



RESEARCH ARTICLE

REVISED Different effects of soybean isoflavone genistein on transforming growth factor levels during orthodontic tooth movement among young and old rabbits [version 2; peer review: 2 approved]

Verastuti Indriasari, Sri Suparwitri , Christnawati Christnawati, Ananto Ali Alhasyimi 

Department of Orthodontics, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta, 55281, Indonesia

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Abstract

Background: Orthodontic treatment to improve aesthetics and for health reasons is performed in children and adults. Elderly individuals have low levels of estrogen, this results in alveolar bone resorption being greater than alveolar bone apposition. Isoflavones present in soybeans may be able to improve the remodeling process through the induction of osteoblastogenesis by increasing transforming growth factor-β1 (TGF-β1) levels. This study aimed to assess the comparative effect of soybean genistein isoflavone to TGF-β1 during orthodontic tooth movement among juvenile and adult rabbits.

Methods: In this study, 12 healthy female rabbits were used. Subjects were divided into four groups (n=3); YG group (young rabbits), YGI group (young rabbits + isoflavones genistein), OG group (old rabbits), and OGI group (old rabbits + isoflavones genistein). Two lower incisors of the rabbit were moved distally using an orthodontic force (50 grams force) delivered by an open coil spring, which was inserted between two brackets. During active movements, the genistein isoflavones were given from the initial installation of the device until days 21, at a dose of 1.2 mg/kg BW once a day. Measurement of TGF-β levels were performed on days 1, 7, 14, 21 after appliance installation. TGF-β1 expression was analyzed using enzyme-linked immunosorbent assay (ELISA) and the optical density (OD) of the sample quantified using a standard curve. The data obtained were analyzed using one-way Anova followed by Tukey HSD test.

Results: The TGF-β1 levels were found to highest in the YGI group, and the TGF-β levels were significantly lower in the OG group (p<0.05). ELISA analysis also revealed that TGF-β1 levels of the OGI group were significantly higher when compared with the OG group (p<0.05).

Conclusion: The administration of soybean genistein isoflavones could improve TGF-β1 levels in old rabbit's during active orthodontic tooth movement.

Keywords

genistein, juvenile, adult, orthodontic tooth movement

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1 **Ida Bagus Narmada** , Airlangga University, Surabaya, Indonesia

Alexander Patera Nugraha , Airlangga University, Surabaya, Indonesia

2 **Lidia Audrey Rocha Valadas** , Federal University of Ceará, Fortaleza, Brazil

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Ananto Ali Alhasyimi (anantoali@ugm.ac.id)

Author roles: **Indriasari V:** Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Software, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Suparwitri S:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – Review & Editing; **Christnawati C:** Data Curation, Formal Analysis, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – Review & Editing; **Alhasyimi AA:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1

This new version includes some minor changes in the background, discussion, and detail of method sections.

Any further responses from the reviewers can be found at the end of the article

Introduction

Today's society is witnessing an increased interest in cosmetic dentistry, thereby making orthodontics a necessity¹. Orthodontic treatment has become one of the most procedures in cosmetic dentistry; it is performed to improve malocclusion, achieve good occlusion and dentofacial harmony^{2,3}. The demand for orthodontic treatment has grown over time, not only in children, but also in adults⁴. The rising number of adults demanding orthodontic treatment presents a new challenge. A previous study found that adults alveolar bone remodeling is much slower than in juveniles due to decreased cellular activity and vascularity, this suggests that orthodontic treatment duration may be longer in adults due to a delay in orthodontic tooth movement (OTM)⁵.

The success of orthodontic treatment depends on the process of alveolar bone tissue remodeling during the treatment which involves bone apposition and resorption by osteoblasts and osteoclasts⁶. For children and juveniles who are in an early stage of growth and development, bone apposition and resorption are balanced. In contrast, in adults, and following the menopause, the rate of bone apposition is less than that of resorption⁷. With increasing age, estrogen levels decrease, resulting an increased incidence of osteoporosis with associated complications⁸. In the context of adult orthodontic patients, it may be beneficial to address this deficiency to improve treatment outcome.

Hughes *et al.*⁹, demonstrated that estrogen replacement may help to inhibit excessive bone loss by restricting osteoclast life span through promotion of apoptosis, mediated by transforming growth factor beta (TGF- β 1). Estrogen effectively modulates TGF- β 1 production in osteoblast and other cells. TGF- β 1 is one of the most significant factors in bone formation, helping to maintain the balance between the dynamic processes of bone formation and bone resorption¹⁰. The development of natural remedies for the promotion of this mechanism, specifically, could be a useful and novel therapeutic approach to enhance bone remodeling by modulating the levels of TGF- β 1 in adults during OTM.

Nowadays, the use of hormones derived from natural ingredients, namely phytohormones, has gained a lot of popularity. One of these is phytoestrogen, a substrate from plants with estrogen-like activity. The isoflavone genistein is a type of phytoestrogen which is the main polyphenol component of the soybean¹¹. Some derivatives of soybeans have been recognized to have a positive effect on bone remodeling without triggering side effects. The effects of isoflavones on the improvement

of osteoblast's proliferation have previously been observed. Genistein administration has shown potential to increase osteoblast numbers during OTM⁴. Further study to explore the effect of genistein in enhancing osteoblastogenesis through improving TGF- β 1 level is needed to validate its advantage in orthodontic bone remodeling. This study aimed establish the comparative effect of genistein on TGF- β 1 during orthodontic tooth movement among juvenile and adult rabbits. The hypothesis of this study is that the administration of soybean genistein isoflavones could improve TGF- β 1 levels during active orthodontic tooth movement, especially in old rabbit. The rabbits were selected as a model as it they have previously used to study the effect of medications on OTM¹². Rabbit also provide an excellent model system to simulate the response of human tissue and are not aggressive, making them easy to handle and observe¹³.

Methods

Animal study

All experimental procedures involving animals were carried out in keeping with guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals to ameliorate any suffering of animals. Ethical clearance was obtained from the Research Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia, with number 00242/KKEP/FGK-UGM/EC/2019.

We used 12 female rabbits (*Oryctolagus cuniculus*) (Integrated Laboratory of Research and Testing, UGM, Indonesia), which were randomly divided into 4 groups (n=3), YG group (young rabbits/controls), YGI (young rabbits + isoflavones genistein), OG (old rabbits/controls), OGI (old rabbits + isoflavones genistein). Young rabbits were 3 months old and weighed approximately 1000 grams, whilst old rabbits were 3 years old and weighed approximately 4000 grams. Sample size (n=3) was determine based on Lemeshow's formula. A sample size of three animals in each group would present more than 85% power to detect significant differences with 0.45 effect size and at a significance level of $\alpha = .05$. Sample groups were chosen utilizing simple random sampling. Each animal was assigned a tag number, the blind-folded researcher then picks numbered tags from the hat.

All the rabbits were housed individually in polycarbonate cages (0.90 × 0.60 × 0.60 m) for a week on a 12-h light/dark cycle at a steady temperature of 25°C and humidity of 50% for acclimatization to compensate for their various origins. Animals were fed a standard pellet diet with tap water *ad libitum*, and were routinely inspected for food consumption and fecal characteristics.

Prior to performing the experimental procedure, rabbits were anesthetized with ketamine (160095, Kepro™, Netherlands), and xylazine (160096, Xyla™, Netherlands) (ketamine dose 35 mg/kg body weight and xylazine 5 mg/kg body weight), intramuscularly on the gluteus muscle during installation of the orthodontic appliance in their mouth. Two lower incisors of the rabbit were moved distally using a NiTi open coil spring 0.010" × 0.030" (O-951-1200, DynaFlex, the Netherlands)

which was inserted between two preadjusted edgewise lower incisor brackets engaged to a 0.016 “x 0.016” rectangular stainless-steel wire (126-029977, American Orthodontics®, USA) (Figure 1). An open coil spring was compressed until it produced 50 grams orthodontic force continuously for 21 days (measured by tension gauge, MedKraft Orthodontics, USA). No reactivation of the appliance was done throughout the experiment. During active tooth movements, the genistein (produced by Prof. Mien Karmini from IPB) were given from the initial installation of the device until days 1, 7, 14 and 21, at a dose of 1.2 mg/kg BW dissolved in 5 ml of distilled water. This dose used was determined from evidence from a previous *in vivo* study¹⁴.

The composition of genistein consists of tempeh, wheat flour, sugar, salt and vegetable oil. The processing process is as follows. Fresh tempeh first cut it into a kind of dice, then boiled in boiling water for 10 minutes. After draining, the tempe is ground with a grinder meat. Tempe that has been ground and then mixed in a mixer that has been light with a little water and all the other ingredients that have been prepared, then wait for 15 minutes or until the dough becomes smooth. Batter it is then flattened in a pan, pressed and made lines as an air cavity. The dough is then baked in the oven with a temperature of 175° up to 200°C for 10 minutes. After that the dough has been shaped like a biscuit is cut into small pieces and then dried use a dryer with a temperature of 70°C for about 16 hours. After dried, the tempe cereal is ground with a kind of flour grinder until it becomes powder.



Figure 1. Design of the orthodontic device: (a) rectangular stainless steel wire, (b) preadjusted edgewise bracket, and (c) open coil spring.

The genistein solution was then given orally using a nasogastric tube once a day. Before gingival crevicular fluid sample collection, all the animals were observed for any general toxicity probability, including edema or deaths, and measured the body weight (using a digital scale, ZB22-P, Zieis®, USA). All these measurements were done by a single blinded observer.

Isolation of gingival crevicular fluid (GCF)

The GCF samples were collected from the two interproximal areas of the maxillary anterior teeth (mesial and distal sides) at four subsequent time points (1, 4, 7, and 14 days after the installation of the orthodontic appliance) (Figure 2). During GCF collection all animals were sedated. Two #15 sterilized paper points (A-022T, Dentsply, Germany) were used to collect the GCF. The paper points were gently inserted approximately 1 mm into the gingival sulcus and were left *in situ* for 30 s after removing the supragingival plaque with cotton swabs. Thereafter, the paper points were isolated with cotton rolls and dried. The dipped paper points were then stored in a sterile 1.5 ml tube comprising 350 µl of physiological saline solution (Nova-Tech, Inc., USA). The tube was centrifuged at 2000 rpm for 5 min at 4°C with the help of a microcentrifuge refrigerator (Eppendorf 5424R, USA) to elute the entire GCF element from the paper points. Paper points were removed, and the supernatant solution was kept at a temperature of –80°C in a refrigerator until further analysis. After the collection of the final samples, all rabbits were sacrificed with an overdosed anesthesia (intravenous injection of 100 mg/kg BW Pentobarbital, 1507002, Pubchem, USA) following Guidelines for the Euthanasia of Animals by American Veterinary Medical



Figure 2. Gingival crevicular fluid isolation.

Association¹⁵ to collect bone samples for further histological analysis (Results not reported).

The TGF- β 1 levels were detected and analyzed by enzyme-linked immunosorbent assay (ELISA). The analysis was done using a quantitative sandwich Rabbit TGF- β 1 ELISA kit (ERB0119, FineTest, China). The TGF- β 1 levels in the sample were found by extrapolating the optical densities of the samples on the standard curve. The optical densities were measured at 450 nm using a microplate reader (Bio-Rad Laboratories Inc., USA), single readings were taken for each animal. TGF- β 1 expression levels were calculated as pg/mL. One-way ANOVA tests followed by Tukey's *post-hoc* test were used to identify possible differences in TGF- β 1 expression between controls and treatment groups. Statistical significance was set at *p* values < 0.05. Statistical analysis was processed with the SPSS 21.0 software system (SPSS Inc., Chicago, Illinois, USA).

Results

In general, giving genistein isoflavones at the selected dose did not cause any general toxicity, edema or deaths, nor did it affect the body weight of rabbits (see underlying data¹⁶).

ELISA analysis revealed that in young rabbits with OTM receiving genistein, TGF- β 1 levels were significantly higher than other groups on day 1, 7, 14 and 21 (*p* < 0.05). In older rabbits with OTM receiving genistein, TGF- β 1 levels were almost the same as those in young rabbits without genistein isoflavones administration on days 1, 7, 14 and 21 (*p* > 0.05). In the old rabbits group without genistein administration, TGF- β 1 levels were significantly lower than the other groups on days 1, 7, 14 and 21 after bonding (*p* < 0.05). The YG and OGI group showed no significant difference in TGF- β 1 levels at all time points (*p* > 0.05) (Table 1–Table 4; underlying data¹⁷).

Discussion

This investigation confirms the hypothesis that soybean isoflavone genistein administration could increase the TGF- β 1 levels during orthodontic tooth movement, especially in older rabbits. In general, results demonstrated that in the subjects receiving soybean isoflavone genistein, TGF- β 1 levels were significantly higher than the other groups. A previous study found that genistein can significantly increase the number of osteoblasts during orthodontic tooth movement¹. Soybeans are the most common source of isoflavones, which have physiological effects that mimic native estrogen in maintaining

Table 1. Descriptive statistics and results of the Anova and Tukey HSD tests comparing the TGF- β 1 in the 4 groups tested at day 1 after orthodontic appliance installation.

Group	N	TGF- β 1 level (pg/ml)	Significance*	p-value		
				YGI	OG	OGI
YG	3	40.34±3.22	<i>P</i> = 0.037*	0.228	0.001*	0.763
YGI	3	49.03±4.02			0.000*	0.058
OG	3	14.44±3.09				0.003*
OGI	3	36.31±3.89				

Values are presented as mean \pm standard deviation or *p*-value only. *by ANOVA, *Significant differences between groups (*p* < 0.05).

ANOVA: Analysis of variance; YG: young rabbit, YGI: young rabbits+isoflavones genistein, OG: old rabbit, OGI: old rabbits+isoflavones genistein).

Table 2. Descriptive statistics and results of the Anova and Tukey HSD tests comparing the TGF- β 1 in the 4 groups tested at day 7 after orthodontic appliance installation.

Group	N	TGF- β 1 level (pg/ml)	Significance*	p-value		
				YGI	OG	OGI
YG	3	31.44±3.92	<i>P</i> = 0.001*	0.998	0.001*	0.098
YGI	3	31.95±2.64			0.001*	0.075
OG	3	15.06±1.22				0.044*
OGI	3	24.03±2.42				

Values are presented as mean \pm standard deviation or *p*-value only. *by ANOVA, *Significant differences between groups (*p* < 0.05)

ANOVA: Analysis of variance; YG: young rabbit, YGI: young rabbits+isoflavones genistein, OG: old rabbit, OGI: old rabbits+isoflavones genistein).

Table 3. Descriptive statistics and results of the Anova and Tukey HSD tests comparing the TGF- β 1 in the 4 groups tested at day 14 after orthodontic appliance installation.

Group	N	TGF- β 1 level (pg/ml)	Significance*	p-value		
				YGI	OG	OGI
YG	3	40.69. \pm 3.25	$P= 0.000^*$	0.634	0.000*	1.000
YGI	3	44.85 \pm 4.63			0.000*	0.590
OG	3	15.15 \pm 3.07				0.000*
OGI	3	40.43 \pm 2.26				

Values are presented as mean \pm standard deviation or p -value only. *by ANOVA, *Significant differences between groups ($p < 0.05$).

ANOVA: Analysis of variance; YG: young rabbit, YGI: young rabbits+isoflavones genistein, OG: old rabbit, OGI: old rabbits+isoflavones genistein).

Table 4. Descriptive statistics and results of the Anova and Tukey HSD tests comparing the TGF- β 1 in the 4 groups tested at day 21 after orthodontic appliance installation.

Group	N	TGF- β 1 level (pg/ml)	Significance*	p-value		
				YGI	OG	OGI
YG	3	27.21. \pm 6.47	$P= 0.045^*$	1.000	0.067	0.984
YGI	3	27.53 \pm 4.98			0.048*	0.973
OG	3	12.63 \pm 2.76				0.042*
OGI	3	25.49 \pm 4.52				

Values are presented as mean \pm standard deviation or p -value only. *by ANOVA, *Significant differences between groups ($p < 0.05$).

ANOVA: Analysis of variance; YG: young rabbit, YGI: young rabbits+isoflavones genistein, OG: old rabbit, OGI: old rabbits+isoflavones genistein).

bone formation rates in rats after ovariectomy-induced osteoporosis¹⁸. A previous *in vitro* study reported that genistein increased alkaline phosphatase expression along with protein and DNA content in osteoblastic MC3T3-E1 cells, indicating an anabolic effect¹⁹. Osteoblastogenesis is induced by TGF- β 1, which is a physiological regulator of osteoblast differentiation and acts as a central component of the coupling of bone formation to resorption during bone remodeling²⁰. TGF- β 1 is osteogenic growth factor that has highly osteogenic attributes, enhancing osteoblast activity (by inducing bone marrow mesenchymal stem cells to differentiate into osteoblasts), stimulating osteoclast apoptosis, restraining osteoclastic activity, and resulting in bone formation²¹. Moreover, TGF- β 1 has been found to perform a critical role in tissue regeneration as it potentially improves the synthesis of connective tissue elements, such as type I collagen, proteoglycans, osteopontin, fibronectin, and osteonectin, during alveolar bone remodeling. The TGF- β 1 superfamily further includes bone morphogenetic proteins (BMPs), which are the most potent inducers of bone formation²².

Our results showed that TGF- β 1 levels in old rabbits are lower than young rabbits. Aging can cause a decreasing in the

osteoblasts number due to an imbalance in bone remodeling²³. Impaired osteoblastogenesis induced by maturation will cause a characteristic of bone loss. It is widely accepted that the loss of bone with aging is a universal phenomenon which is associated with reduced bone strength. In adult individuals, the microarchitecture of trabecular bone becomes thin, while cortical bone becomes thin and porous²⁴. A previous *in vivo* study demonstrated that in response to mechanical stimuli, the alveolar bone of the young rats was more active and sensitive than that of the adult rats⁴. As the aging process progresses, the periodontal ligament becomes more fibrotic and may influence how the tissues react to orthodontic forces²⁵.

The old rabbit group exposed to soybean isoflavone genistein exhibited higher TGF- β 1 levels than the old rabbit's groups without soybean isoflavone genistein and this value was almost similar to the young rabbit's groups without soybean isoflavone genistein. This is caused by administration of genistein to old rabbit's groups which can overcome bone formation problems that are caused by a decrease in estrogen. Genistein is a plant compound with potent estrogenic activity that has similarity in structure with the human female hormone 17- β -estradiol.

This hormone can bind to both alpha and beta estrogen receptors, and imitate the action of estrogens on target organs, and thereby it can provide many health benefits when used in some hormone-dependent conditions, including old age²⁶. Estrogen prevents loss of bone mineral density through a TGF β -dependent mechanism, which stimulate TGF- β 1 production in the bone marrow with a critical “upstream” mechanism. The main role of TGF- β 1 in osteoblastogenesis and bone formation is in recruiting osteoblast progenitors, stimulating their proliferation (increased DNA synthesis) and promoting the early stages of differentiation (bone matrix production) to increases bone formation. In addition, apoptosis of osteoblasts is blocked through maintenance of survival during transdifferentiation into osteocytes by TGF- β 1.

High levels of TGF- β 1 will suppress receptor activator of nuclear factor kappa- β ligand (RANKL) expression, which plays a role in osteoclast differentiation as a osteoclast differentiation factors. In other words, indirectly TGF- β 1 can limit the formation and activation of osteoclasts and increase bone mass^{27,28}. This condition is expected during active orthodontic tooth movement especially in adult.

A limitation of this study was the time points of evaluation limits in 21 days during orthodontic tooth movement. Further studies evaluating alveolar bone and underlying periodontal tissue changes after genistein administration over time during orthodontic tooth movement could further illuminate the underlying biologic processes. Future studies are also needed to confirm the effective dose of material used when applied for humans.

Conclusions

Taken together, the results of this preclinical study suggest that administration of the soybean isoflavone genistein could induce TGF-1 levels during orthodontic tooth movement especially in old rabbits.

Data availability

Underlying data

Figshare: Raw Data TGF beta isoflavone genistein. <https://doi.org/10.6084/m9.figshare.10116704>¹⁷

This project contains the following underlying data:

- TGF LOCKY ANAVA.xlsx (Recorded TGF beta levels)

Figshare: OUTPUT Statistic Isoflavon TGF.docx. <https://doi.org/10.6084/m9.figshare.10117340>²⁹

This project contains the following underlying data:

- OUTPUT Statistic Isoflavon TGF.docx (Output file from statistical analysis)

Figshare: Animal Body Weight of Rabbit. <https://doi.org/10.6084/m9.figshare.10310696>¹⁶

This project contains the following underlying data:

- Animal Body Weight.xlsx (Animal body weights)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](https://creativecommons.org/licenses/by/4.0/).

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Lidia Audrey Rocha Valadas 

Postgraduate Program in Development and Technological Innovation in Drugs, School of Pharmacy, Federal University of Ceará, Fortaleza, Brazil

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 29 May 2020

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Lidia Audrey Rocha Valadas 

Postgraduate Program in Development and Technological Innovation in Drugs, School of Pharmacy, Federal University of Ceará, Fortaleza, Brazil

That's a well designed study and I have written some considerations below:

The introduction is clear and well written;

On the methods session I miss details of how you obtained the isoflavone, for me is the most important

part to assure the reproducibility of the study.

The discussion is small and is necessary to improve it and should add the limitations of the study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology and Dentistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 May 2020

Ananto Ali Alhasyimi, Universitas Gadjah Mada, Sleman, Indonesia

Dear Prof **Lidia Audrey Rocha Valadas**

Thank you for your kind assistance in reviewing our manuscript and providing us with valuable advice for the manuscript and our future work. We have been working throughout the review. The following are our consolidated comments related to the review and some changes we have done based on the reviewer comments.

That's a well designed study and I have written some considerations below:

The introduction is clear and well written;
Thank you so much

On the methods session I miss details of how you obtained the isoflavone, for me is the most important part to assure the reproducibility of the study.

We have edited the methods section and added this information in the present manuscript.

The discussion is small and is necessary to improve it and should add the limitations of the study.

We have edited the discussion section and added other references and limitation of the study as well. Thank you for the punctiliously review. We have added this information in the present manuscript

We thank you again for the valuable insight.

Competing Interests: All the authors involved in this study declare no competing interest.

Reviewer Report 20 January 2020

<https://doi.org/10.5256/f1000research.23351.r58276>

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Ida Bagus Narmada 

Department of Orthodontics, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia

Alexander Patera Nugraha 

Department of Orthodontics, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia

First of all, please allow me to congratulate the authors for attempting to undertake this project which I found very interesting and of valuable additional knowledge. The manuscript itself is well-written and well-structured and I have to also commend the authors for this matter.

However, I may require some clarifications on the following issues:

1. Paper is well organized and easy to follow.
2. Novelty is sufficient and high impact.
3. To improve the readability, It is recommended that the text is checked by a native English speaking person as many of the sentences might be misunderstood. I suggest a revision of the grammar structures by an expert editor in revising manuscripts.

Abstract:

1. Please add the p value used for ANOVA and Tukey HSD ($p < 0.05$) add homogeneity and normality test p-value used ($p > 0.05$) in the abstract section
2. Please add the exact p value obtained after the statistic analysis. ($p = \dots$).
3. There is a minor mistake in keyword: orthodontic orthodontic tooth movement. It would be better to choose the keyword according the MeSH.

Introduction:

1. Please add your hypothesis
2. Please add the statement or sentence about is there any similar studies have been done before or not? or your study is the first study who investigate this topic
3. The Introduction section is OK. It would be better if you mentioned why you only examine the TGF-Beta only in this study?
4. It would be better add the reason why you choose the genistein instead of others natural active compound?

Material and Methods:

1. It would better if you add the detail protocol or cite the protocol reference how to make or obtain the genistein
2. It would better if you add the reason why you choose 50 gram force to deliver the orthodontics tooth movement? please state what kind of force 50gf (light force or heavy force).
3. Please add the detail about the healthy condition of animal model, how to examine the condition of animal model? did you get a help from veterinarian? please state
4. Please explain how to purify the isolated GCF from animal study? how to minimize the GCF contamination and the bias?
5. The author mentioned "During GCF collection all animals were sedated" please add the detail protocol how you sedated the animal study.
6. In this study, the author performed the ANOVA and Tukey HSD. It would be better if the author also describe the Levene's and Shapiro Wilk test.

Results:

1. It would be better to understand and attract the reader if you present the data in the diagrams.
2. Please add the result of Levene's and Shapiro Wilk test.
3. Please indicate whether the difference is significant or not in your graph/ diagrams as well, using some symbols (asteric).

Discussion:

1. Discussion of the results is quite comprehensive. In analyzing the results, the authors also show citations from the previous study to support the explanation of these results.
2. The answer to the hypothesis of this study have been included at the beginning of the discussion section.
3. Please mention the limitation of this study in the discussion section.

References:

The supporting references are OK and adequate.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Orthodontic Research (Orthodontic Tooth Movement)

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 May 2020

Ananto Ali Alhasyimi, Universitas Gadjah Mada, Sleman, Indonesia

Dear Prof **Narmada and Dr Nugraha**

Thank you for your kind assistance in reviewing our manuscript and providing us with valuable advice for the manuscript and our future work. We have been working throughout the review. We have added this information in the present manuscript. We thank you again for the valuable insight.

Competing Interests: All the authors involved in this study declare no competing interest.

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