ORIGINAL ARTICLE

Hazards of Short- and Long-term Administration of Glucocorticoids on the Periodontium in Rats: A Histological and Electron Microscopic Study

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ABSTRACT

The administration of glucocorticoids is proven to cause serious adverse events. Objectives: This study was conducted to compare the possible hazards of the short- and long-term administration of glucocorticoids on the periodontium in rats, using histological examination and scanning electron microscopy. Methods: Fifteen adult male albino rats were included in the study and divided into the 3 groups. Group I served as a control, group II received 7 mg/kg dexamethasone intramuscularly once a week for 5 wk, and group III received 7 mg/kg dexamethasone intramuscularly once a week for 10 wk. The mandibles were dissected and examined histologically as well as with scanning electron microscopy and energy dispersive radiography. Results: Histologically, group I showed normal alveolar process. In group II, bone trabeculae demonstrated obvious Howship’s lacunae of osteoclasts. In group III, bone trabeculae exhibited multiple degenerated osteocytes with apparent vacuolization. Scanning electron microscopy revealed smooth alveolar bone architecture in group I. Groups II and III demonstrated irregular bone architecture with widening of the neurovascular canals. EDX analysis demonstrated the highest calcium-phosphorous concentration in the control group and the lowest in group III. Conclusions: Dexamethasone has a devastating impact on the alveolar bone via the acceleration of bone resorption and decreased activity of osteoblasts. This effect was more pronounced with prolonged drug administration.

Key words: electron microscopy, glucocorticoids, histology, periodontium

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INTRODUCTION

Glucocorticoids (GC) are steroid hormones that are physiologically produced by the adrenal glands and are synthesized for sporadic or uninterrupted systemic and topical use. Numerous biological functions are regulated by glucocorticoids (GCs), such as homeostasis, carbohydrate metabolism, protein catabolism, evolution, awareness, and inflammation. Moreover, GCs play a crucial role in acclimatization to environmental alterations, stress reaction, and immune modification. Synthetic glucocorticoids (GCs) have been widely used in clinical practice for treating autoimmune, rheumatic, and gastrointestinal disorders; neoplasms; and organ transplantation for decades.

Osteoporosis (OP) is a common disorder characterized by systemic bone loss and impaired bone microarchitecture that leads to increased bone fragility and fracture susceptibility. Osteoporosis can ultimately result from reduction of the estrogen level following menopause, extended period of immobilization, increasing age, chronic disorders, and use of certain medicines. Bone loss is a characteristic feature of osteoporosis that may encourage the development and progression of periodontal disease as well as tooth loss. At the bone level, many studies have focused on the link between osteoporosis and periodontitis. Reduction in bone mineral density (BMD) has also been reported in various glucocorticoid treated pediatric disorders, including asthma, organ transplantation, rheumatoid...
arthritis, and systemic lupus erythematosus. In dentistry, inflammatory diseases, such as dental caries and periodontitis, have been documented as a major reason of the tooth loss. However, there has been an annual increase in the number of studies that suggest a strong correlation between systemic osteoporosis and alveolar bone loss or tooth loss. Moreover, the speed of alveolar bone resorption, bone remodeling, and density of bone after tooth extraction are affected by osteoporosis.

Based on the previous studies, Beeraka et al., 2013 studied the clinical picture of the mouth and periodontal status and the radiographic changes in the bone using intra oral periapical radiography and digital orthopantomography. Bouvard et al., 2013 assed the effects of GCs on the alveolar bone and the tibia in a mouse model with histomorphometry and micro computed tomography. In 2015, the effects of dexamethasone on the biomechanical properties of the mandible of rats during the growth phase were assessed using the bending test and computed tomographic analysis. It was concluded that corticosteroid exerted a combined, negative action on bone geometry and volumetric bone mineral density of the cortical bone that would exert independent effects on both the cellular and tissue levels of biological organization of the skeleton in the species. In 2018, hemi mandibles from mice subcutaneously implanted with prednisolone or vehicle-containing pellets for 7, 21, or 55 days were collected for radiographic and histological analyses to assess perilacunar/canalicular remodeling PLR. The authors concluded that in addition to reducing bone mass and suppressing PLR, glucocorticoids reduced the stiffness of the mandibular bone in flexural tests.

Thus, proper clarification of the histological and ultrastructural changes in the mandibular alveolar process, periodontal ligament, as well as the dento-gingival junction upon systemic use of dexamethasone need more investigation and study. Further, the level of calcium and phosphorus were measured using EDX [the trabecular content of calcium (Ca) and phosphorus (P) was analyzed] in the alveolar process of the experimental group and compared to that in the normal control group.

OBJECTIVE

The current work was undertaken to estimate and compare the possible changes that occurred in the mandibular alveolar process, periodontal ligament, and the dento-gingival junction in albino rats following short- and long-term use of glucocorticoids using light microscopy and scanning electron microscopy. Moreover, the quantitative composition of the calcium-phosphorous complex in the studied samples was determined with EDX spot measurement.

METHODS

Animal experimental

Fifteen male adult albino rats weighing 170–200 g were utilized. The animals were obtained from the animal house, the Faculty of Medicine, Cairo University and maintained under the care of a specialized veterinarian. All the experimental animals in all the groups were examined clinically under anesthesia before the administration of glucocorticoids. The animals included in the study were normal. Each group of five animals was kept separately in a plastic cage. They were maintained under optimal temperature at 25°C ± 2°C with 12 h light/dark cycle and had free access to standard rat diet and water. The study was approved by the research ethics committee Faculty of Dentistry, Cairo University. The procedures were designed as per the guidelines for the responsible use of animals in research as a part of the scientific research ethics recommendation of the Ethical Committee, Faculty of Dentistry, Cairo University.

After one week of acclimation to the laboratory environment, the rats were randomly divided into the following three groups (n = 5). In group I (controls), each rat was given subcutaneous injection of 0.5 mL saline once a week for 10 weeks. Group II [glucocorticoid induced osteoporosis (GIOP)] rats received intramuscular injections of 7 mg/kg of dexamethasone (Decadron, 4 mg/mL, Eipico Egypt) once a week for 5 weeks. Group III rats received intramuscular injections of 7 mg/kg of dexamethasone (Decadron, 4 mg/mL, Eipico Egypt) once a week for 10 weeks. Scarification of all the animals was carried out with ketamine overdose, and the mandibles were dissected. We subjected the right side of each mandible for light microscopic examination, while the left sides of the mandibles were used for scanning electron microscopic (SEM) examination as well as analysis of the constituent element (calcium concentration) using an energy dispersive radiography unit attached to the SEM.

Light microscopic examination

Fixation for all the specimens was achieved in 10% neutral formalin for 2 days. Specimens were washed, soaked in 10% ethylene diamine tetra-acetic acid for decalcification for one month, and then rinsed in distilled water. Dehydration of the specimens in ascending grades of alcohol and embedding in paraffin and mesio-distal sectioning of the right side of the jaw were performed. Histological sections of 5 μm thickness were prepared. The sections were stained with hematoxylin and eosin stains using the conventional method, and histopathological examination was performed with light microscopy.
Scanning electron microscopic examination
Fixation of the specimens was performed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 6 h. Thereafter, dehydration of the specimens in ascending grades of ethanol was performed for about 10 min at each passage and 20 min twice in absolute ethanol, subject to critical point drying according to the standard procedure for SEM processing, and left to dry in air at room temperature for 3 days. The specimens were then mounted on scanning electron microscope stubs and studied with a Quanta 250 Field Emission Gun (FEG) SEM (Thermo Fisher Scientific, Waltham, USA).

Energy dispersive X-ray analysis
The quantitative composition of calcium in the studied samples was determined with EDX spot measurement, EDX line scan, and element mapping. The EDX analysis unit works as an integrated feature of the Quanta 250FEG SEM.

Statistical analyses
All the data obtained from the EDX analysis were statistically evaluated. These data are represented as mean ± SD values and were statistically compared among the 3 studied groups. One-way analysis of variance (ANOVA) test was performed for more than 2 independent samples, followed by Tukey’s post-hoc test for pair wise comparisons. The results are expressed as p-values that were considered significant at ≤ 0.05 and highly significant at ≤ 0.01. All the analyses were performed using the following computer programs: Microsoft Excel 2007 and Statistical Package for the Social Science (SPSS) (SPSS Inc., IBM Corp., Armonk, USA) v. 15 for Microsoft Windows.

RESULTS
Histological findings
The control group showed normal architecture of the junctional epithelium and consisted of thin, non-keratinized stratified squamous epithelium with a straight basement membrane supported by dense connective tissue. Histopathological examination of the molar region showed normal histological structure of periodontal ligaments (PDL), alveolar bone, and cementum (Figure 1).

Group II demonstrated marked structural changes in the form of separation of the junctional epithelium from the tooth structure forming a periodontal pocket. Bone trabeculae appeared with randomly distributed osteocytes as well as scalloped reversal lines. Multiple Howship’s lacunae of the osteoclasts were also clearly observed. Disturbed orientation, signs of degeneration, and hyalinization of the collagen fibers of the periodontal ligament with uneven thickness on both sides of the root exhibited area of compression and area of tension. A large dilated blood vessel was also seen in the periodontal ligament. Cellular cementum appeared thicker than that in the control group with multiple empty lacunae of cementocytes. (Figure 2, 3).

Group III exhibited severe loss of attachment of the junctional epithelium from the tooth structure, forming a deep periodontal pocket. Macroscopically, one of the molars (M2) was missing in some experimental rats (Figure 4).

Sections from the osteoporosis groups revealed thinning of the bony trabeculae with marked widening of the marrow spaces along with multiple resorption foci on the bone surface and apparent vacuolization. Lacunae of the osteocytes were empty. Multiple reversal lines and generalized disorganized collagen fibers in the periodontal ligaments were observed. Hypercementosis was obvious in the cellular cementum at the apical region (Figure 5).

Scanning electron microscopy images
The SEM images of the control group showed normal smooth alveolar bone architecture. The neurovascular canals were obvious. However, SEM examination of group II demonstrated alterations in the normal bone architecture. The bone surface revealed roughness and widening of the neurovascular canals. The alveolar bone of group III revealed an irregular rough resorbed surface with a wide neurovascular canal (Figure 6). SEM analysis of the PDL in the control group revealed thick, well-organized collagen fiber bundles. However, the collagen fibers of the PDL of the experimental groups (II and III) were disorganized with areas of degeneration seen in group III (Figure 7).

Energy-dispersive X-ray analysis
The EDX elemental analysis demonstrated the peak of the chemical element – calcium (Ca) phosphorous (ph) concentration – found in the samples in each group. The calcium-phosphorous content in the alveolar bone was evaluated in terms of the percent weight (wt%). EDX quantified the relative contribution of Ca-ph element to 100%. The mean ± SD values for total weight percentage of calcium-phosphorous in groups I, II, and III were calculated (Table 1). The mean total weight percentage of calcium-phosphorous was highest in group I, while the lowest percentage was demonstrated in group III. ANOVA test demonstrated a statistically significant difference among the 3 groups (p-value ≤ 0.01). Post-hoc multiple comparisons between the 2 groups revealed that the difference in the mean values of groups I and II, groups I and III, and groups II and III were statistically significant (p-value ≤ 0.01) (Table 1).
Figure 1. Photomicrograph of group I (control): (A) showing normal architecture of the junctional epithelium (arrow). (B) Normal inter-radicular alveolar bone (AB) and periodontal ligament (PL). (C) Normal cellular cementum (cc), apical fibers of periodontal ligament (AF). (H&E ×200).

Figure 2. Photomicrograph of group II: (A) Showing separation of the junctional epithelium from the tooth structure. (B) Interdental alveolar bone revealed scalloped reversal lines (arrows). (C) Multiple Howship’s lacunae of osteoclasts (arrows). (H&E ×200).

Figure 3. Photomicrograph of group II: (A) exhibited area of compression within the periodontal ligament on both sides of the root (arrows). (B) Thin bone trabeculae (BT) with resorbed alveolar bone surface (arrows). (H&E ×200).

Figure 4. Photomicrograph of group III: (A) Macroscopic picture showing the missing second molar M2. (B) Severe loss of attachment of the junctional epithelium from the tooth structure (arrow). (H&E ×200).

Figure 5. Photomicrograph of group III: (A) showing multiple resorption foci on the bone surface (arrows) and numerous reversal lines. (B) Thinning of the bone trabeculae (BT) and hypercementosis (HC). (C) Disorganized collagen fibers of the periodontal ligaments (PL), multiple resorption foci on the bone surface (arrows) and apparent vacuolization (V). (H&E ×200).

Figure 6. Scanning electron microscope photograph of the alveolar bone surface. (A) Normal architecture of the alveolar bone of the control group with narrow neurovascular canals (white arrows). (B) Rough alveolar bone surface with widening of the neurovascular canal of group II (black arrow). (C) Irregular rough resorbed surface with wide neurovascular canal of group III (yellow arrow). (SEM ×1000).

Table 1. Comparison of the mean values (mean ± SD) of calcium-phosphorous complex weight percentage in the studied groups.

| Studied groups | Ca-ph weight [%] |
|---------------|-----------------|
| Group I       | 87.24 ± 0.1607  |
| Group II      | 72.153 ± 0.2291^a |
| Group III     | 67.516 ± 0.1357^ab |
| p-value*      | ≤ 0.01          |

* ANOVA test; Tukey’s post-hoc test; a statistically significant with group I (p ≤ 0.01); b statistically significant with group II (p ≤ 0.01).

DISCUSSION

Bone biology and physiology are influenced by chronic skeletal disorders, such as osteoporosis. Osteoporosis not only affects the long bones of the body, but also may affect jawbones, leading to alveolar bone alterations that may compromise the handling of periodontal disease and/or its progression.17,18

Postmenopausal estrogen deficiency is regarded as a major cause of osteoporosis.19,20 Nevertheless, the long-term use of glucocorticoids (GCs) may also be considered a secondary cause of osteoporosis. We
used a rat osteoporotic animal model because it is cost-effective, easily available, the bone metabolism of rats is comparable to that of humans, and rats respond to risk factors and medication in manner similar to that in humans. The most utilized animal model for experimental osteoporosis is ovariectomy. It was logical to utilize female rats as the animal models for osteoporosis in the current study. However, there are certain disadvantages. It is applicable only in female rats; a second surgery is required and potentiates the postmenopausal osteoporosis. As well as a sham-operated control group is required and raises the number of rats. Therefore, in the present study, osteoporosis was induced by using dexamethasone (DEX) as an artificial glucocorticoid (GC).

The alterations in the alveolar process were evaluated in the ongoing research following the administration of dexamethasone for 5 and 10 weeks in groups II and III, respectively. These chosen time intervals were based on the data from previous studies and clinical trials. The histological results of the present study coincide with some other results that show that osteoporosis decreases the BMD of jawbones and the height of alveolar bones, resulting in premature loss of posterior teeth without any signs of gingival inflammation or periodontitis. In the current study, multiple vacuoles as well as thinning of the bone trabeculae with widening of the marrow spaces were observed, indicating deteriorated bone structure.

Hypercementosis observed in our study could be explained as an adaptive change in the periodontal ligament, characterized by increased thickness of the cementum on the root surface that exceeds that required to accomplish its normal functions and subsequently leads to abnormal thickening with its histological alterations. Hypercementosis might explain the response of the cementum to the degenerative changes that affect the periodontal ligaments as a trial to enhance the tooth anchorage. This is confirmed by Zhou et al., who explained that the excessive apposition of cementum in the apical half of the root may compensate for the loss of periodontal tissue due to periodontal disease, thus maintaining tooth attachment. Matter Neto et al. also reported that hypercementosis refers to an adaptive change in the periodontal ligament characterized by increased cementum thickness on the root surface much more than that necessary to fulfill its normal functions, resulting in its abnormal thickening. Empty cementocytes lacunae were observed that indicate the degeneration of osteocytes. Owing to this hypercementosis, numerous cementocytes became away from the source of nutrition and so degenerated leaving the observed multiple empty lacunae.

Areas of post-osteoclastic activity were reflected by the presence of multiple reversal lines in both groups II and III. Our results coincide with those reported by Chappard et al.; they showed that the microarchitecture in GIO was characterized by thinning of the bone trabeculae, perforation, and changes in terms of trabecular connectivity. In three-dimensional analyses with reconstructed models, perforation of bone trabeculae was revealed in almost all the patients treated with glucocorticoids.

The results of our study confirmed previous reports that have shown that prolonged administration of glucocorticoids leads to diminution of osteoblastogenesis and accelerated apoptosis of osteoblasts and osteocyte and that osteoclastogenesis and osteoclast survival are provisionally increased. The reduction in the substitution of the resorbed bone by the osteoclasts could be explained by the apoptosis promotion of the osteocytes that are sensors for bone remodeling.

GCs also inhibit osteoblast differentiation via the suppression of bone morphogenic protein II that reinforces the osteoblasts transcription factors. In addition to the repression of osteoblasts differentiation, GCs decrease type I collagen synthesis that principally interferes with the function of the mature cells. The pro-apoptotic effects of glucocorticoids on osteoblasts and osteocytes are attributable to the stimulation of caspase-3, the key mediator of apoptosis. Other results could be also explained by the fact that dexamethasone could down regulate the osteogenic markers, such as alkaline phosphatase, osteocalcin, and Runx-related transcription factor 2 (Runx2).

Dexamethasone motivates the expression of histone deacetylase 6 (HDAC6) that binds to glucocorticoid receptor and may prevent the osteocalcin expression. Other researchers concluded that dexamethasone could reduce the expression of insulin like growth factors I and II because these growth factors increase osteoblast differentiation. Type I collagen synthesis, and bone formation.

The disorganized collagen fibers, signs of degeneration and hyalinization of the periodontal ligament fibers, observed in the current study were similar to previous reports that have shown that osteoporosis and decreased BMD may lead to acceleration of the clinical attachment loss and increase the depth of periodontal pocket postmenopausal women. The association of osteoporosis and periodontitis has been confirmed, primarily based on radiography and to a lesser degree on clinical parameters, in most cross-sectional studies. However, more recent studies have established that severe periodontal disorder is not associated with osteoporosis in postmenopausal women.

The scanning electron microscopy results in our study provided good support for the histological findings. The relative content of calcium is critical for preserving the homeostasis of mineral and the bone metabolism. Ca content was considered a suitable biomarker for assessing bone health. EDX is a sensitive qualitative
and semiquantitative technique for assessing the mineral content variations in calcified tissue.\textsuperscript{43}

Our results were in agreement with those of Lane et al. who studied a murine model and reported that glucocorticoid treatment decreases the mineralization degree of the trabecular bone. GCs may influence the function and metabolism of osteocytes, changing the elastic modulus around the osteocyte lacunae and leading to reduction of the mineral- to-matrix ratios in the same areas with an increase in the size of the osteocyte lacunae.\textsuperscript{44}

Wang et al. stated that microRNAs (miRNAs) are endogenous RNAs made up of 18–25 nucleotides that react with the messenger RNA to alter protein expression. A recent study has stated that several miRNAs have differential expressions in bones treated with GC. For example, decreased miRNA-29a expression reacts with Wnt signaling components and Dkk-1 during differentiation of osteoblasts and is associated with GC-associated bone loss; this could explain the harmful impacts of GC therapy on bone microarchitecture, bone mass, and its biomechanical strength.\textsuperscript{45}

The EDX results in the present study support the SEM findings. These results could be attributed to reduction of intestinal calcium absorption, elevated elimination of urinary calcium, reduction in growth hormone secretion, and alterations in sex steroids metabolism and parathyroid hormone (PTH) as indirect negative impacts of GCs on bone health.\textsuperscript{46} Reduced mineralization was also reported and explained by Lane el al. who reported that glucocorticoids change the osteocyte-canalicular network by altering the elastic modulus surrounding the osteocytes’ lacunae and subsequently decrease mineralization.\textsuperscript{44} Glucocorticoids are known to reduce BMD.\textsuperscript{47,48}

In both, clinical research and animal studies, glucocorticoid use is proven to cause bone loss and reduce BMD. The duration of the glucocorticoid exposure and its cumulative dose determine the degree of these factors.\textsuperscript{47,48} Finally, the prolonged use of glucocorticoids appears to influence the bone in numerous various ways, in sequence of deterioration, dependent on its dose and duration of administration.

CONCLUSION

In conclusion, our results confirmed that long-term administration of glucocorticoids leads to reduced bone formation, decreased mineral apposition rate, and decreased bone volume and trabecular thickness in the alveolar bone of rats. The profound of research mechanisms has allowed worthy results for understanding the mode of action of glucocorticoids, especially if chronically used, at biological bone level. This provides new avenues for further research concerning the treatment and inhibition of the resulting bone disorders.

CONFLICT OF INTERESTS

All author declared that there are no conflict of interest.

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