

Comparative effects of riboflavin, nicotinamide and folic acid on alveolar bone loss: A morphometric and histopathologic study in rats

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SUMMARY

Introduction Periodontitis is a chronic inflammatory and osteolytic disease. Vitamin B complex is a class of water-soluble vitamins that play important roles in cell metabolism.

Objective The aim of this study was to evaluate the effects of riboflavin (RBF), nicotinamide (NA), and folic acid (FA) on alveolar bone loss in experimental periodontitis rat model.

Methods Sixty-four male Wistar rats were randomly divided into the following eight groups: Control, Ligated, RBF50 (RBF, 50 mg/kg daily), NA50 (NA, 50 mg/kg daily), FA50 (FA, 50 mg/kg daily), RBF100 (RBF, 100 mg/kg daily), NA100 (NA, 100 mg/kg daily), and FA100 (FA, 100 mg/kg daily). Periodontitis was induced using silk ligature around the right first mandibular molar. After 11 days the rats were sacrificed. Mandible and serum samples were collected. Changes in alveolar bone levels were measured clinically, and periodontal tissues were examined histopathologically. Serum IL-1 β (pg/ml) levels were analyzed by using ELISA.

Results Mean alveolar bone loss in the mandibular first molar tooth revealed to be significantly lower in RBF100 group than in the Control group. In the Ligated group, alveolar bone loss was significantly higher than in all other groups. The ratio of presence of inflammatory cell infiltration in the Ligated group was significantly higher than in the Control group. The differences in the serum IL-1 β levels between the groups were not statistically significant. Osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups. The osteoblastic activity in the Ligated group, RBF100, and NA100 groups were shown to be significantly higher than those in the Control group.

Conclusion This study has demonstrated that systemic administration of RBF, NA, and FA in different dosages (50–100 mg/kg) reduced alveolar bone loss in periodontal disease in rats.

Keywords: alveolar bone loss; ligature-induced; histomorphometric; micronutrition; vitamin B

INTRODUCTION

Periodontitis is a biofilm-induced chronic inflammatory disease caused by bacterial pathogens and leads to the destruction of the alveolar bone. Although tooth-associated biofilm or dental plaque is required to induce the periodontitis, it is not sufficient to initiate periodontal tissue destruction [1]. Host inflammatory response which is modulated through multiple factors including genetics, smoking, general health, social variables and diet can cause periodontal tissue destruction [1]. Researchers have found strong evidence that macro- and micronutrients could modulate pro- and anti-inflammatory mechanisms effecting individual inflammatory conditions [2]. Some studies have showed that tooth loss is related to poorer nutritional status and poorer quality of life [3, 4]. The association between nutrition intake and periodontal condition has been investigated in many studies. Previously published evidence of the foods and nutrients with antioxidant and anti-inflammatory activity have consistently been linked to a reduced risk of developing periodontal disease such as

vitamin C [5], b-carotene [6], vitamin E [7, 8], folic acid [9], and more recently vitamin B₁₂ [10]. Therefore, research of micronutritional approaches with many vitamins, antioxidants, and therapeutic agents for periodontal treatment has been going on in recent years. Despite the oral symptoms accompanied by vitamin B complex deficiency [11], it remains unknown whether vitamin B complex is associated with periodontitis.

Vitamin B complex is a class of water-soluble vitamins that play important roles in cell metabolism. The vitamin B complex includes eight different vitamins which differ in their chemical composition and pharmacological properties [12].

Riboflavin (RBF), also known as vitamin B₂, helps to provide good vision, releases energy from the foods, promotes healthy skin. Sources of riboflavin are milk, eggs, mushrooms, whole grains, enriched grains, green leafy vegetables, yeast, liver, and oily fish [13]. Clinical riboflavin deficiency is known as ariboflavinosis, and presents as angular stomatitis, cheilosis, and glossitis. Moreover, it is common in developing country populations [14].

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Nicotinamide (NA), known as vitamin B₃ or niacin, has influence on oxidative stress [15], cellular survival, inflammatory cell modulation [16], metabolic disease [17], and energy management [18]. NA deficiency can lead to fatigue, loss of appetite, pigmented rashes of the skin, and oral ulcerations, while more severe types lead to pellagra, characterized by coetaneous rashes, oral ulcerations, gastrointestinal difficulties, and cognitive loss [19].

Folic acid (FA), known as vitamin B₉, is essential for numerous metabolic functions such as synthesizing, repairing, and methylating DNA; it is also especially important in preventing neural tube defects, for example spina bifida [20]. FA is essential for maturation of oral mucosal epithelium and it depresses the hematologic elements which help prevent and fight against the infectious agents in this section of the mouth. Folate deficiency can lead to diarrhea, megaloblastic anemia, peripheral neuropathy, increased oxidative stress, and periodontal disease [9]. Folate supplementation was hypothesized to prevent or decrease fracture incidence by effecting homocysteine metabolism, but some of these studies had inconsistent data about fracture preventing [21].

Vitamin B complex plays an important role in wound healing, gingival health. Effects of vitamin B complex were mostly investigated in terms of soft tissue healing, but some studies have indicated that vitamin B₁₂ [10], vitamin B₉ [22], vitamins B₁, B₂, B₃, B₅, B₆, B₇ reduced the periodontal destruction and tooth mobility [23]. The effects have not been researched comprehensively. The aim of this study was to evaluate these effects in experimental periodontitis rat model, morphometrically and histopathologically.

OBJECTIVE

Vitamin B complex plays an important role in wound healing and gingival health. Effects of vitamin B complex were mostly investigated in terms of soft tissue healing. The aim of this study was to evaluate the effects of RBF, NA, and FA on alveolar bone loss in experimental periodontitis rat model, morphometrically and histopathologically, as these effects have not been researched comprehensively to date.

METHODS

Experimental design

Sixty-four male Wistar rats weighting 250 ± 10 g were used in the study. The animals were kept in temperature-controlled cages (approximately 25°C), exposed to a 24-hour light–dark cycle of equal time, and had access to water and food *ad libitum*. The experimental procedure was approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine. Rats were randomly divided into the following eight groups:

- Non-ligated control (Control) group (n = 8);
- Ligated group (n = 8);

- Riboflavin 50 mg/kg daily (RBF50) group (n = 8);
- Nicotinamide 50 mg/kg daily (NA50) group (n = 8);
- Folic acid 50 mg/kg daily (FA50) group (n = 8);
- Riboflavin 100 mg/kg daily (RBF100) group (n = 8);
- Nicotinamide 100 mg/kg daily (NA100) group (n = 8), and
- Folic acid 100 mg/kg daily (FA100) group (n = 8).

General anesthesia was administered by using ketamine (Eczacıbasi Ilac Sanayi, Istanbul, Turkey) (40 mg/kg). In order to induce experimental periodontitis in Ligated, RBF50, NA50, FA50, RBF100, NA100, and FA100 groups, a 4/0 silk suture (Dogsan Sanayi, Istanbul, Turkey) was placed subgingivally, by the same operator (Karakan NC), around the gingival margin of the right mandibular first molars of the rats. In the Control group, ligature placement was not performed. After application, sutures were checked and loose sutures were tightened again. In the RBF50, NA50, FA50, RBF100, NA100, and FA100 group RBF, NA, and FA (Sigma-Aldrich, Saint Louis, MO, USA) were administered systemically with vehicle solution (physiological saline) by using a gastric gavage, at a rate of 50 mg/kg/d and 100 mg/kg/d [24]. Only physiological saline solution by using gastric gavage was administered in Ligated and Control groups to create sham effect to the rats. Daily systemic treatment with vitamin B groups was continued for 11 days; all rats were sacrificed on the 12th day by using cardiac puncture [25]; blood samples were taken and immediately centrifuged at 3,000 rpm for 10 minutes to obtain serum samples, which were stored at -20°C in microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) until used for enzyme-linked immunosorbent assay (ELISA) analysis. The right mandibles of all rats were dissected and fixed in 10% neutral-buffered solution and stained for histomorphometric analysis.

Measurement of alveolar bone loss

Soft tissues of right mandibular region were defleshed manually and cleaned. The jaws were washed, dried and embedded into 1% aqueous methylene blue solution for identifying cement–enamel junction (CEJ) level. Photographs were taken with a stereomicroscope (×25) and transferred to a computer. The alveolar bone loss was measured in three different sites in the right mandibular molar region by a single examiner (Akpınar A), who marked them to identify the samples. The distance from the CEJ to the top of the alveolar bone crest was measured using Vision Image Analysis Software (Clemex, Longueuil, Quebec, Canada). Mean values of measurements were calculated for statistical analysis (Figure 1).

Laboratory assays

Serum samples were assessed by a commercial ELISA kit (Invitrogen, Camarillo, CA, USA) to determine, IL-1 β (detection range: 31.3–2,000 pg/ml; sensibility or lower limit of detection: 4 pg/ml of recombinant rat IL-1 β) and IL-10

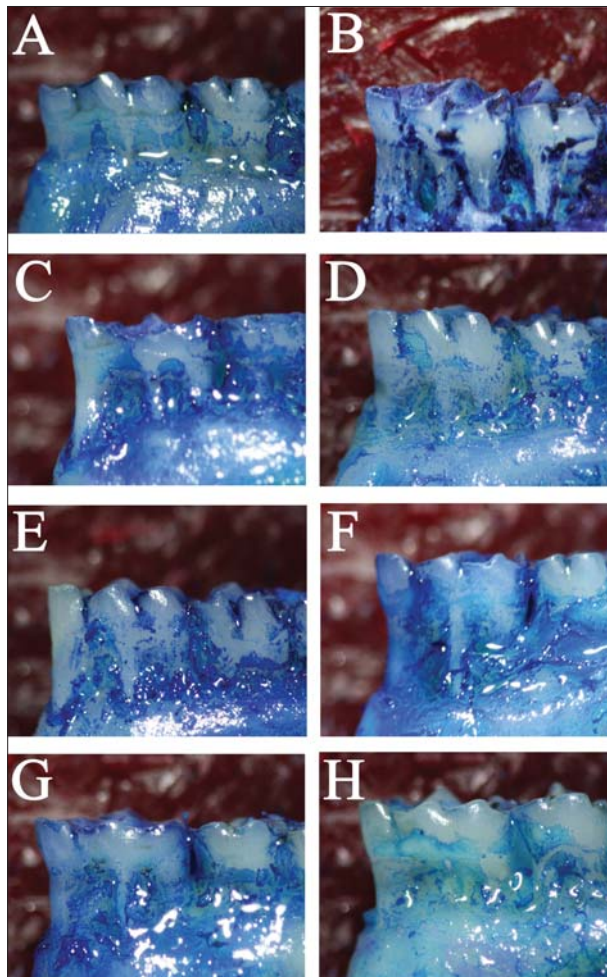


Figure 1. Images of alveolar bone loss in the mandibular first molar in the (A) Control, (B) Ligated, (C) RBF50, (D) RBF100, (E) FA50, (F) FA100, (G) NA50, and (H) NA100 group.

(detection range: 15.6–1,000 pg/ml; sensibility or lower limit of detection: <5 pg/ml of recombinant rat IL-10) in serum samples (samples per group). ELISAs were carried out according to the manufacturer's recommendations; 96-well plates presoaked with appropriate antibodies were used. Standard diluent buffer of 50 μ l was added to 50 μ l samples; 50 μ l of biotin conjugate was added to samples and incubated for two hours at room temperature (37°C). After further incubation at room temperature for two hours, the plates were washed four times, and 100 μ l of streptavidin HRP (diluted 1:5,000) was added at room temperature for 30 minutes. The plates were aspirated and washed four times; 100 μ l of stabilized chromogen was added and incubated for 30 minutes. The amount of 100 μ l of stop solution was added and absorbance of color at 450 nm was measured. The resulting values were expressed in pg/ml. Microwell strips were washed twice with wash buffer. In duplicate 100 μ l sample diluents were added to all standard wells, and 96-well plates presoaked with appropriate antibodies were used. After the plates were coated, the samples and standards were added in various dilutions in duplicate and incubated at 4°C for 24 hours. Antibodies anti-IL-1 β and anti-IL-10 were then added to the wells. Tetramethylbenzidine substrate solution was added to the wells for 15 minutes and activation of blue color develop-

ment in the sample was waited for. Color development was stopped when the color of samples turned yellow. The absorbance of the color at 450 nm was measured.

Histopathological and histomorphometric analysis

Histopathologic sample analyses were performed by a single examiner (Göze F), who masked the samples to identify them to each other. Mandible sections were fixed in 10% formalin, and then in 10% formic acid to demineralize the sample. Next, the specimens were dehydrated, embedded in paraffin, and six- μ m thickness sections were obtained along the molars in a mesio-distal plane for hematoxylin and eosin described. Alveolar bone and interdental septum were analyzed under light microscopy (Eclipse E 600, Nikon, Tokyo, Japan). Inflammatory cell infiltration (ICI) of the periodontal tissues was scored as follows: not visible ICI (score = 0), slightly visible ICI (score = 1), and dense visible ICI (score = 2). A semi-quantitative scoring was used for determining osteoblastic activity as follows: no activity (0), mild/moderate activity (1), and high activity (2) [26]. Osteoclasts, which are large cells including multiple nuclei near border of the resorption surface, were counted morphologically in the histological assessment

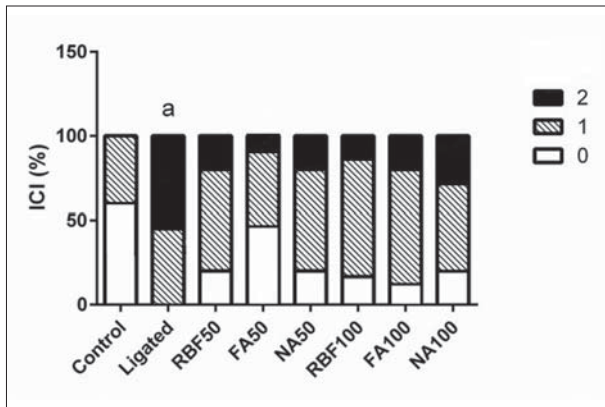
Statistical Analysis

Statistical analyses were performed with SPSS 22.0 for Windows (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov test was performed for determining data distribution. Comparisons between the four groups were performed using the Kruskal-Wallis test. Two independent group comparisons were performed using Mann-Whitney U-test. The data were presented as mean \pm standard deviation and $p < 0.05$ was considered statistically significant. The ratios of the presence of ICI and osteoblastic activity were analyzed using a χ^2 test.

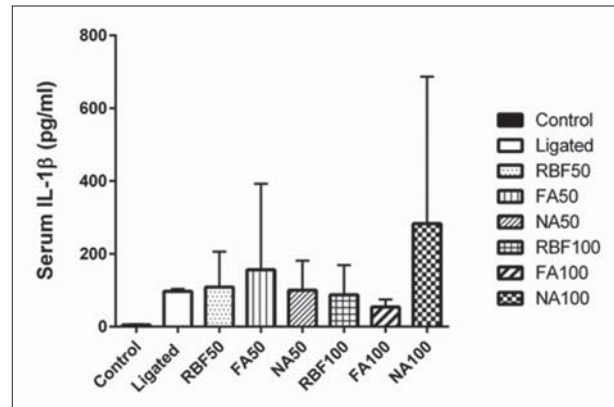
RESULTS

The presence of silk ligature around the first right molars of the rats induced an inflammatory response and periodontitis. The ratio of presence of ICI in the Ligated group ($p < 0.05$) was significantly higher than in the other groups. The ICI in the RBF50, FA50, and NA50 groups and RBF100, FA100, NA100 groups were similar and there was no significant difference between these groups ($p > 0.05$) (Graph 1).

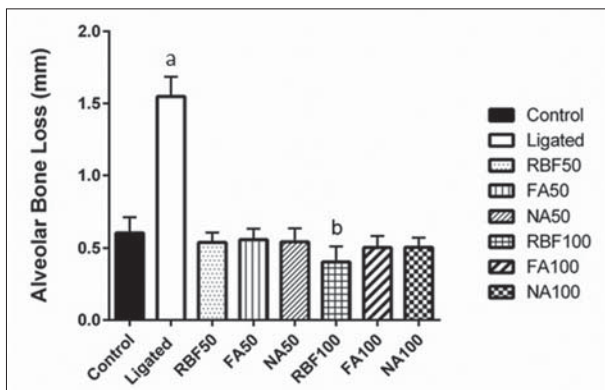
Mean alveolar bone loss measurement with stereomicroscope in the mandibular first molar tooth was revealed to be significantly lower in the RBF100 group than in the Control group ($p < 0.05$). In the Ligated group, alveolar bone loss was significantly higher ($p < 0.05$) compared to all the other groups (Graph 2). The differences in the serum IL-1 β levels between the groups were not statistically significant ($p > 0.05$) (Graph 3). Serum IL-10 levels of all



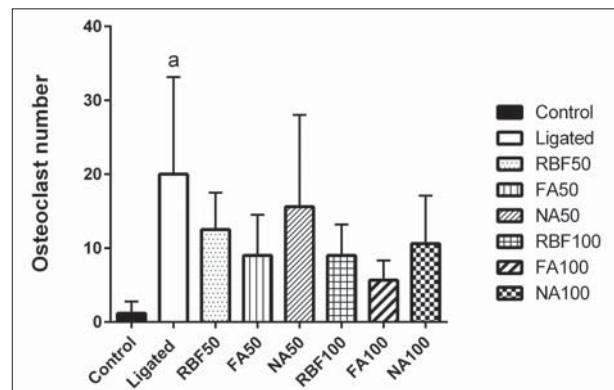
Graph 1. Frequencies (%) of inflammatory cell infiltration (ICI) in groups: (a) Ligated group was revealed as statistically more significant regarding ICI than the Control group ($p < 0.05$).



Graph 3. Mean serum IL-1 β levels with standard deviation bars of the groups; there was no statistical significance between the groups ($p > 0.05$).



Graph 2. Mean alveolar bone loss and standard deviation bars in Control, Ligated, RBF 50, FA50, NA50, RBF100, FA100 and NA100 groups: (a) significant bone loss in Ligated group compared to that of other groups ($p < 0.05$); (b) in the RBF100 group, alveolar bone loss was significantly lower than that in the Control group ($p < 0.05$).



Graph 4. Mean osteoclast numbers in the groups; the osteoclast numbers of all the groups were lower than that in the Ligated group, but this difference was not statistically significant ($p > 0.05$); osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups ($p < 0.05$).

the groups were under the detection range, hence they were not represented in the data.

Graph 4 shows the osteoclast numbers of the groups. The osteoclast numbers of all the groups were lower than the Ligated group, but this difference was not statistically significant ($p > 0.05$). Levels of osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups ($p < 0.05$).

It was revealed that osteoblastic activity in the Ligated group, RBF100, and NA100 groups was significantly higher than that in the Control group ($p < 0.05$). There was no significant difference in term of osteoblastic activity between RBF50, NA50, FA50, and FA100 groups ($p > 0.05$) (Figure 2).

DISCUSSION

The understanding of the nature of periodontal disease causation and pathogenesis has changed dramatically, and as a result the approach of periodontal treatment started evolving 30 years ago, from blocking inflammation to moderating it [27]. There are many studies in literature that refer to links between periodontal disease and dietary intake. Nutrients derived from diet perform as antioxi-

dants, co-enzymes in energy production and metabolic processes and components of tissue structures that keep the body's system functioning properly and maintain good overall health, including oral health. Thus we investigated the effects of different dosages of RBF, NA, and FA on periodontal bone loss in rats and found that the vitamins increased osteoblast activity, and reduced the osteoclast numbers and alveolar bone loss.

In one study, authors researched vitamin B complex supplementation on periodontal wound healing in humans [11]. Patients were given 50 vitamin B complex tablets and 50 placebo tablets for 30 days after the surgery. At the end of the study Vitamin B supplemented subjects demonstrated significantly superior clinical attachment level (CAL) gains, but not in plaque index (PI), gingival index (GI), bleeding on probing (BOP), and BANA test values.

Erdemir and Bergstrom [28] investigated relationship between FA and vitamin B₁₂ serum levels on the one hand, and smoking on the other, in patients with chronic periodontitis. They concluded that mean PI, GI, and CAL were significantly more increased in smokers than in non-smokers, although FA levels of smokers were lowered by 26% in comparison with that of non-smokers. In another study authors tried to evaluate the effects of folate mouthwash on periodontium in experimental gingivitis model

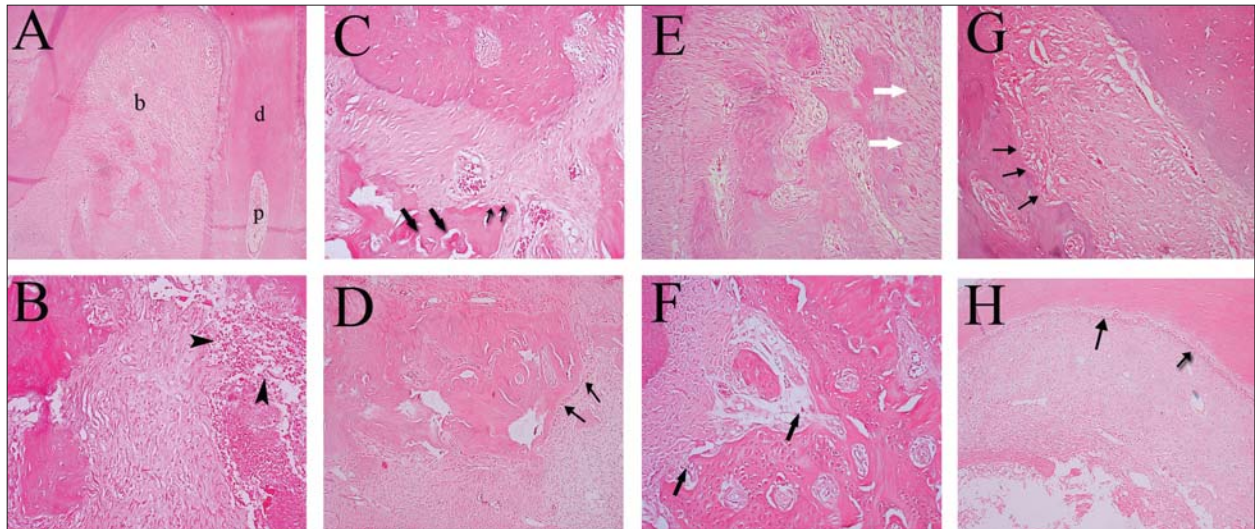


Figure 2. Histopathological sections of mandibular first molar tooth in all the groups (hematoxylin and eosin staining; original magnification: A: $\times 10$; B: $\times 40$; C: $\times 20$; D: $\times 10$; E: $\times 20$; F: $\times 20$; G: $\times 40$; H: $\times 20$): (A) normal mandible showing alveolar bone (b), pulp (p), and dentin (d); (B) in the Ligated group after 11 days, dense inflammatory infiltration area (arrow); (C) RBF50 group showing osteoblastic activity border of alveolar bone (narrow arrows) and ruffled osteoclast border with multiple nuclei (wide arrows); (D) RBF100 group revealed dense osteoblastic activity (arrows); (E) FA50 group, osteocysts in the lacunas (white arrows); (F) FA100 group showing osteoclastic activity (arrow); (G) NA50 and (H) NA100 groups, showing increased osteoblastic activity (black arrows), osteocytes (white arrow), and reduction in osteoclast number, respectively.

[29]. At the end of the study they concluded that the folate mouthwash did not affect plaque accumulation or clinical signs of gingivitis. According to data from the national health and nutrition examination survey 2001/02, serum folate levels are important indicators for periodontal health in older adults and may help maintain oral health properly [9]. Mohammadi et al. [22] aimed to evaluate the protective effect of FA on cyclosporine-induced bone loss in rats. Consequently, they suggested that FA may have a preventive role against cyclosporine adverse effects to bone in rats. In a recent study performed with diabetic rats, authors observed that alveolar bone loss, number of inflammatory cells, and nuclear factor kappa-B ligand positive cells were more decreased in streptozotocin in the NA group than in the streptozotocin group [30]. In our study the results showed that RBF, FA, and NA significantly prevented alveolar bone loss which had been initiated by using ligature on experimental periodontitis model in rats.

There are many experimental periodontitis models which have been applied on rats, such as using ligature on molar teeth, dietary manipulation, or introduction of pathogenic microorganisms. Placing a ligature is a highly presumable model for determining effects of different drugs or agents against periodontal tissues. According to studies, placement of a ligature around the molar teeth of rats leads to appearance an inflammation beneath the ligature [31] and most intense bone loss was achieved by day 11 after ligature placement [32]. We used ligature-induced periodontitis model in our study, rats were sacrificed on day 12 and ligature placement on the first molar tooth caused a significant amount of bone loss.

In this study the dosages were arranged according to studies listed below. No evidence of RBF toxicity was based on limited human and animal studies [33]. Bertollo et al. [34] assessed antinociceptive and anti-inflammatory activ-

ities of RBF in different experimental models with different dosages, i.e. 25, 50, and 100 mg/kg. In an experimental rat model, the authors used 100 mg/kg dosage of RBF to evaluate its antioxidant effect [35]. In another study RBF was administered at 30 and 100 mg/kg by oral gavage to test its protective effect on hepatic injury [36]. The risk of toxicity from FA is low, because folate is a water-soluble vitamin and is regularly removed from the body through urine. Different dosages of FA have been used in many animal studies. In an experimental study on mice, 400–1,200 nmol/kg dosages of FA were used [37]. The dose of FA could be chosen to evaluate its efficiency – a 100-fold dose of its acceptable daily dose, which is considered to be 15 mg/day for a 70-kg healthy adult [38]. In general, NA is given in doses ranging 40–250 mg/day orally when NA deficiency occurs [13]. The median lethal dose for subcutaneous administration of NA in rats is 1.68 g/kg, hence the therapeutic index of NA is wide, but at very high doses it causes reversible hepatotoxicity in animals and humans [39]. No clinical signs, abnormal behaviors, or infections were observed in rats of any group. In our study, 50 mg/kg and 100 mg/kg dosages of RBF, FA, and NA were used on rats. Both of the dosages were effective on alveolar bone loss, although 100 mg/kg dosage of these vitamins revealed more effective results compared to 50 mg/kg dosage; however, these findings are not statistically significant.

IL-1 β plays a key role in periodontal pathogenesis and induces the synthesis and secretion of different mediators such as prostaglandins, chemokines, and other cytokines. IL-1 β exacerbates inflammation and alveolar bone resorption. A development of systemic infection in rats might have increased the levels of IL-1 β . Apart from the serum cytokine analysis, gingival homogenates analysis would be performed to eliminate systemic inflammation effect. In our study, serum levels were the highest in the NA100 group, but the difference between the groups was not sig-

nificant – although NA has an antioxidant effect that may prevent alveolar bone loss in spite of increased IL-1 β level.

CONCLUSION

Within its limitations, this study states that administration of systemic RBF, FA, and NA with different dosages

increased osteoblast activity, decreased osteoclast numbers, and diminished alveolar bone loss in experimental periodontitis in a ligature-induced rat model. Considering this data, RBF, FA, and NA, which are different kinds of vitamin B complex, may provide additional benefits to periodontal therapy for preventing bone loss. More research is needed on effects of vitamin B complex on periodontal bone healing.

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Упоредни утицај рибофлавина, никотинамида и фолне киселине на алвеоларни губитак коштане масе: морфометријска и хистопатолошка студија на пацовима

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КРАТАК САДРЖАЈ

Увод Пародонтопатија је хронична инфламаторна и остео-литичка болест. Комплекс витамина Б је класа витамина растворљивих у води који играју важну улогу у ћелијском метаболизму.

Циљ рада Циљ овог истраживања је био да се процени утицај рибофлавина (РБФ), никотинамида (НА) и фолне киселине (ФК) на алвеоларни губитак коштане масе у експерименталном моделу пародонтопатије код пацова.

Методе рада Шездесет четири мужјака пацова соја вистар насумично су подељена у следећих осам група: контролна, лигатурна, РБФ50 (РБФ, 50 mg/kg дневно), НА50 (НА, 50 mg/kg дневно), ФК50 (ФК, 50 mg/kg дневно), РБФ100 (РБФ, 100 mg/kg дневно), НА100 (НА, 100 mg/kg дневно) и ФК100 (ФК, 100 mg/kg дневно). Пародонтопатија је изазвана везивањем свилене нити око првог десног мандибуларног кутњака. После 11 дана пацови су жртвовани. Сакупљени су узорци доње вилице и серума. Клинички су утврђене промене нивоа алвеоларне коштане масе, док су пародонтална ткива испитана хистопатолошки. Нивои *IL-1β* (pg/ml) серума анализирани су тестом *ELISA*.

Резултати Показало се да је средњи губитак алвеоларне коштане масе у првом мандибуларном кутњаку знатно нижи у групи РБФ100 у односу на контролну групу. У лигатурној групи алвеоларни губитак коштане масе био је значајно виши него у осталим групама. Процент присуства инфилтрације инфламаторних ћелија у лигатурној групи био је знатно већи у односу на контролну групу. Разлике у нивоима *IL-1β* у серуму између група нису биле статистички значајне. Остеокласти уочени у лигатурној групи били су знатно виши од оних у контролној и групи ФК100. Остеобластична активност у лигатурној, групи РБФ100 и групи НА100 била је значајно већа него у контролној групи.

Закључак Ова студија је показала да системска примена РБФ, НА и ФК у различитим дозама (50–100 mg/kg) смањује губитак алвеоларне коштане масе код пародонтопатије код пацова.

Кључне речи: губитак алвеоларне коштане масе; индукована лигатуром; хистоморфометријски; микронутриционизам; витамин Б