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Histology of nonfluorosed and fluorosed bone - An *in vitro* study

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Abstract

Aim: The literature on effect of fluoride on dental caries is well discussed in contrast to periodontal tissues. However a recent review has explored an epidemiological association between fluorosis and periodontal disease and also the influence of fluorosis on periodontal structures along with the comparison of influence of periodontal treatment on fluorosed and non fluorosed teeth. There is a scarcity in literature dealing with effect of fluorosis on biological tissues like bone. Alveolar bone which is an integral part of periodontium similar to extremities has not been studied for mechanical, histologic and mineral aspects of fluorosed bone. Hence the aim was to study the histology of fluorosed and nonfluorosed femoral bone.
Material and methods: A total of 24 healthy nonfluorosed and fluorosed bone (femur) specimens were collected to assess and compare the histology of fluorosed versus non fluorosed bone using light microscope.
Results: Cellularity of cortical and cancellous bone was found to be statistically significant in nonfluorosed group (10.72±4.10, 8.74±2.34) when compared to fluorosed group (6.61 ±3.31, 5.69±1.31) respectively. Trabacular density was same in both nonfluorosed and fluorosed bone [statistically non significant, p= 0.615]. However trabeculae were thick in nonfluorosed bone and short and thin in fluorosed bone.
Conclusion: The observed histologic changes would influence the pathogenesis of periodontal disease and /or outcome of periodontal treatment. Dental fluorosis may soon be designated as environmental risk factor in endemic fluorosed area.
Keywords: Dental fluorosis, periodontitis, histology, femoral bone.

1. Introduction

Fluorine is a common element in the earth's crust and is an essential element for the calcification of bones and teeth. Fluoride ion has played a major role in dramatically reducing dental caries over past 40 years. Excessive systemic exposure to fluoride can lead to disturbances of bone homeostasis, enamel development [dental/ enamel fluorosis] and mineralization. The severity of fluorosis on periodontal hard and soft tissues is dose dependent and also depends on timing and duration of fluoride exposure during development. [1]

Although fluorosis is a global and national problem, there are minimal researchers who are investigating the role of fluoride on periodontal tissues which increases the susceptibility to periodontal disease because of the alteration produced in both hard and soft tissues of periodontium. [2] Before an effective treatment is established, it is pertinent to take into account the non-skeletal tissue involvement in the disease process. It has been stated that fluorine, one of the most reactive elements is found (as fluoride) in many organs and tissues besides the bones and the teeth. [3] It is of importance to investigate the mechanism of action of fluoride ions and the degree of involvement of various other body tissues especially those which do not possess a buffering agent like apatite crystals which are believed to neutralize fluoride ions in bones, before the defluoridation agents like serpentine and magnesite could be employed effectively.

The literature on effect of fluoride on dental caries is well discussed in contrast to periodontal tissues. However, fifteen years of research and a recent review by Vandana K L has presented an epidemiological association between fluorosis and periodontal disease, but also the influence of fluorosis on periodontal structures along with the comparison of influence of periodontal treatment on fluorosed and non fluorosed teeth. There is a scarcity of literature dealing with fluorosis effect on biological tissues like bone and cementum. [2]

Histological aspects of fluorosed bone have been studied scantily. However in a human study [autopsied specimen]showed that fluorotic root surfaces are irregular and have revealed heavy deposits of calcified masses in the form of excessive amounts of fluorine osteocementum at the apical region of the teeth and radiographically osteosclerosis, cementosis and periapical root resorption.[4]

From the above literature, it is understood that the histology of bone from endemically fluorosed belt is not studied. Hence, the comparison of fluorosed versus non fluorosed bone is a new area of interest in fluorosis research. Medline search using keywords fluorosed and non fluorosed bone does not reveal much data. Present study aims to find out changes in histology of fluorosed versus non fluorosed bone.

2. Materials and methods

A total of 24 healthy nonfluorosed and fluorosed bone (femur) samples were collected from orthopaedic section of S. S. Institute of Medical Sciences, Davangere. Subjects with age group of 35 to 55 (for bone) years of both the sexes were included respectively. Written consent was taken from all subjects and ethical clearance was obtained from the Institutional review Board (IRB; Ref No. CODS/ 2184) of College of Dental Sciences, Davangere, Karnataka according to Rajiv Gandhi University of Health Sciences, Karnataka protocols.

The bone samples were required to meet the following inclusion criteria: Bone specimens from systemically healthy patients ; Fluorosed subject selection was based on following criteria – subjects who lived in the endemic water fluoride area for 5 to 10 years consuming water with fluoride levels above 1.2 to 3 ppm (Davangere water fluoride levels 0.2 mg/l to 2.41 mg/l), Subjects with mottled tooth enamel i.e., dental fluorosed stains assessed with the scores C , D E , F of Jacksons simplified fluorosis index (1974); Bone specimens were obtained from subjects who underwent surgical intervention following fracture due to trauma (accident) where in part of the bone (femur) was to be removed. The above criteria used for assessment of fluorosed teeth were considered to select the bone specimens. The exclusion criteria were: Subjects with any metabolic bone disorders (hyperparathyroidism, Paget's disease, hypophosphotasia) and infectious diseases. Sample size was 11.72 using $n= z^2 \sigma / (x_1 - x_2)^2$

2.1 Procedural steps

2.1.1 Collection of bone specimens

Healthy nonfluorosed and fluorosed bone were collected and stored in bottles containing 10 % neutral buffered formalin. [5]

Tissue fixation, decalcification, processing and paraffin embedding

2.1.2 Decalcification of bone

Bone samples obtained were subjected to decalcification process using 10% nitric acid [6] at room temperature with changes of the solution at regular intervals till the end point was reached.

2.1.3 Tissue processing

The decalcified bone were subjected to water wash for 24 hours and routinely processed. Subsequently the processed bone was then embedded in paraffin wax and tissue blocks were prepared. Sections were made using soft tissue microtome and sections were transferred to micro slide.

Tissues were deparaffinized in xylene for 2×8 min, rehydrated in a descending ethanol series (100% 2×5 min, 90% for 5 min, 80% for 5 min) and rinsed in deionized water 2×5 min. For hematoxylin and eosin (H&E) staining, deparaffinized and rehydrated sections were dipped in hematoxylin for 5min, sections were rinsed in running water to remove the excess stain. The hematoxylin stained section were give a single dip in 1% acid alcohol and were then placed under gently running water for 10 min for bluing. Next they were dipped in eosin Y for 2 mins, placed under gently running water to rinse excess stain, then dehydrated in an ascending ethanol series (80% for 1 min, 90% for 1 min and 100% for 1 min), then cleared in clean xylene 2×10 min and allowed to air dry for 60 min before mounting cover slips with DPX for histological assessment.[7]

Following parameters were assessed

- Density of cells in cortical and cancellous bone were counted under 40X magnification taking the average of cells present in 5 fields.
- Presence or absence of resting lines and reversal lines was observed under 40X magnification.
- Presence or absence of osteoclasts was assessed by presence of howships lacunae
- Trabecular density was assessed as sparse trabeculae (+) or packed trabeculae (++)
- Marrow type (fatty or red marrow)

2.2 Statistical analysis

The data obtained from histologic assessment was entered and data was compiled on MS-excel sheet. It was subjected to statistical analysis using SPSS 17.0. Comparision between groups was done using Unpaired t test Mann Whitney U test. P value < 0.05 was considered to be statically significant. NS (p>0.05) = not significant; HS (p<0.001) = highly significant.

3. Results

22 healthy nonfluorosed and fluorosed bone (femur) samples were collected to assess and compare histology of fluorosed versus non fluorosed bone. Cellularity of cortical and cancellous bone was found to be statistically significant in nonfluorosed group (10.72±4.10, 8.74±2.34) when compared to fluorosed group (6.61 ±3.31, 5.69±1.31) respectively.(Table 1**)** Trabecular density of bone: Trabecular density was same in both nonfluorosed and fluorosed bone (statistically non significant, p= 0.615). However trabeculae were thick in nonfluorosed bone and short and thin in fluorosed bone. Resting and reversal lines were more prominent in nonfluorosed bone than in fluorosed bone. Marrow content was fatty in both the groups. Osteoclasts were present in all subjects of nonfluorosed bone whereas osteoclasts were very few to absent in fluorosed bone.

Table 1: Density of cells in nonfluorosed and fluorosed cortical and cancellous bone

Bone	Nonfluorosed	Fluorosed	p value	t value
Cells in cortex	10.72 ± 4.10	6.61 ± 3.31	0.024(S)	- 2.46
Cells in cancellous	8.74 ± 2.34	5.69 ± 1.31	0.010(S)	- 2.88

* p value calculated using unpaired t test (S) = Significant

Table 2: Trabecular density of nonfluorosed and fluorosed bone

non fluorosed	fluorosed
1	1
1	1
2	2
2	2
2	2
2	2
2	2
2	2
2	2
2	2

Mann Whitney u = 45; p= 0.615 (NS)

4. Discussion

In our study, a total of 24 human bones (femur) samples (fluorosed and non fluorosed) were assessed. However, various authors have conducted studies using femur, tibia, fibula, calvaria, rib, vertebra [8], iliac crest, sternum [9], mandible of human bones, rabbits, rats with the sample size varying from 2 [8], 3 to 5[10], 14 [11], 69 [9], 127 [12]. CF Hildebolt in 1997, in a clinical study reported that there exists an association between the bone densities of jaws and metacarpals, forearm bones, vertebrae and femur. [13] Hence, the femoral bone was selected in the current study.

In our study decalcification using 5 % nitric acid was used. Preservation of hard tissues close to the living state is essential for understanding of cellular and subcellular structures and functions. The cutting of thin sections by ordinary methods is impossible in the case of tissues such as teeth and bone. Such tissues must be treated to remove calcium phosphate by a process known as “decalcification”, thereby making the tissue soft enough to be cut by the microtome. Decalcification is carried out by chemical agents, either with acids to form soluble calcium salts or chelating agents that bind to calcium ions. Microwave decalcification is a novel technique was seen to accelerate the decalcification compared to the manual method. [14]

Comparative studies on histologic assessment of nonfluorosed and fluorosed cortical and cancellous bone are not found in literature.

In our study, following parameters were assessed: density of cells in cortical and cancellous, presence or absence of resting lines and reversal lines, presence or absence of osteoclasts, trabecular density, marrow type (red or yellow [fatty]).

Density of cells in nonfluorosed and fluorosed cortical and cancellous bone: In the nonfluorosed cortical and cancellous bone cellularity was significantly higher (10.72±4.10, 8.74±2.34; p= 0.024, 0.010) as compared to fluorosed cortical and cancellous bone (6.61 ±3.31, 5.69±1.31) respectively. As per the authors knowledge this study presents the difference incellular density innonfluorosed and fluorosed cortical and cancellous bone for the first time in literature.

In the current study, various other parameters such as resting and reversal lines, marrow content, osteoclasts were assessed histologically. Resting and reversal lines were more prominent in nonfluorosed bone than in fluorosed bone. Marrow content was fatty in both the groups. Osteoclasts were present in all subjects of nonfluorosed bone whereas osteoclasts were very few to absent in fluorosed bone. The reason for the above observation requires to be elucidated.

Trabecular density of bone: Trebacular density was same in both nonfluorosed and fluorosed bone [statistically non significant, p= 0.615]. However trabeculae were thick in nonfluorosed bone and short and thin in fluorosed bone.

The clinical and experimental results reported in the literature confirm the existence of a direct and specific fluoride action [15] on the cell populations of osteoblasts and osteoclasts. This effect is consistently seen at least in the early stages of exposure [16] and manifests itself through morphological and functional cell changes which are indicative of increased activity in the existing metabolic units. [17]

An experimental *in vitro* and *in vivo* study using sodium fluoride sustained release bone cement has reported , the ability of fluoride to promote bone densification is of dual nature :biochemical : the fluoride rapidly becomes part of the bone hydroxyapatite mineral structure, thus forming fluroapatite which display improved mechanical and biochemical properties; and biological- the fluoride ion exerts a direct action on osteoblasts, promoting their differentiation leading first to an enlarged osteoid wall of bon trabeculae, and in particular of trabecular bone, and subsequently to an increased volume of trabeculae. These effects, if locally achieved by slow release of fluoride from bone cement, should be very useful in the prevention of periprosthetic bone resorption. [17]

The most distinguishing histomorphometric changes concerned the trabecular component [18] and were reflected in an increased total trabecular volume, both in mineralized and in the nonmineralized portion. An overwhelming proportion of the nonmineralized is still the sequel most feared by clinical investigators, since an excess of osteoid tissue may thwart the therapeutic goals or even increase the hazard of fractures of the long bones, particularly of the hip. [17]

The finding in clinical studies, however, appear to bear out Kanis and Meunier's opinion [19] i.e. that such histomorphometric changes are closely related to the local concentration of fluoride depending on daily dose and, secondarily, on the length of treatment. In oral treatment, excess osteoid tissue is typically found in the early stages and at high doses. [18]

Histologic changes in fluorosed bone have been reported.

- Massive accumulation of incompletely mineralized and poorly structured bone makes the bone as radiopaque in x ray , and this radiopaque effect is not due to increased mineralization or failure of resorption.[20]
- Hypermineralization of cortical bone in endemic and industrial fluorosis has also been reported by various authors.[21]
- The unmineralized collagen fibers found in the resorbed areas may be due to the presence of high concentrations of glycosaminoglycans. The presence of high concentrations of sulphated glycosaminoglycans, which are potent inhibitors of mineralization, has been demonstrated in bone. [22] Removal of glycosaminoglycans is a prerequisite for mineralization of collagen fibers. Hence the presence of high concentrations of glycosaminoglycans may be the reason for poorly mineralized collagen fibers. [10] Role of fluoride in increasing GAG: fluoride is known to increase the GAG that is dermatan sulfate content in cortical bone which inhibits mineralization. The presence of high dermatansulphate content in human teeth in dental fluorosis has also been reported. [23]
- The change in the morphology of the collagen fibers and the matrix may possibly be due to reduced cross-link precursors [24] of collagen and collagen biosynthesis. [25] Hence reduced collagen cross-links and biosynthesis along with the significant increase in glycosaminoglycans may be one of the reasons for gross morphological changes in "fluoride-treated" bone. However, it is not clear whether these changes in bone surfaces are a compensation for the increased resorption rate induced by fluoride ingestion reported by Weinmann and Sicher [26], or are associated with a markedly increased osteoblastic activity similar to that described by Schenke *et al*.[27]
- Excessive fluoride deposition in calcified and noncalcified tissues leads to certain specific manifestations. This aspect has been explored with special reference to collagenous and noncollagenous constituents. Hydroayapatite content of cancellous bone is greater than that in cortical bone. These observations suggest that in fluoride toxicity the hydroxyprolin content is reduced both in osseous and nonosseous tissues. This possibly may reflect on the collagen content of the tissues. This indicates that collagen laid down/ synthesized during fluoride ingestion is under hydroxylated and inadequately cross-linked and is rapidly catabolized. Due to excessive ingestion of fluoride, the collagen laid down both in osseous and nonosseous tissues in abnormal.[28]
- Due to Fluoride intoxication, the natures of cells present in osteoid resemble cartilage cells – chondrocytes and the osteoid formed resemble fibrocartilage the chondrocytes appear in trabeculae too. The striking structiural variations observed in the cortical bone due to excessive ingestion of fluoride are 1) increase in the cortical thickness and 2) enhancement of the diameter of the osteon. [29]

In the current study, the cellular density of cortical and cancellous bone has been studied. The effect of fluoride on bone which has been presented in the literature is a good source of information to study biochemical changes in fluorosed bone. The histologic aspects dental cementum of nonfluorosed and fluorosed teeth has been studied. (unpublished data, Dissertation submitted to RGUHS, to

study the mechanical, histological properties and mineral content of fluorosed and nonfluorosed bone and cementum"-an *in vitro* study.)

5. Conclusion

The studies related to the objectives of our current study are not comparable directly as their subjects, age, sex, water fluoride exposure, methodology vary and differ from this study. The pertinent studies are as early as 1950s and there is paucity of studies in an active area of human research till date. This could be owing to the nature of fluoride induced diseases which are chronic in nature and seen in later ages as the cumulative effect. As said, there is no cure for this disease and prevention of occurring fluorosis effects is the ultimate treatment. The government policies should be made compulsory for defluoridation measures and early detection and treatment as the fluoride induced certain changes are reversible.

Further studies on mechanical property i.e., micro/nanohardness and mineral content of fluorosed bone are required. Further studies are required to be done on biochemical aspects of fluorosed and nonfluorosed bone. The role of fluoride on hard tissues is a cumulative effect where in the different doses of fluoride have multiple actions on hard and soft tissues requires to be deciphered.

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