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Effect of Cocoa Administration in VEGF expression during Experimental Orthodontic Tooth Movement in Guinea Pigs

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ABSTRACT

The objective of this study is to investigate the effect of different doses and intake duration of cocoa administration on vascular endothelial growth factor (VEGF) expression during experimental orthodontic tooth movement (OTM) in male guinea pigs (Cavia cobaya). Twelve male guinea pigs were divided into four groups as follows: OTM only control group (ONC), cocoa doses groups: OTM and 1,37 g (OWC1); OTM and 2,05 g (OWC2); OTM and 2,74 g (OWC3). Nickel-titanium open-coil spring installed between both lower incisors generating 35 cN orthodontic force. All groups were further divided into cocoa intake duration as follows: 0,1,7 and 14 days. Gingival crevicular fluid (GCF) extracted on compression side at 4 subsequent time; 0,1,7 and 14 days. The expression of VEGF was examined using a specific enzyme-linked immunosorbent assay (ELISA). Statistical analysis was done using the two-way Anova test and Tukey's Post Hoc LSD. Expression of VEGF in the compression side was significantly different between doses groups and intake duration groups ($p<0.05$). There was significant interaction in VEGF expression between groups of cocoa doses and duration in the compression side of experimental tooth movement ($p< 0.05$). Based on this research, it can be concluded that cocoa administration during OTM influences VEGF expression in the compression side. In this study, a higher dose of cocoa increase VEGF expression, while a longer intake duration influence VEGF expression. Further study of the 2,05 g cocoa dose with immunohistochemistry in both compression and tension side is desired to determine the optimal dose of cocoa to take effect in OTM.

1. INTRODUCTION

An orthodontic force causes growth factors, cytokines, and neurotransmitters in periodontal ligament cells creating osteoclastogenic bone resorption on the compression side and osteoblastic bone formation on the tension side [1]. Orthodontic tooth movement consists of the initial phase, lag phase, and postlag phase. In OTM acceleration, it is important to understand the biological process in each phase [2- 4]. Methods to accelerate orthodontic tooth movement (OTM) are mainly assessed in the orthodontic field due to the length

of orthodontic treatment which usually requires 20-30 months to finish [5]. A substance which generally consumed in daily life researched in OTM is caffeine. Caffeine is a methylxanthines derivatives that cause low calcium intake which leads to decreasing bone density and modulating bone turnover and can be found in tea, coffee, and cocoa. Tea and coffee bring side effects to the central nervous system causing addiction [6], [7].

Cocoa is one of the caffeine sources which contains a lower dose of caffeine with no side effects. Cocoa contains polyphenol and several types of methylxanthine derivatives such as; theobromine, caffeine, and theophylline. Theobromine had been known to stimulate *cyclic adenosine monophosphate* (cAMP) [8]. Polyphenol activates nitric oxide (NO) in endothelial cells [9]. Both cAMP and NO have an important role in orthodontic tooth movement while theophylline is generally used as asthma medication [1], [10].

Cocoa influence angiogenesis by increasing the bioavailability and production of NO in endothelial cells, which induce cytokine promoting angiogenesis. *Vascular endothelial growth factor* (VEGF) expression occurs in endothelial cells and fibroblast cells to induce angiogenesis in response to tissue hypoxia [11], [12]. An in vitro study found that VEGF can stimulate mRNA *RANKL* and VEGF could be detected in *gingival crevicular fluid* (GCF) in a study using *enzyme-linked immunosorbent assay* (ELISA) procedure. A higher level of VEGF expression can be detected in periodontal tissue pathological inflammation such as periodontitis. A study assessing VEGF in OTM was in rats administered with single-dose coffee containing caffeine was once researched by Herniyati [13- 15].

A high and low dose of caffeine was researched in OTM with the animal model by [16], [17] by examining osteoclast count on the compression side and osteoblast count on the tension side, but both had a conflicting result. Thus, the amount of caffeine administered turns out to gave different effects in OTM, and cocoa with no addictive side effects becomes the better option as a caffeine source. Therefore, the purpose of this study is to investigate the effects of different doses and intake duration of cocoa containing caffeine in VEGF expression of compression side during experimental tooth movement in male guinea pigs (*Cavia cobaya*).

2. Materials and Methods

2.1 The Experimental Study Design and Ethical Clearance Approval

The design of this study is an experimental laboratory. The experimental procedures were performed based on the Institutional Animal Care and Usage Committee (ARRIVE guidelines). The health ethical clearance commission, Faculty of Dentistry, Universitas Gadjah Mada approved this study for experimental animal use with approval number 00716/ KKEP/FKG-UGM/EC/2019.

2.2 Determination of Methylxanthine (Caffeine) in Cocoa

Administration of cocoa derived from Hersey's 100% Pure cocoa Natural Unsweetened. In a previous study by [18], Hersey's 100% Pure cocoa Natural Unsweetened was tested using *Fourier transform infrared* (FTIR) and was confirmed to contain methylxanthine (caffeine). Methylxanthines were also detected from the cocoa powder sample under UV light at 254 nm using TLC Analysis.

2.3 Preparation of Cocoa Administration

Three doses of cocoa were given to subjects determined by conversion of maximum caffeine dose for humans (70 kg) derived from the Food and Drug Administration into guinea pigs' body weight. These three doses represent a high dose, medium dose, and low dose of caffeine as follows: 2,3 mg; 3,45 mg; and 4,6 mg caffeine. Since every 5 grams of cocoa powder contain 8,4 mg of caffeine. Thus, three final doses of cocoa

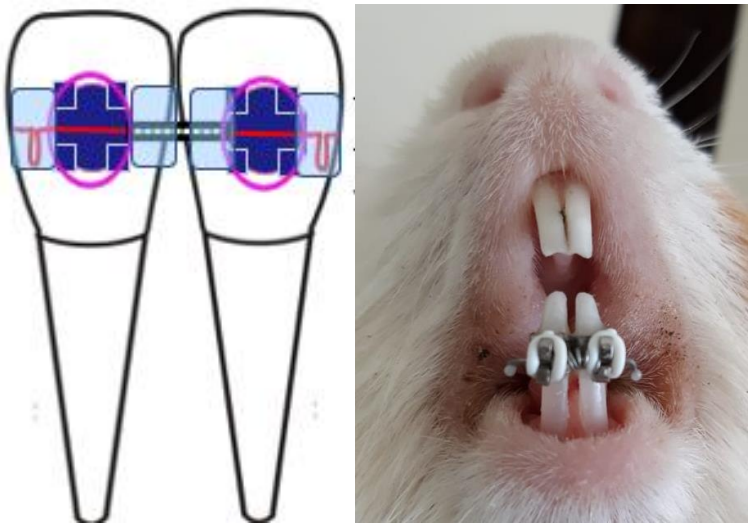
obtained from the conversion were 1,37 g;2,05 g; and 2,74 g of cocoa per day.

2.4 Animal experiments

All experimental animal was adapted under normal laboratory conditions for 7 days to minimize stress due to the new environment. All animals adapted to a 12/12 hr light/dark cycle at 25ºC with a humidity range of 50%. During experiments, the animals were fed standard laboratory pellets and given drinking water ad libitum.

The subjects consisted of twelve male guinea pigs (*C. cobaya*), 3 to 4 months old with 300-350 g body weight were divided into control and three treatments groups randomly. Each group consist of 3 male guinea pigs and the sample size was determined by Federer's sample size formula. The groups were: ONC: OTM only (control); OWC1: OTM and 1,37 g cocoa; OWC2: OTM and 2,05 g cocoa; OWC3: OTM and 2,74 g cocoa. The groups were further divided to be a group of cocoa administration duration as follows: 0,1,7 and 14. Gingival crevicular fluid (GCF) extracted on compression side at 4 subsequent time; 0,1,7 and 14 days.

Ketamine (50 mg/kg BW) (Kepro, Netherlands) and xylazine (Xyla, Netherlands) (5 mg/kg BW) were used to anesthetize all experimental animals intramuscularly on the lower thigh to minimize animal suffering during the installation of the OTM appliance. Modified molar bands created by combining 0.022-inch single-wing straight Roth mini bracket (Marquis™, Ortho Technology®, USA) soldered to the band. This appliance was cemented using *Flowable Composites* (*micro-hybrid composite,* Denfil Vericom, USA) to lower incisors and a 0,016-inch round stainless steel wire (American Orthodontic, USA) was inserted in the bracket slot. Orthodontic Tooth movement on all groups was obtained by activation of nickel-titanium open-coil spring which was installed between lower incisors generating 35 cN (measured with *dynamometer tension gauge* (Medkraft orthodontics, USA)) to move incisors distally. The orthodontic force was applied for 14 days and no re-activation was done.



(1) (2)

Figure 1. Experimental tooth movement appliance design in guinea pigs model (1), The experimental animal model with bonded orthodontic appliance design (2)

Immediately after orthodontic appliance installation, cocoa containing caffeine was administered. Hersey's 100% Pure cocoa Natural Unsweetened (powder) was used as the administered cocoa in all treatment groups. The amount of cocoa powder according to doses conversion was then dissolved in lukewarm distilled water

and administered twice a day using oral gavage.

2.5 Gingival Crevicular Fluid Sample Collection

Gingival crevicular fluid (GCF) was extracted on the compression side at 4 subsequent times; 0,1,7 and 14 days and collected using #15 in size paper point (Sendoline, UK). Before extraction, the gingival crevicular sulcus was gently drained with air jets and isolated using a cotton roll to avoid saliva contamination. Paper point inserted gently into the gingival sulcus, precisely at the distal side of the tooth (compression side) for 30 seconds. Each site was extracted with three dipped paper points and placed into a 1,5 ml Eppendorf tube containing 350µl physiological saline solution. Tubes were centrifuged at 2000 rpm for 5 minutes, 4ºC using a microcentrifuge refrigerator (Eppendorf 5424R, USA) to completely elute all GCF components from paper points. After the centrifugation, paper points were removed and the supernatants were stored at -80ºC until assayed process begin.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The amount of VEGF expression on GCF was examined using a specific enzyme-linked immunosorbent assay (ELISA) with a quantitative ELISA kit (Finetest, China). Collected samples being read at 450 nm wavelength with a microplate reader (Bio-Rad Laboratories, USA). The level of VEGF was determined using the sample's optical density compared to the standard curve. Total VEGF expressions were presented in pg/ml (picogram/milliliter).

2.7 Statistical Analysis

Research data was analyzed by a normality test using the Kolmogorov–Smirnov's test ($p>0.05$). Thereafter, a homogeneity test using Levene's statistic test ($p > 0.05$) was conducted. A two-way Analysis of Variance test was conducted to determine whether there is a difference between groups ($p<0.05$). Furthermore, Tukey's Post Hoc Least significant difference was conducted to know the differences between each group ($p <0.05$). The Statistical Package for Social Science (SPSS) 20.0 version (IBM Corporation; Illinois, Chicago, United States) was used to statistically analyze the data.

3. FINDINGS AND DISCUSSION

The administration of cocoa at all treatment groups did not lead to any general toxicity, edema, death, or extreme changes in the animal model. Enhanced expression of VEGF in the compression side of guinea pigs during experimental OTM was seen in all treatment groups. (Fig.2)

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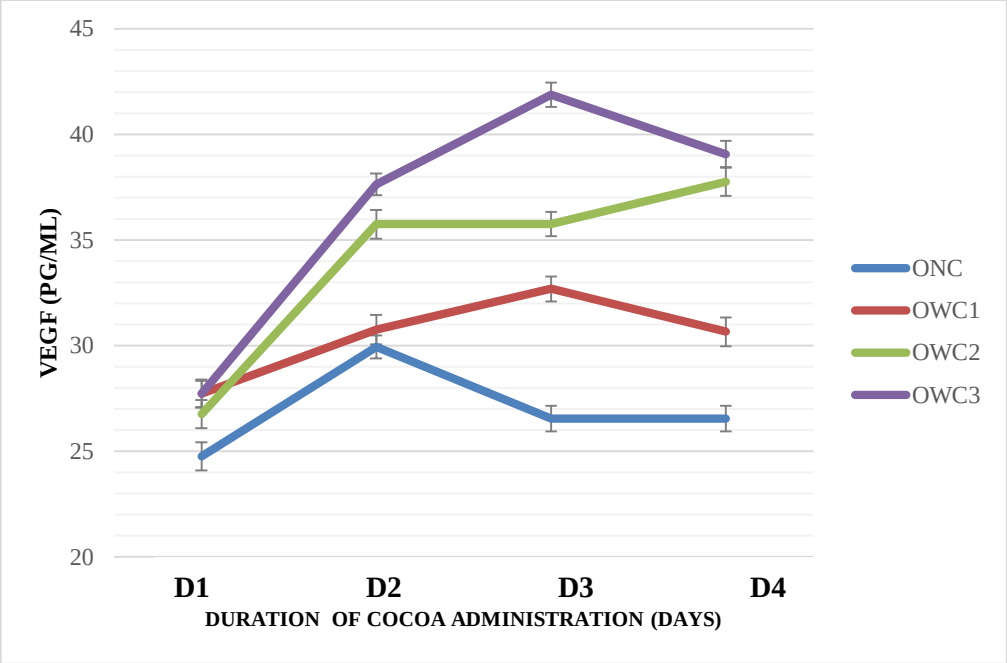

Figure 2. Mean and Standard deviations line diagrams of VEGF expression on the compression side during experimental tooth movement with cocoa administration in guinea pigs between dose groups (ONC, OWC1, OWC2, and OWC3) and duration groups (D0, D1, D7, D14). The two-way ANOVA result shows that there was a significant difference between dose groups and between intake durations ($p<0.05$). This analysis also shows that there was interaction in VEGF expression between doses groups and intake durations ($p<0.05$).

This study was conducted with the administration of three doses (low, medium, and high doses) and intake duration (days 0,1,7, and 14) of cocoa during OTM in an animal model. In the control group, 1,37 g, and 2, 74 g cocoa groups, VEGF expression increase since day 1, and started to decrease on day 7. This result corresponds to a previous study which found that VEGF in GCF increases as soon as 12-24 hours after the orthodontic force was applied which indicates the initial phase of OTM due to local hypoxia and a decrease of VEGF level at day 7 [19- 21]. [22] in a previous study found VEGF reduction from day 7 to 14 in the compression side of the OTM animal model. Decreasing VEGF expression on day 7 was predicted since there was no reactivation of the orthodontic appliance, which means a reduction of orthodontic force. This low orthodontic force may decrease decreased *hypoxia-inducible factor-1* (HIF-1) which resulted in the reduction of VEGF expression [1].

Higher expression of VEGF was found in all treatment groups. The two-way ANOVA result shows that there was a significant difference between dose groups ($p<0.05$) (fig.2). The VEGF expression increased from days 0, 1, and 7 in all treatment groups. This result was influenced by substances in cocoa. Methylxanthines derivatives in cocoa, caffeine, and theobromine were the major factor that influences VEGF expression. Methylxanthine in the previous study could increase Reactive Oxygen Species (ROS) by increasing Cyclic adenosine monophosphate (cAMP) concentration. Reactive Oxygen Species could facilitate the signaling of angiogenic factors, one of which is VEGF [8], [17].

The highest expression of VEGF occurred in the highest dose cocoa group on day 7. This is probably due to methylxanthine in cocoa which has a specific role as a nonspecific adenosine receptor antagonist. This specific role combined with local hypoxia and angiogenesis may regulate VEGF expression [17], [23], [24]. The two-way Anova test reveals a significant difference between intake duration groups ($p<0.05$). The

physiologic concentration of methylxanthine is important to achieve the maximum level of a substance in plasma to produce specific effects. This concentration was regulated by the adsorption rate and clearance rate of a substance inside the body [25]. Therefore, a longer duration of cocoa could produce a bigger impact on target cells.

According to [27], a routine and longer consumption of cocoa would increase the bioavailability of nitric oxide [26]. Nitric oxide in hypoxic conditions had been known to increase VEGF expression to induce angiogenesis. This research is in line with the result of this study that shows an interaction of VEGF expression between doses groups and intake duration groups ($p<0.05$).

Groups	ONC	OWC1	OWC2	OWC3
ONC	-	0.000*	0.000*	0.000*
OWC1	-	-	0.000*	0.000*
OWC2	-	-	-	0.000*
OWC3	-	-	-	

DAYS (D)	D0	D1	D7	D14
D 0	-	0.000*	0.000*	0.000*
D 1	-	-	0.049*	1.000
D 7	-	-	-	0.045*
D 14	-	-	-	-

Figure 3. Tukey's Post Hoc LSD ($p<0.05$) test result of the effect of cocoa administration in VEGF expression (pg/ml) on the compression side during experimental tooth movement in guinea pigs between each dose group and each duration group.

There is no significant difference in VEGF expression on the compression side between day 1 and 14 in guinea pigs administered with cocoa. This data shows cocoa derived a similar effect in VEGF expression on day 1 and 14 (fig.3). The increase of VEGF expression on day 14 in the 2,05 g cocoa group could indicate that this dose has the same effectivity to influence VEGF as the highest dose. Vascular endothelial growth factor may induce neovascularization, tissue oxygenation, and removal of necrotic tissues. The removal of necrotic cells in hyalinized tissue is mandatory to start tooth movement in the lag phase [1], [27]. This study supported a previous study by which concludes that cocoa administration can modulate the OTM rate [18].

Different doses of cocoa given different effects due to their range of compositions, such as polyphenol and theobromine. Previous research by resulted that polyphenol effects in the animal model had conflicting results. Polyphenol decreases VEGF but also modulates nitric oxide in endothelial cells [28], [29]. Theobromine, on the other hand, may reduce inflammation in high concentration, however, its longer half-life (7-12 hours) predominantly affects given effects to target cells [8]. Therefore, further research is needed to assess the effect of each extracted composition of cocoa in OTM, specifically in VEGF expression in compression and tension side.

4. Conclusion

Based on this research's result, it can be concluded that cocoa administration during OTM increases VEGF expression in the compression side. Higher dose cocoa may increase VEGF expression on the compression side and longer intake duration of cocoa influences VEGF expression on the compression side of experimental tooth movement in guinea pigs. Further study of the 2,05 g cocoa dose with immunohistochemistry in both compression and tension side is desired to determine the optimal dose of cocoa to take effect in OTM.

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