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Fluoride affects bone repair differently in mice models with distinct bone densities

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ABSTRACT

We grouped mice [strains: C57BL/6J ($n = 32$) and C3H/HeJ ($n = 32$)] to address the influence of bone density on fluoride's (F's) biological effects. These animals received low-fluoride food and water containing 0 (control group) or 50 ppm of F for up to 28 days. The upper left central incisor was extracted, and the left maxilla was collected at 7, 14, 21, and 28 days for histological and histomorphometric analysis to estimate bone neoformation. Our results showed bone neoformation in all of the evaluated groups, with the presence of bone islets invading the center of the alveoli when replacing the existing connective tissue. Curiously, this biological phenomenon was more evident in the C57BL/6J strain. The histomorphometric analysis confirmed the histological findings in relation to the amount of new bone tissue and showed a decrease in C3H/HeJ mice (control group). Altogether, our results showed differential effects of fluoride bone metabolism, confirming a genetic component in susceptibility to the effects of fluoride.

1. Introduction

Repairing bone loss due to various diseases such as osteoporosis and tumor resection has been a great challenge within regenerative medicine. Because of its importance, several groups worldwide have investigated strategies and biological molecules for bone repair, such as BMPs [1,2] and fluoride (F) [3–5]. In this way, fluoride is known to be deposited in calcified tissues [6]; however, the biological effects of fluoride on bone repair and its effects on bone cells remain unknown [7,8]. Bone cells may have a dose-dependent response to fluoride, which may stimulate bone formation at a low dose or be toxic at a high dose [9,10].

Fluoride's action profiles in alveolar bone repair models would provide information about the chronological evolution of the repair process and the main cellular events at each stage [11]. Biomechanical bone studies performed on different strains of mice have reported differences in bone mass and the capacity of differentiation in osteoblasts [12–14]. In addition, we have shown the preliminary effects of fluoride on the bone-repairing process in vivo [15,16] and on osteoblasts [17].

In order to address fluoride's effects on bone metabolism during alveolar bone repair, we decided to comparatively evaluate two strains of mice (C57BL/6J and C3H/HeJ, presenting differential physiological bone masses) by using histomorphometric analysis. In sum, our results showed a differential effect of fluoride on bone metabolism, confirming a genetic component in susceptibility to fluoride's effects.

2. Materials and methods

2.1. Animals

All experimental procedures were performed according to "The Guiding Principles for the Care and Use of Animals" and the ARRIVE guidelines. The protocol was approved by the Animal Experimentation Committee of University of São Paulo (Process #07/2010). Weanling (21 days old) male C57BL/6J ($n = 32$) and C3H/HeJ ($n = 32$) mice were obtained from the Central Vivarium of Bauru Dental

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School (University of São Paulo), randomly distributed into four groups, and given a low-F diet and water containing either 0 or 50 ppm of F added as NaF. The mice were housed in conventional plastic cages with metal bars and artificial lighting, with a photoperiod of 12 h light/12 h dark and regulated temperature and humidity. After 60 days of treatment, the animals were subjected to surgical procedures. During the entire study (pre-and post-surgery), the animals had free access to drinking water (0 or 50 ppm of F) and food.

2.2. Surgical procedures and treatments

To evaluate alveolar bone repair in different conditions, the animals (total $n = 64$) were divided into 4 experimental groups, as follows: 1. C3H-0 ppm: C3H/HeJ mice that received deionized water ($n = 16$); 2. C3H-50 ppm: C3H/HeJ mice that received water with 50 ppm of fluoride ($n = 16$); 3. B6-0 ppm: C57BL/6J mice that received deionized water ($n = 16$); and 4. B6-50 ppm: C57BL/6J mice that received water with 50 ppm of fluoride ($n = 16$). Firstly, the animals received an intramuscular injection of xylazine chlorhydrate for muscular relaxation and were anesthetized with ketamine chlorhydrate (1:1), with a dose determined according to body weight [18]. The upper left central incisor was extracted [16], and at the end of the experimental periods of 7, 14, 21, and 28 days, the animals were euthanized with an anesthetic overdose and the left maxilla was removed for histological/histomorphometric analysis.

2.3. Histology and histomorphometry

After each experimental period group, the samples (n = 4/period/group) were collected and immediately fixed in phosphate-buffered formaldehyde (10%) for 48 h and then subjected to demineralization by using 0.05 M ethylenediaminetetraacetic (EDTA), pH 7.4 [19]. Then, the samples were dehydrated with graded ethanol, diaphanized in xylene, and embedded in Histosec (Merck KGaA), according to the classical steps of the histological procedure. Transversal 4 μm-thick semi-serial sections were created in a microtome (Microm, model HM 340 E, Germany), and representative sections were mounted on glass slides for hematoxylin/eosin staining.

The microscopic images required an Olympus SC30-CCD camera (Olympus, Hamburg, Germany) mounted on an Olympus System Microscope Model BX43 to take, which were then processed with CellSens software (Olympus, Hamburg, Germany). Quantitative histomorphometry was used to evaluate the formation/resorption of blood clots and the formation of granulations, connective tissue, and new bone. Thereafter, the total surface area (μm^2) of each alveolus and the setting areas with new bone formation, connective tissue, and other tissues (blood clot) or structures were measured. The data (areas: new bone and connective tissue) were converted into percentages (%). For better analysis and description, the hemimaxilla (dental alveolus) was divided into and analyzed as cervical, middle, and apical thirds.

2.4. Statistical analysis

Comparative statistical analyses—specifically, ANOVA and the Tukey test—were applied using Prism GraphPad 5.0 (Prism software, GraphPad, USA). The level of significance was 5% for all cases.

3. Results

3.1. Histological findings

All of mice tolerated the surgical procedure well. There were no complications, and all of the animals could be euthanized as planned. No reductions were observed in body weight, nor were signs of postoperative infections observed.

For the histological analysis, we described the total dental alveolus, i.e., all of the thirds (cervical, middle, and apical) in all of the groups during the different experimental periods. During the 7-day period, for all of the groups, we observed the abundant presence of connective tissue in the alveolar center (Fig. 1A), newly formed bone tissue growing on the walls toward the center (Fig. 1B), and blood vessels distributed at various sites. At 14 days, a similar scenario was observed in both the C3H and B6 groups: the connective tissue occupied a smaller space (Fig. 1C). New bone was observed in the alveolar walls, as was bone formation forming islets toward the center. However, we found a distinct formation in the B6-0 ppm group (Fig. 1D), with the islets of bone formation arranged along the alveoli. In general, for the C3H and B6 groups during the 21-day period, the alveolus was occupied by new bone tissue (trabecular) and connective tissue present between the trabecular bone (Fig. 2A, B). During the last period (28 days), bone neoformation occupied a larger area of the alveoli, with the same pattern formation in all of the groups. In addition, we observed a decrease in connective tissue (Fig. 2C and D). It is relevant to mention that some of the samples presented small pieces of tooth root.

3.2. Histomorphometric analysis

Histomorphometric analysis was performed on the alveolar thirds, as recommended by Accorsi-Mendonça et al. [20] and Vieira et al. [16]. All of the cited data are shown in Tables 1–3 . Some minor areas such as blood clots, small pieces of tooth root, and "technical artifacts" were not included in the tables.

3.2.1. Cervical third

The B6 strain showed higher bone formation according to the period (from 38.63% to 51.42% for the control and 26.83% to 56.72%, 50 ppm). The most significant amount was during the 21-day period ($52.40 \pm 1.44\%$ for B6, 0 ppm, $53.86 \pm 2.97\%$ for B6, 50 ppm; $p < 0.05$). Similar amounts of bone tissue formed among the periods in all of the tested groups. Most of the groups showed a gradual formation of bone tissue during the studied periods, but less new bone formed during the C3H, 50 ppm group at 28 days ($39.07 \pm 14.91\%$ for C3H, 0 ppm, $34.85 \pm 4.07\%$ for C3H, 50 ppm), although this was not significant. Connective tissue decreased gradually during all of the periods, except in the C3H, 50 ppm group during the 28-day period. In general, the B6 strain presented less connective tissue than the C3H strain.

3.2.2. Middle third

The patterns of gradual bone tissue formation were similar among the periods, except for the 28-day period in the C3H strain. The B6 strain had major formation of new bone tissue (42.3%), which was gradual in both groups (0 and 50 ppm), with major formation in the B6-50 ppm group when compared with the C3H-50 ppm group, such as in the 21-day and 28-day periods (Table 2). Consequently, due to the increased bone formation in the B6 strain, a smaller amount of connective tissue was found in this strain compared to in the C3H strain. Finally, we observed that the B6, 50 ppm group presented a gradual decrease by period, with the 28-day period presenting the lowest amount of connective tissue, compared with the other groups ($48.35 \pm 5.68\%$).

3.2.3. Apical third

This region showed the smallest amount of new bone formation. The groups that received 50 ppm of F presented major formations of bone tissue, especially in the 7- and 21-day periods for the B6 strain. However, at 28 days for this same strain, we observed lower bone