

The effects of shark liver oil on fibroblasts and collagen density in the periodontal ligaments of Wistar rats induced with *Porphyromonas gingivalis*

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ABSTRACT

Background: Periodontitis is an infection in tooth-supporting tissues caused by a specific microorganism, *Porphyromonas Gingivalis* (PG), which can trigger collagen destruction. Generally, periodontal therapy employs a combination of mechanical (scaling root planning/SRP) and chemical (antibiotics) remedies, the latter of which can cause bacterial resistance. On the other hand, shark liver oil contains active natural ingredients such as alkylglycerols, squalene, squalamine, and omega-3, which have antibacterial and antioxidant effects. **Purpose:** This study aims to determine the impact of shark liver oil on fibroblasts and collagen density in the periodontal ligament of Wistar rats induced with PG. **Methods:** This study represents a laboratory experiment with post-test only control group design. The research subjects consisted of 35 Wistar rats divided into five groups, namely; a negative control group (K-); a positive control group with PG induction (K+); and three treatment groups induced with PG and shark liver oil once a day for seven days at varying doses of 0.2 g/gBB (P1), 0.3 g/gBB (P2), and 0.4 g/gBB (P3). Following treatment, the subjects were euthanized. The number of fibroblasts was then histologically examined with Hematoxylin Eosin (HE). Meanwhile, the collagen density was histologically analyzed with Masson's Tricrome. Fibroblast cells were observed through a microscope at 400x magnification. Data was statistically analyzed with a one-way ANOVA and post hoc LSD. Collagen Density scoring was then performed. The results were analyzed with a non-parametric Kruskal-Wallis test ($p=0.05$), and subsequently with a Mann-Whitney U test ($p<0.05$). **Results:** The number of fibroblasts in the periodontal ligament areas of each group were 18.6 ± 1.21 for K-; 12 ± 1.26 for K; 16.8 ± 1.72 for P1; 17.1 ± 1.94 for P2; and 23.16 ± 2.78 for P3. The results also indicated that there were significant differences between K- with K+ and P3, K+ with P1, P2, and P3, as well as P3 with P1 and P2. However, there was no significant difference between K- and P1 and P2 or P1 and P2. The results showed that collagen density in the negative control group did not significantly decrease compared to that in the positive control group in which PG was induced. Meanwhile, collagen density in all three treatment groups following doses of 0.2 g/gBB, 0.3 g/gBB, and 0.4 g/gBB being administered significantly increased compared to that in the negative control group and the positive control group subjected to PG induction. **Conclusion:** Shark liver oil can significantly increase fibroblast cells and collagen density in the periodontal ligament of Wistar rats induced with PG.

Keywords: collagen density; fibroblast; periodontitis induced with *Porphyromonas gingivalis*; shark liver oil

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INTRODUCTION

Various oral and dental diseases have recently been found to be widespread among Indonesians. Based on National Basic Health Research statistics issued by the Indonesian Ministry of Health in 2018, the prevalence of such diseases within the country increased from 25.9% in

2013 to 57.6% in 2018.¹ Periodontitis is an infection that occurs in tooth supporting tissues caused by a specific microorganism which triggers progressive damage in periodontal ligaments and alveolar bone resorption.² The dominant bacteria found in chronic periodontitis are gram-negative anaerobic varieties such as *Porphyromonas gingivalis*.³

Porphyromonas gingivalis produces various pathogenic virulence factors such as lipopolysaccharide (LPS).³ LPS are then recognized by Toll-like receptors-4 (TLR-4) present in adjacent cells, namely; Junctional Epithelium (JE), Macrophages, and dendritic cells.⁴ Macrophages function to secrete anti-inflammatory mediators, pro-inflammatory mediators, and growth factor. The activated TLR 4 pathway can subsequently affect the release of pro-inflammatory mediators including IL-1, IL-8, IL-12, and TNF- α .⁵ TNF- α and IL-1 causing neutrophils and monocytes to be attracted to the site of bacterial invasion.⁴ Furthermore, untreated periodontitis can cause periodontal pocket formation, damage to periodontal ligaments, and alveolar bone density changes.⁶ Certain types of collagen responsible for maintaining tissue structure¹ are present in the periodontal ligaments, the most common of which are type I collagen (80%) and type III collagen (20%).^{7,8}

Current periodontitis therapy usually employs a combination of mechanics, including scaling root planning, and chemicals such as antimicrobials.² Unfortunately, at the specified dose, the use of antimicrobials, especially antibacterials (antibiotics), is ineffective, while the passage of time can cause resistance. As a result, several experts conducted various studies to identify an antibacterial in order to overcome the problem.^{9,10} However, the wound healing process is influenced by local and systemic factors. Hence, periodontitis therapy is expected to not only eliminate disease-causing bacteria (local factors), but also to suppress damage to the host cell inflammatory response components (systemic factors).¹¹

Sharks can be found in almost all Indonesian waters, be they territorial, oceanic, or the country's Exclusive Economic Zone (EEZ).¹² *Centrophorus moluccensis* is a type of shark whose liver oil is commonly extracted for subsequent use for medicinal purposes.¹³ Previous research conducted by Agustina (2015)¹⁴ indicated that shark liver oil has inhibitory properties in relation to *Porphyromonas gingivalis* bacteria. The alkylglycerol contained in sharks can even protect the structure and function of white blood cell membranes and macrophages.¹⁵ Macrophages play a role in the phagocytosis of bacteria and secrete anti-inflammatory cytokines, thus triggering fibroblast proliferation and collagen synthesis.¹⁶ In addition, another investigation conducted by Hafez *et al.* (2011),¹⁷ argued that the safe dose of orally-administered shark liver oil for rats is one of 1,000-2,000 mg/kg/day. Therefore, this study aims to reveal the effects of shark liver oil on fibroblast cells and collagen density in the periodontal ligaments of rats induced with *Porphyromonas gingivalis* at certain doses, namely; 0.2 g/g BW, 0.3 g/g BW, and 0.4 g/g BW, as an supplementary therapy for seven days.

MATERIALS AND METHODS

This study was approved by the Dental Research Ethics Commission of Hang Tuah University (certificate number:

EC/008/KEPK-FKGUHT/VII/2019). Its conduct followed the guidelines of the Dental Research Ethics Commission, Universitas Hang Tuah and constituted a true laboratory experiment incorporating post-only group design. The research subjects comprised 35 rats aged 4-6 months and weighing 200-250 grams which were divided into five groups, namely; a negative control group (K-) which received no treatment; a positive control group (K+) induced with *Porphyromonas gingivalis*, but without shark liver oil; and three treatment groups induced with *Porphyromonas gingivalis* and shark liver oil at specific doses, namely; 0.2 g / g BW (P1); 0.3 g / g BW (P2); and 0.4g / g BW (P3).

Wistar rats were acclimatized to the experimental laboratory in cages with sufficient air and light for seven days.¹⁸ On the eighth day, all groups of subjects were given 20 mg of kanamycin and 20 mg of ampicillin in drinking water. Their oral mucosa was then smeared with chlorhexidin gluconate 0.12% once daily for four days.¹⁹ On day 12, Groups 2, 3, 4, and 5 were orally induced with up to 2 ml of 1×10^9 CFU/ml of *Porphyromonas gingivalis* bacteria using feeding tubes three times in four days at 32-hour intervals, before being applied topically to their oral cavity and anus in the colorectal section using a cotton bud or microbrush. The subjects were subsequently incubated for three weeks following the first induction. Periodontitis is characterized by a change in the periodontal tissue during which blood cells from the vasa migrate into the extravagant tissue as part of the healing process.^{20,21}

After three weeks, the Wistar rats in Groups 3, 4, and 5 were orally fed shark liver oil (*Centrophorus Sp* obtained from factory X) at various concentrations for seven days. On the 41st day, all the subjects were sacrificed and their mandibles extracted. These were then fixed in a formalin buffer solution (PBS pH 7.0 and formalin 10%). The number of fibroblasts was observed with HE staining technique, while collagen density was examined by means of MT painting technique.

Fibroblast cells in the periodontal ligament were examined with a microscope from three fields of view and their number calculated based on an average magnification of 400x. Fibroblast cells appeared with a large chromatic

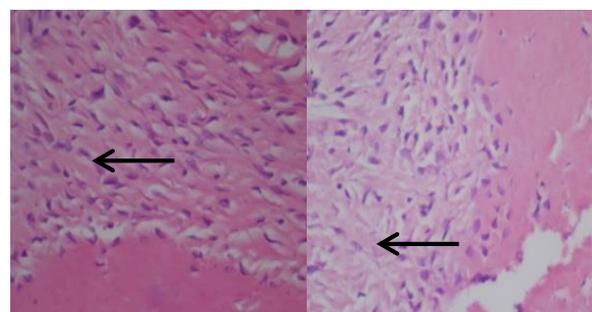


Figure 1. Histopathological picture of fibroblasts in a periodontal ligament at 400x magnification. Fibroblast cells are indicated by black arrows.

nucleus and eosinophilic or spindle-shaped cytoplasm. Meanwhile, the assessment of collagen density in periodontal ligaments was observed from five fields of view using an Olympus cx 22 light no.5 microscope at 400x magnification. MT staining produced a light blue or greenish blue colour in type 1 collagen and red in the core, keratin, and cytoplasm. All research data was then tabulated and analyzed statistically with a one-way ANOVA test to calculate the number of fibroblasts, followed by a Kruskal Wallis test. Meanwhile, a Mann-Whitney test was performed to assess collagen fiber density using 2016 SPSS version 23.

RESULTS

The examination results of fibroblasts with HE painting were examined by two observers with their mean values being subsequently calculated. Figure 1 shows the results of HE painting on the mandibular after decalcification. Moreover, based on the calculation results of the mean number of fibroblast cells, the highest number of fibroblasts

was found in Group P3 which had been administered a 0.4 g / g BW dose of shark liver oil (Figure 2). The data was analyzed with a one-way ANOVA test the results of which revealed that there were differences ($p < 0.05$) within each group. A post-hoc LSD test was then performed to analyze differences between the groups.

Based on the results of the LSD post-hoc test (Table 1), there were no significant differences between K- with P1 and P2, or between P1 and P2 ($p > 0.05$). The observation results of collagen density based on the data in Figure 3 were as follows: the highest collagen density value occurred in Group P3 which had received shark liver oil at a concentration of 0.4 g / g BW. Histochemistry result of collagen density was shown in Figure 4. Collagen density was assessed by two observers with a value mode to identify the highest score. The second and third highest collagen density values were found in Group P2 and Group K, while the lowest was that in Group K+ which had been induced only with Pg bacteria. The data obtained was then statistically analyzed with a Kruskal Wallis test. Since the significance value obtained was 0.001 ($p < 0.05$), a Mann-Whitney analysis was completed to identify the differences between the groups.

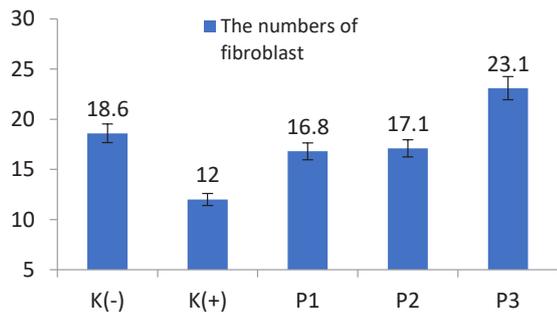


Figure 2. The graph of the mean number of fibroblast cells in periodontal ligament suffering from periodontitis in each group.

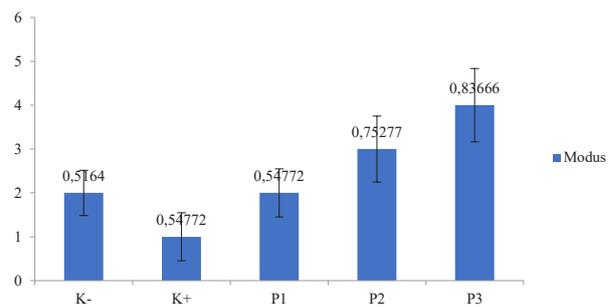


Figure 3. A graph of collagen density.

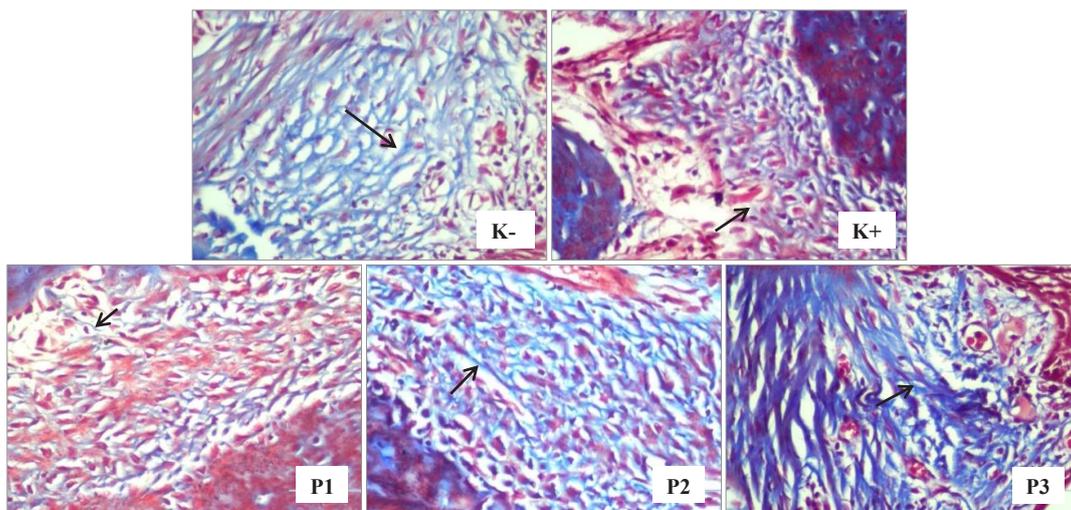


Figure 4. The results of collagen fibrous density preparations in periodontal ligaments at 400x magnification using MT staining (collagen fibers are indicated by black arrows).

Table 1. Results of post-hoc LSD test on the mean number of fibroblasts in periodontal ligament suffering from periodontitis

| Groups/Mean | K+ | P1 | P2 | P3 |
|-------------|-------|-------|-------|-------|
| K- (18.6) | .000* | .103 | .178 | .000* |
| K+ (12) | | .000* | .000* | .000* |
| P1 (16.8) | | | .761 | .000* |
| P2 (17.16) | | | | .000* |

Note *: p<0.05

Based on the results of Mann-Whitney (Table 2), no significant difference existed between Group K- and Group K+ (p=0.575). However, there was a significant difference between Group K- and Group P1(0.030), Group P2(0.016), and Group P3(0.007). Similarly, a significant difference was identified between Group K+ and Group P1 (0.019), Group P2 (0.011), and Group P3 (0.006). In contrast, there was no significant difference between Group P1 and Group P2 (0.423). While a significant difference existed between Group P1 and Group P3 (0.042), there was none between Group P2 and Group P3 (0.147).

DISCUSSION

The bacterial induction procedure for *Porphyromonas gingivalis* in this study was conducted for three weeks based on previous research by Praptiwi (2008)²² and Mulawarmanti et al. (2014).²³ The condition of periodontitis is detected by an anatomic pathological examination of periodontal tissue that shows the existence of extravation. Periodontitis is characterized by changes in periodontal tissue that indicate the migration of blood cells from the vasa to the extravagant tissue as part of the healing effort.²⁴

Shark liver oil was applied orally to the Wistar rats (*Rattus norvegicus*) in the treatment groups which had been induced with *Porphyromonas gingivalis* bacteria potentially triggering collagen destruction. The incidence of such destruction was found in Group K+ induced with *Porphyromonas gingivalis* bacteria since the results of collagen density in this group confirmed that the number of fibroblast cells was the lowest.

The LPS component in the outer membrane layer of the *Porphyromonas gingivalis* bacterium will usually bind to the periodontal tissues, thereby causing the release of TRL-4.^{5,25} The activation of TRL-4, in turn, triggers the release of pro-inflammatory mediators, such as IL-1 and TNF- α , with the result that it influences the attempts of the host to fight infection by activating immune cells, for example; PMNs, macrophages, and lymphocytes.^{25,26} Excessive PMN activity can increase the ROS to levels that damage various cell mechanisms. Such harm can cause the destruction of gingival tissue, periodontal ligaments, and alveolar bone.^{25,27}

The results of this study show that the number of fibroblast cells in the Group K members, i.e normal rats,

Table 2. The results of a Mann-Whitney test

| | K+ | P1 | P2 | P3 |
|----|------|-------|-------|-------|
| K- | .575 | .030* | .016* | .007* |
| K+ | | .019* | .011* | .006* |
| P1 | | | .423 | .042* |
| P2 | | | | .147 |

was not significantly different to that in the treatment groups. This means that the administration of shark liver oil can render the condition of the subjects induced with *Porphyromonas gingivalis* bacteria normal again, as those in Group K-. Furthermore, this also indicates that its provision can prevent further damage by stimulating fibroblast cell regeneration.

The results of this study also reveal that a significant increase in the number of fibroblast cells occurred in those subjects induced with *Porphyromonas gingivalis* and subsequently treated with shark liver oil at doses of 0.2 g / g BW, 0.3 g / g BW, and 0.4 g / g BW. The strongest antibacterial properties were found in the treatment group following the administration of shark liver oil at a dose of 0.4 g / g BW compared to those in the other treatment groups whose members received doses of 0.2 g / g BW and 0.3 g / g BW. This indicates that the high antibacterial content of shark liver oil at a dose of 0.4 g / g BW enables it to inhibit inflammatory cytokines and also increase fibroblast proliferation in the periodontal ligaments.

An additional finding of this study is the significant difference in the density of collagen between Group K-, i.e. normal mice, and the treatment groups which had received shark liver oil at doses of 0.2 g / g BW, 0.3 g / g BW, and 0.4 g / g BW. The density of collagen in Group K was lower than that in the treatment groups following the administration of shark liver oil at doses of 0.2 g / g BW, 0.3 g / g BW, and 0.4 g / g BW. This indicates that the provision of this oil can inhibit further collagen damage by stimulating the formation of new collagen.

In general, collagen begins to form on the third day and increases until the 21st day at the end of the proliferation process.¹⁶ High-speed collagen synthesis returns the wound to normal tissue within a period of six to twelve months. Early in the proliferation process, fibroblasts will proliferate binding to extracellular matrix to form scar tissue and accelerate wound healing. Collagen accumulation caused by excessive fibroblast synthesis will reorganize causing regular tissue to form along the wound. Synthesis of collagen by fibroblasts is controlled by collagenase and other factors that damage new collagen.²⁸

Collagen remodeling during the maturation phase actually depends on collagen synthesis by fibroblasts and collagen degradation in the maturation phase (remodeling).³⁹ The maturation phase is the longest within the healing process and lasts approximately one year.

The excessive production of collagen by fibroblast cells will be degraded by collagenase and metalloproteinase enzymes resulting in collagen being more organized.¹⁶ Fibronectin gradually disappears and both hyaluronic acid and glycosaminoglycans are replaced by proteoglycans. At this time, collagen fibers close together causing collagen cross-linking and ultimately degrading excess collagen.²⁸ Type III collagen will then be replaced by type I collagen, thereby increasing the strength of collagen fibers (tensile strength). However, the strength of collagen fibers can only recover up to 80% of the strength of normal collagen fibers which prevailed before wound formation.¹⁶

This study also proved that subjects induced with *Porphyromonas gingivalis* bacteria had a significant difference from the treatment groups induced with *Porphyromonas gingivalis* bacteria and then administered specific doses of shark liver oil, namely; 0.2 g / g BB0.3 g / g BW, and 0.4 g / g BB up to a maximum of 2 ml orally to subjects once a day for seven days. The shark liver oil therapy provided was expected to increase fibroblast cells and restore collagen density leading to inhibition of further damage.

Previous research conducted by Alhanout *et al.* (2010)¹⁰ indicated that squalene and squalamine can act as antibacterials. They were both tested on both gram-negative bacteria, for example; *Escherichia coli* and *Pseudomonas aeruginosa*, in addition to gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. Similarly, another investigation undertaken by Agustina (2015)¹⁴ showed that shark liver oil has inhibitory properties in relation to *Porphyromonas gingivalis* bacteria. Alkylglycerol can bind to phospholipid cell membrane changing its structure to membrane fluidity and antioxidants and protecting the structure and function of membranes in white blood cells and macrophages.¹⁵ Moreover, squalene and omega 3 can be considered as scavengers of radical peroxide (antioxidant) capable of inhibiting ROS, thereby preventing oxidative stress.^{29,30} This intensive antioxidant activity can, in turn, reduce ROS and increase collagen synthesis.³¹

The group of subjects given shark liver oil therapy through a 0.2 g / g dose of BW experienced a significant difference to that administered a 0.4 g / g dose of BW. However, there was no significant difference between the group of subjects given shark liver oil therapy at a dose of 0.2 g / g BW to that administered a 0.3 g dose of / g BW. This might be due to subject susceptibility to small doses. In other words, the higher the dose administered, the more active the ingredients of the drug compound in the connective tissue will be. Hence, the inhibition of inflammation and destruction progression is higher.³²

The group of subjects given shark liver oil therapy through a 0.2 g / g dose of BW did not demonstrate a significant difference from the group given one of 0.3 g / g BW. However, there was a significant difference in collagen density between the group of subjects given shark liver oil therapy at the dose of 0.2 g / g BW and the group

administered one of 0.4g / g BW. The highest number of fibroblasts was also found in the group of subjects which received shark liver oil therapy at a dose of 0.4g / g BW. This indicates that this form of therapy at a dose of 0.4g / g BW is effective in treating *Porphyromonas gingivalis* bacteria since it possesses the ability to increase the number of fibroblasts and stimulate the formation of new collagen.

Shark liver oil also contains squalene with a high level of oxygen carried throughout cell membrane.²⁸ The nature of squalene is contrary to the that of *Porphyromonas gingivalis* bacteria which are anaerobic gram-negative bacteria. As a result, it can render unfavourable the environmental conditions of the *Porphyromonas gingivalis* bacteria. The mechanism of squalamine contained in shark liver oil can even change the integrity of the bacterial cell membrane by increasing its permeability characterized by the release of ATP and also directly encountering gram negative bacteria which cause damage to the outer cell membrane.

Shark liver oil contains alkylglycerol that can release proteases which transform autolytic enzymes from an inactive to an active state. This compound can even inhibit the synthesis of peptidoglycan in the bacterial cell wall causing it to experience lysis, leading to bacterial cell death.^{33,34} Changes to the cell membrane will be evident from the wrinkled membrane structure of empty cells. Stunted bacterial growth can then reduce the activity of macrophages in the phagocytic process.¹⁶

Shark liver oil contains Omega 3 composed of EPA and DHA which share the same physical and structural properties.³⁵ Squalene and omega 3 can also be considered scavengers of radical peroxide (antioxidants) which can inhibit ROS, thereby preventing oxidative stress.^{29,30} Moreover, omega 3 plays an important role as a useful anti-inflammatory in the wound healing process.³⁵

In addition, alkylglycerol can also protect the structure and function of white blood cell membranes and macrophages.¹⁵ Macrophages are cells that play the most important role in the wound healing process since they promote the phagocytosis of bacteria, thereby assuming the role of PMN. Macrophages will turn into macrophage efferocytosis (M2) which secrete anti-inflammatory cytokines such as IL-4, IL-10, and IL-13. IL-4 plays a role in fibroblast proliferation and collagen synthesis.¹⁶

Macrophages also produce growth factors, for example PDGF, FGF and TGF- β , which induce fibroblasts to proliferate, migrate, and form an extracellular matrix.^{16,36} Over time, this extracellular matrix will be replaced by type III collagen which is also produced by fibroblasts. The type III collagen will be subsequently replaced by type I collagen during the maturation phase.¹⁹ Increased collagen fibers can signal the process of wound healing.³⁷

Ultimately, the wound healing process is influenced by both local and systemic factors with the result that therapy not only removes disease-causing bacteria (local factors), but also suppresses damage to the host cell inflammatory response component (systemic factors).¹¹ In other words,

given its antibacterial properties, antioxidants, and immune cell abilities, shark liver oil promotes the healing process of chronic periodontitis at doses of 0.2 g / g BW; 0.3 g / g BW; and 0.4 g / g BW.

In conclusion, shark liver oil at doses of 0.2 g / g BW; 0.3 g / g BW; and 0.4 g / g BW can increase fibroblasts and collagen density in the periodontal ligaments of Wistar rats induced with *Porphyromonas gingivalis* bacteria. Nevertheless, shark liver oil at a dose of 0.4 g / g BW has the most profound effect on increasing fibroblasts and collagen density in the periodontal ligaments of Wistar rats induced with *Porphyromonas gingivalis* bacteria.

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