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Manuscript Number: 21-1-1355

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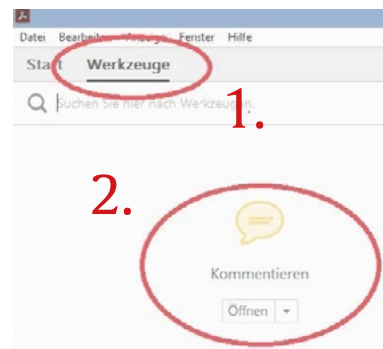
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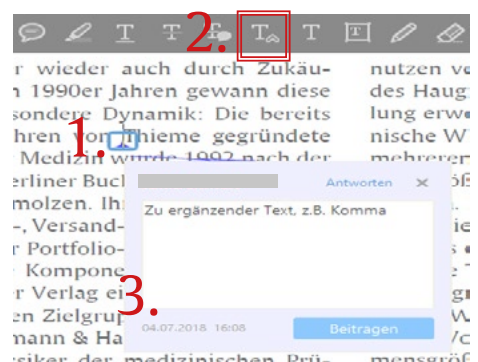
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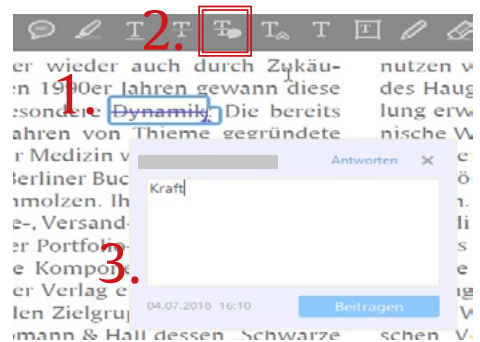
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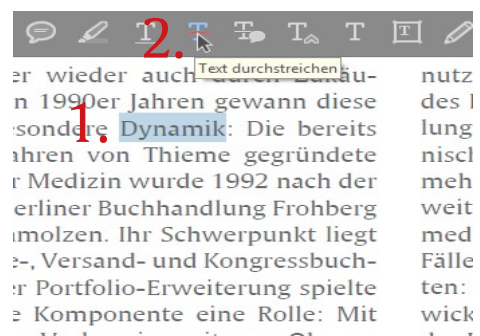
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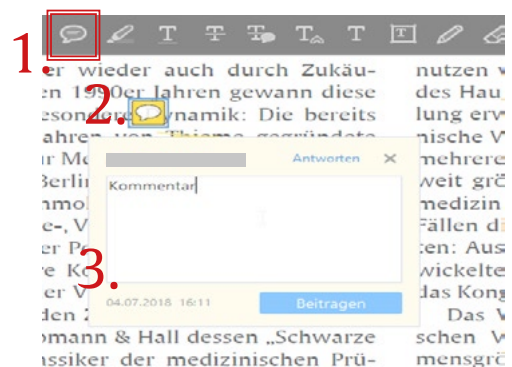
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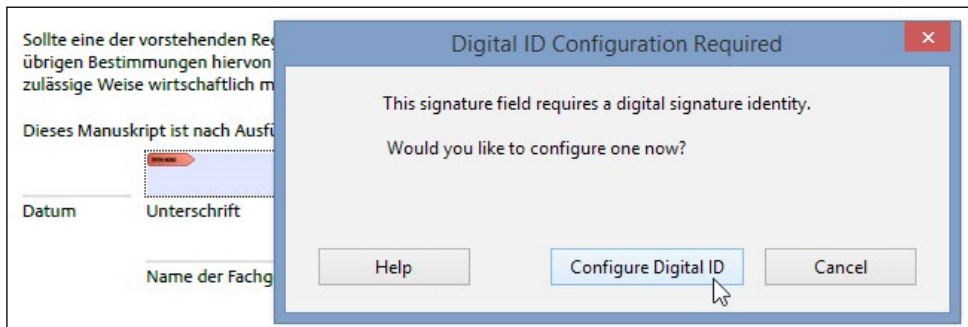
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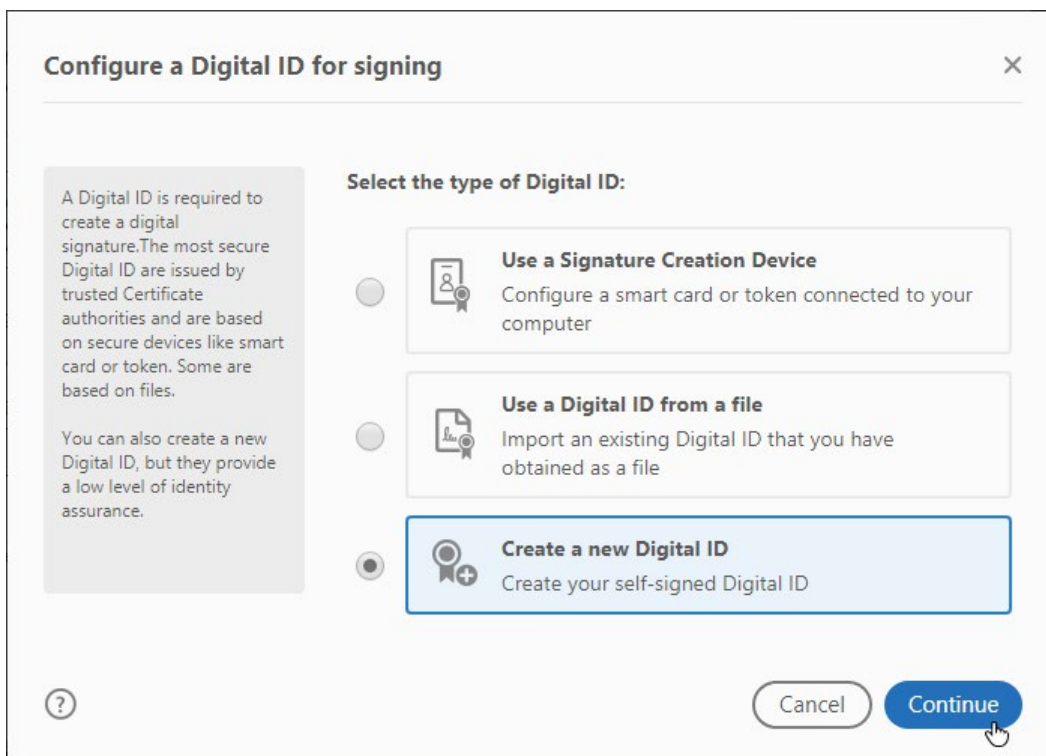
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The Effects of Rice Husk Liquid Smoke in *Porphyromonas gingivalis*-Induced Periodontitis

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Abstract

Objectives The purpose of this study is to analyze the effects of rice husk liquid smoke in *Porphyromonas gingivalis*-induced periodontitis in the inflammatory and proliferation marker such as nuclear factor kappa β (NF- κ B), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), transforming growth factor- β (TGF- β), fibroblast growth factor 2 (FGF2), collagen type 1 (COL-1) expression, and the number of macrophages, lymphocytes, and fibroblasts.

Materials and Methods Rice husk liquid smoke is obtained by the pyrolysis process. *Porphyromonas gingivalis*-induced periodontitis in 20 μ L phosphate-buffered saline containing 1×10^9 CFU was injected into the lower anterior gingival sulcus of Wistar rats. The periodontitis was then treated with 20 μ L/20 g body weight of rice husk liquid smoke once a day for 2 and 7 days, respectively. After treatment, the bone and lower anterior gingival sulcus were analyzed with immunohistochemistry and hematoxylin-eosin staining.

Results The treatment of periodontitis with rice husk liquid smoke showed a lower NF- κ B, TNF- α , and IL-6 expression and a higher TGF- β , FGF2, and COL-1 expression than the control after treatment for 2 and 7 days ($p < 0.05$), respectively. The number of macrophages and fibroblasts was also higher when compared with the control group ($p < 0.05$), but the number of lymphocytes was lower than the control ($p < 0.05$).

Conclusion Rice husk liquid smoke showed its effects on *Porphyromonas gingivalis*-induced periodontitis with a decrease in inflammatory markers and an increase in proliferation markers. The development of a rice husk liquid smoke periodontitis treatment is promising.

Keywords

- ▶ *Porphyromonas gingivalis*
- ▶ periodontitis
- ▶ liquid smoke
- ▶ rice husk

Introduction

Liquid smoke is a result of the wood-burning liquefaction part of the pyrolysis process.¹ Liquid smoke is traditionally used as a food preservative, especially with fish

products,^{2,3} because it maintains protein content⁴ and nutrients and, due to a particular organoleptic characteristic,⁵ prevents bacterial contamination.⁶ Liquid smoke has antibacterial properties because it contains phenolic

DOI <https://doi.org/10.1055/s-0041-1727554>
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compounds⁷ and carbonyls.¹ In lower concentrations,^{6,8} liquid smoke is able to inhibit Gram-negative and Gram-positive and fungal growths, such as *Escherichia coli*, *Staphylococcus aureus*,⁶ and *Candida albicans*.⁹

Most types of liquid smoke have a high phenolic compound content and possess antioxidant and antibacterial properties.¹ The total phenolic compound content in the liquid smoke is dependent on the pyrolysis, wood, and final temperature. In Indonesia, liquid smoke is produced from coconut shells¹⁰ and rice husks.^{11,12} This liquid smoke has shown a low toxicity^{11,12} and a potential to be beneficial to human health.¹³ Furthermore, rice husk liquid smoke has shown efficiency in the inflammatory process of oral wound healing through the increased recruitment of inflammatory cells and decreased recruitment of proinflammatory cytokines and inflammatory mediators, such as the tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), IL-6, prostaglandin E, and leukotriene B4 (LTB4),^{14,15} by inhibiting reactive oxygen species (ROS)¹⁶ and nitric oxide.¹⁵

One of the oral diseases caused by bacteria is periodontitis. The *Porphyromonas gingivalis* is a Gram-negative bacterium able to produce lipopolysaccharides, which trigger responses from the host.¹⁷ The host's response to this bacterium includes the activation of fibroblasts and endothelial cells, recruitment of inflammatory cell, such as macrophages and lymphocytes, and the subsequent release of inflammatory mediators and cytokines, which play a crucial role in periodontal tissue destruction.¹⁸ The role of *Porphyromonas gingivalis* is to upregulate the nuclear factor kappa B (NF- κ B)¹⁹ and increase proinflammatory cytokines, such as IL-6,²⁰ TNF- α , and IL-1 β ,²¹ through inducing the ROS to activate the JAK2 and c-Jun.²²

Based on the potential of using liquid smoke against bacteria, we conducted a study on the use of rice husk liquid smoke for the treatment of diseases caused by *Porphyromonas gingivalis*. The study was conducted by analyzing the expressions of NF- κ B, TNF- α , IL-6, transforming growth factor β (TGF- β), fibroblast growth factor 2 (FGF2), and collagen type 1 (COL-1) as well as the responses of inflammatory cells, such as macrophages, lymphocytes, and fibroblasts.

Material and Methods

Liquid Smoke Production

The use of liquid smoke in this study was based on previous research.^{11,14} Liquid smoke is obtained from rice husks through the pyrolysis process, with a combustion temperature of 400°C over 8 hours. The smoke is then condensed using a condenser, then submitted to the distillation process performed using a temperature of 120 to 150°C.¹⁴

Animals

In this study, the experimental animals used were male Wistar rats (*Rattus norvegicus*) aged 5 to 6 months, with a weight ranging from 250 to 300 g. The rationale for the choice of age was based on previous studies where the sizes of the mandibles and gingiva were considered sufficiently accessible for bacteria. Research on the experimental animals

was performed in the Animal Laboratory at the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

This research protocol has been approved by the Health Experiment Committee of the Faculty of Dentistry, Airlangga University in Surabaya, with the registration number 160/HRECC.FODM/III/2020.

Porphyromonas gingivalis-Induced Periodontitis

Periodontitis is obtained by injecting *Porphyromonas gingivalis* (Pg ATCC 33277 PK/5, Thermo Scientific) with the formulation of 1×10^9 CFU in 20 μ L phosphate-buffered saline into the gingival sulcus between the two lower mandibular incisors using a 0.5-mL syringe.²³

The *Porphyromonas gingivalis* injection is performed every 3 days for 14 days.²⁴ On the fourteenth day and after, clinical signs of periodontitis will be seen. The criteria of these signs are a reddish coloring of the gingiva, swelling of the interdental incisive central mandibular area, and interdental resorption by histopathology examination.

Treatment Using Rice Husk Liquid Smoke

Rice husk liquid smoke was slowly inserted into the gingival sulcus,²⁵ using a 1-mL syringe with a unbeveled needle (13 mm \times 0.04 mm) (Terumo, Japan), while the control group was given sterile water. During the application process, the oropharyngeal regions of the experimental animals were covered with sterile gauze to prevent the ingestion of rice husk liquid smoke. This treatment was performed once a day for 2 and 7 days. The dosage used was 20 μ L/20 g body weight.¹⁵

NF- κ B, TNF- α , IL-6, TGF- β , FGF2, and COL-1 Expression

Lower central incisive and alveolar bone tissue was collected after preceded euthanasia by CO₂ inhalation on days 2 and 7 after rice husk liquid smoke treatment. The immunohistochemistry staining used the standard method of streptavidin-biotin-peroxidase complex to bind primary antibodies, using the LSAB System Universal Kit. Diaminobenzidine tetrahydrochloride was used as chromogen and counterstained with Mayer's hematoxylin.

The primary antibody used was NF- κ B (*anti-p65 antibody [nuclear Factor-KB P65], polyclonal, anti-body online GmbH, Germany*), TNF- α (*anti-TNF α antibody, polyclonal, anti-body online GmbH, Germany*), IL-6 (*anti-IL6 antibody, polyclonal, anti-body online GmbH, Germany*), TGF- β (*anti-TGF β 1 antibody, polyclonal, anti-body online GmbH, Germany*), FGF2 (*anti-FGF2 antibody, polyclonal, anti-body online GmbH, Germany*), and COL-1 (*anti-collagen, type I antibody, monoclonal, anti-body online GmbH, Germany*). All measurements were made using a light microscope (Nikon H600L microscope; Nikon, Japan) with a magnification of 1,000 \times at five fields of view with a single blind operator.

Number of Macrophages, Lymphocytes, and Fibroblasts

The number of macrophages, lymphocytes, and fibroblasts was analyzed using hematoxylin and eosin. All measurements were made using a light microscope (Nikon H600L

microscope; Nikon, Japan) with a magnification of 400× at five fields of view with a single blind operator.

Statistical Analysis

The differences of each expression and the cell numbers were analyzed using with an independent t -test. A p -value of $p < 0.05$ is considered as a significant difference for each group.

Results

NF- κ B, TNF- α , and IL-6 Expression

After 2 days of treatment with rice husk liquid smoke, there were no differences in NF- κ B, TNF- α , and IL-6 expressions ($p = 0.146$; $p = 0.139$; $p = 0.198$) ($p < 0.05$) compared with the control group. Meanwhile, after 7 days of rice husk liquid smoke treatment, the NF- κ B, TNF- α , and IL-6 expressions were all lower when compared with the control group ($p = 0.000$) ($p < 0.05$) (►Table 1). The NF- κ B, TNF- α , and IL-6 expressions are presented in ►Fig. 1.

TGF- β , FGF2, and COL-1 Expression

The 2-day rice husk liquid smoke treatment of the periodontal pocket showed that the TGF- β , FGF2, and COL-1 expressions ($p = 0.002$; $p = 0.003$; $p = 0.000$) ($p < 0.05$) were higher when compared with the control group. The same results also showed higher TGF- β , FGF2, and COL-1 expressions ($p = 0.004$; $p = 0.001$; $p = 0.001$) ($p < 0.05$) when compared with the control group after the 7-day rice husk liquid smoke treatment of the periodontal pocket (►Table 1). The TGF- β , FGF2, and COL-1 expressions are presented in ►Fig. 2.

Number of Macrophages, Lymphocytes, and Fibroblasts

The 2-day rice husk liquid smoke treatment showed a higher number of macrophages compared with the control group

($p = 0.004$) ($p < 0.05$). The same results also showed a higher number of macrophages when compared with the control group ($p = 0.003$) ($p < 0.05$) after the 7-day rice husk liquid smoke treatment (►Table 1; ►Fig. 3).

In contrast, the 2-day rice husk liquid smoke treatment showed a lower number of lymphocytes when compared with the control group ($p = 0.013$), and after the 7-day rice husk liquid smoke treatment the experimental group showed a lower number of lymphocyte cells when compared with the control group ($p = 0.001$) ($p < 0.05$) (►Table 1; ►Fig. 3).

The 2-day rice husk liquid smoke treatment showed a higher number of fibroblasts when compared with the control group ($p = 0.000$) ($p < 0.05$). The same results also showed a higher number of fibroblasts when compared with the control group ($p = 0.001$) ($p < 0.05$) after the 7-day rice husk liquid smoke treatment (►Table 1; ►Fig. 3).

Discussion

The role of *Porphyromonas gingivalis* in the tissue destruction related to periodontitis is connected to Toll-like receptor 4 (TLR4). The activation of TLR4 can activate Myd88-dependent pathways. When the Myd88 is activated, this protein will activate the NF- κ B and activator protein-1 as well as the inflammatory gene.²⁶ The activation of NF- κ B increases the production of proinflammatory cytokines, such as TNF- α and IL-6, resulting in the tissue destruction related to periodontitis. The treatment of periodontitis with rice husk liquid smoke is able to interfere with the inflammatory process, including the activation of NF- κ B and proinflammatory cytokines, such as TNF- α and IL-6.

Rice husk liquid smoke contains several types of phenolic compounds, in the form of 2-methoxyphenol (guaiacol), mequinol, phenol, and 4-ethylguaiacol,¹¹ which can increase the expression and activation of the Nrf2 signaling pathway²⁷ and inhibit the expression activation of the NF- κ B/I κ B α signaling pathway.²⁸ 4-Ethylguaiacol increases the production

Table 1 The NF- κ B, TNF- α , IL-6, TGF- β , FGF2, and COL-1 expression and the number of macrophages, lymphocytes, and fibroblast after treatment with rice husk liquid smoke

Marker	Treatment for 2 days (X \pm SD)			Treatment for 7 days (X \pm SD)		
	RHLS	Control	p -Value	RHLS	Control	p -Value
NF- κ B expression	5.20 \pm 1.92	7.00 \pm 1.58	0.146	2.60 \pm 1.14	10.20 \pm 1.92	0.000 ^a
TNF- α expression	9.20 \pm 1.48	11.40 \pm 2.51	0.139	5.20 \pm 1.92	13.40 \pm 2.51	0.000 ^a
IL-6 expression	7.80 \pm 1.79	9.80 \pm 2.59	0.198	2.80 \pm 0.84	12.60 \pm 2.07	0.000 ^a
TGF- β expression	11.20 \pm 2.16	4.60 \pm 2.41	0.002 ^a	13.40 \pm 1.43	6.00 \pm 1.87	0.004 ^a
FGF2 expression	11.20 \pm 1.92	3.20 \pm 1.92	0.003 ^a	13.40 \pm 2.86	4.60 \pm 2.07	0.001 ^a
COL-1 expression	11.40 \pm 2.30	3.10 \pm 1.14	0.000 ^a	13.40 \pm 2.41	5.40 \pm 2.30	0.001 ^a
Macrophages	10.60 \pm 1.52	5.40 \pm 2.30	0.004 ^a	12.40 \pm 2.07	6.60 \pm 2.30	0.003 ^a
Lymphocytes	7.80 \pm 1.92	12.80 \pm 2.77	0.013 ^a	3.40 \pm 1.14	14.60 \pm 3.21	0.001 ^a
Fibroblast	10.20 \pm 1.92	3.40 \pm 1.67	0.000 ^a	13.40 \pm 2.51	6.20 \pm 1.92	0.001 ^a

Abbreviations: COL-1, collagen type 1; FGF2, fibroblast growth factor 2; IL-6, interleukin-6; NF- κ B, nuclear factor kappa β ; RHLS, rice husk liquid smoke; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α .

Note: X \pm SD: mean \pm standard deviation

^aSignificant difference using independent t -test with $p < 0.05$.

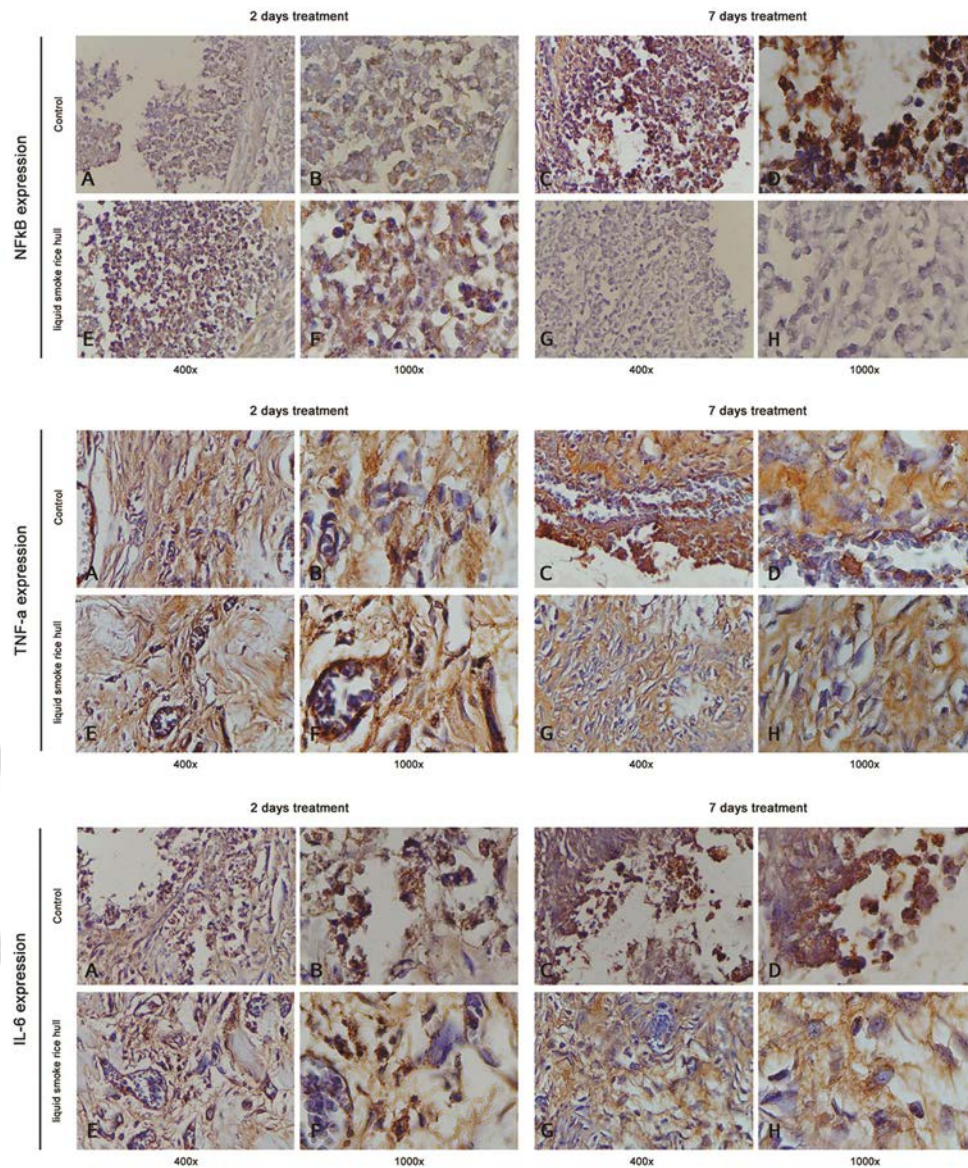


Fig. 1 The nuclear factor kappa β (NF- κ B), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) expression. (A, B) Control for 2 days; (C, D) control for 7 days; (E, F) treatment with liquid smoke rice hull for 2 days; (G, H) treatment with liquid smoke rice hull for 7 days.

and releases Nrf2 into the nucleus to prevent the phosphorylation of NF- κ B/I κ B α , thereby reducing inflammatory activation and the release of inflammatory cytokines. The antioxidant properties of 4-ethylguaiaicol can be contributed to the inhibition of inflammation by scavenger of ROS and increase inflammatory response,²⁹ by inhibiting the activation of NF- κ B to produce proinflammatory cytokines, such as TNF- α and IL-6.¹⁰

On a cellular level, rice husk liquid smoke treatment is able to interfere with the responses of inflammatory cells, such as macrophages and lymphocytes. The 2- and 7-day rice husk liquid smoke treatments showed an increased number of macrophages. The same results also showed a higher number of macrophages after the 7-day rice husk liquid smoke treatment. In contrast, the 2- and 7-day rice husk liquid smoke treatments showed a lower number of lymphocytes. This condition is a result of the inhibition of proinflammatory

cytokine production. A previous study also confirmed this condition. This is because the main content of phenolic compounds in distilled liquid smoke rice can inhibit free radicals, thereby accelerating the recruitment of inflammatory cells and accelerating the inflammatory process.¹⁴ In the condition of periodontitis, rice husk liquid smoke can inhibit tissue damage with the toxins produced by periodontal bacteria accelerating the inflammatory response.

Not only the inflammatory process, but also the proliferation stage, is affected by rice husk liquid smoke. The TGF- β , FGF2, and COL-1 expressions, as indicators of proliferation, also change. The mechanism involved is resolution of the inhibition of the inflammation process. With the inhibition of free radical formation, the transcription factor for the formation of anti-inflammatory cytokines and growth factors, namely Nrf2, will be activated. Previous studies have shown that application of rice husk liquid smoke can increase the

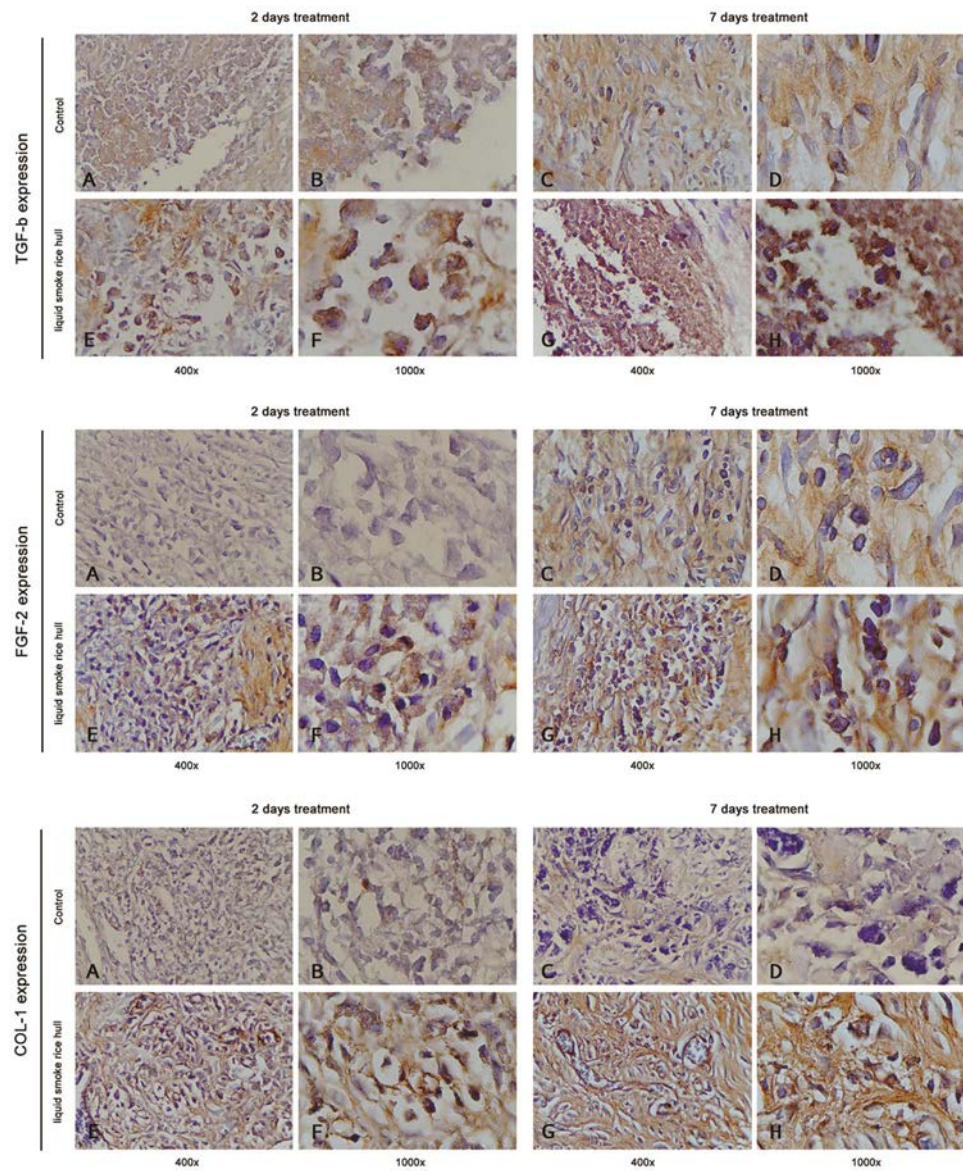


Fig. 2 The transforming growth factor- β (TGF- β), fibroblast growth factor 2 (FGF2), and collagen type 1 (COL-1) expression. (A, B) Control for 2 days; (C, D) control for 7 days; (E, F) treatment with liquid smoke rice hull for 2 days; (G, H) treatment with liquid smoke rice hull for 7 days.

formation of growth factors, such as TGF- β ,¹⁴ FGF, and vascular endothelial growth factors.^{14,30} In this research, it can also be seen that growth factors, such as TGF- β , FGF, and COL-1 expressions, increased with rice husk liquid smoke treatment.

The increase of growth factors after rice husk liquid smoke treatment also affected the fibroblast number. As a healing marker, fibroblast was also affected after treatment. The 2- and 7-day rice husk liquid smoke treatments showed a higher number of fibroblasts. The increased number of fibroblasts was due to the decrease in the inflammatory process and the increase in the number of growth factors. The phenolic compound is responsible for this process because it is able to increase the inflammatory cell response and inhibit the production of proinflammatory mediators.^{31,32}

Another reason for this effect of rice husk liquid smoke is its ability to inhibit Gram-negative and Gram-positive

bacteria, such as *Porphyromonas gingivalis*, in the progression of periodontitis.¹ Phenolic compounds are known to disturb the cytoplasmic membranes of bacteria and cause the intracellular fluids to leak.^{33,34} Carbonyls inhibit microbial growth by penetrating the cell wall and inactivating enzymes located in the cytoplasm and the cytoplasmic membrane. Carbonyls act by condensing the free primary amino groups in the polypeptide chains, primarily in the side chains of basic amino acids. These amino groups may be an essential part of the active site of the enzyme, or they may function as a binder for the substrate by hydrogen bonding.¹

Conclusion

Rice husk liquid smoke showed its effect on the periodontitis condition by decreasing the inflammatory markers, such as lymphocytes and NF- κ B, TNF- α , and IL-6 expressions,

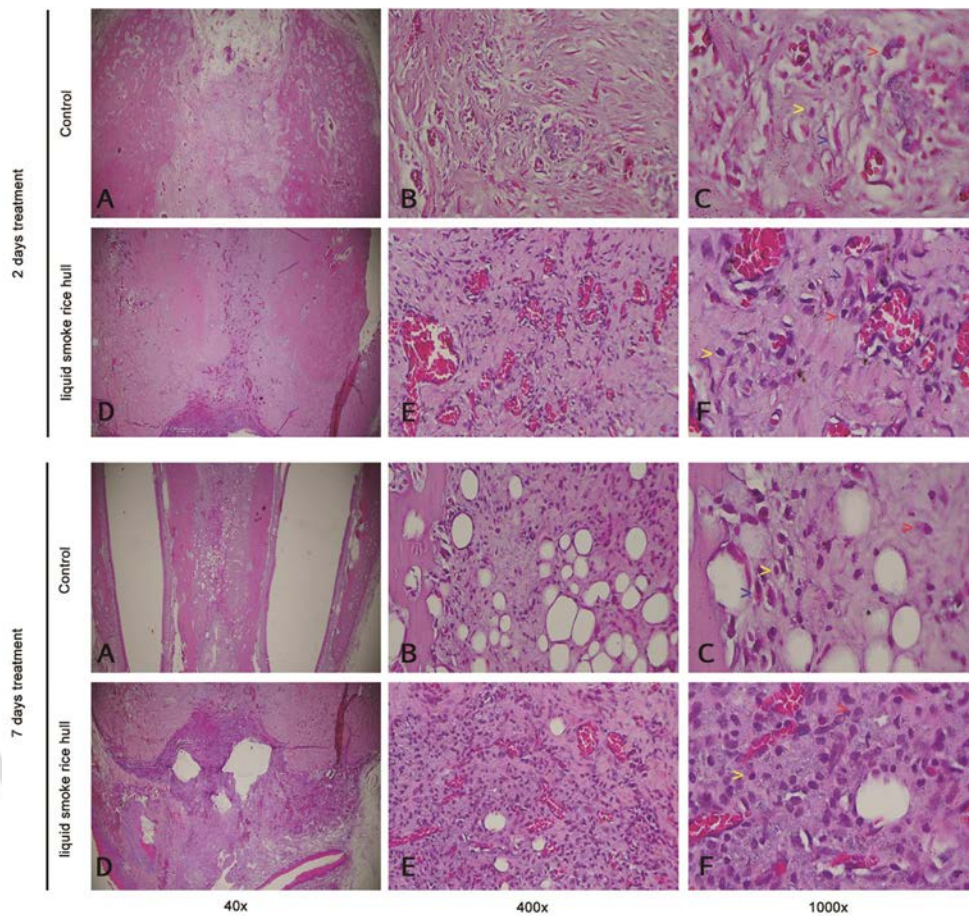


Fig. 3 The number of macrophages (red arrow), lymphocytes (yellow arrow), and fibroblast (blue arrow). (A, B) Control for 2 days; (C, D) control for 7 days; (E, F) treatment with liquid smoke rice hull for 2 days; (G, H) treatment with liquid smoke rice hull for 7 days.

and increased the proliferation markers, such as the number of macrophages, fibroblasts, and TGF- β , FGF2, and COL-1 expressions. The use of rice husk liquid smoke is promising in periodontitis treatment.

Funding

This study was funded by Ministry of Higher Education, Republic of Indonesia 2020 in the schema PENELITIAN DASAR UNGGULAN PERGURUAN TINGGI (PDUPT) (funding number 630/UN3.14/PT/2020).

Conflict of Interest

None declared.

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