EFFECT OF ACUTE AND CHRONIC FLUORIDE ADMINISTRATION ON BONE HISTOPATHOLOGY, BONE FLUORIDE ACCUMULATION, AND LOCOMOTOR ACTIVITY IN AN ANIMAL MODEL OF PALEOPATHOLOGICAL FLUOROSIS

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ABSTRACT: Fluorosis may be identified in skeletal materials from ancient civilizations by macroscopic signs in teeth and bone and hard tissue fluoride levels. In the present study, human teeth, femoral, and rib specimens from the Van Fortress excavation, in Turkey, were examined for the presence of fluorosis. In addition, an animal study in rats was conducted as a model of human fluorosis, by examining the effects of fluoride administration, in various doses and for different durations, on weight loss, locomotor activity, fluoride accumulation, and deformation in bone and teeth. Fifty-six adult male Wistar albino rats, weighing 150–200 g, were divided into 7 different groups of 8 rats. Four acute groups were treated with 0 (control), 5, 15, and 50 mg/L of fluoride in drinking water for 7 days and three chronic groups were treated with 5, 15, and 50 mg/L of fluoride for 90 days. The results of the analysis of the human samples from the Van Fortress excavation showed that none of the dental, femoral, or rib samples had a fluoride content that was significantly greater than that of the surrounding soil. The results of the rat study showed that no significant differences between the groups were found in body weight on days 1, 30, 60, and 90. The rotarod locomotor test showed a significant ($p<0.05$) dose- and time-dependent reduction in locomotor activity as a result of the fluoride administration in the 50 mg/L chronic fluoride group compared to the control, 5 mg/L acute fluoride, 15 mg/L acute fluoride, and 5 mg/L chronic fluoride groups. Significant fluoride accumulation was found in the femoral neck (cortical tissue), the femoral head (trabecular tissue), and in rib bone. Light microscopy showed a severe thinning of the epiphyseal growth plate and bone trabeculae in the femoral bone tissue. We concluded that femoral bone (cortical and trabecular parts) and ribs are good sites for assessing the effects of fluoride exposure in animal models of human fluorosis.

Keywords: Anthropology; Dental fluorosis; Histopathology; Paleopathology; Skeletal fluorosis; Van.

INTRODUCTION

Toxic effects from fluoride exposure in an organism is called fluorosis.[1,2] According to the duration of the fluoride exposure, fluorosis may be acute and chronic. Acute fluoride intoxication may occur after a short period of exposure to a high dose of fluoride, while long-term exposure to a lower dose of fluoride may result in chronic fluorosis. Fluorosis may vary according to the duration and amount of fluoride intake, nutritional status, age, and gender of the subject. It is stated that chronic fluorosis, whose pathophysiology is not fully understood, seriously damages many systems in the human body.[3,4] Bone deformations,[5] tooth discoloration, erosion, and hypoplasia[6] are some of the signs of fluorosis that may occur. In addition, some bones can become hard and break easily.[7] Chronic fluorosis occurs very slowly in the body. This toxicity occurs particularly in teeth and bones due to the

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affinity of fluoride for calcium.[8,9] Dental fluorosis refers to a dental condition that is caused by a systemic intake of fluoride in an amount causing a toxic effect. Dental fluorosis creates color changes along the tooth surface which may include opaque white lines, white spots, and yellowish-brown stains.[10,11] More than 95% of the fluoride accumulated in the body is found in the skeletal tissue. Life-time exposure to fluoride through water and nutrients may cause a continuous fluoride accumulation. Due to differences in metabolic activity, the rate of fluoride deposition differs in the various bony tissues of the skeleton. From this point of view, researchers reported that spongy bones (ribs, skull bone, and pelvis) were more affected than compact bones. In addition, it is stated that fluoride accumulation alters the developmental period of the organism.[12] Fluoride accumulation in bone can be determined radiologically. Fluorosis may cause skeletal complaints. The most important changes of skeletal fluorosis are osteosclerosis and osteoporosis in bones, calcifications in the tendons and spine, deformities in the hips and knees, disruption of neck and lumbar movements, pain in lower extremities, arthritis, and ankyloses.[13,14] The severity of the lesions in the bone varies in different cases.[15] Fluorosis also causes negative effects on soft tissues such as the lungs, liver, nervous system, and heart.[16] In addition, fluorosis has been reported to cause degenerative changes in skeletal muscles.[15] Skeletal material comprises the most extensively studied human remains in anthropological studies.

In paleopathology, skeletal tissue is evaluated to reveal the presence of disease in the deceased individual. Fluorosis is an important issue in paleopathological studies. The bone fluoride levels varies in different parts of the skeleton. Animal models are frequently used as models for fluorosis to humans. The aims of the present study were: (i) to examine human skeleton remains (femoral head, femoral neck, rib, and teeth), obtained in 2015, from the Van Fortress excavation in Van, Turkey, for the presence of fluorosis by inspection and the measurement of fluoride levels, and (ii) to evaluate the effects of both acute and chronic fluoride accumulation in rats, using different concentrations of fluoride, by measurement of fluoride levels, histopathological examination, and locomotor activity tests.

MATERIALS AND METHODS

The experimental study was conducted with the permission of the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee. In the study, healthy adult male Wistar albino rats weighing 150–200 g were used. They are obtained from the Experimental Medicine Application and Research Unit of Van Yüzüncü Yıl University. Fifty-six experimental animals were divided into 7 different groups. No fluoride was administered to the control group. The acute fluoride groups were administered 5, 15, and 50 mg/L fluoride for seven days while the chronic fluoride groups received 5, 15, and 50 mg/L fluoride for 90 days. The fluoride was administered as sodium fluoride (NaF, Sigma) dissolved in tap water and available *ad libitum* as drinking water for the animals.

Sample preparation and trace element analysis: Following the termination of the experimental study, the right femoral bone, ribs, and teeth of each experimental animal were extracted for analysis of fluoride accumulation. For the histopathological analysis, the left femoral bone was obtained. In addition, the campus tap water and the standard pellet feed given to the animals were also

evaluated for fluoride content. The bones prepared for trace element analysis were burnt at 550ºC with the dry ashing method. After removal from the ash furnace, the samples were made ready for analysis by adding 1.25 mL of 3N hydrochloric acid solution and making the volume up to 12.5 mL with deionized water. The Extech FL 700 fluoride measuring instrument was used for the fluoride measurement of the samples after calibration as described in the instruction manual. Ten mL of TISAB solution and 10 mL of the prepared sample were added to a disposable polyethylene container. The fluoride content of the sample was then measured with the Extech FL 700 fluorometer. After each measurement, the fluoride meter was readied for the next measurement by washing with distilled water and drying with tissue paper.

Locomotor activity and weight analysis: The locomotor activity of the experimental animals was measured with a device, the rotarod, with a grooved rotating rod on which an animal can stay for a period of time before falling off, on the day on which the trial was terminated: day 7 for the control and acute groups and day 90 for the chronic groups. With this device, a practice period was first given to the experimental animals. Three tests were then applied to each experimental animal for 60 seconds each, and the locomotor activity of the experimental animals was measured and analyzed. In addition, the body weights were also measured on day 7 for the control and acute groups, and on days 7, 30, 60, and 90 for the chronic groups.

Histopathological analysis: After the necropsy, the femoral tissues were fixed in formalin solution (10%) for 48 hours. For decalcification, they were kept in the solution for decalcification (Osteosot, Merck, HC313331, made in Germany) for 96–210 hours. Following the softening of the tissues, they were washed with running tap water for 24 hours. The tissues were then processed with 80% alcohol (12 hours × 2 times), 90% alcohol (12 hours × 2 times), 96% alcohol (12 hours × 2 times), 100% alcohol (12 hours × 2 times), chloroform (5 hours × 3 times), and liquid paraffin (12 hours) and they were then buried in paraffin blocks. Four µm thick sections were taken from each block and the preparations were prepared on the slide. Preparations for histopathological examination were stained with hematoxylin-eosin (HE) and examined with light microscopy (Leica DM 1000). According to the histopathological findings, the sections were evaluated as (–), mild (+), moderate (++), severe (+++) and very severe (++++).

Statistical analysis: The descriptive statistics used in the study were expressed as mean and standard error of mean. Statistical analysis was performed by using the Kruskal-Wallis test and post hoc tests for continuous variables and relevant tests for semi-quantitative variables. The significance level of the statistics in the calculations was taken as 5% and SPSS (ver. 20) statistical package program was used for the calculations.

Human material and soil: Femoral bone, ribs, and teeth of human skeletons, together with soil, were obtained from the Van fortress excavation. The fluoride levels were measured with the same device, the Extech FL 700 fluorometer, after processing with the thermal and chemical sample preparation procedures.

RESULTS

Weight loss: The Wistar albino rats initially weighed 150–200 g and no statistically significant difference was present in body weight between the control group and the

experimental groups (acute and chronic fluoride exposure) in terms of weight loss from the beginning to the termination of experimental procedures (data not shown).

Locomotor activity: The persistence time in the rotarod test was significantly reduced ($p<0.05$) as a result of the fluoride administration in the 50 mg/L chronic fluoride group compared to the control, 5 mg/L acute fluoride, 15 mg/L acute fluoride, and 5 mg/L chronic fluoride groups (Figure 1).

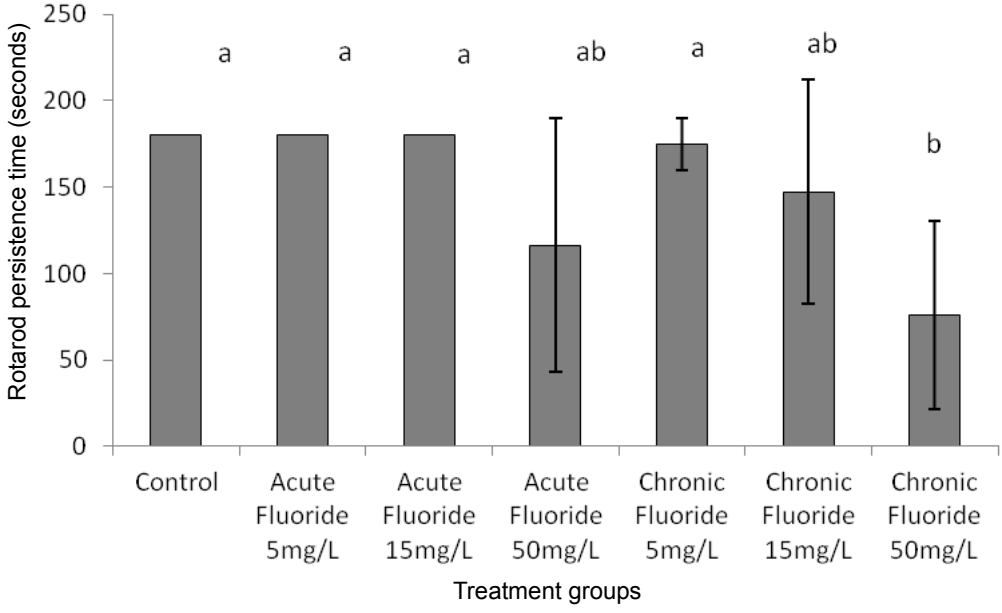


Figure 1. Rotarod persistence time of the rats at the end of experimental procedure (7 days for the control and the acute groups; 90 days for the chronic groups). Different letters indicate statistically different groups $p<0.05$.

Analysis of the fluoride levels in bone and teeth of the rats: The fluoride content was significantly higher ($p<0.05$) in the cortical tissue of the femoral neck and the rib bone of the 50mg/L chronic group compared to the control group (Figure 2). The present study contributes to the current literature by showing that the fluoride level in the femoral head is important in assessing fluoride accumulation in rats and also by demonstrating the fluoride level in the femoral neck is also important. In addition, the rib bone was found to have a significant level of fluoride accumulation. The fluoride content of some bone tissues could not been measured because their level of fluoride accumulation was lower than the detection limit of our device.

Analysis of the human skeletons from the Van Fortress excavation: The analysis of the human skeletons from the Van Fortress excavation showed that the fluoride content of the tooth samples were below the lower limit of detection of the Extech FL 700 fluorometer. Six femoral neck samples were analysed and one sample had a fluoride content of 0.278 mg/L. Six femoral head samples were examined and the mean of two samples was 1.371 mg/L. Of six rib samples, two had a fluoride level of 0.901 mg/L. The analysis of six soil samples showed the mean for three samples was 1.190 mg/L. None of the excavation samples had statistically significant differences because of the small sample size. Thus none of the dental, femoral, or rib samples had a fluoride content that was significantly greater than that of the surrounding soil.

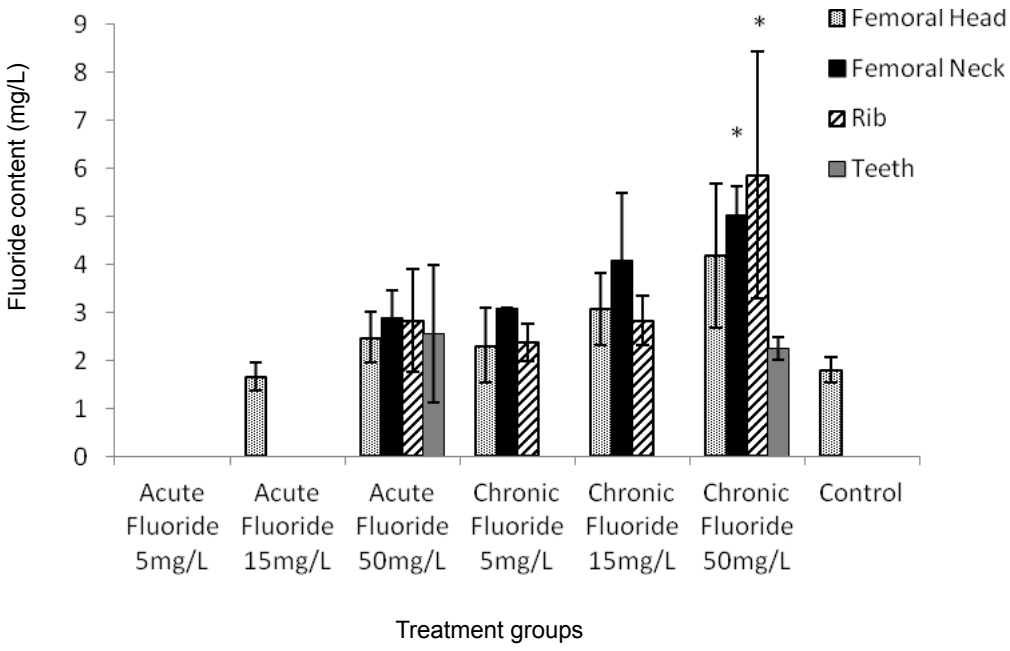


Figure 2. Fluoride accumulation in the femoral bone and tooth tissue of the control (0 mg/L for 7 days), acute fluoride (5, 15, and 50 mg/L for 7 days) and chronic fluoride (5, 15, and 50 mg/L for 90 days) groups. *p<0.05 indicates statistically significant fluoride accumulation compared to the control group.

Bone histopathology: In the 0 mg F/L control group, the bone histopathological evaluation showed that the femoral bone tissue was normal in appearance.

In the 5 mg F/L acute fluoride group, the femoral bone tissue, bone trabeculae, bone marrow, and epiphyseal growth plate were similar in appearance to that of the control group.

In the 15 mg F/L acute fluoride group, the bone trabeculae were of normal appearance but a thinning of the epiphyseal growth plate and moderate fat accumulation in the bone marrow were observed.

In the 50 mg F/L acute fluoride group, a slight thinning in the bone trabeculae was visible together with a moderate thinning in the epiphyseal growth plate and intense fat accumulation.

In the 5 mg/L chronic fluoride group, there was a marked thinning in the bone trabeculae and the epiphyseal growth plate but the bone marrow was normal in appearance.

In 15 mg/L chronic fluoride group, there was an intense thinning in the bone and the epiphyseal growth plate and a slight fat accumulation in the bone marrow.

In the 50 mg/L chronic fluoride group, a very intense thinning was present in the bone trabeculae and the epiphyseal growth plate together with a slight fat accumulation in the bone marrow.

The histopathological findings in the femoral bone tissue are summarized in the Table and shown in Figures 3 and 4.

Table. Histopathological findings in femoral bone tissue

	Epiphyseal growth plate thickness	Fat accumulation in bone marrow	Bone trabecular thickness
Control group	++++	–	++++
5 mg/L acute fluoride group	++++	–	++++
15 mg/L acute fluoride group	+++	+++	+++
50 mg/L acute fluoride group	++	++++	++
5 mg/L chronic fluoride group	+++	–	+++
15 mg/L chronic fluoride group	++	+	++
50 mg/L chronic fluoride group	+	+	+

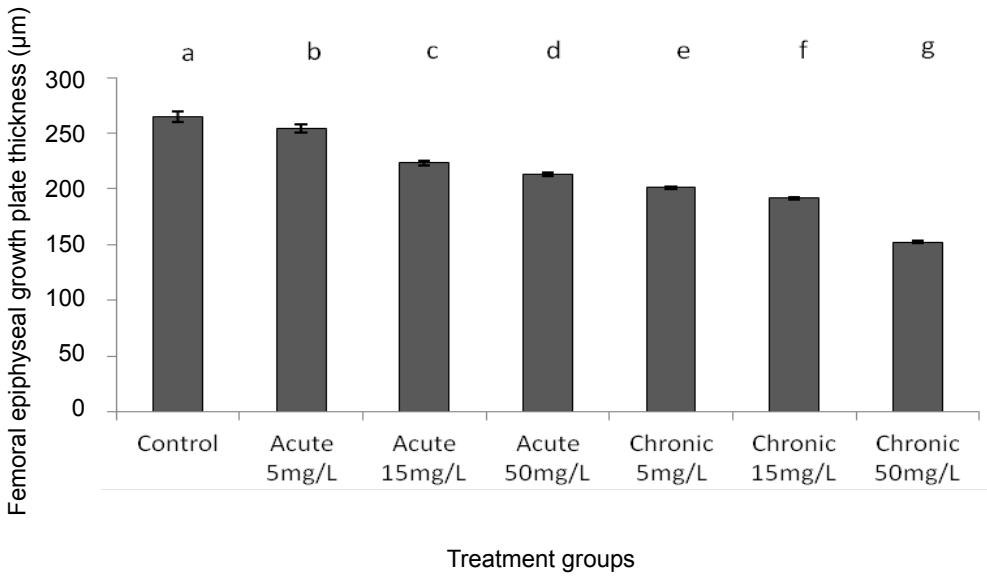
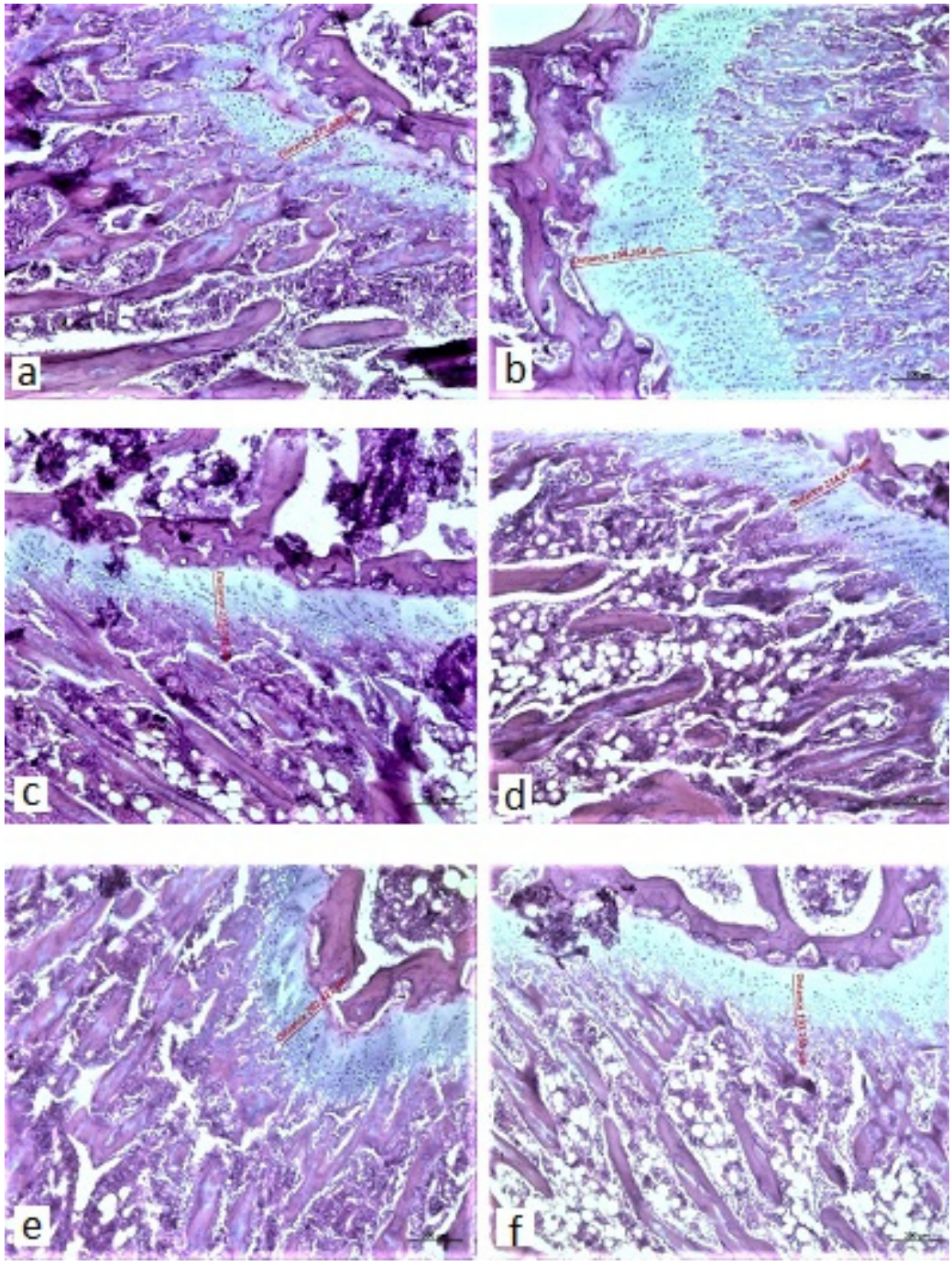


Figure 3. Measurement of femoral epiphyseal growth plate thickness in micrometers (µm) (Zen blue edition measurement program). Different letters indicate statistically different groups ($p<0.05$).



Figures 4a–4f. Femoral bone tissue histopathological findings.
4a: control group; 4b: 5 mg/L acute fluoride group; 4c: 15 mg/L acute fluoride group; 4d: 50 mg/L acute fluoride group; 4e: 5 mg/L chronic fluoride group; and 4f: 15 mg/L chronic fluoride group. H&E, Bar: 200 µm.

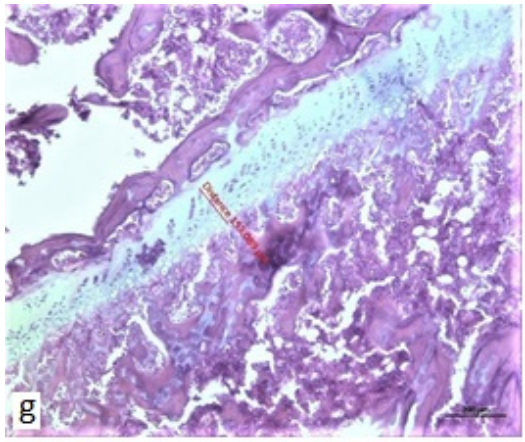


Figure 4g. Femoral bone tissue histopathological findings. H&E, Bar: 200μm.
4g: 50 mg/L chronic fluoride group. H&E, Bar: 200μm.

DISCUSSION

In paleopathological studies the main research material is skeletons. This tissue provides important information for anthropologists on subjects such as health, nutrition, and physical development. However, in paleopathological studies, especially those involving trace element analysis, more studies are needed to evaluate which bone in skeletal tissue is more affected from pathological impacts such as fluorosis. For this reason, a study aiming to model the accumulation of fluoride and the evaluation of different pathological parameters in the fluoride-exposed experimental animals was performed.

In this modeling study, it was observed that the neck (cortical tissue) as well as the head (trabecular tissue) of the femur bone were significant fluoride accumulation sites. In addition, the rib bone was also found to be a significant area of fluoride accumulation, and therefore a significant tissue for assessing the presence of fluorosis. The effect of fluoride on bone changes according to the bone structure. It has been reported that the effects of fluoride on trabecular bones have an earlier onset and are more widespread than the effects on cortical bones. Inoue et al. also reported that fluoride was stored more in trabecular bone than in cortical bone.[17] Our results are in parallel with the literature findings. As the duration and amount of fluoride exposure increased, a remarkable increase was observed in the fluoride content of femoral head, femoral neck, and rib bones.

Dental fluorosis, with discoloration of the teeth due to exposure to toxic levels of fluoride while the teeth are developing, up the age of approximately 8 years, is well recorded in the literature.[18-21] Yoshimura et al. reported that people who lived in historic periods experienced a color change in the teeth originating from fluorosis.[22] From these studies, it can be stated that prolonged exposure to a high level of fluoride can cause color changes in the teeth in both modern and historic populations. Based on this information, we tried to observe the situation in our modeling study. However, for both the control group and the experimental group, with both acute and chronic fluoride exposure, no color change occurred in the teeth. This is thought to be due to

all our animals being adult at the beginning of the experimental procedure. The period during which fluorosis may cause color change in the teeth is the pre-adult period. For adults, the dental accumulation of fluoride decreases and does not cause any observable color change on macroscopic analysis and that for this reason no color change can be observed in the teeth of our experimental animals. Everett,[23] supplied drinking water with 50 mg/L (50 ppm) fluoride to 5–6-week-old mice for 60 days and observed dental color changes due to fluoride. However, in our study, although the fluoride accumulation in the 50 mg F/L-exposed acute and chronic groups was significant in both the femoral neck and rib bone, there was only a slight non-significant accumulation in the teeth (Figure 2). It has been reported that fluoride exposure for a long period in high dose causes fluoride accumulation in the bones with structural changes that can be detected by macroanalysis.[7,24-27] Our current study aimed to model the accumulation and deformation of the skeletal material during fluoride exposure by exposing rats, both acutely (7 days) and chronically (90 days) to fluoride in drinking water at concentrations of 5, 15, and 50 mg/L. However, although fluoride accumulated in the hard tissues (significantly in femoral neck and rib, non-significantly in femoral head and teeth), no deformation was observed in the bones (femur, humerus, ribs, and teeth) of the experimental groups. Numerous studies have shown that severe osteofluorosis needs to be develop in order to observe a structural change on macroanalysis. However, with the duration of the fluoride exposure and the dosage used in our experimental study, we did not expect to see the development of severe osteofluorosis.

The fluoride level of the Van Yüzüncü Yıl University campus tap water was found to be 0.3 mg/L. Cavus et al., in another study performed in the same period, obtained a similar value (0.32 mg/L).[28] The fluoride content of the pellet feed was lower than that of all the examined tissues (0.055 mg/L) and did not significantly disrupt the study. However the fluoride in the campus tap water (0.3 mg/L) and pellet feed (0.055 mg/L) were a source of the relatively small amount of fluoride received by the control group.

Although the main purpose of the study was to model fluorosis in an animal model, bone and dental tissues of human skeletons from the Van fortress excavation were also examined. Since these samples were selected from skeletal remains of individuals who did not show any sign of fluorosis on their teeth, no significant fluorosis was expected. For the teeth samples, the fluoride levels were less than the lower limit of detection of our fluoride-specific electrode and this finding supported our assessment that severe fluorosis was not present in the individuals. Although no statistically significant difference was found between the small number of samples examined, the highest fluoride level found, a mean of 1.371 mg/L in two femoral heads, was also similar the mean of three soil samples of 1.1990 mg/L. This suggests a diagenetic effect (post-mortem interaction with buried soil) rather than cases of fluorosis.

Weight loss has been shown by many researchers as one of the most prominent symptoms of chronic fluorosis. However, no weight loss was observed in the experimental animals. The lack of weight loss may have been due to relatively short duration of the experiment (7–90 days) and the relatively low dose of fluoride used (5–50 mg/L in drinking water). The relationship between weight loss and fluorosis

has been discussed by many researchers. Mullenix et al. obtained a similar result, of no significant weight loss, in an experiment on 90-day-old rats who were given 100 ppm in drinking water for 6 weeks compared to a control group who did not receive added fluoride.[29] Long et al. performed an experiment that lasted for 7 months with Wistar rats weighing 100–120 g.[30] This study consisted of 3 different groups: (i) a control group who did not receive additional fluoride; (ii) a group who received 30 ppm fluoride; and (iii) a group who received 100 ppm fluoride. The 100 ppm group had a significant weight loss compared to the control group. The lack of significant weight loss in both in our study and the Mullenix et al. study, may be due to the age of the animals, the dose of fluoride administered, and the study duration.[29] In the studies by Long et al.[30] and Çenesiz,[31] the weight loss occurred in immature animals after the fluoride was administered in high dosage for long periods of more than 6 months.

The rotarod test was applied to measure the effect of fluoride exposure on the locomotor activity of rats. The results showed that the fluoride had an effect on the movement system and that the decreased locomotor activity was dose- and exposure duration-dependent. The results of the rotarod test also support the doses and durations of fluoride exposure used in our study protocol. We found that in order to affect locomotor activity, fluoride should be applied for a long period of time in a high dose. Many studies have reported that fluorosis causes a decrease in motility. However, there is no clear information about the dose and exposure duration required for this to occur and many researchers have conducted experiments to clarify this. Agustina et al. administered 5, 10, and 20 mg/kg NaF for 30 days to 12–16-week old Wistar rats weighing 150–250 g for 60 days.[32] On the 6th, 39th, and 60th days of the study the rotarod test was performed and no statistically significant difference in locomotor activity was observed between the groups. In another experimental study, Balaji et al. applied 100 and 200 ppm NaF for 30 days to mice weighing 25–27 g.[33] The rotarod test was applied to the experimental animals on the second to last day of the experiment and a statistically significant difference in locomotor activity was observed between the experimental and the control groups. Fluorosis was found to have had a negative effect on locomotor activity in the 200 ppm group.

Although no structural changes were observed by macroanalysis, a severe thinning was observed, on microanalysis in the femoral bone tissue with light microscopy, in the bone trabeculae and the epiphyseal growth plate (Table and Figures 3 and 4). In the present study, it can be said that fluorosis causes structural changes at the micro level. The histopathological findings showed a time- and dose-dependent negative impact on the bone trabecular thickness, the epiphyseal growth plate, and the accumulation of fat in bone marrow. The reduction in epiphyseal growth plate thickness, which can be used as a parameter indicating growth retardation, was mainly observed in the 50 mg/kg chronic fluoride group. All the values obtained indicate a hampering effect of fluoride according to the dosage and time schedule. The reductions in bone trabecular thickness occurred in parallel with time- and dose-dependent reductions in the epiphyseal growth plate thickness. The fat accumulation in the bone marrow was highest in the 50 mg/kg acute fluoride group. Histopathological methods are also used widely in parallel with computerized tomography in animal studies.[34] Some diet restrictions or treatment with

chemotherapeutic agents such as doxorubicin may cause rat epiphyseal growth plate thinning, as has been shown in a study by Noguchi et al.[35] Our study revealed that fluorosis had a prominent impact on the epiphyseal growth plate. This finding also supports our result of attenuated locomotor activity performance by the rats in the rotarod test.

Fluorosis is a prominent disease in various parts of the world.[36] The widespread occurrence of a high intake of fluoride, via drinking water and food sources, makes fluorosis a common and unavoidable health problem.[37] Animal modelling studies of fluoride exposure can lead to understanding of the different physiological aspects of this situation and will help to clarify some of fluoride-related health problems that have been observed in burial samples from ancient civilizations.

CONCLUSIONS

We concluded that femoral bone (cortical and trabecular parts) and ribs are good sites for assessing the effects of fluoride exposure in animal models of human fluorosis. The modeling study revealed that trabecular bone as well as cortical bone and rib bone were important areas for the accumulation of fluoride. Further anthropological studies on this topic will provide valuable information. In addition, the rotarod study showed the negative effects of long-term high-dose fluoride exposure on locomotor activity. Histopathological study shows that fluorosis caused severe thinning in the trabecular thickness and the epiphyseal growth plate. Both the histopathological and the rotarod data suggest that fluorosis causes adverse effects on the living organism. Similarly, it would be appropriate to further evaluate individuals who were exposed to fluorosis in the ancient societies.

ACKNOWLEDGEMENTS

Authors would like to thank Ufuk Komurcuoglu, Zehra Satar, Yildiray Basbugan, M Hamdi Demirkol, and Van Yyubat for their contributions during the experimental procedures. This study was supported by the Van Yüzüncü Yıl University Scientific Research Coordination Unit with project number SYL-2017-5953.

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