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ANTIOSTEOPOROTIC OUTCOME OF FOSAMAX AND KLIMADYNON IN OSTEOPOROTIC RATS DURING TOOTH ERUPTION

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ABSTRACT

Objective: The study aimed to assess histologically and histomorphometrically the antiosteoporotic outcome of Fosamax and Klimadynon (black cohosh) in osteoporotic albino rats during tooth eruption. **Methods:** 12 female albino rats were divided into 4 equal groups: Negative Control group, Osteoporotic group: animals were injected by methyl-prednisolone subcutaneously for two months. Fosamax group: osteoporotic rats were treated simultaneously with oral Fosamax. Klimadynon group: osteoporotic rats were treated simultaneously with oral klimadynon. Pregnancy and delivery occurred during experiment, 40 offspring rats were divided into 4 equal groups, labelled from I to IV, each group was related to its corresponding mother' s group. Five rats from each offspring group were sacrificed at 7 and 14 days after their birth, the mandibles dissected and prepared for histopathological evaluation. **Results:** significant wearing away of the bone and diminution in the thickness of bone trabeculae were spotted in Group II in analogy to the control one. Group III showed remarkable improvement of the bone thickness over the erupting molars. While in group IV, the alveolar bone trbeculae were thin in structure. The highest mean values of bone thickness at 7 & 14 days were evident in the Fosamax group. **Conclusion:** The remarkable bone loss induced by Glucocorticoids could be compensated by Fosamax. Since black cohosh is a natural product, it is it is proposed to be used as a to be used as a favourable osteoprotective medication, however, it could cause delay in eruption but in a lesser degree than Fosamax.

KEY WORDS: black cohosh, Fosamax, tooth eruption, osteoporosis

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INTRODUCTION

Osteoporosis is a serious bone disease at which bone density is reduced, bone construct is deteriorated, and alteration of the total non-collagenous proteins. It is a result of years of bone loss, due to imbalance between bone formation and resorption. On the global scale, it is reported that many millions of fractures are annually results from osteoporosis, this means that every 3 seconds there is an osteoporotic fracture. [1]

Eruption of teeth is a developing event by which teeth move from where they begin to develop to where they function in the oral cavity. Various systemic disorders can influence the eruption development such as renal abnormality, bone ailments, tumours, and osteoporosis (OP) which described as decreased bone strength which prompts to a higher risk of fracture. [2, 3]

In aggregate, glucocorticoids are used frequently and extensively. Glucocorticoids raise the risk of fracture even when daily doses are low, and risk sustained to increase as exposure levels increased. Although fracture risk is mostly age related, use of glucocorticoids increases fracture risk in both younger patients and the eldery. [4]

Bisphosphonates drugs are the most widely used treatment for osteoporosis and Fosamax is one of them. [5] Alendronate is the active ingredient of Fosamax and is considered to be the global measure care for GIOP. [6] That being said, the prolonged use of the forementioned drugs could provoke necrosis of the jaw bones specially during the intake of GCs. [7]

Many mechanisms contributing to the bone loss are related to estrogen deficiency, so the appropriate estrogen supplement is considered the primary treatment. [8] Due to the long-term harm associated with hormone replacement therapy, and the growing interest in alternative treatment, one such a safe alternative is black cohosh. [9]

Klimadynon (black cohosh) is a dry extract of the rhizome of Cimicifuga Racemosa (CR BNO 1055) in the form of coated tablets. It was reported in a recent comparative study that black cohosh has equally effective therapeutic effects as conjugated estrogens. Moreover, recent studies have indicated the favourable effects of black cohosh on bone mineral density as well as bone metabolism. [10-12] Thus, the objective of the current experimental in vivo study was to assess histologically and histomorphometrically the antiosteoporotic outcome of Fosamax and klimadynon in osteoporotic albino rats during tooth eruption.

MATERIALS AND METHODS

Experimental Animals

The current blinded prospective comparative study conducted on 12 albino virgin female rats free of systemic diseases aged 16-19 weeks and weighing 180-220 grams. The rats were maintained under controlled temperature and light and allowed access to food and water in a well-ventilated animal shelter within the Faculty of Medicine, Assuit University, Egypt.

The rats were divided into 4 equal groups:

Negative control: 3 normal rats were kept under the same conditions as the other experimental rats.

Osteoporotic group: 3 rats were injected subcutaneously by 0.5 mg/kg methyl-prednisolone three times per week for two months to trigger osteoporosis and kept without treatment. [13]

Fosamax group: 3 rats were injected subcutaneously by 0.5 mg/kg methyl-prednisolone three times per week for two months to trigger osteoporosis, and treated simultaneously with oral Fosamax in a dose of 0.84 mg/kg five times a week by oro-gastric-tube. [14]

Klimadynon(black cohosh) group: 3 rats were injected subcutaneously by 0.5 mg/kg methyl-

prednisolone three times per week for two months to trigger osteoporosis and treated simultaneously with oral klimadynon in a dose of 15 mg/kg/ five times a week by oro-gastric-tube. [15]

Every rat was weighted every week and the dose adjusted according to its weight. The doses calculated according to the following formula. [16]

$$\text{Animal dose} = \text{Human dose} \times 18 \times \frac{\text{animal weight in gram}}{1000 \times 200}$$

Pregnancy occurs during the experiment; the virgin female rats were timed mated with male rats. Females were housed together with males in a single cage until expultion of vaginal plug was noted; female rats were examined through vaginal smears at the next day. Mating was confirmed by The presence of sperm in these smears, and the date of mating was detrmined as gestational day (GD) 0. Pregnant females were then kept singly throughout gestation period until delivery, that occurred on days 20–22, then their offspring were used in the study.

Sample size was modeled by version.3.1.9.2, of G*Power programme Faul et al,. [17, 18]

$$f = \frac{\sigma_\mu}{\sigma}$$

$$\sigma_\mu^2 = \frac{\sum_{i=1}^{k} n_j (\mu_i - \mu)^2}{N}$$

Where, f is the effect size which was 0.70; α= 0.05; β= 0.05; Power= 1- β = 0.95 and the estimated sample size (n) should be 40 samples which was divided equally 10 samples each.

Forty offspring of rats were evenly distributed into 4 groups; each group consisted of 10 offspring belong to their corresponding mother' s group:

G I: 10 offspring from control mothers' group.

GII: 10 offspring from Osteoporotic mothers' group.

GIII: 10 offspring from Fosamax mothers' group.

GIV: 10 offspring from Klimadynon (black cohosh) mothers' group.

Histological evaluation:

After the end of the experiment, 5 offspring rats from each group were sacrificed at 7 and 14 days after their birth. The mandible was dissected from each rat and kept in 10% neutral formalin, decalcified in EDTA. The specimens were then passed in alcohol through various concentrations ordered from70% to 100% then rinsed with xylene. Samples were then placed in paraffin wax for 24 hours and immersed in paraffin blocks. Using a microtom, All tissues were cut mesiodistally in a serial manner at a thickness of 5 mm. The specimens were incubated overnight at 60C after placing them on glass slides, then immersed it in xylene, then ethanol to rinse them off, and finally water. The last stage performed was a Hematoxylin & Eosin stainning for general histological examination. [19]

Analysis of bone histomorphometry:

The thickness of the formed alveolar bone in all groups was determined by histomorphometric analysis. Measurements of bone thickness were evaluated in photomicrographs at x200 magnification, using Image J analysis 1.48v software program. Measurement of bone thickness in micrometres (μm) by the distance between the reduced enamel epithelium and the outer surface of the bone crypt. For each section, the thickness was measured 5 times, and the mean was calculated and recorded for each specimen. [20]

Statistical Analysis

Data were computed and analysed through the tests mentioned later. Normal distribution of the samples were checked by a normality test (Shapiro-Wilk). Mean ± and Standard deviation (SD) were calculated as a descriptive statistics. Time interval comparison made by Paired sample t-test. Four groups compared each time by one-way ANOVA. Pairwise comparisons were made by Bonferroni post hoc tests. All analysis were done using SPSS software, version 26.0, Armonk, NY: IBM Corp. P-value was significant at levels ≤ 0.05.

RESULTS

i. The histologic results

Group I (control): at 7 days, the alveolar bone showed a normal architecture, bordered by osteoblasts and osteocytes embedded each within its own lacuna. Some empty osteocytes lacunae were noted. The interconnected trabeculae enclosing medullary spaces contained loose connective tissue. (**FIGURE** 1) While the eruption pathway above the erupting molar was established at 14 days (**FIGURE** 5).

Group II: irregular alveolar bone trabeculae with sporadic osteoblasts were evident. Thin bone trabeculae were seen surrounding extensive marrow spaces. Scattered fibroblasts with randomly oriented collagen fibres and few inflammatory cells were observed. The neighbouring connective tissue displayed inflammatory cells as well as the diffuse spread of spindle shaped fibroblasts with varying in size. (**FIGURES** 2& 6)

Group III: ingrowth of dense remodelled bone trabeculae above the erupting molar was observed in addition to a more evident rate of bone development. Active osteoblasts bordered the bone trabeculae with linear arrangement along the bone edges in some areas of newly formed bone. Randomly distributed osteocytes were clearly visible enclosed singly inside their lacunae surrounded by intercellular calcified matrix. Also, sparse narrow bone marrow spaces were clearly identified that contained loose

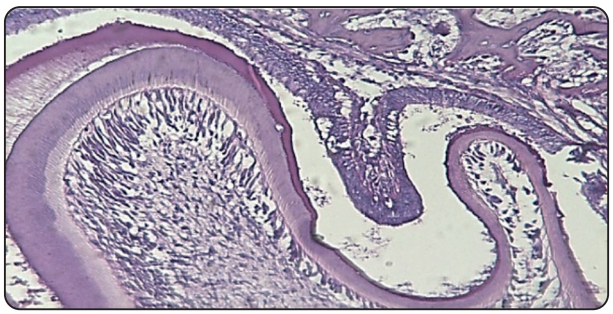

Fig. (1) Photomicrograph of rat lower molar at 7 days, GI control group (coronal part) showing advanced bell stage with relatively normal bone thickness, normal trabeculation and arrangement in lamellar pattern. (H&E 200X)

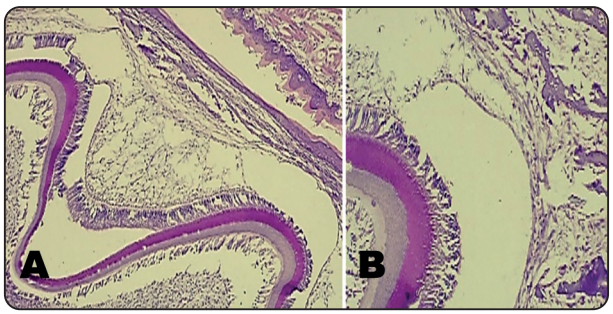

Fig. (2 a & b) Photomicrograph of rat lower molar at 7days, GII osteoporotic group showing reduced bone thickness than normal, however the present bone characterized by abnormal architecture over the cusp tip. (H&E a 200, b 400X)

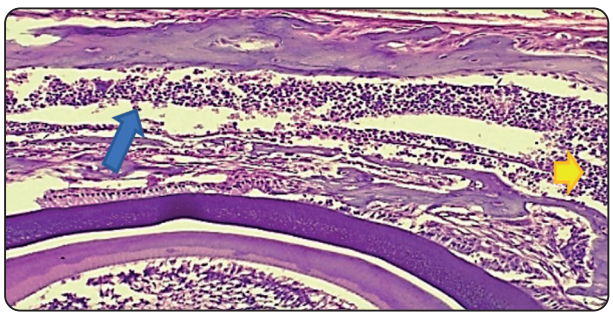

Fig. (3) Photomicrograph of rat lower molar at 7 days, G III Fosamax group (coronal part) showing advanced bell stage, presence of both enamel and dentin matrix with increase with areas of extravasated RBCs (blue arrow) and inflammatory cells in bone marrow spaces (yellow arrow). (H&E 200X)

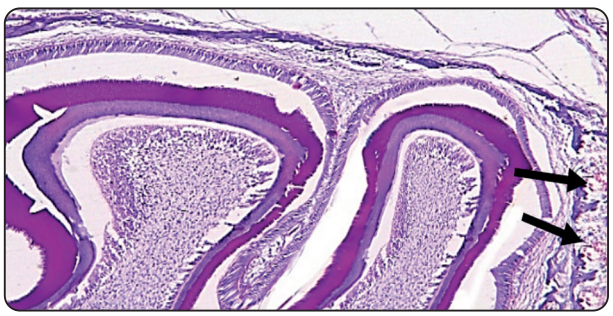

Fig. (4) Photomicrograph of rat lower molar at 7 days, GIV black cohosh group (coronal part) showing advanced bell stage, reduced bone thickness at coronal portion (remarkable thinning in bone trabeculae), areas of hemorrhage (extravasated RBCs) inside bone marrow cavities. (arrows). (H&E 200X)

connective tissue. Areas of extravasated RBCs and inflammatory cells in bone marrow spaces are observed. (**FIGURES** 3&7)

Group IV: the results revealed randomly oriented new bone that formed in thin trabecular architecture over the erupting molars. Areas of extravasated RBCs inside the wide marrow spaces were observed. With the trabecular bone thickness increased at 14 days as compared to 7 days. (**FIGURES** 8&4)

ii. The histomorphometric results

In table 1, the results are presented noticeable difference between the bone thickness in the four groups at 7 days and 14 days using one-way ANOVAs at $P<0.05$. Pair-wise comparisons between the groups showed a significant difference between each group to another except between GI with G IV after 14 days (**FIGURE 9**). After 7 days the high mean values were recorded in GIII (132.55±8.06) followed by GI (42.04 ±4.52) while GIV was the lowest one (20.96±2.08). On the other side after 14 days the high mean values were recorded in GIII (77.39±5.99) followed by GII (24.88 ±1.51) then G IV (14.41± 1.40) while GI was the lowest one (10.64±1.19).

As regards to changes within each group, there was a statistically significant decreased in bone thickness with increasing time. The changes were decreased significantly about 74.7%, 27.6%, 41.6% and 31.3% in GI, GII, GIII and GIV respectively (**TABLE 1**)

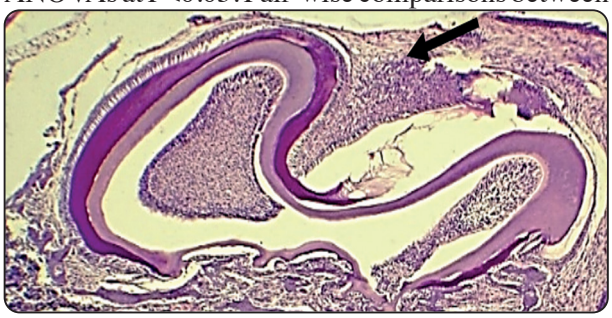

Fig. (5) Photomicrograph of rat lower molar at 14 days, GI control group showing resorption of the bony crypt to creat a path for eruption (arrow). (H&E 200X)

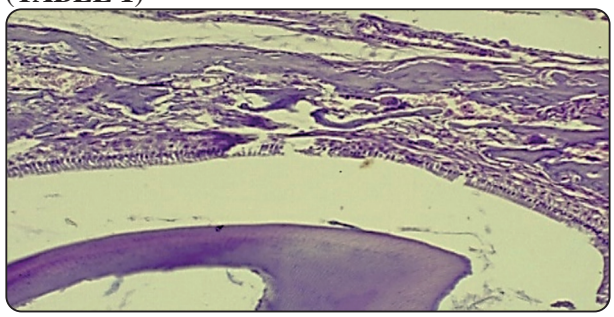

Fig. (6) Photomicrograph of rat lower molar at 14 days, GII osteoporotic group (coronal part) showing advanced bell stage thinning of bone thickness, loss of normal trabeculation and arrangement in lamellar pattern, the marrow spaces widened. (H&E 200X)

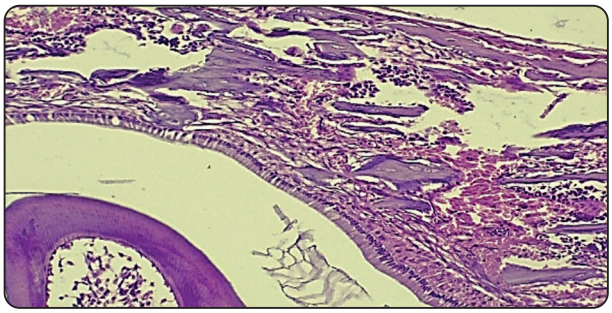

Fig. (7) Photomicrograph of rat lower molar at 14 days, GIII Fosamax group (coronal part) showing advanced bell stage with increased bone thickness and increased number of bone trabeculae with abnormal arrangement (random orientation) rich in randomly distributed osteocytes (highly cellular which resemble woven bone, no lamellar structure) areas of extravasated RBCs and inflammatory cells in bone marrow spaces. (H&E 200X)

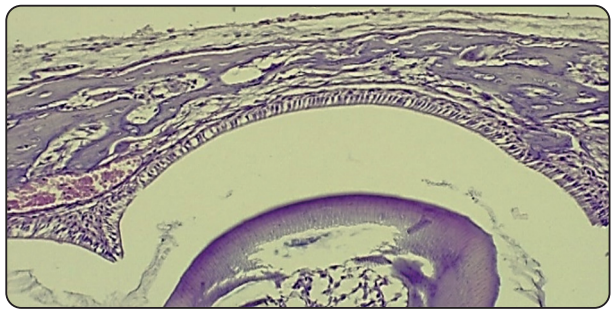

Fig. (8) Photomicrograph of rat lower molar at 14 days, GIV black cohosh group (coronal part) showing enamel space in advanced bell stage, wearing off reduced enamel epithelium, reduced bone thickness at coronal portion (remarkable thinning in bone trabeculae) and areas of hemorrhage inside bone marrow cavities. (H&E 200 X)

TABLE (1), The four groups comparison under study for bone thickness at 7 and 14 Days

	7 days		14 days		%	Paired t-test	P value
	Mean	SD	Mean	SD	change		
GI	**42.04** ^{b}	4.52	**10.64** ^{c}	1.19	-74.7 (d)	15.85	<0.001**
GII	**34.35** ^{c}	1.38	**24.88** ^{b}	1.51	-27.6 (d)	8.016	0.001**
GIII	**132.55** ^{a}	8.06	**77.39** ^{a}	5.99	-41.6 (d)	10.11	0.001**
GIV	**20.96** ^{d}	2.08	**14.41** ^{c}	1.40	-31.3 (d)	12.02	<0.001**
ANOVA Test (F)	563.56		461.90				
P value	<0.001**		<0.001**				
Multiple Comparisons using Bonferroni post-hoc							
GI vs GII	0.13 ns		<0.001**				
GI vs GIII	<0.001**		<0.001**				
GI vs GIV	<0.001**		0.50 ns				
GII vs GIII	<0.001**		<0.001**				
GII vs GIV	<0.001**		<0.001**				
GIII vs GIV	<0.001**		<0.001**				

; and different letters at the same column means significant difference at P<0.05 (d); means decreased

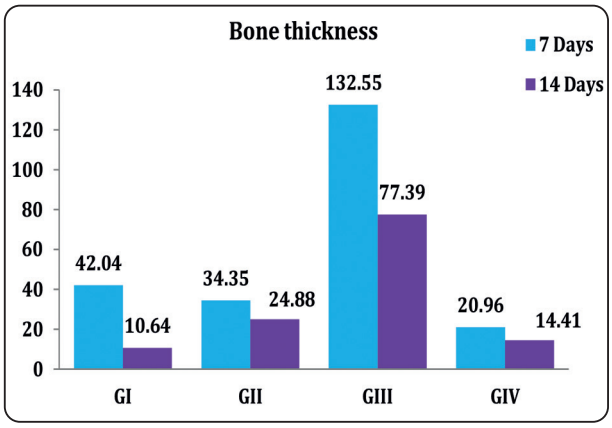

Fig. (9): comparison between the four groups under study for bone thickness at 7 and 14 days

DISCUSSION

Osteoporosis is one of the most common complications of Glucocorticoids therapy, and thus presents a major global health issue due to the increased tendency to bone fractures and morbidity. [21]

In the current study, osteoporosis was induced by dexamethasone hence it is the most common causative agents for osteoporosis, among other corticosteroids, in animal models. Furthermore, it has a long-acting effect so the daily injections could be avoided. [22]

Easy to handle, low- cost, short study time, relatively disease resistant, moreover it is a versatile model suitable for a wide range of research purposes. All these factors have contributed to making this animal model is most commonly used for glucocorticoids induced osteoporosis. [23, 24]

Eruption of teeth is one of the most important indicators for the development and growth of the children. The tooth crown pierces the bone and gingiva to emerge in the oral cavity, this scene is accompanied by osteoclastic bone resorption. So the tooth eruption is affected by antiresorptive agents when they are introduced during this period. [25]

Thus, the current study evaluates histologically and histomorphometrically the antiosteoporotic

outcome of Fosamax and klimadynon (black cohosh) in osteoporotic rats during tooth eruption.

The current histological findings showed a notable resorption of the bone trabeculae that becomes thin and surrounding dilated marrow spaces, this was the effect of glucocorticoids administration (Figure 2). Glucocorticoids augments bone resorption; by inhbiting Osteoprotegerin (OPG) expression, and improvement of activator of NF-kappa b ligand (RANKL) and macrophage colony stimulating factor (M-CSF) receptors expression. Furthermore, by suppressing the crucial Wnt/β-catenin pathway and its influence on the differentiation of osteoblasts. [21,26]

Shata et al showed similar results in their study, severe bone resorption of the femoral bone manifests as a noticeable thinning and irregular trabecular bone. Compared with the control group, the areas of bone marrow became more wide and increased in the fatty areas. [27]

In this study, it was noted that Fosamax has proven capability to enhance trabecular bone volume, density, increase number of randomly oriented alveolar bone trabeculae with reduced trabecular separation over the erupting molars (Figures 3,7). The histomorphometric results were inconsistent with the histological results that revealed highest mean values of bone thickness in the Fosamax group at 7 & 14 days as compared to the other groups. (Table 1 & figure 9) These were in coincident with the results of Fujita et al that showed the improvement effect of Risedronate on retarded trabecular and cortical bone growth due to prednisolone intake. [28]

Another study came into this context stated that the Bisphosphonates bind to the hydroxyapatite on the bone surfaces particularly at the active remodelling sites suppressing bone resorption and blocking osteoclast activity through reducing their recruitment and activity, and increasing their apoptosis. On the other hand they prevent osteoblast apoptosis. [29]

Fosamax stimulates pro-inflammatory cytokines, TNF-α and IL-1ß response resulting in inflammation, therefore the involvement of the NF-ϰB signalling pathway is strongly suggesting. This was consistent with the findings of the others who revealed the gastric mucositis of alendronate-treated rats due to the up-regulation of NF-ϰB and the proinflammatory factor COX-2. [30]

In the current study, the black cohosh induced bone formation in relatively normal pattern of architecture over the erupting molars (figures 8). The current results could be explained due to the ability of the black cohosh to stimulate bone formation through activating the osteoprotegerin production. [31] Cyclooctane, a tripentoid glycoside extracted from black cohosh, suppresses NFϰB and ERK signalling pathways that inhibits RANKL-induced osteoclast differentiation. [32] At 7 days, the mean values of bone thickness in G IV were lower than that of group III which recorded the highest mean values (Table 1).

It has been reported that Remifemin (which has the same main active ingredient of black cohosh) that limited the release of pro-inflammatory cytokines and thus inhibited the development of osteoporosis. [33] Cui et al also reported that Remifemin has an obvious influence against bone loss, enhance the biomechanical aspects of bone and prevents bone loss in ovariectomized rats. [34] Remifemin induces the bone formation through its ability to produce osteoprotegerin and to improve both serum osteocalcin and bone alkaline phosphatase. [30] This comes within the highlights of Shawky, et al study, they proposed to use Remifemin as a favourable medication against osteoporosis. [35]

Further studies were conducted on the ovariectomized rat model which claimed that the Remifemin had a positive effect on the bone structure by inhibition of osteoclastic bone resorption by suppressing both the formation of osteoclast-like cells and their resorbing activity. [36,37] Additionally, it prevents the oxidative damage of the osteoblasts synchronized with raising the growth of cells,

collagen content, alkaline phosphatase action, and mineralization in the cells. Furthermore, it exerts estrogenic properties in the bone tissue (mainly in osteoblasts). [38] Therefore, all of the above led to an increase in bone minerla density in the ovariectomized mice model. [39-42]

On the other hand, different results were exported from a comparative study by Kolios et al which revealed that the osteoporotic metaphyseal fracture healing was enhanced by estrogen more than by Cimicifuga Racemosa. By given short-term osteoporosis prophylaxis CR, the metaphyseal cortical bone was not affected, and callus density was not enhanced. Also, CR could not prevent the typical destruction of the trabecular structure of the metaphyseal bone. [43, 44]

Although certain extracts of *Cimicifuga Racemosa* had already been described as safely effective treatment of some gynaecological disorders, this current study did not evaluate the biocompatibility or safety of black cohosh.

CONCLUSION

Within the highlights of the current study, glucocorticoids induced remarkable bone loss over erupting molars, which could be compensated by Fosamax that promoted alveolar bone formation, so could delay the tooth eruption. Since black cohosh is a natural product, it may be recommended as an appropriate medication for osteoporosis, however, it could cause delay in eruption but in a lesser degree than Fosamax. Still more studies are needed to fully explain the specific mechanisms of black cohosh that influence bone protection, as well as regarding the optimal dose and duration of treatment.

Ethical Statement:

Ethical Committee of Faculty of medicine, Assuit University has considered and approved the presented research as conducted in Ethical principles ((IRB NO:17300880, date of approval: 25 October 2022).

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