albumin and alkaline phosphatasewere found to be unchanged in the early stages of Vitamin D deficiency in this rat model.

- The study found that with increasing levels of water fluoride levels there was a significant increase in BMD ($p<0.05$). Alkaline Phosphatase, serum and bone fluoride content and Osteocalcin were found to be increased in the Vitamin D deficient rats when compared to the control group rats, as the water fluoride levels increased. On the other hand, increased levels of fluoride was found to have a stimulating and then an inhibitory effect on the activity of the osteoclasts as evidenced by the elevated C terminal telopeptide levels with exposure to moderate fluoride levels and a fall in the levels on exposure to high levels of fluoride . Thus, highlighting that fluoride may affect the osteoblast activity and fluoride deposition in bone while Vitamin D deficiency may accentuate this effect. This could possibly lead to the causation of fluorotoxic metabolic bone disease.
- Pathological changes of mild bone thickening and increased osteoid in the bone were noticed among five (80%) of the Vitamin D deficient rats exposed to high levels of fluoride. A lesser percentage (40%) two of the rats belonging to the control group exposed to high levels of fluoride showed these same changes in bone.
- Renal function was assessed by analysing the serum creatinine, urine fluoride and urine Cystatin C which were higher among the rats treated with high levels of fluoride when compared to those treated with low and moderate levels of fluoride. Renal tubular changes may not have been found in all the cases but it was observed that fluoride does play a role in renal damage in selected cases.

CONCLUSION

In conclusion, IC is a reliable method of analysing water fluoride levels. Using the IC method for analysis, Jolarpet taluk of Vellore district was found to have high levels of ground water fluoride which may predispose the population to develop dental and skeletal fluorosis. Researcher found that, Vitamin D deficiency affectsBMD,BMC and fat mass in individuals. Fluoride also does have an effect on the osteoblastic activity as well as fluoridedeposition in bone and these effects are accentuated in the presence of Vitamin D deficiency, perhaps leading to the causation of FMBD. Though high fluoride intake is known to deteriorate renal tubular function but it may do so only in selected cases. This paves way for future research to analyse other factors involved in causation of FMBD.

RECOMMENDATIONS

RECOMMENDATIONS AND FUTURE RESEARCH IMPLICATIONS

- As the researcher found the Jolarpet taluk to have high ground water fluoride levels, further investigation of the co-existing Vitamin D deficiency and incidence of dental and skeletal fluorosis among the population must be carried out.
- Further investigations may also be carried out among children belonging to the various socio-economic backgrounds in the Jolarpet taluk to assess the relationship of age, socio-economic background and nutritional status to Vitamin D deficiency and development of FMBD.
- To study further the role of fluoride in causing renal tubular damage among the adults and school-going children at the Jolarpet taluk.

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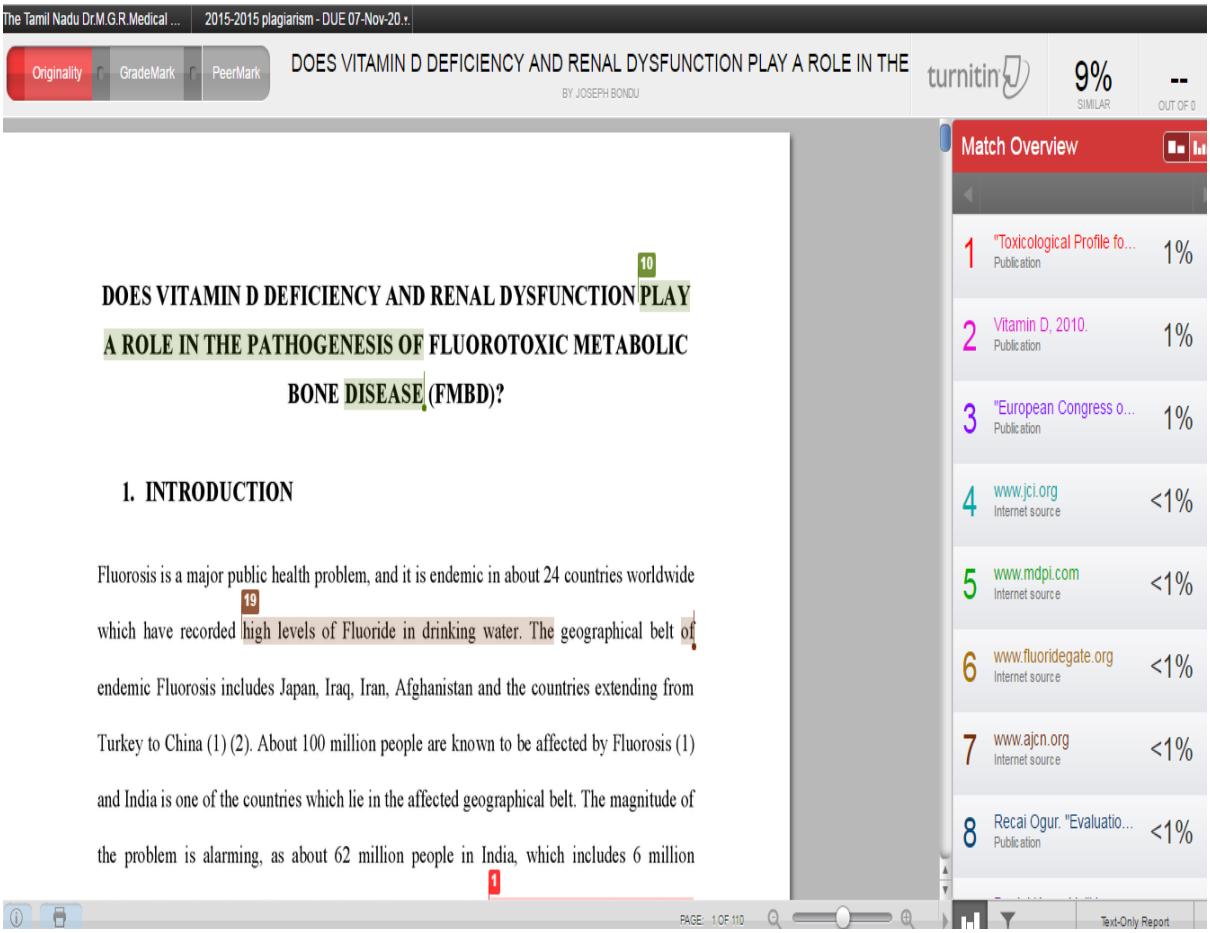
APPENDIX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1. INTRODUCTION Fluorosis is a major public health problem, and it is endemic in about 24 countries worldwide which have recorded high levels of Fluoride in drinking water. The geographical belt of endemic Fluorosis includes Japan, Iraq, Iran, Afghanistan and the countries extending from Turkey to China (1) (2). About 100 million people are known to be affected by Fluorosis (1) and India is one of the countries which lie in the affected geographical belt. The magnitude of the problem is alarming, as about 62 million people in India, which includes 6 million children, have developed Fluorosis, due to the consumption drinking water with high levels of fluoride. The states which are worst affected are Rajasthan, Bihar, Jharkhand, Punjab, Chhattisgarh, Maharashtra, Haryana, Andhra Pradesh, Karnataka and Tamil Nadu (3). Thus the researcher has aimed to study the prevalence of high ground water fluoride levels in the various villages in the district of Vellore, Tamil Nadu . The toxic manifestations of Fluorosis are Dental Fluorosis, Skeletal Fluorosis and Genu Valgum which is found among children (4). Dental fluorosis occurs when there is excess Fluoride ingested over the years of tooth calcification (especially during the first 7 years of life) (5).The early signs may be "mottling" of dental enamel (6), loss of the shiny appearance and chalky white patches on the teeth. These

white patches then turn yellow and sometimes even brown or black (7). Skeletal Fluorosis is associated with intake occurring all through life of water with a fluoride content of 3.0 to 6.0 ppm or more (6). The is deposition of fluoride in the skeleton may lead to disabilities like musculoskeletal dysfunction, arthritis, ankylosis of spine with radiculopathy , osteosclerosis with ligament calcifications as well as peripheral neuropathy (8). Genu valgum is the bowing of lower limbs occurring with the bone disorders of osteomalacia, osteosclerosis and osteopenia (9). This has been reported from some districts of Andhra Pradesh, Rajasthan, Bihar, Karnataka and Tamil Nadu. Studies have shown that it is children with a higher intake of fluoride that exhibit this syndrome (10) (11). The World Health Organization (WHO) has prescribed the acceptable levels of Fluoride to be <1.5ppm in drinking water (12), while the Indian standard of permissible drinking water levels is less than 1 ppm(12).But some states in India, have markedly high levels of fluoride. Water fluoride levels of more than 1.5 ppm have been recorded in about 17 states in India (13). The literature states that eventhough high levels of Fluoride consumption can lead to dental and skeletal fluorosis an optimal amount of consumption is essential for adequate bone mineral deposition. Fluorosis is also known as Fluorotoxic Metabolic Bone Disease (FMBD). Teotia studied the prevalence of disorders of calcium and bone metabolism among the Indian population from 1963 to 2005. Among the 4, 11,744 subjects found to have these disorders, 52% had nutritional bone diseases, 43% had endemic Fluorosis