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The effect of cacao bean extracts on the prevention of periodontal tissue breakdown in diabetic rats with orthodontic tooth movements

Nuzulul Hikmah [a,b,*], Mohamad Hidayat [c], Nur Permatasari [d], Aulanni'am Aulanni'am [e], Achmad Rudijanto [f]

[a] Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[b] Department of Dentistry-Biomedical Sciences, Oral and Maxillofacial Pathology, Faculty of Dentistry, Universitas Jember, Jember, Indonesia
[c] Department of Orthopaedic, Saiful Anwar General Hospital, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[d] Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[e] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia
[f] Department of Internal Medicine, Saiful Anwar General Hospital, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

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ABSTRACT

Objective: Proper management of orthodontic treatment in diabetic patients is essential due to the heightened risk of periodontal tissue breakdown associated with hyperglycemia. Cacao bean extracts (CBE) are known to reduce the inflammatory response and increase synthesis and angiogenesis in periodontitis. Therefore, this study aims to examine the effect of CBE on preventing periodontal tissue breakdown in diabetes with orthodontic force.

Methods: A total of 25 Wistar rats were divided randomly into 5 groups, including non-diabetes, diabetes, diabetes cacao 125, 250, and 500 mg/kg BW. Diabetic rats were induced with the stratified dose of Streptozotocin, and a 30-g-force from orthodontic device was applied in all groups. Diabetes cacao group was given CBE for 7 days using a gastric probe. GCF samples were used to analyze the eNOS level through the ELISA method. NFκB, Collagen-1, and FGF-2 expression were then assessed using the immunohistochemical method, while the number of fibroblasts and blood vessels was observed using hematoxylin-eosin stained tissue. The data obtained were analyzed with one-way ANOVA and post hoc tests, with $p < 0.05$.

Results: CBE at a dose of 250 mg/kg BW significantly increased eNOS level, Collagen-1, and FGF-2 expression, and the number of fibroblasts and blood vessels in diabetes groups. Meanwhile, the treatment decreased NFκB expression in diabetes groups ($p < 0.05$).

Conclusion: This study proved that CBE increased periodontal ligament synthesis and angiogenesis and decreased inflammatory response, thereby preventing periodontal tissue breakdown in diabetic rat models with tooth movement.

1. Introduction

Hyperglycemia is widely known to accelerate periodontal tissue damage by increasing the production of reactive oxygen species (ROS) and inflammation, decreasing angiogenesis, and delaying gingival wound healing.[1] Consequently, orthodontic treatment in diabetic patients typically requires appropriate management to obtain optimal tooth movements through periodontal tissue remodeling.

Several studies have shown that orthodontic force used during treatment can cause injury to periodontal ligament, leading to the formation of pressure and tension sides. Strain in periodontal ligament also leads to a change in blood flow and hypoxia, triggering inflammation, angiogenesis, periodontal ligament remodeling, and osteogenesis.[2,3] The inflammatory response is mainly regulated by nuclear factor-kappa B (NF-κB), which is widely known for its role in osteoclastogenesis.[4] Meanwhile, angiogenesis comprises several angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and nitric oxide (NO) synthesis through the enzyme endothelial nitric oxide synthase (eNOS).[5,6] Periodontal ligament remodeling is characterized by the degradation and synthesis of

* Corresponding author. Department of Dentistry-Biomedical Sciences, Oral and Maxillofacial Pathology, Faculty of Dentistry, Universitas Jember, Jl. Kalimantan 37, Jember, 68121, Indonesia.
E-mail addresses: nuzulul@unej.ac.id (N. Hikmah), mohamadhidayat43@gmail.com (M. Hidayat), nungky.permatasari@gmail.com (N. Permatasari), aulani@ub.ac.id (A. Aulanni'am), achmad_rudijanto.fk@ub.ac.id (A. Rudijanto).

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extracellular matrix (ECM), primarily collagen, mediated by fibroblasts.[7]

According to previous studies, hyperglycemia can increase inflammatory response and disrupt tissue reorganization during orthodontic tooth movement. This condition can also lead to a decrease in the number of fibroblasts, causing impaired periodontal tissue healing and angiogenesis.[8,9] Therefore, interventions such as supporting therapy that regulates blood glucose levels, inflammatory responses, and periodontal ligament remodeling can be considered in diabetes conditions.

In line with these reports, cacao (Theobroma cacao L.) is a widely preferred and consumed commodity in various countries around the world. Cacao bean has also been reported to contain several macro and micronutrients with bioactive compounds such as methylxanthines (theobromine, caffeine) and flavanols (catechin, epicatechin, and procyanidins), which are higher compared to other plants and fruits.[10] Despite the potential of the commodity, investigations regarding its effect on diabetes with orthodontic tooth movement are limited. However, previous studies showed that phenolic compounds in cacao play a role in reducing blood glucose levels, improving glucose metabolism, modulating insulin secretion, and preventing vascular damage.[11,12] In experimental animals with orthodontic tooth movement, its administration accelerated tooth movement by increasing osteoclastogenesis on the pressure side.[13] Cacao has also been shown to play a role in healing periodontal tissue through anti-inflammatory effects by inhibiting the NFκB signaling pathway and suppressing gingival oxidative stress.[14,15] Therefore, this study aims to examine the effect of CBE in preventing periodontal tissue breakdown in diabetic rats with orthodontic tooth movements.

2. Material and methods

2.1. Sample preparation and experimental animals

Dry fermented Edel cacao beans from PT. Perkebunan Nusantara XII, Banyuwangi, East Java, Indonesia were extracted based on previous methods with few modifications.[16] This study was carried out after obtaining approval from the Ethics Committee of the Faculty of Medicine, Brawijaya University, Malang, Indonesia No. 182/EC/K-EPK/07/2022. A total of 25 male, four-month-old, and 250–300 g Wistar rats were divided into 5 groups namely nondiabetes, diabetes, as well as diabetes with 125, 250, and 500 doses of cacao extract. In addition, experimental animals were induced with Streptozotocin (SC-200719, Santa Cruz) for 5 consecutive days at doses of 40, 35, 30, 25, and 20 mg/kg BW intraperitoneally followed by incubation for 7 days.[17] Diabetes in experimental animals was characterized by blood glucose levels >300 mg/dL.

Rats were anesthetized using ketamine HCl (Ilium Ketamil Injection, Troy Laboratories Pty Ltd, Australia) and Xylazine (Xyla, Interchemie) at a dose of 10 mg/kg BW intramuscularly. A 30-g force of orthodontic appliance was applied in line with a previous study for 7 days.[17] In diabetes cacao groups, cacao extract was given orally using a gastric probe for 7 days after the orthodontic appliance was inserted. The doses of cacao extract were 125, 250, and 500 mg/kg BW.[18]

2.2. Blood glucose level measurement and gingival crevicular fluid (GCF) analysis

Blood samples for measuring blood glucose levels were taken from the tail vein using a 26Gx0.5-inch needle and quantified using a Glucometer (Easy Touch GCU). Therefore, the experimental animals were anesthetized and orthodontic appliance was removed. GCF sample in the tension and pressure sides was collected by inserting a paper point no-15 into the gingival sulcus at the tension and pressure sides for 50 s. The paper points were then placed in a microcentrifuge tube containing 350 μL phosphate buffer solution and centrifuged for 20 min at a speed of 1000×*g* and a temperature of 4°C. The supernatant was taken and placed into a new tube and stored at −80°C for eNOS level analysis. This analysis used the sandwich enzyme-linked immunosorbent assay (ELISA) method (Rat NOS3/eNOS ELISA Kit, Catalog No. E-EL-R0367, Elabscience) based on the manual kit procedures.

2.3. Histological and immunohistochemical analysis

Experimental animals were euthanized using 40 mg/kg BW of ketamine intramuscularly. Tissue samples were fixed with a 10 % buffered formalin solution for 24 h and decalcified using 14 % EDTA for 30 days at room temperature. After tissue processing histologically, the samples were cut using a microtome in a transverse direction in the selected area, namely the cervicoapical two-thirds. The samples were cut with a thickness of 5 μm and 3 μm. Slides with a thickness of 5 μm were subjected to hematoxylin-eosin staining. The number of fibroblasts and blood vessels in the tension and pressure sides were counted manually using a light microscope with 400× magnification in 3 selected fields of view and calculated as average.

Slides with a thickness of 3 μm were stained immunohistochemically to observe the expression of NF-κB, Collagen-1, and FGF-2. After being deparaffinized, the slide was immersed in a 3 % solution of hydrogen peroxide in absolute methanol for 20 min at room temperature. Subsequently, the samples were incubated with NF-κB (mouse monoclonal antibody NF-κB p65, sc-8008, Santa Cruz), Collagen-1 (mouse monoclonal antibody COL1A1, sc-293182, Santa Cruz), and FGF-2 (mouse monoclonal antibody FGF-2, sc-74412, Santa Cruz) overnight at 4°C. Incubation was carried out with a secondary antibody conjugated to an enzyme-labeled polymer (N-Histofine Simple Stain MAX PO (MULTI), Nichirei Biosciences Inc., Japan) for 20 min, chromogenic substrate for 5 min, and Mayer solution for 5 min in room temperature. Positive NF-κB and FGF-2 expression was showed by counting the number of cells in periodontal ligament, which were brown with light microscopy at 400× magnification. Meanwhile, positive Collagen-1 expression was suggested by the amount of brown extracellular tissue in periodontal ligament and calculated with Image G Software from slides photographs at 400× magnification using a light microscope connected to an Optilab camera in 3 selected fields of view in the tension and pressure sides and counted as average.

2.4. Statistical analysis

Data consisted of mean and standard deviation, while analysis was performed using SPSS Statistics 26.0 (SPSS Inc., Chicago, IL, USA) software. In addition, a one-way ANOVA test was carried out to determine the differences between groups and a post hoc test to evaluate the differences between each group with a p-value of $p < 0.05$.

3. Results

3.1. Blood glucose level of rats with orthodontic force

Based on the results, blood glucose levels in diabetes group suggested the presence of hyperglycemia with a mean of 382 mg/dL. In addition, diabetes cacao 125 and 250 groups showed normal blood glucose levels

Table 1
Blood glucose levels of rats with orthodontic force.

Groups	Blood glucose level (mg/dL)
Nondiabetes	115.2 ± 13.88[a]
Diabetes	382 ± 68.38
Diabetes cacao 125	195.2 ± 81.48[a]
Diabetes cacao 250	164.2 ± 74.56[a]
Diabetes cacao 500	204.6 ± 14.73

Note: Data are presented as mean and standard deviation.
[a] Blood glucose level categorized in normal levels (n = 5/group).

of <200 mg/dL, as presented in Table 1.

3.2. NF-κB, Collagen-1, and FGF-2 expression in periodontal ligament

The expression of NF-κB was observed in the tension and pressure sides (Fig. 1A). On the tension side, NF-κB expression in diabetes group significantly increased compared to the non-diabetes ($p < 0.05$), but the results were not significantly different on the pressure side. Meanwhile, the expression of NF-κB in diabetes group treated with CBE showed no significant difference compared to the non-diabetes group in the tension and pressure sides ($p > 0.05$) (Fig. 1C).

Collagen-1 expression was also observed in the tension and pressure sides, as shown in Fig. 1B. The expression of Collagen-1 in diabetes group decreased significantly compared to the non-diabetes ($p < 0.05$). Meanwhile, diabetes group treated with 250 and 500 CBE showed no significant difference compared to the non-diabetes group in the tension and pressure sides (Fig. 1D).

Fibroblast growth factor-2 (FGF-2) expression in the tension and pressure sides (Fig. 2A) decreased significantly in diabetes group compared to the non-diabetes ($p < 0.05$). Meanwhile, FGF-2 expression in diabetes group treated with 250 CBE showed no significant differences from the non-diabetes group on both sides ($p > 0.05$) (Fig. 2C).

3.3. eNOS levels in periodontal ligament of rats with orthodontic force

Table 2 showed that the eNOS level in the pressure and tension sides decreased significantly in diabetes group compared to the non-diabetes ($p < 0.05$). Meanwhile, eNOS levels in diabetes group treated with 250 mg/kg BW CBE did not differ from the non-diabetes group ($p > 0.05$).

3.4. Fibroblast and blood vessels in periodontal ligament of rats with orthodontic force

The number of fibroblasts and blood vessels in the tension and pressure sides (Fig. 2B) was observed through hematoxylin-eosin staining. Based on the results, there was no significant difference in the number of fibroblasts on the tension side between the non-diabetes group and diabetes cacao 250 group ($p > 0.05$). However, on the pressure side, the results differed significantly (Fig. 2D). The number of capillary blood vessels in diabetes cacao 250 group significantly

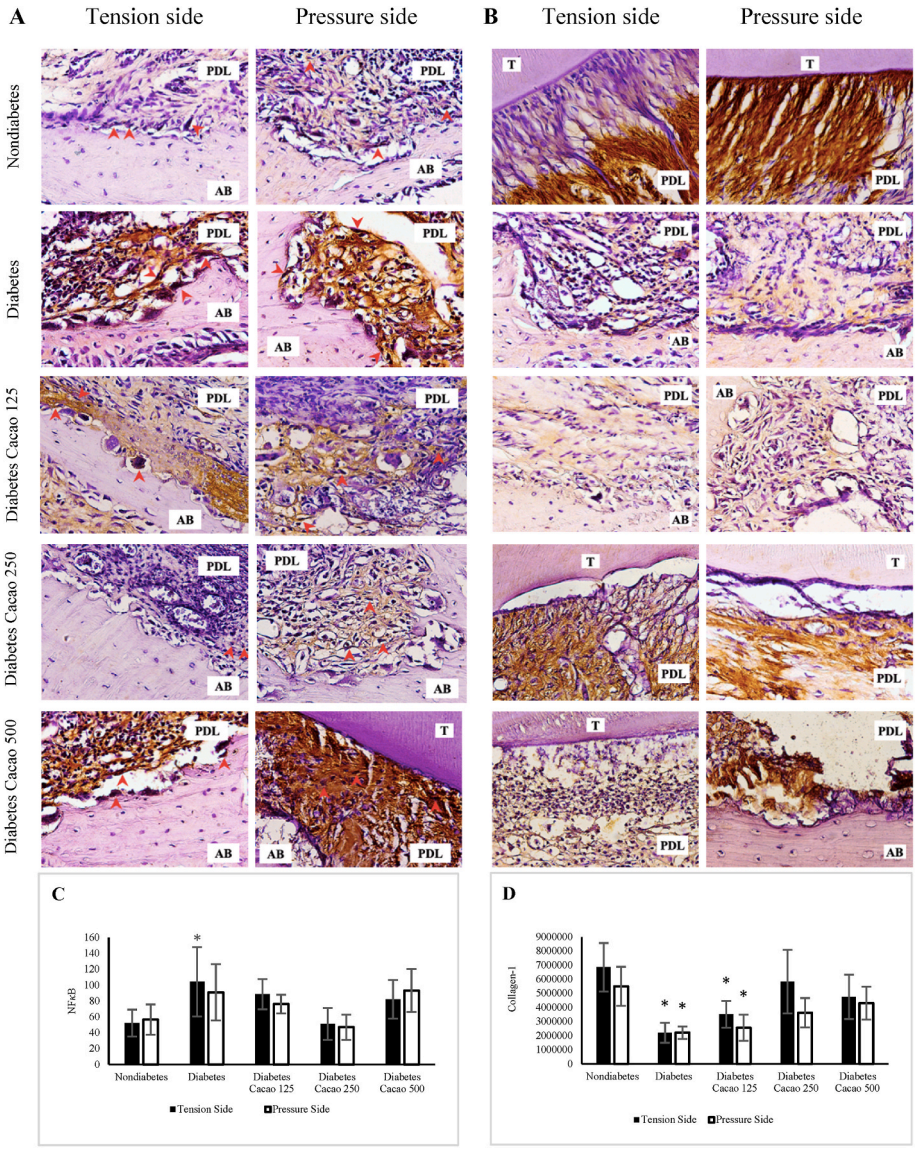

Fig. 1. The NF-κB and Collagen-1 expression in periodontal ligament of Wistar rats. (A) The NF-κB (red arrow) and (B) Collagen-1 were stained with immunohistochemistry methods and examined at a magnification of 400x using a light microscope. The expressions of (C) NF-κB and (D) Collagen-1 presented as mean and standard deviation. *$p < 0.05$, compared with nondiabetes group ($n = 5$/group). PDL: periodontal ligament, AB: alveolar bone, T: tooth, scale: 50 μm.

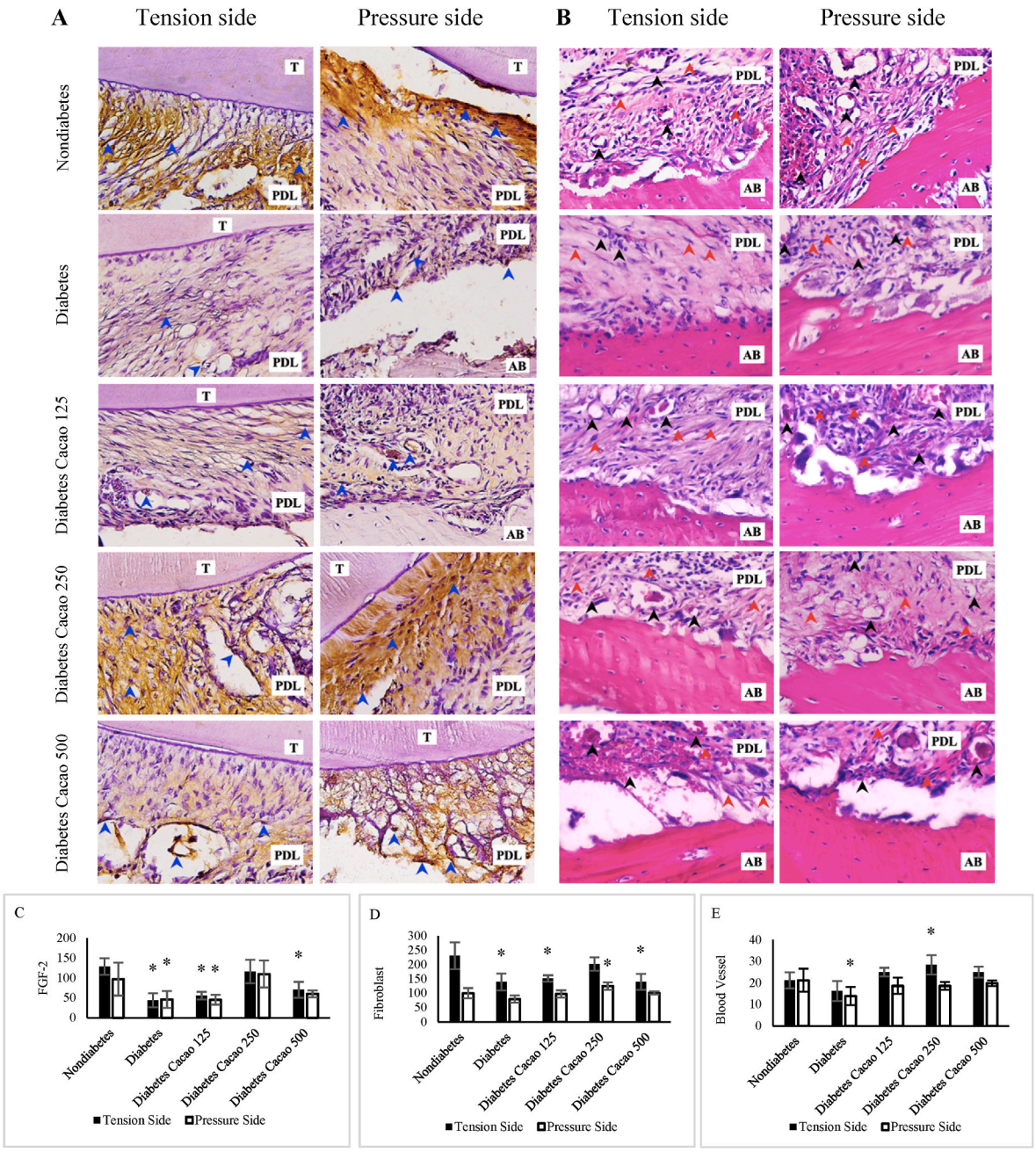

Fig. 2. The FGF-2 expression and histological image in periodontal ligament of Wistar rats. (A) The FGF-2 expressions (blue arrow) were stained with immunohistochemistry methods and (B) fibroblasts (red arrow) and capillary blood vessels (black arrow) with hematoxylin-eosin and examined at a magnification of 400x using a light microscope. (C) The FGF-2 expressions, the number of (D) fibroblasts and (E) blood vessels are presented as mean and standard deviation. *$p < 0.05$, compared with nondiabetes group (n = 5/group). PDL: periodontal ligament, AB: alveolar bone, T: tooth, scale: 50 μm.

Tabel 2
The eNOS levels in GCF of rats with orthodontic force.

Groups	eNOS levels (pg/mL)	
	Tension side	Pressure side
Nondiabetes	62.10 ± 8.50	73.22 ± 12.46
Diabetes	34.81 ± 4.42[a]	39.27 ± 2.64[a]
Diabetes cacao 125	50.45 ± 4.30[a]	58.74 ± 6.96[a]
Diabetes cacao 250	54.34 ± 6.31	63.96 ± 3.52
Diabetes cacao 500	41.40 ± 1.69[a]	50.48 ± 8.05[a]

Note: Data are presented as mean and standard deviation.
[a] $p < 0.05$, compared with nondiabetes group (n = 5/group).

increased compared to the non-diabetes ($p < 0.05$). On the pressure side, there was no significant difference between the two groups (Fig. 2E).

4. Discussion

This was the first study to evaluate the effect of CBE on preventing periodontal tissue breakdown in diabetic rat models with orthodontic forces. The application of 30 g-force of orthodontic appliance for 7 days damaged the periodontal tissue. The experimental rats were treated with cacao extract and administered systemically. The treatment caused a reduction in blood glucose levels and inflammatory response, increasing angiogenesis, and regenerating periodontal ligament of diabetic rats with orthodontic forces. Theobromine compounds in cacao beans played

an essential role in glucose metabolism.[19] Moreover, polyphenolic compounds were known to effectively inhibit the formation of advanced glycation. This occurred primarily through the inhibition of ROS formation, Schiff base, Amadori products, and the subsequent formation of dicarbonyl groups, activating the glyoxalase system, and blocking AGEs-RAGE interactions.[20]

Several studies have used diabetic experimental animal models with orthodontic force. The results obtained were consistent with previous studies stating that hyperglycemia induced an inflammatory response by increasing NF-κB expression in tension and compression sides. However, the administration of CBE decreased the expression of NF-κB on both sides. Various studies had also confirmed the effect of CBE on improving the inflammatory response in periodontal tissue through the NF-κB signaling pathway.[14] In vitro and in vivo studies showed that flavanols, epigallocatechin gallate, procyanidin, and theobromine had anti-inflammatory effects on periodontitis and inflammatory disorders.[21–23]

To assess the effect of CBE on periodontal tissue repair, a histopathological examination was conducted, and the results were analyzed quantitatively. Diabetes group showed decreased Collagen-1 and FGF-2 expression, eNOS levels, as well as the number of fibroblasts and blood vessels in the tension and compression sides. This study also showed an increase in angiogenesis, where eNOS levels and FGF-2 expression in diabetes cacao 250 group had the same profile as the non-diabetes group. The number of blood vessels on the pressure side also yielded similar results. The increase in Collagen-1 expression and the number of fibroblasts in the tension side in diabetes group with cacao administration showed periodontal ligament regeneration.

The type I collagen fibers in periodontal ligament responded to mechanical forces during tooth movement, playing a crucial role in maintaining tooth position stability.[7] Flavanol compounds in cacao beans, specifically catechins and procyanidins, contributed to decreasing the degradation of Collagen-1 in periodontal tissue. As stated in a previous study, epigallocatechin gallate played an essential role in periodontal disease healing by reducing osteoclast activity and collagen breakdown.[24] La et al. (2010) reported that administering type A proanthocyanidins in cranberry extract inhibited the degradation of periodontal tissue collagen in periodontitis.[25]

The mechanism of cacao flavanols and methylxanthines in controlling molecular pathways that regulate cell signaling and tissue function included using the activation of MAPK and PI3K/Akt pathways through several growth factors, such as IGF-1 and FGF.[26] In cacao treatment group, an increase in FGF-2 expression, as well as the number of fibroblasts and blood vessels on the tension and pressure sides showed a heightened increase in angiogenesis in the diabetic rats with orthodontic tooth movement. A previous study showed that epigallocatechin gallate from green tea extract stimulated the expression of VEGF and FGF-2 during tooth movement in Wistar rats.[27] Caffeine compounds also affected tooth movement by increasing the number of osteoclasts and blood vessels.[28]

The decrease in eNOS levels in diabetes group was observed in another study where a reduction in NO levels occurred in aging endothelial cells.[1,29] In this study, cacao extract increased eNOS levels in periodontal ligament across the diabetes group. Several supporting studies showed that flavanols and theobromine compounds in cacao and dark chocolate were associated with regulating NO.[26,30] According to investigations on endothelial cell aging and blood vessel function, epicatechin could stimulate NO levels and improve vascular function.[29]

5. Conclusion

In conclusion, this study proved that the administration of CBE reduced blood glucose levels in diabetic rats. Moreover, treatment with a dose of 250 mg/kg BW prevented periodontal ligament breakdown. The proposed mechanism comprised the regulation of inflammatory responses by reducing blood glucose levels and NFκB expression as well as increasing angiogenesis through the enhancement of eNOS levels, FGF-2 expression, and blood vessel numbers. The mechanism also comprised the repair of periodontal ligament through increased collagen-1 expression and fibroblast numbers. However, this study had limitations because it only used experimental animals treated for 7 days. Further investigations with longer interventions were needed to determine the mechanism of remodeling periodontal ligament and alveolar bone.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Statement form

The authors declare that this research used experimental animal subjects, so the author did not obtain the patient's or guardian's consent.

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