SUMMARY: The aim of this report is to analyze the effects of high fluoride (F) intake on matrix metalloproteinase-2 (MMP-2), tissue inhibitors of metalloproteinase-1 (TIMP-1), and transforming growth factor-β (TGF-β) levels. Twenty-seven rabbits were divided into control and F groups. The control group comprised 11 rabbits that received distilled drinking water; the experimental group consisted of 16 rabbits that received distilled water with excessive F for 70 days. At the end of 70 days, serum F concentrations were evaluated, and the rabbits were then sacrificed. Bucco-lingual histological sections were obtained bilaterally from the mandibular bone. MMP-2, TIMP-1, and TGF-β levels in the bone matrix were determined by immunohistochemical staining. The difference between the groups was evaluated with the Mann–Whitney U test, and the intragroup difference in staining intensity was evaluated with Friedman and Wilcoxon tests. Increased mineralization and high bone turnover was observed in the fluorosis group. TGF-β immunostaining of the mandibular bone in the experimental group was significantly more intense compared with that in the control group (p=0.03). MMP-2 and TIMP-1 staining were not statistically different between the experimental and the control groups (MMP-2, p = 0.110; TIMP-1, p=0.479). Intra-group comparison revealed significant differences at MMP-2/TIMP-1 and TIMP-1/TGF-β ratios only in F group (p=0.02 for MMP-2 and p=0.01 for TGF-β). Excessive F uptake may affect bone remodeling process by increasing TGF-β levels and TIMP/MMP and TIMP/TGF-β ratios. Further studies including other bone regulating molecules are needed in order to explain bone metabolism in the presence of high doses of F.

Key Words: Bone turnover with high F; MMP-2; Rabbit; TIMP-1; TGF-β.

INTRODUCTION

High fluoride (F) levels cause toxicity.1-3 At very high doses, skeletal and dental fluorosis occurs, characterized by debilitating changes in the skeleton and marked mottling and discoloration of the teeth.4 High F intake increases the accretion, resorption, and calcium turnover rates of bone tissue, thereby affecting the homeostasis of bone mineral metabolism.5 F has also been shown to increase bone metabolic turnover and stimulate bone cell proliferation.6

A number of factors affect bone turnover, including hormones, cytokines, and mechanical stimuli. All of these factors affect the amount and quality of the tissue produced.7 Studies have shown that osteoblast-derived transforming growth factor-β (TGF-β), matrix metalloproteinase (MMP) and tissue inhibitors of
metalloproteinase (TIMP) play a role in bone metabolism by degrading the bone matrix. TIMP-1 has been used as a marker of inhibition of bone resorption. Investigators have found that low concentrations of TIMP-1 stimulate osteoclastic bone resorption, whereas higher concentrations inhibit the resorption process. It is well known that an imbalance between MMPs and TIMPs leads to the destruction of connective tissue, which occurs in a number of pathological events. TGF-β has been implicated in regulating a variety of cellular events involved in bone growth and turnover. TGF-β is produced by osteoblasts and chondrocytes and mainly stored in the mineralized extracellular bone matrix.

There is no evidence yet about the possible effects of excessive F intake on osteogenic markers. The aim of this study was to investigate the effects of excessive F intake on remodelling of mandibular bone via analyzing bone matrix concentrations of MMP-2, TIMP-1 and TGF-β.

MATERIALS AND METHODS

The animal experiment protocol was approved by Ondokuz Mayıs University Ethics Committee. Twenty-seven skeletally mature adult male New Zealand white rabbits (body weight: 2.5–3kg) were divided into a control (n=11) and a F (n=16) group. The animals were maintained in a dedicated animal holding facility in the laboratory animal unit of the University. They were housed individually in stainless steel cages and fed with standard laboratory rabbit chow and water ad libitum for 70 days. The control group received distilled drinking water. The experimental group received distilled drinking water with excessive F (40 mg/L). Following feeding, blood samples were collected from the marginal ear vein and serum F levels were measured. Animals were sacrificed using a high dose of pentobarbital at the end of the 70th day. Following sacrifice, bucco-lingual histological sections were obtained bilaterally from the mandibular bone at the molar teeth region.

Serum analysis of fluoride: A F-specific ion electrode (ORION 9609BN, Thermo Electron Corp, MA, US) connected to a digital ion analyzer (ORION EA 940, Orion Research, Inc., US) was used to measure the F ion concentration. The electrode was previously calibrated with four standard F solutions of 0.19 mg/L, 1.9 mg/L, 19 mg/L, and 190 mg/L, respectively. Before measurement, 1 mL of each serum sample solution was pipetted into a clean plastic test tube, and 1 mL of total ionic strength adjustment buffer II (TISAB II, Orion Research, Inc., Beverly, MA, USA) concentrate with 1,2-cyclohexylenedinitrotetraacetic acid (Thermo Orion, MA, USA) was added to each solution. The concentration (millivolt) of each solution was directly read out on the instrument display. Data concerning F were recorded in mg/L.

Histochemistry and immunohistochemistry: The mandibles were dissected and fixed in buffered 10% formalin solution and decalcified in formic acid and sodium citrate solutions. Sections 5 µm thick were then cut and mounted on organosilane (3-aminopropyl) triethoxysilane-coated glass slides. The sections stained with hemotoxylin-eosin (HE) and examined under a light microscope.
Commercially available immunohistochemistry kits (Zymed Laboratories, Inc., USA) were used to perform the immunohistochemical analysis of the sections using rabbit-specific polyclonal antibodies against MMP-2 (sc-53630, 4D3 Santa Cruz Biotechnology, Inc., CA, USA) TIMP-1 (Ab-2 Clone 102-D1 Thermo Scientific, Fremont, CA, USA), and TGF-β (Santa Cruz Biotechnology, Inc., CA, USA). All of the sections were preincubated in 10% goat nonimmune serum (Zymed Laboratories, Inc., USA) at room temperature for 10 min to block nonspecific binding of second-step antibodies (Zymed Laboratories, Inc., USA). The tissue sections were then reacted with primary antibodies and then reacted with biotin-conjugated second-step antibody (Zymed Laboratories, Inc., USA) for 10 min at room temperature and rinsed in phosphate-buffered saline (PBS). To inactivate endogenous peroxidase, the sections were incubated in 0.3% H2O2/methanol for 60 min. The sections were again rinsed with PBS and reacted with the streptavidin-biotin-peroxidase complex (Zymed Laboratories, Inc., USA) for 10 min at room temperature. Following additional rinsing with PBS, the sections were incubated with 3-amino-9-ethylcarbazole (Zymed Laboratories, Inc., USA) for 15 min and then counterstained with Mayer’s hematoxylin. Four negative controls were prepared by omitting the primary antibodies, and then by replacing them with PBS or unrelated rabbit antibodies. The bone sections were evaluated, and the immunoreactivity against the antibodies was determined by a score of 0 to 3 (0: no immunoreactivity; 1: light immunoreactivity; 2: moderate immunoreactivity; 3: strong immunoreactivity). Two separate examiners performed the scoring immunostaining procedure on both the right and left mandibular bones of the molar region for each rabbit. They used the same magnification in all the examinations. The mean of the two scores were determined as the final score.

Statistical analysis: The Mann-Whitney U test was used to determine the difference between the groups. The intragroup difference in the staining intensity was evaluated with Friedman and Wilcoxon tests. The statistical significance level was determined as p<0.05, and the results were expressed as the median (min-max) of the values.

RESULTS

The serum F levels in the experimental group (301.3±52.18 µg/L) were significantly higher than those in the control group (18.20±1.58 µg/L) (p<0.05).

HE-staining revealed high cellularity in the experimental group, but there was no evidence of high cellularity in the control group. Increased number of osteoblasts and osteoclasts were observed in bone formation and resorption areas in the experimental group (Figure 1A) compared with the control group (Figure 1B). These features pointed to high bone turnover in the experimental group.
TGF-β immunostaining of the mandibular bone in the experimental group was significantly higher than that in the control group (p=0.03). The osteoblasts in the experimental group showed high TGF-β expression, whereas the osteoclasts showed less TGF-β expression (Figure 2A). Both the osteoblasts and the osteoclasts in the control group showed low levels of TGF-β expression (Figure 2B).

Although MMP-2 and TIMP-1 immunostaining of the mandibular bone was slightly more dense in the experimental group, the difference was not statistically significant (p=0.110 and p=0.479, respectively). The experimental group exhibited slightly higher MMP-2 expression, especially in the osteoblasts of the bone resorption areas (Figure 3A-3B).
The osteoblasts and osteoclasts in experimental group also showed slightly higher TIMP-1 expression (Figure 4A) than those in the control group (Figure 4B).

The intra-group comparison of the immunostaining of MMP-2, TIMP-1, and TGF-β were evaluated and significant differences were found in the experimental group. MMP-2 and TGF-β showed more dense staining than TIMP-1 (p=0.02 for MMP-2 and p=0.013 for TGF-β) while TGF-β and MMP-2 staining intensities were similar (p=0.076) in the experimental group.

**DISCUSSION**

The action of F on bone has been extensively studied and shown to have an effect on bone minerals, bone cells, and bone architecture. Sodium fluoride (NaF) promotes the recruitment of active, normally functioning osteoblasts independent of any coupling between bone resorption and formation. Several studies have investigated the pathogenesis and mechanical properties of bone under excessive F intake. At high concentrations, F may cause the formation of abnormally mineralized bone and impaired quality of bone. It may also result in hypermineralized bone, as evidenced by a shift toward denser bone and...
increased hardness.\textsuperscript{20} Consistent with the literature, our study revealed increased cellular activity in the fluorosis group.

Although, the dose-dependent effects of F on the mechanical properties of bone are well known, the underlying mechanisms are still not fully understood. TGF-\(\beta\) is a very potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation, and differentiation in committed osteoblasts.\textsuperscript{13} The concentration of TGF-\(\beta\) in the human skeleton appears to be related to bone turnover.\textsuperscript{21} We detected increased TGF-\(\beta\) expression in the fluorosis group compared with the control group. This finding suggests that excessive F intake may induce the production of TGF-\(\beta\). We also found increased cellular activity in this group. This finding could possibly be attributed to the high TGF-\(\beta\) levels.

In an \textit{in vitro} study, Farley et al have reported that F increases bone turnover even at normal therapeutic doses.\textsuperscript{17} On the other hand, Pepene et al. evaluated the effects of F salts on the concentrations of three growth factors that including TGF-\(\beta\) in patients with osteopetrosis and found no significant relationship.\textsuperscript{22} Lütfioglu et al. suggested that fluorosis altered the homeostasis of periodontal soft tissue by increasing TGF-\(\beta\) and also TIMP-1 and MMP-2 balance in animal model.\textsuperscript{16} We also used an animal model and evaluated the TGF-\(\beta\) levels under excessive F intake. Consistent with Pepene et al. and Lutfioglu et al., we conclude that it is likely that F intake influences the composition of the remodeling of matrix, possibly in relation to TGF-\(\beta\) levels.\textsuperscript{16,22}

Only a few studies have analyzed the possible mechanism by which F acts on bone turnover.\textsuperscript{15,16,22,23} Buyukkaplan and Guldag evaluated the amount of glycosaminoglycan of patients diagnosed with systemic fluorosis and reported decreased glycosaminoglycan in these patients.\textsuperscript{23} Farley et al. demonstrated for the first time \textit{in vitro} that NaF can increase the proliferation rate of bone cells.\textsuperscript{15} They also showed that the effect of NaF on [3H] thymidine incorporation can be modulated by parathyroid hormones and human skeletal growth factors.\textsuperscript{15} Waddington and Langley investigated the influence of F on MMP and TIMP on bone matrix remodeling.\textsuperscript{24} They reported an altered profile of MMP species in the presence of F.\textsuperscript{24} They concluded that F is likely to have serious consequences for the subsequent formation of the extracellular matrix, the prime function of which is the regulation of mineral deposition and the control of crystal morphology. Nevertheless, they did not detect immunoreactivity for TIMP-1 and MMP-2. Consistent with the results of Waddington and Langley, we found no significant differences in MMP-2 or TIMP-1 levels in the fluorosis and control groups in our study. However, we found altered TIMP/MMP and TIMP/TGF-\(\beta\) ratios in the fluorosis group. TIMP-1 staining was more intensive than MMP-2 and TGF- \(\beta\) in the fluorosis group. This finding could be attributed to an up-regulation effect of TGF-\(\beta\) on TIMP-1.\textsuperscript{25} In our opinion, increased TGF-\(\beta\) levels alters the balance between TIMP-1 and MMP-2 and leads to an inhibition of proteolytic activity.

Although F is known to alter bone metabolism, the mechanism underlying its action remains unclear. To the best of our knowledge, this is the first \textit{in vivo} study to evaluate TGF-\(\beta\), MMP-2, and TIMP-1 levels in mandibular bone in response to
high F concentrations. Increased levels of TGF-β could serve as an important marker in the evaluation of the pathological action of F in bone tissue. Further studies that include other bone regulating molecules are required to elucidate the molecular events controlling the formation of bone matrix under excessive F intake.

ACKNOWLEDGEMENT

This research was supported by the Ondokuz Mayıs University Scientific Research Fund [Project number: DHF.058].

The study was presented at the 92nd Annual Meeting of the American Association of Oral and Maxillofacial Surgeons (AAOMS) in Chicago, Illinois, (Muğlalı M. Poster board number 64. Effects of excessive fluoride intake on MMP-2, TIMP-1, and TGF-β expressions of mandibular bone. J Oral Maxillofac Surg 2010; 68(9 Suppl):e95-6).

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