Vol 9, Special Issue 2, 2017

INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

ISSN - 0975-7058

Research Article

IMPACT OF ZOLEDRONATE BISPHOSPHONATE GEL IN VIRGIN COCONUT OIL ON THE INCREASE OF OSTEOCLAST APOPTOSIS

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Received: 16 September 2017, Revised and Accepted: 3 October 2017

ABSTRACT

Objective: Drug has the potential to support the anchorage in orthodontic treatment through the inhibition of tooth movement resulting from bone resorption due to the apoptosis of osteoclasts. A subperiosteal injection of zoledronate (ZOL) can increase the apoptosis of osteoclasts. Several researchers have reported that a subperiosteal injection of ZOL bisphosphonate (BP) (ZOL) can act against orthodontic tooth movement by increasing the apoptosis of osteoclast cells. However, subperiosteal injection is invasive and painful, so a gel emulsion of ZOL in virgin coconut oil (VCO) was used to substitute the subperiosteal injection.

Objective: This study aimed to show the impact of the ZOL in VCO gel (Ge-ZOL) on the extent of osteoclasts apoptosis.

Methods: The study used 27 Sprague-Dawley rats which were divided into three groups: Nine rats in the experimental group were given 40 µg of Ge-ZOL, nine rats in the control group were given VCO emulsion gel without ZOL (Ge-), and nine rats in the normal group were not given any treatment. The gel was applied to the buccal mucosa using a cotton bud for 2 min at hour of 0, 4, and 8 on days 0, 1, 2, 3, and 4. The rats were sacrificed on days 1, 3, and 5, and then, evaluated by immunohistochemical caspase-3 staining.

Result: The number of apoptotic osteoclast cells in the experimental group was significantly higher than in the control and normal groups ($p<0.05$). The number of apoptotic osteoclast cells in the experimental group on the day 1 was significantly higher than on the days 3 and 5 ($p<0.001$).

Conclusion: The application of Ge-ZOL to the buccal mucosa proven to improve the number of apoptotic osteoclast cells in the experimental group on the day 1, and this number was higher than on the days 3 and 5.

Keywords: Zoledronate bisphosphonate, Virgin coconut oil, *Caspase-3*, Osteoclast apoptosis.

 DOI: http://dx.doi.org/10.22159/ijap.2017.v9s2.07

INTRODUCTION

Orthodontic treatment aims to improve the function of mastication, speech, and facial appearance as well as to maintain stability after treatment [1]. Orthodontic treatment works on the principle of moving and then securing some teeth, which is called anchorage [2,3]. There are several methods of anchorage such as using the dentition as an anchorage unit, using additional extraoral anchorage such as headgear, using additional intraoral anchorage such as a lingual bar and a Nance holding arch, or using the mini-implant or mini-screw [4-10].

Besides the mechanical tools mentioned above, there are pharmaceutical methods that have good potential in supporting anchorage through the inhibition of bone resorption. Bisphosphonate (BP) and prostaglandin inhibitors are two types of drug that are known to inhibit orthodontic tooth movement [3]. BP is a drug commonly used to treat osteoclastic bone resorption diseases, such as osteoporosis, cancers that can metastasize to the bone (breast, prostate, lung, and kidney cancer), bone marrow cancer (multiple myeloma), and Paget's disease [11]. Zoledronate (ZOL) is the newest generation of BP, nitrogen-containing heterocyclic imidazole BP that has a greater ability to inhibit bone resorption compared to other BP [11-18]. BP can bind hydroxyapatite crystals in a mineralized bone matrix so that the bone is more resistant to the osteoclast function. BP also prevents bone marrow from differentiating precursor cells through the inhibition of the mevalonate pathway of cholesterol biosynthesis and by inducing osteoclast apoptosis. BP works specifically on the osteoclasts, causing the apoptosis of the osteoclasts, thus preventing the occurrence of osteoclastic bone resorption and inhibiting orthodontic tooth movement [18-20]. Researchers have reported several experimental animal studies that show that a subperiosteal injection of BP can inhibit orthodontic tooth movement through the anti-osteoclast effect of BP [18-22]. It is therefore anticipated that the administration of Ge-ZOL in virgin coconut oil (VCO) will have the same effect as the subperiosteal ZOL injection. Ge-ZOL in VCO is suggested expected as a way of improving the function of orthodontic anchorage by promoting osteoclast apoptosis [18,19].

The application of orthodontic force produces an inflammatory process and forms a chemical mediator that stimulates osteoclast formation in the pressure area. The osteoclasts will then resorb the alveolar bone so that the tooth can move. The mechanism of orthodontic tooth movement thus involves the process of resorption and bone apposition through osteoclast apoptosis [1-3]. Apoptosis is programed cell death that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells [21]. The caspases are a family of genes important for maintaining homeostasis through their regulation of cell death. Immunohistochemical caspase-3 contains an antibody that can detect and bind to antigen caspase-3 in osteoclasts undergoing apoptosis [23].

Subperiosteal injection of BP is invasive and painful. Thus, it is necessary to develop BP that can be applied topically. Ge- has several advantages over a regular gel as it is one of the hydrophobic and mucoadhesive drug delivery systems that can attach and penetrate easily through the oral mucosa without any pain [24-26]. The Ge- used in this study is VCO and carboxymethyl cellulose (CMC). VCO is pure coconut oil, derived from fresh coconut flesh, processed without

using any chemicals or heating so that the active components such as the vitamins and polyphenols are not damaged. More than 60% of the compounds within VCO consist of medium chain saturated fatty acids; these are lauric acid (53%) and capric acid (7%). These are easily absorbed by the body due to their molecular size, which is not too big [27]. This study aimed to determine the impact of Ge-ZOL in preventing osteoclastic bone resorption. This was achieved through the observation of the extent osteoclast apoptosis in the rat alveolar bone using immunohistochemical (IHK) caspase-3.

METHODS

Twenty-seven Sprague-Dawley rats were included in this study. The inclusion criteria were male, 3 months old, 180-200 g, derived from the same strain, and in good condition. The study was done under the supervision of a veterinarian of the Health Research and Development Division of the Indonesian Ministry of Health. The study was approved by Research Ethical Committee of the Faculty of Medicine, Universitas Indonesia (Approval Number: 248/UN2.F1/ETIK/2015).

Subjects were divided into three groups: Nine rats in the experimental group were given Ge-ZOL with VCO, nine rats in the control group were given Ge- with VCO, and nine rats in the normal group were not given any treatment. The normal group was used to show the validity of the control group. 25 mg gel was made fresh, consisting of 40 µg ZOL. The gel was applied to the buccal mucosa of the lower right first molar on the days 0, 1, 2, 3, and 4 at hours 0, 4, and 8 using a cotton bud with the circular movement for 2 min. Three rats from each group were sacrificed on the days 1, 3, and 5, respectively. After sacrificed, the specimens were cut in the sagittal direction so that the right side of the mandible was obtained. Preparation of the histological specimens and the IHK caspase-3 staining was done at the histology laboratory of the Faculty of Medicine, Universitas Indonesia. The specimens were fixated in neutral buffer formalin 10% at 4°C for 24 h, and then, demineralized using Rapidcal-Immuno until soft. Samples were put into alcohol in sequence, xylol alcohol, mixture of xylol and paraffin, and liquid paraffin. The specimens were then cut with microtomes in a transverse direction to a thickness of ±4 µm and then dyed with IHK caspase-3 [23].

The specimens were observed using the Olympus IX73 research inverted microscope with ×20 magnification, captured with an Olympus DP80 camera, and then the area of the medulla was measured using the Olympus cellSens V.1.11 program at the UI-Olympus Bioimaging Center. A calibration test was performed inter- and intra-observer on 20% of the samples by the histological experts of the Faculty of Medicine, Universitas Indonesia, and the researcher.

Intra- and inter-observer reliabilities were analyzed using the Bland-Altman test. All data were analyzed using the one-way ANOVA. The results were considered statistically significant when $p<0.05$.

RESULTS

The result of the interobserver reliability test with Bland-Altman showed a good reliability between the researcher and the histological expert. The result of the intraobserver reliability test with Bland-Altman showed a good reliability between the first and second examination results done by researcher with interval 1 week.

The results of the one-way ANOVA test showed that there were significant differences in the extent of osteoclasts apoptosis between the groups ($p<0.001$). Based on the *post hoc* Bonferroni test, there were significant differences between the experimental and the control groups on the day 1 and between the experimental and the normal groups on the day 1 ($p<0.05$) (Fig. 1).

Table 1 shows that the number of apoptotic osteoclasts cells in the experimental group on the 1st day was significantly different than on the 3rd and 5th days ($p=0.001$ and $p<0.001$).

The result from the histological examination results using the IHK caspase-3 from each group on days 1, 3, and 5 are presented in Figs. 2-4.

DISCUSSION

Several studies have proved that an injection of BP in the buccal mucosa, combined with orthodontic force, could inhibit the orthodontic tooth movement arising from the apoptosis of osteoclasts cells [18,19,21,24]. However, the injection may cause discomfort, pain, and a systemic effect. Thus, there is a need to develop a new type of BP in the form of a gel emulsion to overcome these effects [28]. BP consists of two families: Non-nitrogen-containing BP and nitrogen-containing BP. The newest generation of BP is ZOL, a nitrogen-containing heterocyclic imidazole BP, which has been shown to have potential as an inhibitor of bone resorption [11-18]. The gel form was chosen because its usage

Table 1: Analysis of the number of apoptotic osteoclast cells in the experimental group on the days 1, 3, and 5

Experimental groups (days)	p value
1–3	0.001
1–5	<0.001
3–5	0.251

Post hoc bonferroni test. *p<0.05

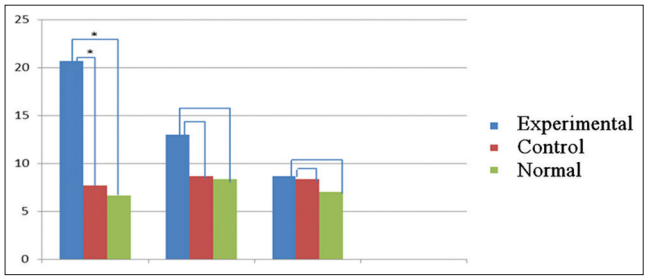

Fig. 1: Number of apoptotic osteoclast cells in experimental, control, and normal groups on the days 1, 3, and 5 with one-way ANOVA test. *p<0.05

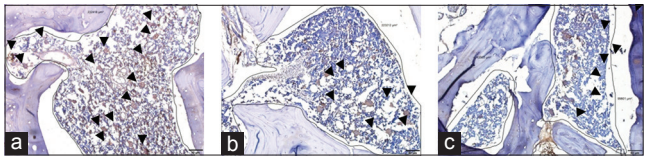

Fig. 2: Histological view of alveolar bone from experimental, control, and normal groups on the day 1, stained with IHK caspase-3, ×20 magnification, bar 50 µm. The arrows showed osteoclast apoptosis (brown multinucleated giant cells). (a) Experimental group on the day 1, (b) control group on the day 1, (c) normal group on the day 1. The number of apoptotic osteoclast cells in figure a is higher than that in b or c

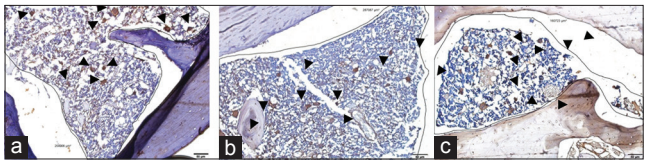

Fig. 3: Histological view of alveolar bone from experimental, control, and normal groups on the day 3, stained with IHK caspase-3, ×20 magnification, bar 50 µm. The arrow shows osteoclast apoptosis (brown multinucleated giant cells). (a) Experimental group on the day 3, (b) control group on the day 3, (c) normal group on the day 3. The number of apoptotic osteoclast cells in figure a is almost same as that in b and c

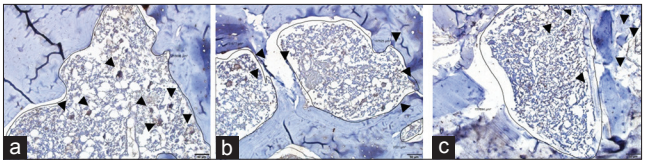

Fig. 4: Histological view of alveolar bone from experimental, control, and normal groups on day 5, stained with IHK caspase-3, ×20 magnification, bar 50 µm. The arrow shows osteoclast apoptosis (brown multinucleated giant cells). (a) Experimental group on the day 5, (b) control group on the day 5, (c) normal group on the day 5. The number of apoptotic osteoclast cells in figure a is almost same as that in b and c

in oral medication is well-known. Therefore, it was expected that the topical application of ZOL in a gel emulsion would have the same effect as the BP injection. The use of the gel emulsion is very simple, and it can be applied to the oral mucosa without pain and is, therefore, more comfortable for the patients. VCO and CMC were the gel emulsions chosen for this study: CMC is known as a mucoadhesive polymer that is capable of attaching and penetrating oral mucosa. VCO is pure coconut oil, derived from fresh coconut flesh, consisting of medium chain saturated fatty acids such as lauric acid and capric acid, which are easily absorbed by the body [27]. Until now, there has been no ZOL BP in the form of a gel emulsion.

The ZOL BP dose used in this study was 40 µg, which is 3 times the dose used in an injection. The epithelial layer of rats' oral mucosa is not different from the epithelial layer of human oral mucosa. However, the thickness of rats' oral mucosa is less than that of the human, with about 40 and 140 µm thickness, respectively. A study using a PGE_2 gel at a dosage of 25 µg PGE_2 which was 3 times the dose of a PGE_2 injection, was performed in Sprague-Dawley rats at 0, 2, and 4 h [29]. The results showed that a repeated gel application could penetrate the rats' oral mucosa layer and increase the inflammatory cell count. The present study used 25 mg of Ge-ZOL, consisting of 40 µg ZOL, applied to the buccal mucosa lower right first molar of nine rats in the experimental group at hours 0, 4 and 8. Fig. 1 shows that topical application of gel ZOL had the effect of increasing osteoclast apoptosis. This means that Ge-ZOL penetrated deep into the oral mucosa layer to the alveolar bone.

In the present study, IHK Caspase-3 was used in the histological staining to count the number of apoptotic osteoclast cells. Osteoclast apoptosis was indicated by the brown cells that could be seen with the IHK caspase-3 staining (Figs. 2-4). IHK caspase-3 staining contains antibodies that detect and bind to antigen caspase-3 on osteoclast undergoing apoptosis [23]. An apoptotic osteoclast cell count was then performed using the Olympus IX73 research inverted microscope with 20x magnification. The number of apoptotic osteoclast cells on the 1st day shown in Fig. 2a is higher than that in Fig. 2b and c. The result shown in Fig. 2a-c is reflected in Fig. 1 and indicates that Ge-ZOL with VCO is capable of causing osteoclast cells apoptosis 24 h after the application of Ge-ZOL. The result of this study is in accordance with the study by Benford *et al*., which found that administration of ZOL to cell cultures was able to increase the number of apoptotic osteoclast cells after 24 h, using the biomarkers caspase-3 [23].

A *post hoc* Bonferroni test showed that the number of apoptotic osteoclast cells in the experimental group on the day 1 had a statistically significant difference when compared to the number of these cells on the days 3 and 5 ($p=0.001$ and $p<0.001$). However, the number of apoptotic osteoclast cells on the days 3 and 5 was not significantly different from each other ($p=0.251$) (Table 1). The number of apoptotic osteoclast cells in the control and normal groups was almost the same throughout, so the control group was validated by the normal groups. It can be concluded that Ge- with VCO alone is not capable of increasing the number of apoptotic osteoclast cells and that is the addition of ZOL that increases the apoptotic of the osteoclast. A cotton bud was used with a circular movement for 2 min to apply the gel in the control group. There may be some pressure applied during this twisting motion, but the pressure did not increase the osteoclast apoptosis (Fig. 1).

From this result, we can conclude that VCO is a good delivery system because it can deliver ZOL through the oral mucosa and increase the number of apoptotic osteoclast cells.

This study showed that a repeated application of Ge-ZOL with VCO to oral mucosa, at a dose of 40 µg, can increase the number of apoptotic osteoclast cells on the day 1. Therefore, Ge-ZOL has potential for use as a locally applied medicine to inhibit osteoclastic bone resorption. This present study is a pilot study; further research is needed to identify the effect of Ge-ZOL in orthodontic tooth movement. Moreover, further experiments are needed to explore the biologic, physical, and chemical stability of Ge-ZOL with VCO, the optimal dose of Ge-ZOL with VCO, and the best time for application time of Ge-ZOL with VCO.

CONCLUSIONS

It is concluded that a gel emulsion ZOL BP in VCO can penetrate into the alveolar bone of rats based on the increase in apoptosis of the osteoclast cells. The number of apoptotic osteoclast cells in the experimental groups on the day 1 was higher than on the day 3 or day 5.

ACKNOWLEDGMENT

The publication of this manuscript is supported by Universitas Indonesia.

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