Effect of pearl millet extract versus alendronate on mandibles and salivaryglands of rats subjected to cafeteria diet associated with corticosteroids

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Abstract

Many studies suggested that glucocorticoids and cafeteria diet (CD) are among the risk factors involved in osteopenia. The aim of this study is to evaluate the effect of aqueous extract of *Pennisetum glaucum* (AEPG) compared to a bisphosphonate, the Alendronate (ALN) on biochemical composition and histological of mandibles and parotid salivary glands of rats fed cafeteria diet associated with methylprednisolone (MPS) intraperitoneal (ip) administration. Thirty six 6week-old-females were divided into six groups: control group; CD group, CD-MPS group [CD, MPS (9 mg.kg^{-1} of body weight (b.w.) ip]; CD-AEPG group [CD, (250 mg.kg^{-1} b.w per os (po)], CD-MPS-AEPG group [CD, MPS (9 mg.kg^{-1}b.w (ip)), AEPG (250 mg.kg^{-1}, b.w. po)]; CD-MPS-ALN group [CD, MPS (9 mg.kg^{-1}b.w., ip), ALN (2 mg.kg^{-1} b.w., po)]. These products were administered for 5 days/week. After 6-weeks treatment period of the study, rats were weighted. Mandibles and maxillary salivary glands (MSG) were isolated and cleaned of all soft tissue and weighted. The mandibles were analyzed for biochemical composition, histology and ultrastructure.The salivary glands were just analyzed by histological technics. In our study, we demonstrated that AEPG treatment obtains better amelioration than ALN without significant decrease calcium content. We noted also an increase of manganese content. Remarkably, trabecular bone appeared intact for groups treated with AEPG compared to the others groups, except the group of ALN where we noted real amelioration but less than AEPG group. These findings suggest that the AEPG could be used to prevent bone diseases and accelerate bone formation in rats feeding cafeteria diet associated with methylprednisolone.

Keywords: Pearl millet, mandible, methylprednisolone, cafeteria diet, alendronate, rat.

1. Introduction

Bone growth in adolescent is a crucial period. However, long-term glucocorticoid treatment increases the risk of several serious diseases, such as osteoporosis, bone fragility and fractures events [1], and knows to alter oral homeostasis [2]. Osteoporosis is a public health problem principally involving postmenopausal women and older people. Corticosteroids interfere with the coupling of resorption and deposition cycle in normal bone, which results in reduced bone formation and increased bone resorption [3]. Many disorders are linked to a high fat diet. Fat and calorie-dense foods and sedentary life styles provoke long-term imbalances between energy uptake and expenditure. Numerous studies indicate that increase high fat diet may be directly responsible of both increase marrow adipogenesis and decrease osteoblast differentiation [4, 5].

Bisphosphonates (BPs) are widely used in the treatment of osteonecrosis [6], and osteoporosis therapy [7]. They are strong antiresorptive drugs. Bisphosphonates suppress bone resorption by abolishing mature osteoclasts and hindering osteoclast formation from precursors [8]. It is known that these drugs can be used at lower dose and defined period to provide valuable protection concerning the physiology bone or alveolar bone alteration [9].

Pearl millet (*Pennisetum glaucum*) belongs to the family of *poaceae*. In Africa and Asia countries, pearl millet is cultivated as edible seeds.This plant has several types of medicinal values as antidiabetic [10]. Its dried seeds were widely used as a folk medicine in Morocco to improve traumatic pains and bone' fractures, but they were not evaluated scientifically.

The aim of this study was to evaluate the effect of aqueous extract of *Pennisetum glaucum*on biochemical composition and histological of mandibles and maxillary salivary glands (MSG) of rats fed a cafeteria diet associated with methylprednisolone intraperitoneal administration.

2. Materials and methods

2.1. Animals

Six week-old-female Wistar rats (50 to 60 g) were used for these experiments respecting all the Rabat Medical Pharmacy School IRB. They were housed three per plastic cages ($42 \times 26 \times 18$ cm) in a room at 25°C controlled temperature, 50% relative humidity and in 12 h light/ 12 dark cycles. All animals were provided with filtered tap water and standard diet ad libitum and allowed to acclimate for one week. All procedures performed throughout the experiment conformed to the guidelines of Council instructions about the protection of living animal used in scientific investigations.

2.2. Sample preparation plant

Whole grains of pearl millet (*Pennisetum glaucum* L.) were obtained from Rabat herbalist (Morocco). The grains identified by the botanic department of the National Scientific Institute, were dried at room temperature and grounded to obtain a fine powder using an electric blender. For the aqueous extract of *Pennisetum glaucum* (Aepg), 1 kg of fine powder was mixed with distilled water (3 volumes) in a glass jar and left for 2 days at room temperature in an orbital digital agitator (Rotatest 560VIT. 15-300 T/MIN). The solvent was filtered through Whatman pleated filter paper number 3. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator [13] and stored at + 4°C.
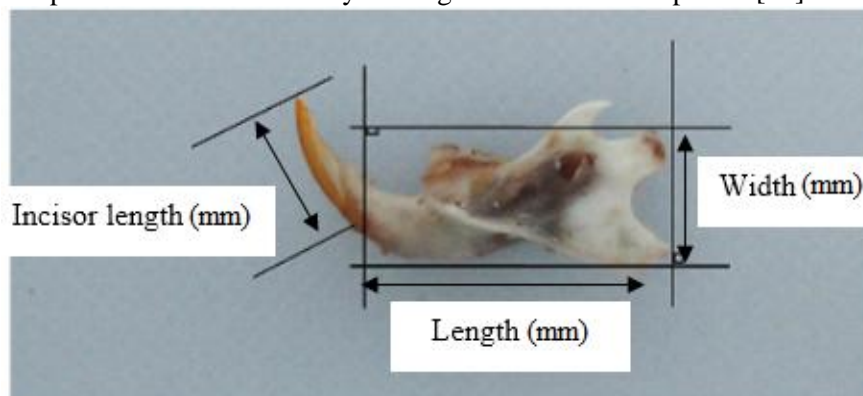
2.3. Experimental protocol

The rats of this experimentation were randomly divided into six groups (n=6) for 36 days. The experiments were conducted between 10h00 and 12h00, and various products were tested as follows: control group; CD group; CD-MPS group [CD, MPS (9 mg.kg^{-1}of body weight (b.w.) intraperitoneacly (ip)]; CD-AEPG group [CD, (250 mg.kg^{-1} b.w per os (po)]; CD-MPS-AEPG group [CD, MPS (9 mg.kg^{-1}b.w (ip)), AEPG (250 mg.kg^{-1}, b.w po)]; CD-MPS-ALN group [CD, MPS (9 mg.kg^{-1}b.w. (ip)), ALN (2 mg.kg^{-1} b.w. po)]. MPS, AEPG and ALN were administered for 5 days/week. At the end of the 6-wk treatments period of the study, rats were weighed, then anesthetized with overdose of diethyl ether. After sacrifice, all mandibles and maxillary salivary glands (MSG) were isolated and cleaned of all soft tissue. The MSG and mandible were immediately weighted by electronic balance (Redwag, PS 110/C/1) to determine weight. The mandible length and width were measured with vernier caliper (Eclipse, Farnell) (figure 1). The samples were fixed in 10% neutral buffered formalin for 24 hours. The left mandibles were used for histological evaluation. The right mandibles were used for scanning electron microscopy and mineral composition by inductively coupled plasma emission atomic spectrometry (ICP-AES).

2.4. Cafeteria diets

The cafeteria diet (CAF) adopted, providing modified versions of Harris high fat cafeteria diet [11]. It consisted of 3 variants: (i) condensed milk + bread + peanuts + pellet chow (4:1:4:1), (ii) chocolate + biscuits + dried

coconut + pellet chow (3:2:4:1), and (iii) cheese + boiled potatoes + beef tallow + pellet chow (4:2:4:1). The different variants were presented on alternate days throughout the treatment period [12].

Figure1. Macroscopic measurement of mandibles of rats

2.5. Mineral analysis of bone
The right mandible was chosen for mineral composition analysis. The mandible was weighted to determine the wet weight (WW), dried to eliminate water and weighted to determinate the percentage of water content (% water composition = [(WW-DW)/WW] ×100). Mandible was placed in a 70°C incubator (Schutzart DIN 40050 IP-20, Germany) for 48 hours. After recording the dry weights, the mandible was digested by placing them in solution of 5 mL concentrated nitric acid (NHO_3), 3 mL 70% perchloric acid ($HClO_4$) and 2 mL 35% hydrogen peroxide and microwaving at 180 PSI and 180°C. Mineral concentrations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, JobinYvonUltima2) [14].

2.6. Histological evaluation
Histological evaluation was performed using a magnifying glass. After fixation, the left mandibles were decalcified in 5% nitric acid for 27 days. All the samples were dehydrated by graded series of alcohols (Schrlau, Scharlab S.L, Spain) from 50-100%. The specimens were cleared and impregnate in Toluene (Prolabo, AnaloR Norma pur, VWR International SAS, French). Following dehydration, the mandible and salivary glands were embedded in paraffin and 5 μm thick slices were taken from the length, and the stained with Hematoxylin-eosin and Manson's Trichrom [15]. The slides were observed by light microscope (Leica Microsystems DM2500, Germany).

2.7. Scanning electronic microcopy
Bone structure was evaluated by scanning electronic microscopy (SEM). After fixation, undercalcified bone samples were dehydrated through a graded series (70%, 90%, 95%, 100%) of alcohol [16], then evaluated in environment SEM operating (Philips XL Series, Fei Quanta 200) at 30 kV.

2.8. Statistical analysis
Data was presented as the mean ± standard error mean (SEM) for six groups (n = 6). Multiple comparisons were analyzed between groups using one way analysis of variance and Bonferroni's post-hoc test. Significance was established at $p<0.05$ comparing all treatment groups to control. Data analyses were performed using Graph Pad Prism, version 6.
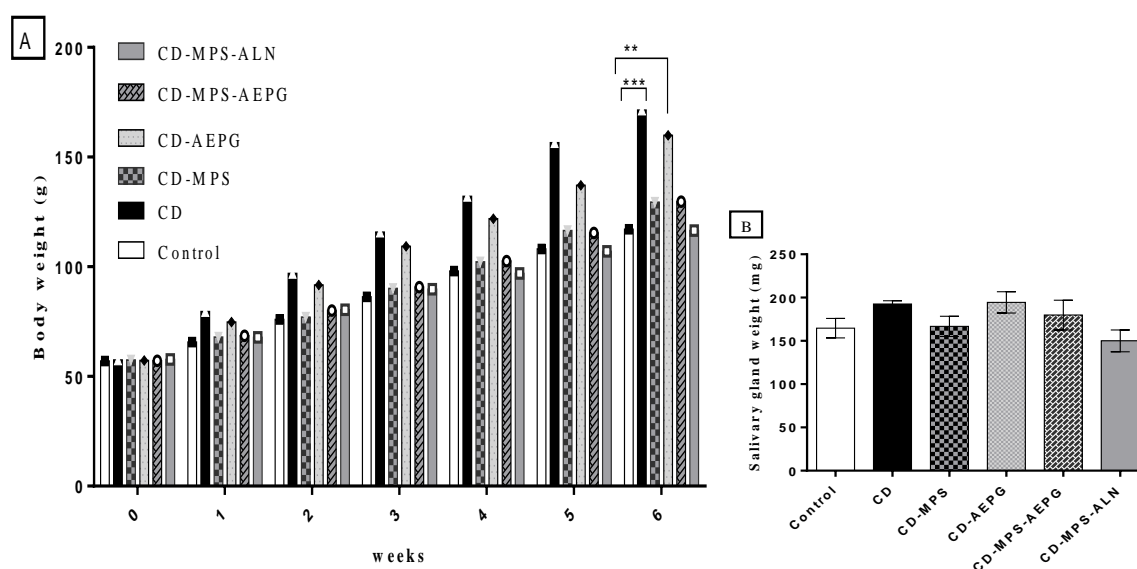
3. Results and discussion
The aim of our study is to demonstrate that the AEPG has a very important role in preventing the consequent bone abnormality of methylprednisolone administration in rats previously feeding the cafeteria diet. In addition, we compared the results with the action of alendronate which is a bisphosphonate whose action is known and documented concerning its role in the prevention and treatment of osteoporosis.

We considered the following parameters:

3.1. Macroscopical parameters

Figure 1 shows the methodology used to measure the macroscopic parameters of mandible. At the beginning of the study, the body weight shows no significant difference in all groups, as shown in figure 2. So, the CD and MPS effects on body weight revealed no significant difference in the first five weeks. As shown in figure 2A, body weight gain was significantly increased in CD and CD-AEPG groups compared to the control group ($p < 0.001$, $p < 0.01$ respectively) after week 6.

MSG weight no significantly increased in both CD and CD-AEPG groups compared to the other groups (figure 2).Thus, combination of MPS and ALN showed insignificantly reductions in the weight of MSG compared to the control group. Mandible weight shows no significant difference in all animal's groups. As shown in Table 1, CD or the combination of AEPG and CD for 6-week shows no significantly increase of the length and width jaw growth. The water content mandible was no significantly decreased in MPS treatment. We noted that the incisor length was no significantly increased in the MPS-AEPG or MPS-ALN groups.

Figure 2. (A) Changes in body weight during the treatment period; (B) body of parotid salivary gland. **p<0.01, ***p<0.001, compared with control group.

The relationship between glucocorticoid and osteoporosis was demonstrated by several authors. In the present study, we have showed that MPS intraperitoneally administration reduced body weight in rats, but no significantly. Similar studies have demonstrated a negative association between glucocorticoid and body weight or growth [17]. The mandible weight was decreased in ALN group compared to the control group, but no significantly. Some studies reported this result [18]. According to other study, dexamethasone administration induced significant loss of body weight in rat [19]. Our results showed that six weeks treatment of MPS decreased no statistically significant weight of maxillary salivary glands. Our findings reveled that MPS had no significantly difference on either mandible length or width of rat. The knowledge about glucocorticoid effect on mandible was demonstrated [17].

When adjusted for ALN, the weight, length and width of mandible were decreased compared to MPS groups, but we observed no significant differences between the groups. This difference can be explained that ALN plays an important role in the jaw osteonecrosis if used for long time. It's admitted that bisphosphonate effect is associated with the development of osteonecrosis in the jaws of rodents after tooth extraction [18, 20, 21]. Generally, we don't find significant changes concerning the rat body mass, mandible and MSG weight, as well as the length and width of mandible.

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Table 1. Descriptive macroscopical parameters of mandibles of rats

Groupes	Jaw weight (mg)	Jaw length (mm)	Jaw width (mm)	Incisor length (mm)	Water content jaw (%)
Control	275.800±15.560	20.670±0.210	9.500±0.316	9,333±0.166	0,373±0.012
CD	297.500±11.870	21.250±0.250	10.000±0.000	9.500±0.182	0,385±0.011
CD-MPS	258.500±10.720	20.670±0.333	9.750 ±0.250	8.833±0.307	0,353±0.010
CD-AEPG	270.000±12.040	21.000±0.447	9.917±0.153	9.330±0.477	0,361±0.006
CD-MPS-AEPG	269.800±9.116	20.920±0.327	9.830±0.210	10.000±0.258	0,410±0.022
CD-MPS-ALN	250.000±9.903	20.500±0.408	9.660±0.166	10.000±0.223	0,409±0.018

Data are expressed as mean ± SEM. Control group, CD: cafeteria diet, CD-MPS: cafeteria diet + methylprednisolone, CD-AEPG: cafeteria + *Pennisetum glaucum*, CD-MPS-AEPG: cafeteria diet + methylprednisolone + *Pennisetum glaucum*, CD-MPS-ALN: cafeteria diet + methylprednisolone + alendronate.

3.2. Mineral analysis

Mandible mineral content is reported in table 2. No difference in the concentration of calcium, magnesium and iron content were seen between control group and experimental groups. The AEPG administration shows no significantly decrease calcium and manganese content than the others groups.

As shown in **table 2**, we revealed that zinc content of CD-MPS-AEPG group was significantly increased ($p<0.05$) compared to the CD-MPS group. This result showed that MPS diminished mineral content of mandible. Both of the AEPG and ALN treatment were significantly attenuated MPN effect of manganese content ($p<0.01$, $p<0.01$ respectively). Our results showed that CD has significant increased manganese content in mandible than in the control group, but no significant increased calcium, phosphorus and iron content compared to the control group respectively. Similar findings for bone mineral content were reported that high fat diet may have long-term consequences for skeletal health and skeletal pathologies such as osteopenia [22]. In our case, we have seen an increase of manganese for groups treated by AEPG and ALN significantly.

Table 2. Analysis mineral concentration of right mandible in rats measured by inductively coupled plasma atomic emission spectrometry.

Parameters	Control	CD	CD-MPS	CD-AEPG	CD-MPS-AEPG	CD-MPS-ALN
Ca (%)	23.920±2.064	25.77±2.443	26.48±0.441	22.820±2.383	26.470±0.967	26.670±0.703
Mg (%)	0.481±0.038	0.479±0.028	0.476±0.006	0.416±0.043	0.441±0.016	0.447±0.011
P (%)	12.180±1.145	18.16±2.644	15.700±3.30	14.240±2.517	19.640±0.612[a]	19.720±0.427[a]
Fe (%)	0.023±0.002	0.043±0.012	0.036±0.005	0.033±0.003	0.038±0.004	0.038±0.005
Zn (%)	0.055±0.006	0.060±0.007	0.036±0.002	0.043±0.002	0.043±0.001[c]	0.048±0.000
Mn (≤mg/L)	2.630±0.221	4.453±0.227[a]	4.007±0.053[a]	3.682±0.138	4.300±0.065[ad]	4.313±0.109[a]

Data are expressed as mean ± SEM. Control group, CD: cafeteria diet, CD-MPS: cafeteria diet + methylprednisolone, CD-AEPG: cafeteria + Pennisetum glaucum, CD-MPS-AEPG: cafeteria diet + methylprednisolone + Pennisetum glaucum, CD-MPS-ALN: cafeteria diet + methylprednisolone + alendronate. Ca: calcium; Mg: magnesium; Fe: iron P: phosphorus; Zn: zinc; Mn: manganese

[a] $p<0.05$ vs control group, [b] $p<0.05$ vs CD group, [c] $p<0.05$ vs CD-MPS group, [d] $p<0.05$ vs CD-AEPG group, [e] $p<0.05$ vs CD-MPS-AEPG group.

[a] $p<0.05$, [a] $p<0.05$ vs CD-MPS-AEPG, CD-MPS-ALN (P) respectively. [c] $p<0.05$ vs CD-MPS (Zn).

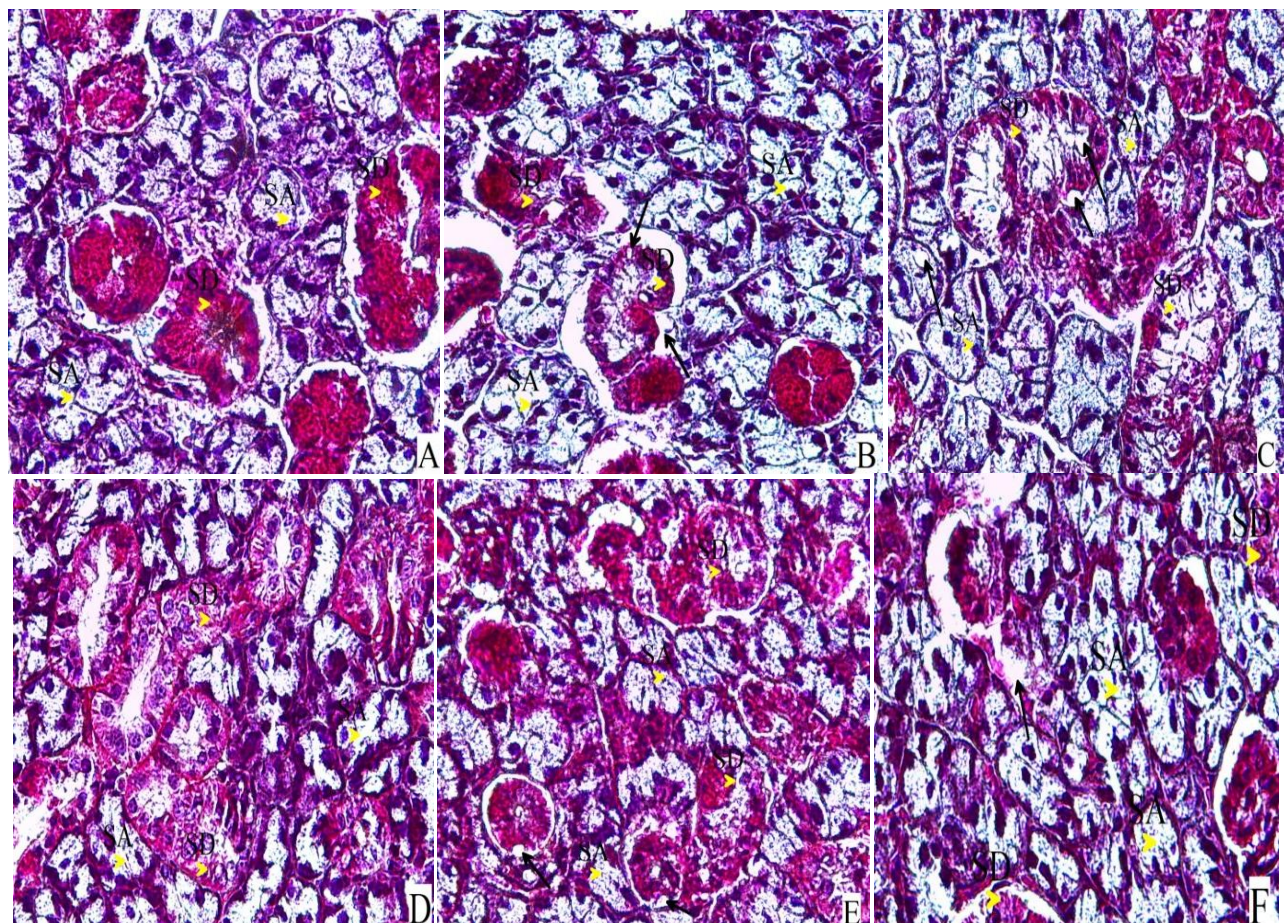[a] $p<0.001$, [a] $p<0.05$, [a] $p<0.01$, [a] $p<0.01$ vs CD, CD-MPS, CD-MPS-AEPG, CD-MPS-ALN (Mn) respectively, [d] $p<0.05$ vs CD-MPS-AEPG (Mn).

Our investigations showed that MPS treatment was significantly decreased phosphorus and zinc content compared to be combination treatment groups. Many studies have confirmed that glucocorticoid administration induced bone loss [23]. We indicate that CD did not affect magnesium content. It was demonstrated that GCs directly and indirectly influence bone mineralization at multiple levels [24]. In this study, we revealed that combination treatment of CD and AEPG don't bring real effect for calcium, magnesium, iron and phosphorus content compared to the control group.

3.3. Histologic evaluation

Histological analysis of maxillary salivary glands was presented in figure 3. The group receiving CD showed higher fat vacuole than those in the corresponding control group. The combination of CD and MPS increased the expression of fat vacuole and decreased acinar cell volume in comparison to alendronate and AEPG treatment groups, where we see real decrease of fat vacuoles. Our data showed that administration of MPS caused a decrease in the masses of maxillary salivary glands. It is noted that MPS showed the reductions of the acinar cells volume. Combination CD treatment with AEPG marked higher amelioration, which decreased fat vacuole in the acinar cells. ALN treatment was revealed some cellular alteration as striated duct. It is known that that bisphosphonates induce alterations and disturb functional salivary glands [25]. Furthermore, circumferences of acinar cells were normal and regular in the experimental groups (CD-AEPG, CD-MPS-AEPG respectively). In the two groups where we used AEPG, we noted better amelioration than alendronate.
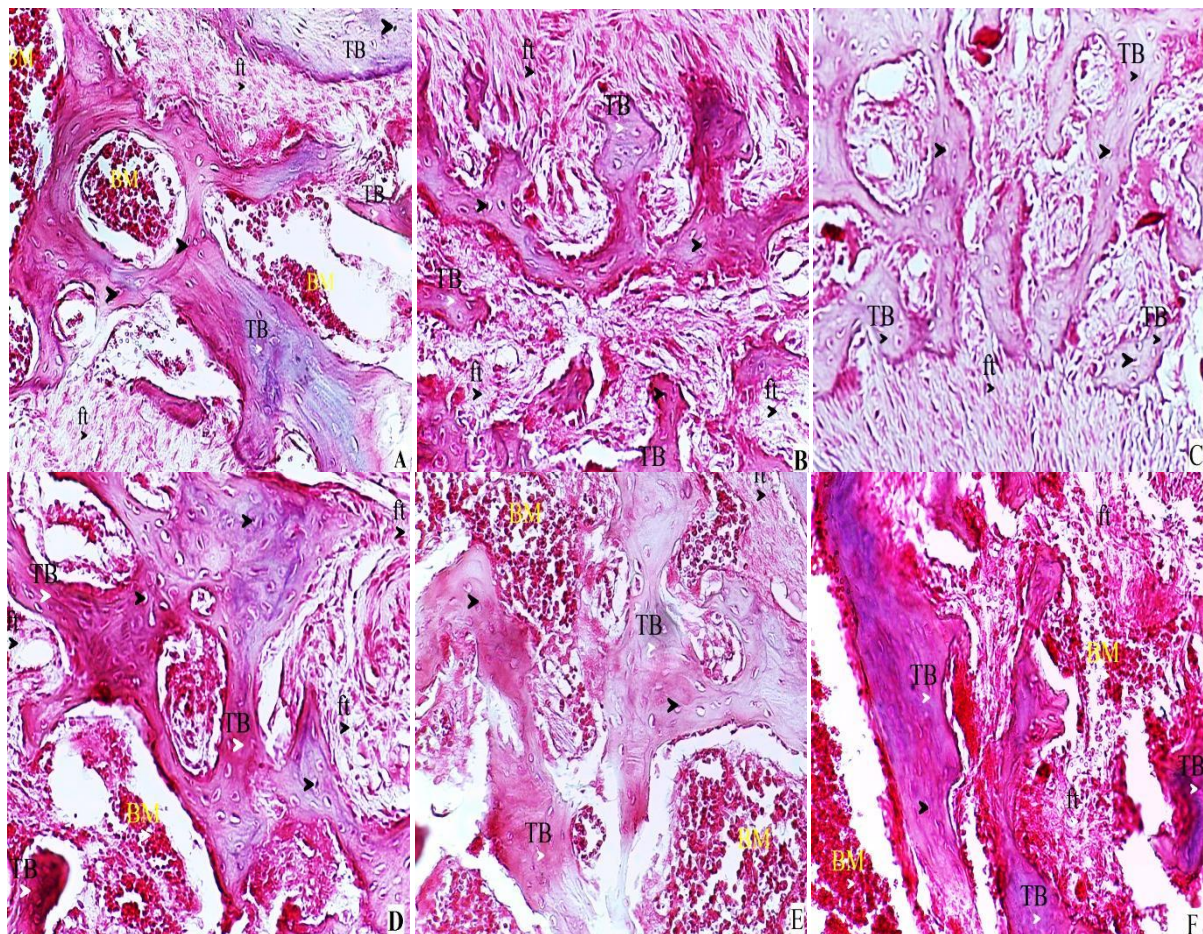

Figure 3. Masson's trichrom staining (200× magnification) of maxillary salivary gland: (A): control group; (B): CD group; (C): CD-MPS group; (D): CD-AEPG group; (E): CD-MPS-AEPG group; (F): CD-MPS-ALN group. SA, serous alveolus; SD, striated duct. Black arrows denote the fat vacuole.

In this investigation, our research focalized on trabecular bone of mandible. HE staining showed the bone quality. In both CD and MPS groups, bone size was decreased.

Hematoxylin-eosin stains (200×, magnification) examination shows regular trabecular bone in control group. In the present study, we evaluated antiosteoporosis activity of AEPG against MPS. We noted higher trabecular bone separation and loss of jagged trabecular in CD and CD-MPS groups. Combination treatment of CD-AEPG or CD-MPS-AEPG showed an increase of the trabecular size with intact structure. Several studies showed that glucocorticoid therapy decreases bone formation rate, decreased wall thickness of trabeculae and in situ death of portions of bone as causal factor for the bone pathology and structure alteration [23, 24]. Bone marrow was moderately reduced in CD group and replaced by fat vacuole. Our data showed many irregularly osteocytes and osteoid cells, which osteocytes lacunae was observed in MPS group compared to the control group. Combination treatment of alendronate and MPS increased trabecular bone, but higher degree of irregular structural was noted with increased osteocytes cells.
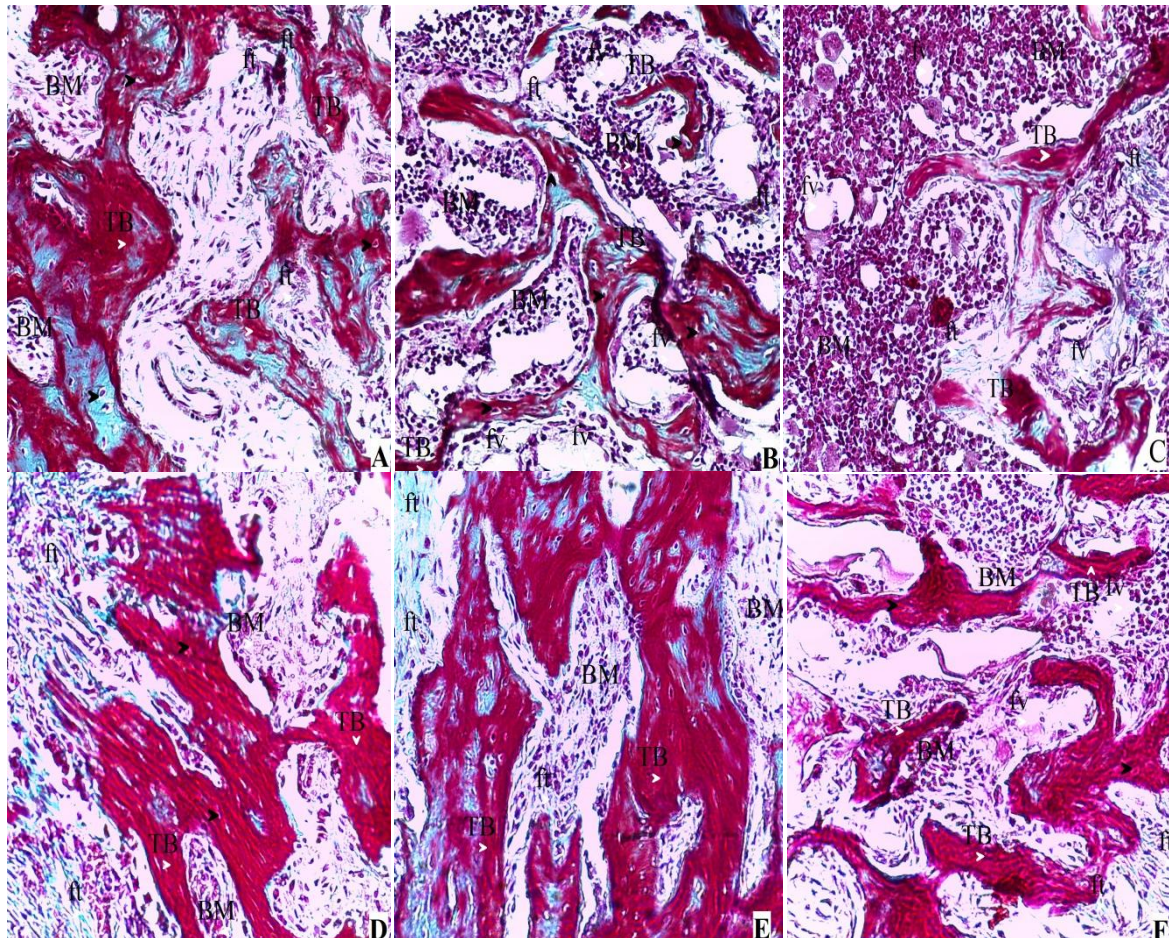

Figure 4. Hematoxylin and eosin staining (200× magnification) of mandibular bone: (A) control group; (B): CD group; (C): CD-MPS group; (D): CD-AEPG group; (E): CD-MPS-ASPG group; (F): CD-MPS-ALN group. BM: bone marrow; ft: fibrous tissues; fv: fat vacuole; TB: trabecular bone.

Analysis of the sections of the left mandible of control group revealed normal trabecular bone structure and bone marrow in inter-trabecular spaces (figure 4). We also noted that the animals from CD and CD-MPS groups showed a decrease trabecular size of mandible. In the CD and CD-MPS groups, the numerous fat vacuoles observed were elevated in bone morrow.

Masson's Trichrom reveals some parameters as fibrous tissue. It shows highly specialized cells, mineralized and unmineralized connective tissue matrix, and spaces that include the bone marrow cavity, vascular canals, canaliculi, and lacunae. Osteocytes have emerged as key regulators of skeletal and mineral homeostasis. Thus, the number of these cells could be influenced by the presence of osteoporosis and osteopenia [26]. Besides the

density of fibrous tissue, we have revealed the abundant content of fibrous in both AEPG-treated animals compared to control group (figure 5). However, in the CD-MPS group, the few new bone and fibrous tissue were reduced and arranged dispersedly. The treatment of osteoporosis provides not only usage of antiresorptive drugs such as bisphosphonates but also drugs characterized by a stimulating action on osteoblastic component and therefore on neoplasm, like teriparatide [27]. MPS reduced fibrous tissue that is replaced by necrotic bone. The action mechanism of AEPG is still unknown, but it seems to mitigate osteoclasts cells. Several studies have reported that BPs could be impairing molecular signaling not only of osteoblasts and osteoclasts [28], but also of fibroblasts and keratinocytes [29].
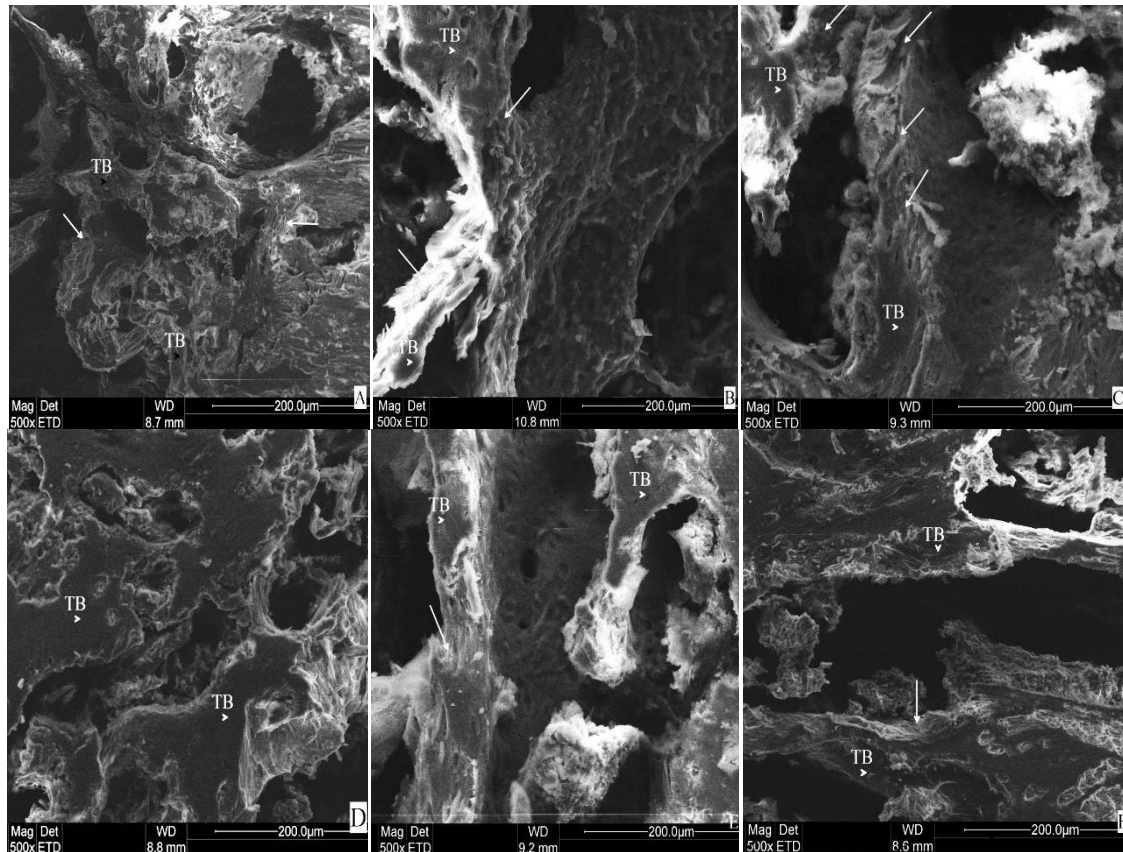

Figure 5. Effect of AEPG on mandible histological examination. Masson's trichrom staining (200× magnification) of submandibular gland: (A) control group; (B) CD group; (C) CD-MPS group; (D) CD-AEPG group; (E) CD-MPS-AEPG group; CD-MPS-ALN group. BM: bone marrow; ft: fibrous tissues; fv: fat vacuole; TB: trabecular bone.

3.4. Scanning electronic microscopy

On SEM images obtained from mandible revealed the severe bone destruction in CD and MPS groups (Figure 6). Our observations demonstrated that CD or CD-MPS provoked a remarkable resorption areas and a decrease in the thickness of the trabecular bone of the mandible. Our data indicate that methylprednisolone aggravates alter and loss bone in the cancellous bone in CD group. MPS administration revealed important bone destruction in CD-MPS group. It known that glucocorticoid decreases the bone production and increases the bone destruction [30]. Remarkably, trabecular bone appeared intact for groups treated with AEPG compared to the others groups, except the ALN group where we noted real amelioration but less than AEPG group.

Figure 6. Scanning electronic microscopy images (500× magnifications) revealed mandibular bone defect: (A) control group; (B) CD group; (C) CD-MPS group; (D) CD-AEPG group; (E) CD-MPS-AEPG group and CD-MPS-ALN group. White arrows denote the remodeling areas defect.

Conclusion

1. The administration combined of corticoid and high-fat diet in rat altered mineral composition, salivary gland structure and trabecular bone
2. In our study, we demonstrated that AEPG treatment obtains better amelioration than ALN without significant decrease calcium content. We noted also an increase of manganese content.
3. Remarkably, trabecular bone appeared intact for groups treated with AEPG compared to the others groups, except the group of ALN where we noted real amelioration but less than AEPG group.
4. The action mechanism of AEPG is still unknown, but it seems to mitigate osteoclasts cells.
5. It seems that AEPG has real advantage effect, so it can be used in the prevention of osteopenia induced by methylprednisolone treatment in rats.

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