Original Article

Combination of Dental Pulp Stem-Cell Secretome and Robusta Coffee Bean Extract (*Coffea canephora*) in Enhancing Osteocalcin and Alkaline Phosphatase Expression in Periodontitis-Induced Wistar Rats

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Abstract

Introduction: Indonesia riches with many beneficial herbal ingredients; one of them is coffee obtained from Jember which has antibacterial and anti-inflammatory properties for treating periodontitis. Meanwhile, dental pulp stem cells (DPSCs) during culture secrete various advantageous secretome for tissue regeneration. This investigation intended to examine the expression of osteocalcin (OCN) and alkaline phosphatase (ALP) after the administration of the combination between DPSC secretome and the Robusta coffee bean extract (RCBE) in periodontitis-induced animal model. **Materials and methods:** Thirty-five Wistar rats were randomly selected and divided into seven groups accordingly; group K0, group K1-7 (untreated periodontitis rats for 7 days), group K1-14 (untreated periodontitis rats for 14 days), group K2-7 (periodontitis rats administered with RCBE for 7 days), group K2-14 (periodontitis rats administered with RCBE for 14 days), group K3-7 [administered with both RCBE and stem-cell secretome (SCS) for 7 days], and group K3-14 (administered with both RCBE and SCS for 14 days). Periodontitis was induced by implementing wire installed in the rat's first mandibular molar. The combination of RCBE and DPSC secretome was administered intrasulcus in the rat's first mandibular molar gingiva. Moreover, least significant difference was performed after the analysis of variance test to investigate the significant difference between groups ($P < 0.05$). **Results:** The highest OCN and ALP were expressed in group K3-14, whereas the lowest OCN expression was found in K1-7 group and lowest ALP expression was displayed in K0 group. Additionally, there was significant difference in OCN and ALP between groups. **Conclusion:** The administration of the combination between dental pulp stem-cell secretome and RCBE (*Coffea canephora*) can enhance OCN and ALP expression as documented immunohistochemically.

Keywords: *Alveolar bone, bone regeneration, herbal medicine, osteoblast, stem cell*

Introduction

Periodontitis is an inflammatory disease in the tooth-supporting tissue caused by the periodontopathogen bacteria or chronic traumatic force producing progressive damage to alveolar bone and the loss of periodontal tissue adhesions, resulting in the periodontal pocket and gingival recession.[1-4] The pathologic process of periodontal disease caused by *Porphyromonas gingivalis* is the most common bacteria in the periodontal pocket.[5] These bacteria have a virulence factor which will stimulate proinflammatory cytokines.[6] Following this, proinflammatory cytokines will increase vascular permeability and the expression of adhesion molecules such as intercellular adhesion molecule-1 and P-selectin on the surface of endothelial cells which causes polymorphonuclear neutrophilic to enter the tissue and releases lysosomal enzymes that contribute to the proliferation of osteoclast resulting in alveolar bone damage and tooth avulsion.[7]

The bone damage caused by a higher number of osteoclasts over a long time can lead to severe bone resorption. On the other hand, bone regeneration will occur when the number of osteoblasts is higher than osteoclasts.[5,8] Concurrently, the high osteocalcin (OCN) can be an indicator of

Received: 16 July 2021
Accepted: 10 November 2021
Published: 14-January-2022

How to cite this article: Martin M, Sari DS, Mantika RA, Praharani D. Combination of Dental Pulp Stem-Cell Secretome and Robusta Coffee Bean Extract (*Coffea canephora*) in Enhancing Osteocalcin and Alkaline Phosphatase Expression in Periodontitis-Induced Wistar Rats. J Orofac Sci 2021;13:136-41.

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Access this article online
Website: www.jofs.in
DOI: 10.4103/jofs.jofs_157_21
Quick Response Code:

bone regeneration which can act as a chemoattractant or chemotaxis marker of osteoblast proliferation.[9] Additionally, it was known that OCN expression is directly proportional to the rate of bone formation in rats due to osteoblast activity.[9,10] In addition to OCN, another indicator which has role in bone formation is alkaline phosphatase (ALP). ALP is secreted at the time of osteoblast differentiation. The deposition of calcium and phosphate into the ALP bone matrix has an important participation, which is to be a good indicator of the level of bone formation after bone damage.[11,12]

Earlier study by Sari *et al.* showed that the bovine tooth bone graft combined with adipose stem cell can treat any alveolar bone defect.[13] Regeneration therapy based on current research has been developed using cell-based materials, that is, dental pulp stem cells (DPSCs). DPSCs have a prominent role in regenerating the damaged tissue by regenerating the cells through their ability to proliferate and differentiate into various types of cells.[14,15]

Currently, herbal therapy is being developed due to the cheap cost and the easiness to obtain. The herbal ingredient is widely available in Indonesian, especially Robusta coffee (*Coffea canephora*) from the Coffee and Cocoa Research Center (Puslitkoka) in Jember, East Java. Coffee naturally contains caffeine, phenolic compounds, trigonelline, and chlorogenic acid (CGA) that possesses antibacterial and anti-inflammatory activities. Robusta coffee has a high caffeine content of 1.6% to 2.4%.[16] Furthermore, Robusta coffee contains about 7.0% to 10.0% CGA, whereas Arabica coffee has a CGA content of around 1.9% to 2.5%. The CGA content is higher in Robusta coffee than Arabica coffee.[17] CGA can serve as hepatitis B's antiviral, antihypertensive, antidiabetic, antioxidant, and hepatoprotector. Thus, the potential of this content can be an alternative and material for the development of new drugs.[17]

Related to this matter, the contents of CGA from Robusta coffee beans and stem-cell secretomes (SCSs) are known for stimulating the periodontal tissue stem-cell differentiation.[18-20] Additionally, the studies regarding the combination of Robusta coffee bean extract (RCBE) and DPSC secretome treating periodontitis are still limited. Based on the description above, the aim of this study is to investigate the expression of OCN and ALP after the administration of the combination between DPSC secretome and RCBE in periodontitis-induced Wistar rats (*Rattus novergicus*) as animal model.

Materials and Methods

Ethical approval for this study (Ref. Number 998/UN25.8/KEPK/DL/2020) was provided by the Ethical Committee of Medical Research at the Faculty of Dentistry in Jember University, Jember, on September 10, 2020. This type of research was laboratory experimental, with a post-test only control group design.

In the beginning, RCBE was made at the State Polytechnic of Malang by dissolving the extract in methanol. The test solution was homogenized for 30 minutes and filtered by applying 0.45 mm polytetrafluoroethylene, a filter paper, before using it. The determination of CGA in RCBE was performed by thin-layer chromatography (TLC). Silica gel plate was made from stationary phase G60 F254/TLC with a length of 8 cm and a width of 2 cm, washed with methanol, then activated in an oven at 100°C for 10 minutes. Furthermore, a total of 10 mg of the extract was dissolved in 1 mL of ethanol.[21]

Meanwhile, DPSC secretome was obtain by freshly extracting the intact human teeth (<6 hours). A combination of RCBE and DPSC secretome with a concentration of 0.125 µM was generated from the Center of Stem Cell Research and Development in Airlangga University, Surabaya. Moreover, the gel base was created by mixing sodium methyl cellulose with Aquadest. The concentration of each gel dilution contained with 75% of an active substance.[22] Then, RCBE will be formed into a gel in the similar way.

For the animal model, 35 healthy male Wistar rats aged around 12 to 14 weeks old with weight of between 200 and 250g were divided into seven groups accordingly: group K0, group K1-7 (untreated periodontitis rats for 7 days), group K1-14 (untreated periodontitis rats for 14 days), group K2-7 (periodontitis rats administered with RCBE for 7 days), group K2-14 (periodontitis rats administered with RCBE for 14 days), group K3-7 (periodontitis rats administered with both RCBE and SCS for 7 days), and group K3-14 (periodontitis rats administered with both RCBE and SCS for 14 days). General anesthesia was administered which was ketamine (0.4 mL/kg) and xylazine (0.2 mL/kg) via the intramuscular route. Furthermore, the periodontitis model was created using a 0.7-mm stainless steel wire. The wire must be in compliance with to the cervical curve of the M1 crown of rats. It was completed by inserting the wire on the mesial M1 using an artery clamp.[23] After the ligation for 2 weeks, RCBE gel with a volume of 0.05 mL was irrigated in the gingival sulcus. The combination of RCBE and DPSC secretome gel with a volume of 0.05 mL was administered in the gingival sulcus using a single dose. Finally, all samples were sacrificed with ketamine and xylazine in a lethal dose, accordingly.

After being sacrificed, mandibular bone from all the samples was generated and immersed in 10% formalin buffer solution for at least 8 hours before decalcification; thus, the tissue can be observed with no damage for the tissue fixation process. Then, the sample was decalcified using a formic acid solution for 14 days and observed to make sure there were no air bubbles occurred. The bone tissue was sliced with a microtome and deparaffinized. Sections were then incubated with the primary antibody antiosteocalcin antibody (G-5): sc-365797 and anti-ALP antibody (ab224335). The positive number of OCN and ALP expression in osteoblast at periodontal tissue was

performed by three observers in three different visual fields by employing a light microscope at 400× magnification.[24]

Additionally, the data were analyzed by applying Statistical Package for the Social Sciences 20.0 software (SPSS for Windows; SPSS, Chicago, IL, USA). The data obtained were then analyzed with the normality test using the Shapiro–Wilk test to determine whether the data were normally distributed or not, then continued with the variant homogeneity test to examine the population variations employing the Levene test ($P > 0.05$). A one-way analysis of variance (ANOVA) test then followed up with the least significant difference (LSD) test were performed to determine the significance of differences between groups ($P < 0.05$).

Results

The highest OCN was displayed in K_3-14 group, whereas the lowest OCN expression was expressed in K1-7 group [Table 1]. The results of LSD test in OCN expression revealed a significant difference ($P < 0.05$) between groups except for the K-0 group with K1-7, K1-14, K2-7, K3-7; K1-7 with K1-14, K2-7, K3-7; K1-14 with K2-7, K2-14, K3-7; and K2-7 with K2-14, K3-7 [Table 2]. Immunohistochemical features of the periodontal tissue expressed OCN in osteoblast of first molar left mandible [Figure 1].

Meanwhile, the highest ALP was presented in K_3-14 group, whereas the lowest ALP expression was exhibited in K-0 group [Table 3]. The results of LSD test in ALP expression displayed a significant difference between groups ($P < 0.05$), except for the K-0 group with K1-7, K1-14, K3-7; K1-7 with K1-14, K2-7, K2-14, K3-7; K1-14 with K2-7, K3-7; K2-7 with K2-14, K3-7; and K2-14 with K3-7 [Table 4].

Table 1: Mean number (*X?*) and standard deviation (SD) of osteocalcin expression released by osteoblasts in the mesial alveolar crest of first molar in Wistar rats

No	Group	*N*	*X?* ± SD	ANOVA
1	K-0	4	8.08 ± 1.00	0.001*
2	K1-7	4	7.91 ± 1.60	
3	K1-14	4	10.08 ± 1.52	
4	K2-7	4	9.91 ± 1.52	
5	K2-14	4	12.75 ± 1.10	
6	K3-7	4	8.75 ± 1.95	
7	K3-14	4	19.16 ± 3.89	

Group K0: control group; group K1-7, untreated periodontitis rats for 7 days; group K1-14, untreated periodontitis rats for 14 days; group K2-7, periodontitis rats administered with RCBE for 7 days; group K2-14, periodontitis rats administered with RCBE for 14 days; group K3-7, periodontitis rats administered with both RCBE and SCS for 7 days; group K3-14, periodontitis rats administered with both RCBE and stem-cell secretome for 14 days. ANOVA, analysis of variance; RCBE, Robusta coffee bean extract. *There is a significant difference ($P < 0.05$).

Immunohistochemical features of the periodontal tissue expressed OCN in osteoblast of first molar left mandible [Figure 2].

Discussion

The model of periodontitis rats in this study employed the ligation technique for 14 days. The reason for using Wistar rats is because Wistar rats have periodontal tissue characteristics that are almost the same as humans, where the periodontal tissue structure of rats has oral gingival epithelium, oral sulcular, and junctional epithelium. Wistar rats are also easy to get model periodontitis, easier to handle, and good for therapeutic models in periodontitis.[25] The results of our study after 14 days of ligation rat models were the presence of alveolar bone migration in an apical direction. This is in line with the earlier studies which stated that giving ligation with a span of 14 to 21 days will produce a chronic periodontitis model with a decrease in the alveolar crest of 1 to 1.2 mm.[25] Other studies have also proven that ligation using silk suture wires on M1, M2, and M3 mandibular rats for 15 days results in a decrease in the bone of 0.5 mm. Several other studies have proven that the 14-day ligation technique is very effective in inducing periodontitis, it is explained that periodontitis induced by ligation can also increase the likelihood of systemic disease.[25,26]

The proinflammatory cytokines should be regulated to increase wound healing rate. Mesenchymal stem cell (MSC) secretome may beneficial to control proinflammatory cytokine through enhancement of anti-inflammatory cytokines cascade. Furthermore, it is known that SCS contains a high protein, growth factors, angiogenic factors, cytokines, extracellular matrix proteins, extracellular matrix proteases, hormones, lipid mediators, and exosomes. They also contain prominent proteins in the process of osteogenic regulation towards bone cells including osteoblast cells which can trigger the bone formation process.[20,27-29]

The CGA can increase the osteogenic differentiation of human adipose tissue-derived MSCs (hAMSC) by

Table 2: Least significant difference test results of osteocalcin expression released by osteoblasts in the mesial alveolar crest of first molar in Wistar rats

Kelompok	K-0	K1-7	K1-14	K2-7	K2-14	K3-7	K3-14
K-0	–	1.000	0.159	0.159	0.002*	0.473	0.000*
K1-7		–	0.159	0.159	0.002*	0.473	0.000*
K1-14			–	1.000	0.058	0.43	0.000*
K2-7				–	0.058	0.473	0.000*
K2-14					–	0.012*	0.000*
K3-7						–	0.000*
K3-14							–

*There is a significant difference ($P < 0.05$).

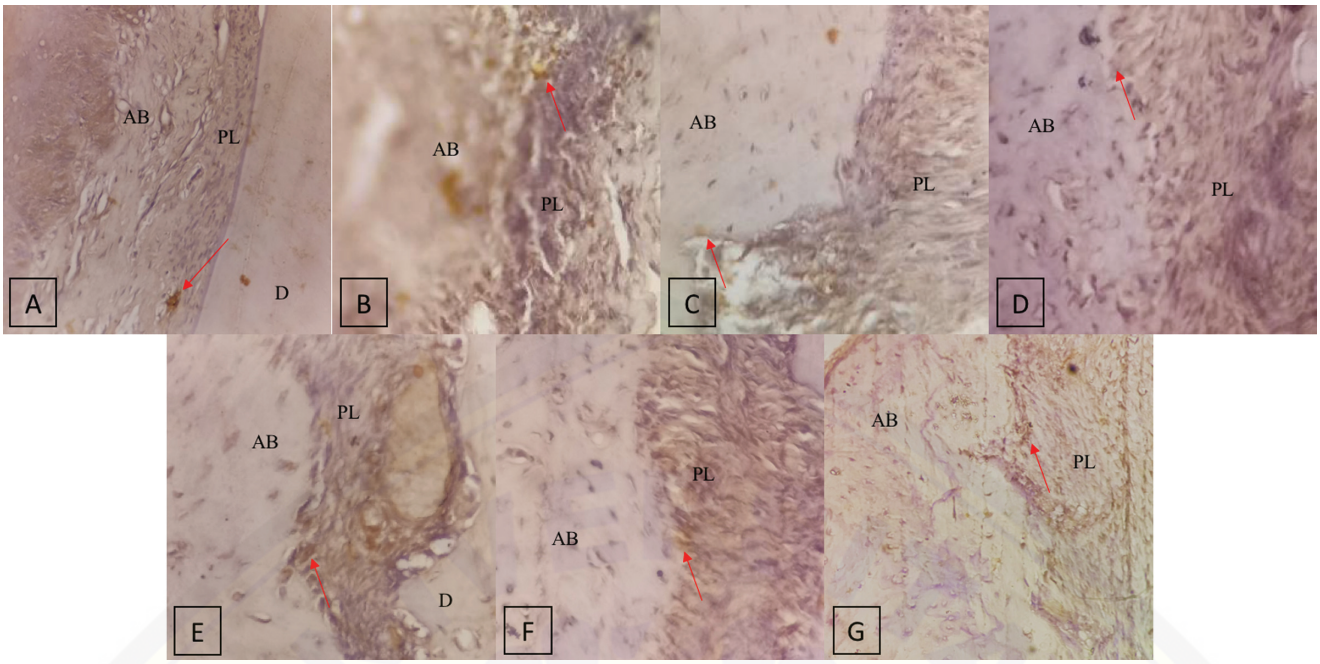

Figure 1: Histologic sections of the Wistar rats afflicted chronic periodontitis. Immunohistochemistry with monoclonal antibody (anti-rat-osteocalcin) and DAB were performed. (A) K0; (B) K1-7; (C) K1-14; (D) K2-7; (E) K2-14; (F) K3-7; (G) K3-14. The positive cells were stained brown (red arrow) with 400× magnification using a light microscope. AB, alveolar bone; D, dentin; PL, periodontal ligament;.

Table 3: Mean number (*X?*) and standard deviation (SD) of alkaline phosphatase expression released by osteoblasts in the mesial alveolar crest of first molar Wistar rats

No	Group	*N*	*X?* ± SD	ANOVA
1	K0	4	7.16 ± 1.60	0.001*
2	K1-7	4	9.08 ± 0.69	
3	K1-14	4	8.08 ± 0.69	
4	K2-7	4	10.00 ± 0.98	
5	K2-14	4	10.91 ± 2.04	
6	K3-7	4	9.33 ± 1.44	
7	K3-14	4	19.5 ± 2.33	

ANOVA, analysis of variance. *There is a significant difference ($P < 0.05$).

Table 4: Least significant difference test results alkaline phosphatase expression released by osteoblasts in the mesial alveolar crest of first molar Wistar rats

Kelompok	K-0	K1-7	K1-14	K2-7	K2-14	K3-7	K3-14
K-0	–	0.108	0.349	0.015*	0.003*	0.069	0.000*
K1-7		–	0.480	0.349	0.108	0.813	0.000*
K1-14			–	0.108	0.026*	0.349	0.000*
K2-7				–	0.480	0.480	0.000*
K2-14					–	0.165	0.000*
K3-7						–	0.000*
K3-14							–

*There is a significant difference ($P < 0.05$).

increasing the mineralization of bone tissue. This shows that RCBE can increase the potential for osteogenesis. This study expressed that the combination of CGA gel and SCS can increase the amount of OCN expression from osteoblast cells which proves the presence of alveolar bone regeneration. Meanwhile, both DPSC and its secretomes can increase osteogenic differentiation.[20,27]

Meanwhile, the inflammation that occurs in chronic periodontitis involves many inflammatory mediators [tumor necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β), etc.] and chemokines. Tissue damage can also be caused by the infiltration of leukocytes.[1-3] The activity of biologically active substances in bone resorption is influenced by cytokines that induce a local inflammatory response in the periodontium. After the inflammatory response increases, it causes the release of proinflammatory cytokines and other proinflammatory mediators such as TNF-α, IL-1, and IL-6 which further causes the inflammatory process through the RANKL-RANK-OPG pathway which stimulates bone resorption. These inflammatory cytokines inhibit osteoblast differentiation resulting in bone resorption which is a hallmark of periodontal disease.[30]

Several components in RCBE such as caffeine, volatile and nonvolatile organic acids, phenols, and aromatic compounds were reported to have antimicrobial activity. CGA and caffeic acid, which are nonvolatile organic acids found in RCBE, inhibit the growth of several gram-positive and gram-

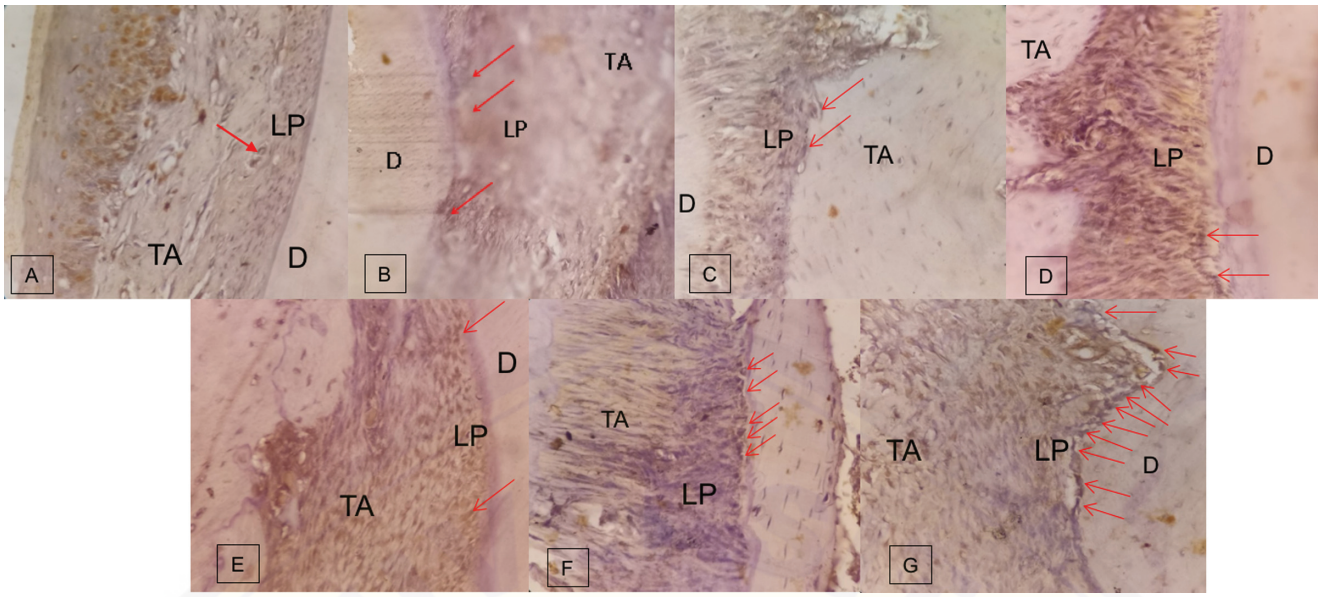

Figure 2: Histologic sections of the Wistar rats afflicted chronic periodontitis. Immunohistochemistry with monoclonal antibody (anti-rat-alkaline phosphatase) and DAB were performed. (A) K0; (B) K1-7; (C) K1-14; (D) K2-7; (E) K2-14; (F) K3-7; (G) K3-14. The positive cells were stained brown (red arrow) with 400x magnification using a light microscope. AB, alveolar bone; D, dentin; PL, periodontal ligament.

negative microorganisms. The content of coffee bean extract can increase the number of osteoblasts and decrease osteoclasts in the alveolar bone of periodontitis rats.[31] CGA from coffee can increase IL-6 synthesis in osteoblasts which can initiate the bone formation. Simultaneously, CGA can enhance osteogenic differentiation of hAMSC by increasing the mineralization in bone tissue. Therefore, this proves that CGA can increase the potential for osteogenesis.[17] The groups divide by 7 and 14 days cause the soft callus bone regeneration occur in 7 to 14 days. Another study also reported that SCS improved bone healing in a mouse model with bone damage in calvaria. A follow-up study of the *in vitro* confirmed that SCS significantly increased the early bone regeneration in calvarial defects (after 2 and 4 weeks) compared to the control group.[19] DPSCs are able to differentiate into cells such as odontoblasts, osteoblasts, adipocytes, and smooth and skeletal muscle cells.[19] Indeed, DPSC secretome has a direct therapeutic effect on the bone formation process. It is because SCS has an immunomodulatory activity that can increase various stages of bone formation.[27,28] Therefore, these findings support the results of this study that the composition in the combination of RCBE with DPSC secretomes can improve the bone regeneration but there still needed to investigate others marker.

Conclusion

The combination of RCBE and dental pulp stem cells' secretome in the gel form could increase OCN and ALP expressions in rat models of periodontitis compared to other groups as documented immunohistochemically. Nevertheless, further study is still needed to investigate others marker of alveolar bone regeneration in the periodontitis-induced animal model after the administration of combination between RCBE and dental pulp stem cells' secretome in the gel form with various examination methods.

Acknowledgments

The authors thank The University of Jember, Faculty of Dentistry, for providing laboratory resources and guidance in performing this study. The authors also thank Alexander Patera Nugraha DDS, MSc, PhD for the discussion of this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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