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*Communication*

# Preventive Effects of Chlorogenic Acid on Alveolar Bone Loss in Ligature-Induced Periodontitis in Mice

**Yuka Nishida [1], Katsumitsu Shimada [2], Kanji Horibe [3], Kousuke Seki [4], Yoshinori Murai [5], Chiharu Sogawa [6], Satoshi Murakami [2], Hiroaki Nakamura [3], Yuji Masuda [7] and Norio Sogawa [1,*]**

1 Department of Molecular Engineering and Drug Developmental Sciences, Graduate School of Oral Medicine, Matsumoto Dental University, 1780 Hirooka Gobara, Shiojiri 399-0781, Japan
2 Department of Clinical Pathophysiology, Matsumoto Dental University, 1780 Hirooka Gobara, Shiojiri 399-0781, Japan
3 Department of Oral Histology, Matsumoto Dental University, 1780 Hirooka Gobara, Shiojiri 399-0781, Japan
4 Nagano Vegetable and Ornamental Crops Experiment Station, 1066-1 Tokoo, Souga, Shiojiri 399-6461, Japan
5 Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba 305-0005, Japan
6 Department of Clinical Engineering, Faculty of Life Sciences, Hiroshima Institute of Technology, 2-1-1 Miyake, Saeki-ku, Hiroshima 731-5193, Japan
7 Division of Maxillofacial Biology, Institute for Oral Science, Matsumoto Dental University, 1780 Hirooka Gobara, Shiojiri 399-0781, Japan
* Correspondence: norio.sogawa@mdu.ac.jp; Tel.: +81-263-51-2254

**Abstract:** Chlorogenic acid (CGA) is a polyphenol that is present in coffee beans, many vegetables, and fruits. Since CGA has been reported to exert antioxidant and anti-inflammatory effects, it is expected to protect against periodontitis. In the present study, we used a ligature-induced experimental periodontitis model and investigated the beneficial effects of CGA against alveolar bone resorption caused by experimental periodontitis. To examine the inhibitory effects of CGA on bone loss, a ligature was wrapped around the maxillary right second molar, and CGA was intraperitoneally injected once a day for 2 weeks. In another experiment to investigate the restorative effects of CGA on bone loss, a ligature was wrapped around the maxillary right second molar for 2 weeks, it was then removed, and CGA was intraperitoneally injected once a day for 2 weeks. At the end of the experiments, the maxillae were removed, and CT images were taken. Alveolar bone loss was measured as the distance from the cement–enamel junction to the alveolar crest. The statistical analysis was performed using GraphPad Prism6 (Dunn's multiple comparison test). The results revealed that the ratio of the buccal alveolar bone loss (vs. the bone loss on the nonligated side) induced by ligation was significantly decreased by the administration of CGA (5 mg/kg) for 2 weeks. Moreover, the bone loss ratio on the buccal and palatal sides after 2 weeks of ligation was significantly decreased by the 2-week administration of CGA (5 mg/kg). The present results revealed that CGA exerted preventive effects against alveolar bone loss caused by experimental periodontitis.

**Keywords:** Chlorogenic acid; periodontitis; alveolar bone loss; protective effects; dentistry; periodontology



**Citation:** Nishida, Y.; Shimada, K.; Horibe, K.; Seki, K.; Murai, Y.; Sogawa, C.; Murakami, S.; Nakamura, H.; Masuda, Y.; Sogawa, N. Preventive Effects of Chlorogenic Acid on Alveolar Bone Loss in Ligature-Induced Periodontitis in Mice. *Appl. Sci.* **2023**, *13*, 4129. https://doi.org/10.3390/app13074129

Academic Editor: Andrea Scribante

Received: 23 December 2022
Revised: 20 March 2023
Accepted: 21 March 2023
Published: 24 March 2023



## 1. Introduction

Chlorogenic acid (CGA) is a polyphenol that is present in plants, particularly vegetables, fruits, and some plant seeds [1–3], such as coffee (~42.4 mg/g dry weight) [4], lettuce (~1.04 mg/g fresh weight) [5], carrot (~0.15 mg/g fresh weight) [6], burdock (~0.48 mg/g dry weight) [7], apple (~2.10 mg/g dry weight) [8], and blueberries (~0.44 mg/g fresh weight) [9]. CGA has been reported to exhibit beneficial activities, including the inhibition of blood glucose elevation [10,11], the reduction in blood pressure [12,13], and improvements in cognition [14,15], as well as anticancer [16] and antioxidant effects [17,18]. Furthermore, CGA exerts anti-inflammatory [19] and antibacterial effects [20].

Periodontitis, an inflammatory condition that affects the tissues surrounding teeth, is the main cause of tooth loss in adults and is highly prevalent worldwide [21,22]. Periodontitis is caused by periodontopathic bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*), *Tannerella forsythia* (*T. forsythia*), *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia* [23–26]. Among these bacteria, *P. gingivalis*, *T. denticola*, and *T. forsythia* are classified as red-complex bacteria because of their close involvement in the onset of periodontitis [27,28]. Periodontopathic bacteria form biofilms and produce proteolytic enzymes and leukotoxins. Their products induce an inflammatory reaction in the local tissues surrounding teeth. Osteoclasts are then activated and resorb alveolar bone [29].

A number of local approaches have been adopted against these biological events, such as the administration of antibiotics and surgery. However, each approach has some limitations, such as resistant bacteria and the invasiveness of surgery. Therefore, the use of natural products present in plants may exert beneficial effects against periodontopathic bacteria and the symptoms of periodontitis.

CGA has been suggested to prevent bone resorption in periodontitis. Kwak et al. investigated the effects of CGA on the receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclast differentiation by inhibiting RANKL signaling in an *in vitro* study and lipopolysaccharide-induced bone erosion at the femur in an *in vivo* study [30]. The effects of CGA in an *in vivo* experimental periodontitis model currently remain unclear, and only a few studies have investigated its pharmacological effects on periodontitis in a ligature-induced experimental periodontitis model [31]. Moreover, limited information is currently available on whether CGA has the ability to restore alveolar bone that had already decreased (or resorbed). Thus, there is no evidence to support the beneficial effects of CGA for patients with periodontitis [32].

Therefore, the present study examined the inhibitory effects of CGA on the development of alveolar bone loss and its reparative effects on such bone loss. The aim of this study was to confirm the pharmacological effects of CGA on alveolar bone loss in a ligature-induced experimental periodontitis model. This model has a number of advantages over other models in studying periodontal tissue and alveolar bone regeneration, such as the rapid induction of the disease and predictable bone loss [33].

## 2. Materials and Methods

### 2.1. Animals

Mice (ddY strain, male, 6-week-old, Japan SLC, Inc., Hamamatsu, Japan) were used after a one-week acclimation period in the Animal Quarters of Matsumoto Dental University. All mice were housed under a 12 h light/dark cycle at a constant temperature (23 ± 2 °C) and 50–60% humidity with ad libitum access to food and water. After ligation, the mice were fed food softened with water to prevent the ligature from coming off.

Fifty-seven mice were used. However, the mice in which the silk blade sutures threaded around the teeth came off within 2 weeks were excluded. Therefore, the number of mice in each experimental group is described in each figure.

All experimental procedures were conducted in accordance with the Guidelines for Animal Experiments of Matsumoto Dental University and were approved by the Animal Care and Use Committee of Matsumoto Dental University. Briefly, all experimental treatments were carefully performed to not inflict pain on the mice, and the number of mice used was limited to the minimum needed in the control and experimental groups. The minimum sample size was set to five and calculated assuming the ligature residual rate of 60%.

### 2.2. Chemicals

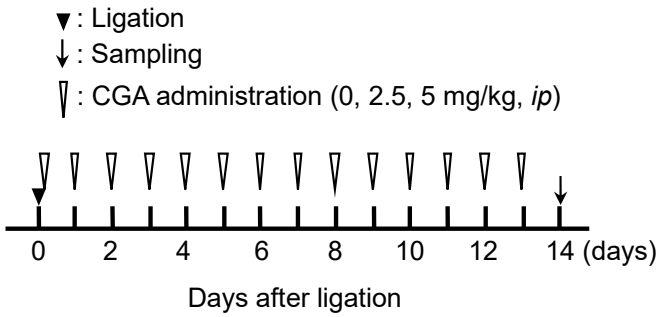
CGA (Chlorogenic acid) was purchased from MP Biomedicals (Irvine, CA, USA). Pentobarbital sodium (Somnopentyl) was obtained from Kyoritsu Seiyaku, Co. (Tokyo, Japan). The tartrate-resistant acid phosphatase (TRAP)/alkaline phosphatase (ALP) stain

kit (TRAP/ALP Stain Kit), eosin (1% Eosin Y Solution), and paraformaldehyde were supplied by FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Hematoxylin (HX87717419) was purchased from Merck & Co. (Kenilworth, NJ, USA). Tetra-sodium ethylenediaminetetraacetate and di-sodium dihydrogen ethylenediaminetetraacetate dihydrate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

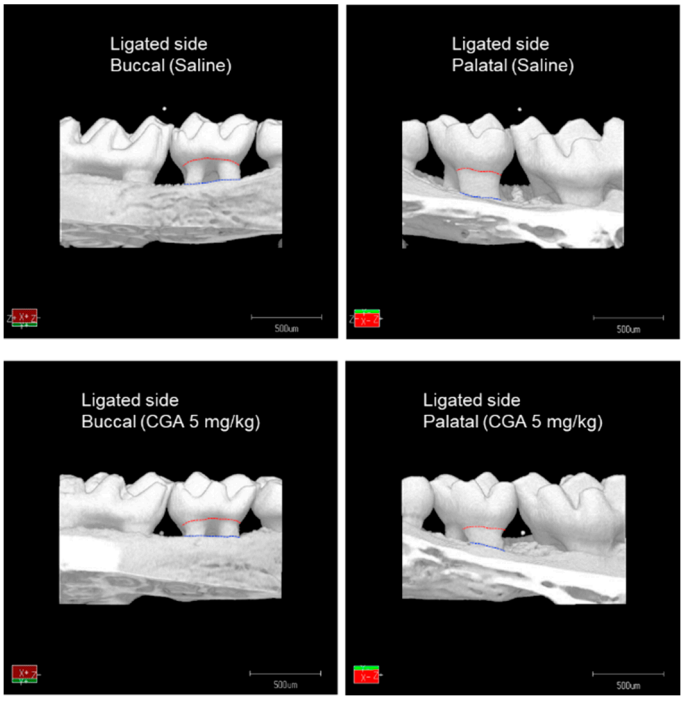
### *2.3. Creating a Ligature-Induced Periodontitis Model and Chemical Administration*

The mice were anesthetized with pentobarbital (50 mg/kg) by an intraperitoneal (*ip*) injection. The placement of the ligature was performed according to the method of Abe et al. [34]. Under anesthesia, the mouths were kept open, and a 7–0 silk blade suture (Natsume Seisakusyo Co., Ltd., Tokyo, Japan) was threaded around the right maxillary second molar for 2 weeks to induce periodontitis.

In the experiments to investigate the inhibitory effects of CGA, the mice were administered saline (0.1 mL/10 g) or CGA (2.5 and 5 mg/kg, 0.1 mL/10 g, CGA was dissolved in saline) immediately after ligation. Saline and CGA were administered once a day for 2 weeks by an *ip* injection. The experimental design is shown in Figure 1A.

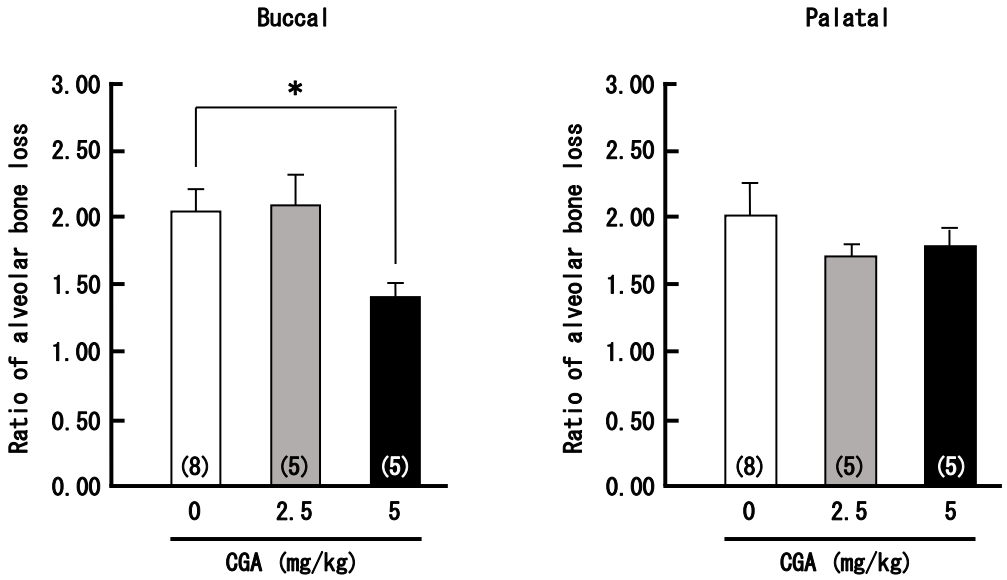



(**A**)




(**B**)

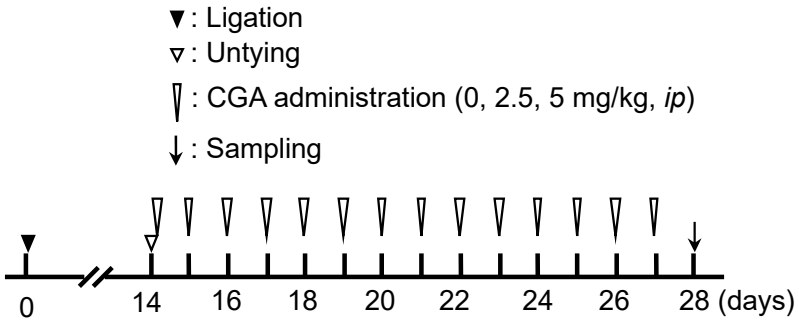
**Figure 1.** *Cont.*




(**C**)

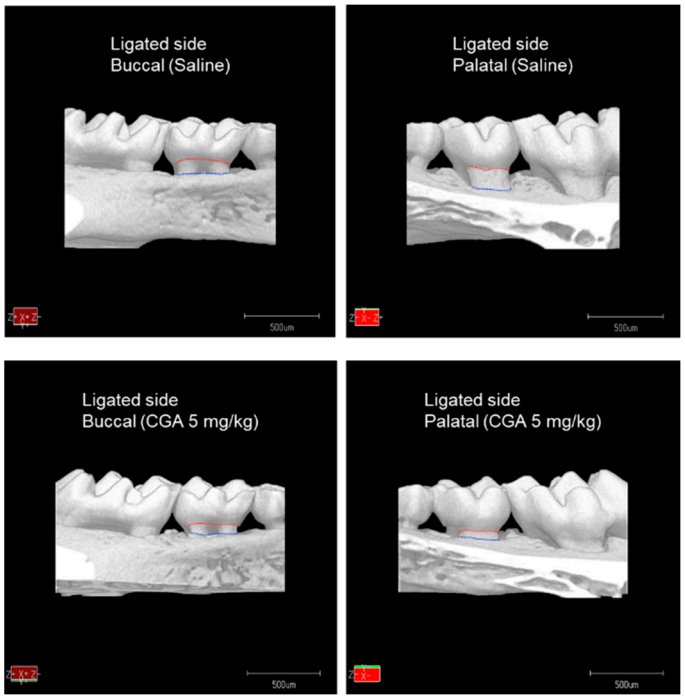
**Figure 1.** (**A**) Experimental design to examine the inhibitory effects of CGA on bone loss in the ligature-induced periodontitis model. (**B**) 3D images of the inhibitory effects of CGA on bone loss in the ligature-induced periodontitis model. The CEJ and ABC were marked using a red line and a blue line, respectively. (**C**) The inhibitory effects of CGA on bone loss in the ligature-induced periodontitis model. Data are expressed as the ratio of the CEJ–ABC distance (CEJ–ABC distance on the ligated side/CEJ–ABC distance on the nonligated side). Each value shows the mean ± S.E., $n = 5$–8. * $p < 0.05$ (significantly different from the CGA 0 mg/kg [saline] group using the Kruskal–Wallis test followed by Dunn's multiple comparison test).

In the experiments to examine the restorative effects of CGA on bone loss, ligation was performed for 2 weeks, and mice were then administered saline (0.1 mL/10 g) or CGA (2.5 or 5 mg/kg or 0.1 mL/10 g) immediately after the ligature was removed. CGA was dissolved in saline and administered once a day for 2 weeks. The experimental design is shown in Figure 2A.

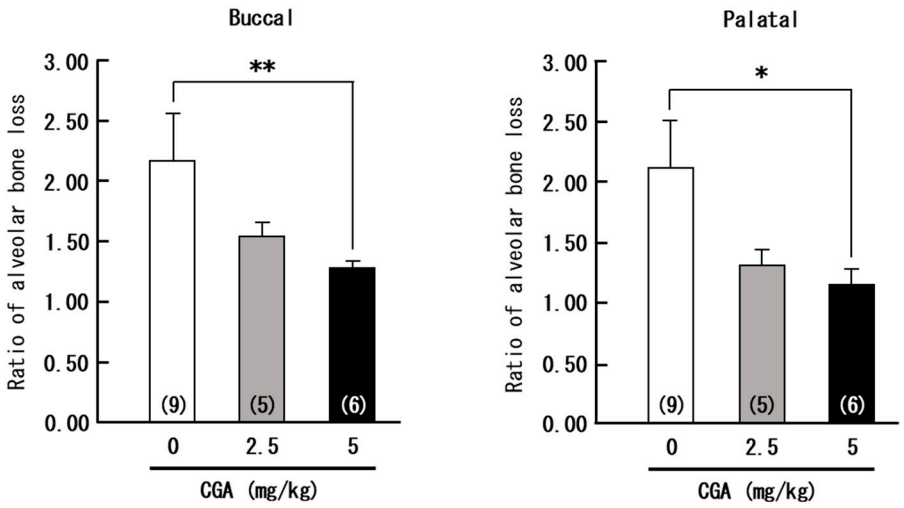



(**A**)

**Figure 2.** *Cont.*




(**B**)




(**C**)

**Figure 2.** (**A**) Experimental design to examine the restorative effects of CGA on bone loss in the ligature-induced periodontitis model. (**B**) 3D images of the restorative effects of CGA on bone loss in the ligature-induced periodontitis model. The CEJ and ABC were marked using a red line and a blue line, respectively. (**C**) The restorative effects of CGA on bone loss in the ligature-induced periodontitis model. Data are expressed as the ratio of the CEJ–ABC distance (CEJ–ABC distance on the ligated side/CEJ–ABC distance on the nonligated side). Each value shows the mean ± S.E., $n$ = 5–9. * $p < 0.05$, ** $p < 0.01$ (significantly different from the CGA 0 mg/kg [saline] group using the Kruskal–Wallis test followed by Dunn's multiple comparison test).

### *2.4. Microcomputed Tomography (μCT) Analysis*

Perfusion fixation with 4% paraformaldehyde (PFA) was performed on mice under anesthesia, and the maxillae were immediately dissected. The fixed maxillae were scanned using microfocus X-ray CT (ScanXmate-A080; Comscantecno, Yokohama, Japan), and two-dimensional (2D) images of the buccal and palatal sides were generated using the bone

analytic software TRI/3D-BON (Ratoc System Engineering, Tokyo, Japan). The μCT was set to 30 kilovolts with 250 microamperes and yielded a series of 480 consecutive slices.

The distance from the cement–enamel junction to the alveolar bone crest (CEJ–ABC distance, Figure S1) was measured by ImageJ (version 1.52a, NIH, Bethesda, MD, USA, open-source software, https://github.com/imagej/ImageJ (accessed on 18 June 2015) to estimate the alveolar bone loss on the 2D images. The CEJ–ABC distance analyzed was the average of 3 measurements at the center of the mesial root of the second molar on the buccal side and at the center of the palatal root of the second molar on the palatal side.

### 2.5. Histological Analysis

In addition to perfusion fixation, the maxillae were fixed in 4% PFA at 4 °C overnight and then transferred to a decalcifying solution with 10% ethylenediaminetetraacetic acid (pH 7.0) for 4 weeks. The maxillae were embedded in paraffin, and serial sections (thickness of 5 μm) were prepared in the sagittal plane and were stained with hematoxylin and eosin (HE). TRAP staining and ALP staining were performed to observe osteoclasts and osteoblasts, respectively, according to the manufacturer's instructions.

### 2.6. Statistical Analysis

The data were expressed as the mean ± standard error. The statistical analysis was performed using GraphPad Prism (version 6, GraphPad Software, San Diego, CA, USA). Differences between the control and each CGA group were compared using the Kruskal–Wallis test followed by Dunn's multiple comparison test. Differences were considered to be significant at $p < 0.05$.

## 3. Results

### 3.1. Inhibitory Effects of CGA on Alveolar Bone Loss in the Ligature-Induced Periodontitis Model

The ratio of the CEJ–ABC distance (CEJ–ABC distance on the ligated side/CEJ–ABC distance on the nonligated side) was significantly decreased on the buccal side (CGA 0 vs. CGA2.5: $p > 0.999$, CGA 0 vs. CGA5: $p = 0.0167$) but not on the palatal side (CGA 0 vs. CGA2.5: $p = 0.3720$, CGA 0 vs. CGA5: $p = 0.4672$) by the administration of 5 mg/kg CGA (Figure 1B,C).

In the HE-stained sections (Figure S2A,B), the ABC was lower in the control group than in the CGA 5 mg/kg group. Furthermore, the number of osteoclasts was higher in the control group than in the CGA 5 mg/kg group (Figure S2C,D); however, the number of osteoblasts did not significantly differ between the two groups (Figure S2E,F).

### 3.2. Restorative Effects of CGA on Alveolar Bone Loss in the Ligature-Induced Periodontitis Model

The ratio of the CEJ–ABC distance was significantly decreased on the buccal (CGA 0 vs. CGA2.5: $p = 0.2301$, CGA 0 vs. CGA5: $p = 0.0012$) and palatal (CGA 0 vs. CGA2.5: $p = 0.1622$, CGA 0 vs. CGA5: $p = 0.0103$) sides by the administration of 5 mg/kg CGA (Figure 2B,C).

## 4. Discussion

Some polyphenols have been reported to exert potent inhibitory effects on inflammation and osteoclast activity [35]. For example, ellagic acid in berries, pomegranates, and nuts exhibited anti-inflammatory activity in various organs and tissues [36–39] as well as anti-osteoclast activity [35].

CGA, a polyphenol, also exerts anti-inflammatory [19], anti-osteoclast [30], and antibacterial effects [20]. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CGA against *P. gingivalis* were previously reported to be 4 and 16 mg/mL, respectively [40]. Among the red-complex bacteria considered to be closely involved in the onset of periodontitis, *P. gingivalis* has attracted the most attention because it has frequently been detected in chronic periodontitis, produces many types of proteases, such as collagenase and gingipains, and plays a role in gingival inflammation and alveolar

bone resorption [41]. However, we used 1 mg/mL CGA solution for systemic administration in the present study. Since this concentration of CGA was lower than the MIC and MBC against *P. gingivalis*, the direct effects of the CGA on the periodontopathic bacteria may not have been significant.

On the other hand, CGA has been reported to inhibit the NF-kappa B signaling pathway and promote the Nrf2/HO-1 antioxidant pathway [42–45]. Therefore, the anti-inflammatory activity of CGA [46,47] may have been further enhanced by its systemic administration in the present study because Nrf2 has been identified as a negative regulator of NF-kappa B.

We herein demonstrated that CGA exerted preventive effects against alveolar bone loss in a ligature-induced experimental periodontitis model. As shown in Figure S2, the decreases in the number of osteoclasts may be attributed to the inhibition of osteoclast differentiation, as demonstrated by Kwak [30].

We also found that CGA promoted alveolar bone repair (Figure 2B,C). The pharmacological effects of CGA during the convalescent phase after the removal of ligatures in a ligation-induced experimental periodontitis model currently remain unknown. In the present study, the two-week administration of CGA after the removal of ligatures decreased the ratio of the alveolar bone loss. This indicates that CGA restored alveolar bone loss more than the control treatment. However, since we did not find a significant difference between the CGA (5 mg/kg) administration group and the saline administration group in the number of osteoblasts (Figure S2), the observed decrease in the bone loss ratio may be attributed to the shift in the balance of the bone metabolism in which the suppression of osteoclasts led to the predominance of osteoblast activity.

The limitation of this study is that the data were phenomenal contents, and the mechanisms underlying the reported phenomenon remain unclear. However, only a few studies have investigated the effects of CGA on periodontitis in a ligature-induced experimental periodontitis model [29]. Moreover, the present study is the first to suggest its beneficial effects on alveolar bone that had already decreased (or resorbed). The results obtained will contribute to the development of treatments for periodontitis.

The content of CGA in ordinary plants, such as vegetables and fruits, may not be sufficient to exert antiperiodontitis effects. However, materials in vegetables and fruits are considered to be less harmful to the living body than other chemicals, such as antibacterial agents and anti-inflammatory medicines. Therefore, the topical application of a treatment using a mixture with base materials is appropriate for periodontitis [31]. In addition to the plant-derived ingredients, the beneficial actions of live microorganisms and their metabolic products, such as probiotics and postbiotics [48–50], have been attracting public attention due to the increase in health consciousness. For example, Butera et al. reported that the postbiotics-based material was effective for the domiciliary treatment of periodontitis [50]. Therefore, the combined application of CGA and substances associated with microorganisms might be more effective in improving the symptoms of periodontitis. Further studies are needed to clarify the mechanisms responsible for the beneficial effects of CGA on alveolar bone loss prior to its clinical application.

## 5. Conclusions

The present study showed the inhibitory effects of CGA on periodontitis and the symptoms associated with periodontal diseases in a ligature-induced experimental periodontitis model and suggested its beneficial effects on resorbed alveolar bone. The results obtained herein demonstrated that CGA prevented the development of alveolar bone loss in ligature-induced experimental periodontitis, which may be attributed to its inhibitory effects on osteoclasts.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13074129/s1, Figure S1: The cement-enamel junction (CEJ) and alveolar bone crest (ABC). Figure S2. Inhibitory effects of CGA on bone loss in the ligature-induced periodontitis mode.

**Author Contributions:** Conceptualization, K.S. (Kousuke Seki) and N.S.; methodology, S.M. and H.N.; validation, S.M. and H.N.; formal analysis, Y.M. (Yoshinori Murai); investigation, Y.N., K.S. (Katsumitsu Shimada), K.H., and N.S.; resources, K.S. (Kousuke Seki) and Y.M. (Yoshinori Murai); data curation, C.S.; writing—original draft preparation, N.S. and C.S.; writing—review and editing, K.H., Y.M. (Yoshinori Murai), and K.S. (Kousuke Seki); visualization, K.S. (Katsumitsu Shimada) and S.M.; supervision, N.S.; project administration, Y.M. (Yuji Masuda); funding acquisition, Y.N., K.S. (Kousuke Seki), Y.M. (Yoshinori Murai), and N.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by a Grant-in Aid for Scientific Research from the Nagano Society for the Promotion of Science (Grant No. NPS2020318).

**Institutional Review Board Statement:** The animal study was approved by the Animal Care and Use Committee of Matsumoto Dental University (No. 258-14), and conducted in accordance with the Guidelines for Animal Experiments of Matsumoto Dental University.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data is contained in Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflict of interest.

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