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Effects of Administering the Soybean Isoflavone Genistein on Alkaline Phosphatase Levels During Orthodontic Tooth Movement in Young and Old Rabbits

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ABSTRACT

The goal of this study was to investigate differences in alkaline phosphatase (ALP) levels in young and old rabbits after administering the soybean isoflavone genistein during orthodontic tooth movement. Twelve rabbits were used and assigned to four groups (n = 3); OG (old rabbits), OGS (old rabbits + soybean), YG (young rabbits), and YGS (young rabbits + soybean). The rabbit mandibulary incisors were distalised using a nickel-titanium open coil spring (50 g force). Genistein was administered from the initial orthodontic force delivery until day 21, at a dose of 1.2 mg/kg BW once a day. ALP levels (U/mg) were measured on days 1, 7, 14, 21 after orthodontic force delivery using a UV-Vis 6300 spectrophotometer at a 405 nm wavelength. The results were analysed by one-way analysis of variance followed by Tukey's Honest Significant Difference (HSD) test ($p < 0.05$). The ALP levels between the young and old age groups were significantly different. ALP levels were highest in the YGS group, and significantly lowest in the OG group ($p < 0.05$). Moreover, the ALP level of the OGS group was significantly higher than that in the OG group ($p < 0.05$). In conclusion, daily consumption of soybean isoflavone genistein could enhance ALP levels during orthodontic tooth movement, particularly in older rabbits.

Keywords: *Alkaline phosphatase; orthodontic tooth movement; soybean genistein*

INTRODUCTION

Today malocclusion occurs in the majority of the population (Hassan & Rahimah, 2007). The principal purpose of orthodontic treatment is to fix malocclusion through orthodontic tooth movement (OTM), which occurs as a result of mechanical forces being applied to the teeth and is characterized by alveolar bone remodelling (Suparwitri *et al.*, 2018). Bone remodelling during OTM is a continuous and balanced turnover process in which the newly formed bone is substituted for old bone, managed by osteoclasts and osteoblasts. Osteoclasts play a role in bone resorption, while osteoblasts play a role in bone formation (Zhang *et al.*, 2014). Osteoblasts secrete serum alkaline phosphatase (ALP) during bone formation (Stucki *et al.*, 2001). ALP in gingival

crevicular fluid (GCF) is an indicator or biological marker of the bone remodelling (specifically bone formation) process during OTM (Alhasyimi *et al.*, 2018a).

Bone formation and resorption are balanced during the remodelling process in healthy young adults (Suparwitri & Noviasari, 2020). The increase in the number of adults seeking orthodontic treatment indicates that the biological differences among young and old periodontal tissues need to be adequately understood. The literature confirms that chronological age is a relevant risk factor for bone loss (osteoporosis) and periodontal problems (Rody *et al.*, 2014). Age-related bone loss occurs in humans and animals due to imbalanced bone remodelling. The balance of the remodelling process during treatment is associated with completed orthodontic treatment. The amount of new bone formed decreases with aging because the decreasing number of osteoblasts cannot compensate for the speed of bone resorption (Monroe *et al.*, 2003).

Many pathological processes related to oxidation occur in elderly subjects. Oxidative stress leads to increased activity of intracellular reactive oxygen species, which suppress bone remodelling (Hagiwara *et al.*, 2011). Therefore, the antioxidant properties of natural resources have received special attention. Using natural remedies as a source of antioxidants could be a valuable and novel therapeutic strategy to improve bone remodelling during OTM. Soybeans are food that contain many antioxidants, including the isoflavone genistein (Alsherbiney *et al.*, 2020). Isoflavones are present in soy products in particularly high concentrations; thus, the dietary intake of these compounds may affect the deposition of minerals in bone tissue (Liang *et al.*, 2018). Genistein contains many phenolic compounds that stimulate the proliferation of osteoblasts (Ming *et al.*, 2013). Phenolic compounds act as antioxidants and eliminate free radicals (Visioli & Bernardini, 2011). Soybeans also contain phytoestrogens, which are natural plant compounds that have an effect similar to estrogens produced in the body. Estrogens have receptors in periodontal tissues and bone. Estrogens in periodontal tissue and bone aid in the process of bone remodelling and the repair of periodontal tissue, particularly during bone formation (Hughes *et al.*, 1996). The present study examined the effect of the soybean isoflavone genistein on ALP levels during OTM in young and old rabbits. The hypothesis of this study was that administration of the soybean isoflavone genistein could increase ALP levels during OTM especially in an older individual. A rabbit model was chosen to test this hypothesis because this animal provides a clear representation of the bony structural changes that occur under stress, and they have been used to examine the effect of medications on OTM (Alhasyimi *et al.*, 2018b).

MATERIALS AND METHODS

This study was quasi-experimental research with 12 healthy female New Zealand rabbits used as research subjects, which were allocated into four groups (n = 3). A young rabbit group (± 3 months, weight 1,000 g) was not given genistein (YG), a young rabbit group was given genistein (YGS), an old rabbit group (± 3 years, weight 4,000 g) was not given genistein (OG), and an old rabbit group was given genistein (OGS). Use of these animals was granted by the Institutional Research Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada (No. 00242/KKEP/FKG-UGM/EC/2019), and all experiments were conducted according to the guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rabbits were kept in individual polycarbonate cages. Standard environmental requirements of a 12-h light/dark cycle at 25°C, and a humidity level of 50 ± 15% were maintained. To minimise the risk of displacing the orthodontic appliance

during chewing, the animals were fed soft food (finely ground standard pellets) with tap water *ad libitum*.

The rabbits were anesthetised with ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight) intramuscularly before bonding the brackets. The lower incisors were cleaned with a rubber cup that was smeared with pumice for 15 seconds until clean. The teeth were washed with clean water for 10 seconds, dried with a push spray, acid etching was applied to the labial surface of the lower incisor for 15 seconds, rinsed with a cotton pellet soaked in water, and dried with push spray. A 0.022" straight-wire bracket slot was bonded, and the lower incisors were distalised bodily with a 0.010" × 0.030" nickel-titanium open coil spring, which was compressed between two brackets attached to a 0.016" × 0.016" wire (3M Orthodontics, St. Paul, MN, USA) to deliver a continuous force of 50 g. The length of the SS wire was 4 mm longer than the open coil spring as tolerance if OTM occurred. The force was exerted for three weeks, and the springs were not reactivated during the experiment. The soybean isoflavone genistein was administered from the beginning of OTM to days 1, 7, 14, and 21, at a dose of 1.2 mg/kg BW suspended in 5 ml of distilled water. The dosage used was determined from a previous *in vivo* study. The dosage used was determined from a previous *in vivo* study (Indriasari *et al.*, 2020). Soybean isoflavone genistein was administered via the oral route using a nasogastric tube once a day to the OGS and YGS groups.

GCF samples were taken with paper points inserted into the gingival sulcus at a depth of 1 mm for 60 seconds on the mesial and distal sides of the lower incisors on days 1, 7, 14, and 21 after orthodontic force delivery (Fig. 1). Three paper points were used at intervals of 60 seconds to boost the volume of GCF. The paper points were placed immediately in 1.5 ml Eppendorf tubes with 350 µl of physiological saline solution. The tubes were centrifuged for five minutes at 2,000 rpm to completely elute the GCF component. The paper points were taken, and the supernatant solution was stored at −80°C until ALP levels were determined.

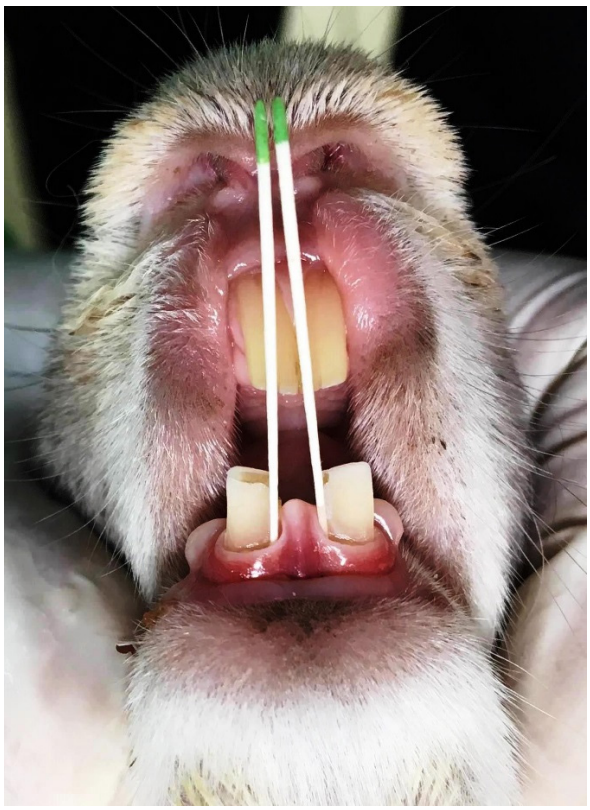

Fig. 1 The #15 sterilised paper points were inserted 1 mm into the gingival sulcus and were left in situ for 60 second.

Fifty µl of 40 mM carbonate buffer (pH 9.8) was incorporated with 3 mM $MgCl_2$, which was placed in a microplate. Fifty µl of GCF sample and 50 µl of 3 mM p-nitrophenylphosphate were added to the same well, and the microplate was incubated for 30 minutes at 37°C. The enzyme reaction was stopped by adding 50 µl of 0.6 M sodium hydroxide. The absorbance was determined at a wavelength of 405 nm using a spectrophotometer. ALP activity was expressed in enzyme units (U) as the amount of p-nitrophenol released per minute at 37°C. ALP levels were determined based on units (U) of activity vs. the total protein content (mg) and were expressed as U/mg.

All data were analysed using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA). The ALP levels were compared among the groups at four subsequent times by one-way analysis of variance, followed by Tukey's post-hoc Honest Significant Difference (HSD) multiple comparison test. All data is presented as mean ± standard deviation (SD). The *p*-value < 0.05 was deemed as statistically significant.

RESULTS

The mean and standard deviation values of ALP (U/mg) on days 1, 7, 14, and 21 are shown in Tables 1–4. The data were homogenous and normally distributed according to the Shapiro-Wilk normality and the homogeneity test. Based on the results of both tests, the data were eligible for parametric testing. The results of the one-way ANOVA test could be seen in Tables 1–4. ALP levels were significantly higher in the young rabbits receiving genistein than the other groups on the four subsequent observation days ($p < 0.05$). ALP levels in the old rabbits consuming soybean (OGS group) were nearly equal to those in the young rabbits without taking soybean consumption (YG group) on days 1, 7, 14, and 21 ($p > 0.05$). In contrast, the OG group exhibited significantly lower ALP levels than the other groups on the four subsequent time points after the initial delivery of force ($p < 0.05$). ALP levels were not different between the YG and OGS groups at any of the time points ($p > 0.05$) (Tables 1–4).

DISCUSSION

The results showed that the ALP levels between the different age groups were significantly different and that younger rabbits exhibited higher ALP levels than older rabbits. It is known that older aged spinal cord cells have a maximum life span that differs from that of younger aged spinal cord cells, as apoptosis increases and

Table 1 Mean, SD and results of the ANOVA and Tukey's HSD tests comparing the ALP in the four groups analysed at day 1 after orthodontic force delivery

Group	*N*	ALP level (U/mg)	Significance*	*p*-value		
				YGS	OG	OGS
YG	3	20.34 ± 2.93	$p = 0.049$*	0.096	0.022*	0.062
YGS	3	19.03 ± 3.98			0.001*	0.053
OG	3	14.44 ± 3.76				0.049*
OGS	3	16.31 ± 3.14				

Note: *Significant differences between groups ($p < 0.05$).
YG: young rabbit, YGS: young rabbits+ soybean isoflavones genistein, OG: old rabbit, OGS: old rabbits+soybean isoflavones genistein.

Table 2 Mean, SD and results of the ANOVA and Tukey's HSD tests comparing the ALP in the four groups analysed at day 7 after orthodontic force delivery

Group	*N*	ALP level (U/mg)	Significance*	*p*-value		
				YGS	OG	OGS
YG	3	21.44 ± 4.01	$p = 0.031$*	0.121	0.023*	0.063
YGS	3	23.52 ± 4.12			0.002*	0.076
OG	3	15.06 ± 3.73				0.045*
OGS	3	19.03 ± 3.79				

Note: *Significant differences between groups ($p < 0.05$).
YG: young rabbit, YGS: young rabbits+ soybean isoflavones genistein, OG: old rabbit, OGS: old rabbits+soybean isoflavones genistein.

Table 3 Mean, SD and results of the ANOVA and Tukey's HSD tests comparing the ALP in the four groups analysed at day 14 after orthodontic force delivery

Group	*N*	ALP level (U/mg)	Significance*	*p*-value		
				YGS	OG	OGS
YG	3	30.69 ± 4.18	$p = 0.000$*	0.058	0.001*	0.167
YGS	3	35.85 ± 4.01			0.001*	0.231
OG	3	21.95 ± 3.72				0.001*
OGS	3	30.93 ± 3.94				

Note: *Significant differences between groups ($p < 0.05$).
YG: young rabbit, YGS: young rabbits+ soybean isoflavones genistein, OG: old rabbit, OGS: old rabbits+soybean isoflavones genistein.

Table 4 Mean, SD and results of the ANOVA and Tukey's HSD tests comparing the ALP in the four groups analysed at day 21 after orthodontic force delivery

Group	*N*	ALP level (U/mg)	Significance*	*p*-value		
				YGS	OG	OGS
YG	3	27.41 ± 3.26	$p = 0.048$*	0.198	0.059	0.543
YGS	3	31.53 ± 3.77			0.041*	0.536
OG	3	16.33 ± 2.91				0.035*
OGS	3	23.19 ± 3.29				

Note: *Significant differences between groups ($p < 0.05$).
YG: young rabbit, YGS: young rabbits+ soybean isoflavones genistein, OG: old rabbit, OGS: old rabbits+soybean isoflavones genistein.

proliferation and differentiation of osteoblasts in bone marrow stromal cells decrease with increasing age (Zhou *et al.*, 2008).

The aging process is associated with increased reactive oxygen species (ROS), which include peroxides and free radicals that affect the formation and life span of osteoblasts. ROS stimulates tissue oxidation, which can kill cells. ROS also increase bone resorption indirectly by stimulating osteoclastogenesis via the receptor activator of nuclear factor-κB (RANK)-receptor activator of nuclear factor-κB ligand (RANKL) pathway (Lee *et al.*, 2005). RANK competes with osteoprotegerin (OPG) produced by osteoblasts to bind RANKL. Thus, as RANK binds RANKL, it inhibits the RANKL-OPG bound for osteoblast proliferation (Alhasyimi *et al.*, 2017). Osteoblasts synthesize and secrete ALP during bone formation (Alhasyimi *et al.*, 2018a), so differences in osteoblast activities between the young and old age groups caused differences in ALP levels between the two groups.

This study provides evidence that consuming soybean isoflavone genistein increases ALP levels during OTM, particularly in older rabbits. A prior *in vivo* study determined that genistein significantly enhances the quantity of osteoblasts during OTM (Suparwitri *et al.*, 2016). Osteoblast proliferation is stimulated by the phenolic acids contained in soybeans. Phenolic compounds act as antioxidants that eliminate free radicals (Król-Grzymała & Amarowicz, 2020). Free radicals stimulate oxidative stress and differentiation of osteoclasts, resulting in increased bone resorption (Lee *et al.*, 2005). In addition, the phenolic compounds in soybeans modulate Runx2, which is an essential molecule for inducing osteoblast differentiation, including the proliferation and maturation capacity of osteoblasts and increased ALP activity (Król-Grzymała & Amarowicz, 2020).

The old rabbits that received soybean isoflavone genistein daily had higher ALP levels than the old rabbits that were not exposed to soybean, and this value was almost similar to the young rabbits that were not given soybean. Soybeans are the most popular source of isoflavones, which simulate estrogens in advancing bone formation rates in an animal model (Brandi, 1997). One study reported that genistein improves ALP expression along with DNA and protein contents in osteoblastic MC3T3-E1 cells, indicating an anabolic effect (Sugimoto & Yamaguchi, 2000). Genistein is the main isoflavone in soybeans and is known as a phytoestrogen. Phytoestrogens have estrogenic effects because they have the same chemical structure as 17β-estradiol, so they bind estrogen receptors (Turner *et al.*, 2007). Estrogens inhibit cytokines, which inhibit bone resorption and ultimately increase osteoblast proliferation and enhance ALP level (Keiler *et al.*, 2014). Estrogens stimulate OPG production through osteoblasts, which can inhibit osteoclast differentiation and activities. Estrogens are known to inhibit glucocorticoid-induced osteoblast apoptosis, consequently lengthening the life span of osteoblasts (Gohel *et al.*, 1999).

The results of this study revealed a decrease of ALP levels in the old groups (OG and OGS). Lower levels of estrogen are observed in old age (Suparwitri *et al.*, 2016). Estrogen receptors in human osteoblasts have an anti-resorption effect on bone. Estrogen receptors in human osteoblasts have an anti-resorption effect on bone. Estrogens suppress osteoclast differentiation by inhibiting the interaction between RANK and RANKL (Shevde *et al.*, 2000). In addition, estrogens inhibit the production of IL-6, IL-1, TNF-α, IL-11, IL-7, and TGF-β, which are important for osteoclast differentiation (Bezzera *et al.*, 2005). An increase in the interaction between RANK and RANKL was detected in the older groups along with the decrease of estrogen, which later inhibited the interaction between OPG and RANKL, so ALP levels decreased. Decreased differentiation of osteoblasts in the older age groups was also due to a shift in the differentiation of mesenchymal stem cells to adipocytes rather than osteoblasts (Hu *et al.*, 2018). The decreased number of osteoblasts decreased the ALP levels in the older age groups.

ALP levels in all groups tended to increase from days 1 to 7 after delivery of orthodontic force. The 7-day enzyme activity definitively coincided with a lag phase of tooth movement when hyalinization occurred (Kumar *et al.*, 2019). The ALP level then peaked on day 14 after orthodontic force was applied. The enzyme levels indicated the beginning of the post-lag phase two weeks after OTM, and usually the enzyme activity tended to peak on day 14, which is an indication of the highest cellular (osteoblast) activities (Alhasyimi *et al.*, 2018a).

A limitation of this study was the observation time limit of three weeks after the installation of orthodontic appliances. Further research is needed to assess the effect of the soybean isoflavone genistein in a longer time frame during OTM for evaluation of the biological effect of soybean consumption over time during orthodontic tooth movement.

CONCLUSION

Within the limitations of the study, it can be concluded that administration of the soybean isoflavone genistein increased ALP levels in older aged rabbits to be the same as ALP levels in younger aged rabbits that were not given genistein during OTM.

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