Immune Response Mechanism of the Gingival Epithelium in the Tissue Repair Exposed to Porphyromonasgingivalis Toxin on Topical Administration of Nigella sativa Extract

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Immune Response Mechanism of the Gingival Epithelium in the Tissue Repair Exposed to Porphyromonasgingivalis Toxin on Topical Administration of *Nigella sativa* Extract

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Abstract

Background and Objectives: Periodontal disease is not the typical cause of pain experienced by sufferers. This discomfort is commonly observed in advanced stage. P. gingivalisbacteria infections, and Lipopolysaccharides (LPS) have been identified as the main virulence factors. Furthermore, numerous attempts have been exploited during treatment with unsatisfactory outcomes, including the administration of local or systemic antibiotics, periodontal curettage and flap surgery. Therefore, early prevention is one of the effective and efficient approaches, particularly involving the use of medical plants, and the natural material selected was black cumin (Nigella Sativa). The aim of this research, therefore, is to investigate the effects of Nigella sativa extract against the inflammation reaction and beta defensin-2 (BD-2) mechanism in gingival epithelium exposed to P. gingivalis LPS. Materials and Methods: This is an experimental research performed in the laboratory, using Randomized Posttest-Only Control Group Design. The animals used include white 45 Wistar rats (Rattus norvegicus) randomly allocated to 3 research groups, comprising K (LPS P. gingivalis), P1 (N. sativa extract + LPS), and P2 (LPS + N. sativa). Furthermore, each set was subsequently divided into 3 on the basis of time span, at 4, 7, and 21 days. The tissue expressions of BD-2, and MMP-8 were examined using immunohistochemical techniques. Results: This investigation showed a significant

difference between K, P1, and P2 groups at any span of time. Moreover, lower MMP-8 expression were observed in the P1 and P2 groups, compared to K on day 4, 7, and 21, while the BD-2 expression was higher in P1 (31.3) and P2 (32.2) than K (19.8). Conclusion: Based on the study results, Nigella sativa was determined to be capable of decreasing MMP-8 expressions, and increase BD-2, as part of the host innate response.

Keywords: TLR-4, NFkB, IL-17, MMP-8, BD-2, Nigella sativa, gingivitis

Introduction

The human health is possibly assessed based on the oral cavity conditions. Therefore, poor hygiene is assumed to aggravate a disease [1]. The tooth-supporting tissue also become damaged, and consequently cause unsteadiness and periodontal diseases [1,2].In addition, periodontal disease occurs irrespective of age, sex, race, or economic status [2]. This manifestation is irreversible and attributed to poor knowledge or ignorance. Therefore, prevention is necessary to avoid aggravated damage, and tooth loss [3].

Various previous studies have shown efforts made to alleviate this phenomenon, including through scaling and root planning, administration of local and systemic antibiotics, curettage, and flap surgery [4,5,6]. Moreover, each of these procedures is characterized by individual drawbacks, as observed with recession after flap surgery, extensive gingival tissue damage at curettage, drug allergy, and resistance to antibiotic implicated in poor compliance [7,8,9]. Therefore, it is necessary to have a biocompatible natural material with a low allergen factor as well as the capacity to prevent periodontal tissue damage.

In addition, there has been an upsurge in plant studies, due to the frequent use in traditional medicine for over thousands of years 10. These resources are also easily accessed and developed with minimal side effects [10,11]. The medicinal black cumin (Nigella sativa) is one of the numerous examples, characterized by thymoquinone as the most commonly found content, alongside alkaloids, saponins, flavonoids, protein, and fatty acids [12].

Specifically, thymoquinone is an anti-microbial agent with broad spectrum activity, and is known to annihilate gram-positive, gram-negative bacteria, viruses, parasites, and fungi [11,12]. In addition, the alkaloid and saponin contents also play a crucial role in disrupting bacterial defenses [12,13]. Meanwhile, several bacteria forms are known to influence periodontal disease, including Porphyromonasgingivalis as the most instrumental, Treponema denticola, and Tannerella forsythia [14,15]. Particularly, P. gingivalis is a gram-negative anaerobic bacteria[16], with product characterized by the

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potential to damage periodontal tissue, through lipopolysaccharide (LPS) production. This output is acknowledged to be pathogenic and capable of inducing the proinflammatory response of cytokines[17].

The IL-1α, IL-1β, TNF, IL-6, and IL-8 generated subsequently stimulate an increase in polymorphonuclear leukocyte production [18,19]. Therefore, a greater yield positively influence the the release of reactive oxygen species (ROS), which results in periodontal tissue damage [19].

Furthermore, increased pro-inflammatory cytokines also triggers matrixmetallo-proteinase (MMP) and the consequent damage of extracellular matrixes [20]. Specifically, MMP-8 plays the most significant role during collagenase processes, and the excessive pathological activity generated have been implicated in periodontal tissue damage[21].

This outcome is possibly prevented by reinforcing the innate immunity of the oral cavity, as the front line antagonist against pathogenic bacteria. These include antimicrobial peptides (AMP), which significantly contributes to the maintenance of homeostasis. In addition, beta-defensin (BD) was the first form to be identified in the epithelium in the oral cavity. This immunity quickly and properly adapts to most epithelial surfaces, including the oral cavity, and easily kills trapped microbes. Previous studies have shown a change in the BD-2 expression of epithelial cells infected by treponema denticola bacteria. This is one of the microorganisms implicated in periodontal disease, following a stimulation by bacterial and proinflammatory products, including interleukin (IL) 1β and IL-6.[22].

Therefore, the aim of is study was to discuss the effect of Nigella sativa extract administration on inflammatory reactions. This manifestation is particularly initiated by MMP-8 and the beta defensin-2 (BD-2) mechanisms in the gingival epithelium exposed to P. gingivalis, which is a periodontitis-causing LPS bacteria.

Material and Methods

Preparation of experimental animals

The animals were clinically evaluated and placed in a suitable environment for 14 x 24 hours prior to use in this study. The selected Wistar rats include all males at an age range of 8-10 weeks and weighing 120-150 grams. Furthermore, the animals were observed for specific behaviors and important clinical signs with potential application in health monitoring. The environmental conditions, including food supplies was according to the standard requirement, while the cage control, and waste management was performed by experienced and trained personnel from the Biochemistry Laboratory, Faculty of Medicine, Airlangga University.

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Preparation of LPS P. Gingivalis

Lipopolysaccharide1435 / 1450 (tetra-acetylated) Porphyromonasgingivalis (Astarte Biologics, WA, USA, catalog number 7010) were prepared for immunoneuromodulatory induction by intracellular injection

Preparation of Nigella Sativa

This involved diluting a 3% Nigella sativa extract as required

Method

This study involved 3 treatments, performed by injecting porphyromonasgingivalis LPS as a control, while the second group was administered Nigella sativa extract for 4 days before porphyromonasgingivalis LPS. In addition, the third was provided with both treatments on the same day.

LPS Administration

The PgLPS1435 / 1450 injection was administered intrasulcularly on the buccal part of the rat's upper right molar. Furthermore, a dose of 1.0 µg / ml was provided to initiat the gingivitis effects

Nigella Sativa Extract Administration Procedure

Therefore, 0.5 ml of a 3% Nigella sativa extracts solution was administered on the gums around the teeth twice a day for 21 days. This was accompanied by regular evaluations on days 4, 7, and 21, using immuno-histochemical peroxidase. Furthermore, this technique was applied to determine the expression of BD-2 and MMP-8 at the Microbiology Laboratory, Faculty of Dentistry, Airlangga University.

Statistical Analysis

The group mean values and significant differences between groups were compared and verified using one-way ANOVA. Therefore, the results were considered significant at $p \leq 0.05$.

ResultThe Control group is represented by K, while P1 was treated with Nigella sativa (Ns) extract before LPS bacteria Porphyromonasgingivalis (Pg), and P2 was administered both treatments simultaneously.

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Table 1 shows the manifestation of significant differences between K, P1, and P2 groups on day 4 of BD-2 and MMP-8 expression. The outcome was respectively higher and lower in both P1 and P2 groups compared to K.

Table 1 Mean and standard deviations of BD-2 and MMP-8 expressions on gingiva exposed to LPS bacteria Pg before and after the topical administration of nigella sativa extract on the 4th day of observation

Variable	Group	Mean	Standard deviation	p
MMP-8	K	8,6	1,14	
	P1	8,2	0,84	0,000*
	P2	7,8	0,84	
BD-2	K	11,8	1,48	
	P1	24,8	2,28	0,000*
	P2	31,6	2,07	

Description: * = p <0.05 is significantly different

Table 2 shows the significant differences in BD-2 and MMP-8 expression between all groups on the 7th day. The mean outcomes were respectively decreased, and increased compared to K.

Table 2 Mean and standard deviations of BD-2 and MMP-8 expressions on gingiva exposed to LPS bacteria Pg before and after the topical application of Nigella sativa extract on the 7th day of observation

Variable	Group	Mean	Standard deviation	P
MMP-8	K	9,6	1,14	
	P1	6,8	0,84	0,0
	P2	6,2	0,84	00
				*
BD-2	K	16,6	2,6	
	P1	27,4	1,95	0,0
	P2	26	1,58	00

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*

Description: * = p <0.05 is significantly different

Table 3. Mean and standard deviations of BD-2 and MMP-8 expressions on gingiva exposed to LPS bacteria Pg before and after the topical application of Nigella sativa extract on the 21st day of observation

Variable	Group	Mean	Standard deviation	P
MMP-8	K	12,6	2,3	
	P1	6,8	0,84	0,000*
	P2	5	0,71	
BD2	K	19,8	3,83	
	P1	31,6	2,07	0,000*
	P2	32,2	3,7	

Description: * = p <0.05 is significantly different

Table 3 highlights the significant differences between the BD-2 and MMP-8 expressions in all groups on the 21st day. The mean outcome respectively increased and decreased with the P1 and P2 groups compared to K.

The results from measuring BD-2 expression on the gingiva

Figure 1 shows the results of immunohistochemical examination on the gingival epithelium of rats, in order to detect BD-2 expression at 400x magnification.

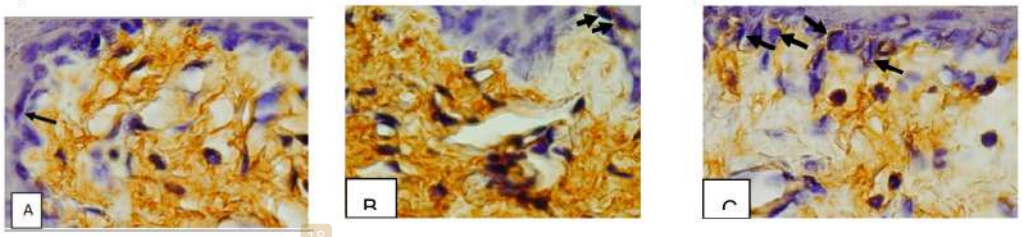

Figure 1. An overview of BD-2 expression in rats' gingival epithelial cells. (A) control, (B) Nigella sativa extract + P. gingivalis LPS exposure, (C) P. gingivalis LPS exposure + Nigella sativa extract.

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Table 4 shows the differences in BD-2 expressions from the gingiva of all treatment groups.

This demonstrated the existence of significant variations with respect to the group and time. In addition, increased output was reported for BD-2 in all groups, indicating the positive impact of Nigella sativa extract.

Table 4. Mean and standard deviation of BD-2 expression in gingiva for each group and time

Group	Time						P
	Day 4		Day 7		Day 21		
K	11,8 1,48	±	16,6 2,6	±	19,8 3,38	±	0,000
P1	24,8 2,28	±	27,4 1,95	±	31,6 2,07	±	
P2	31,6 2,07	±	26 ± 1,58		32,2 3,7	±	

Figure 2. The mean of BD-2 Expressions based on Time

Figure 2 demonstrates an increase in the mean quantity of BD-2 expressed. However, P2 showed a lower value on day 7, which consequently increased on day 21.

Results from measuring the MMP-8 expression in the gingiva

Figure 3 shows the immunohistochemical examination results obtained from the gingival epithelium. This assessment was used to detect MMP-8 expression at 400x magnification.

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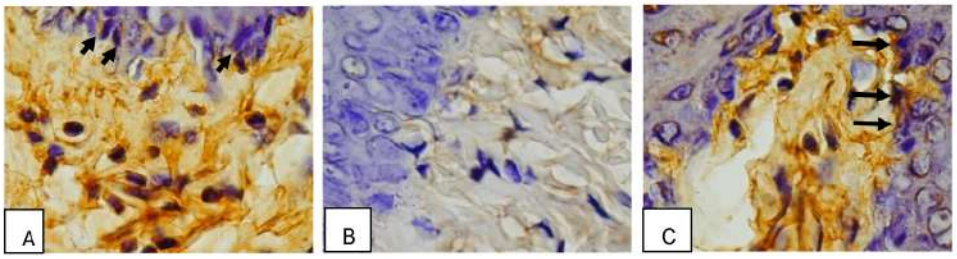

Picture. 3. An overview of the MMP8 expression in rats' gingival epithelial cells. (A) control, (B) Nigella sativa extract + P. gingivalis LPS exposure, (C) P. gingivalis LPS exposure + Nigella sativa extract

Table 5 shows the differences in measurement results between each treatment group.

Table 5. Mean and standard deviation of MMP-8 expression in gingiva for each group and time

Group	Time			P
	Day 4	Day 7	Day 21	
K	8,6 ± 1,14	9,6 ± 1,14	12,6 ± 2,3	0,000
P1	8,2 ± 0,84	6,8 ± 0,84	6,8± 0,84	
P2	7,8 ± 0,84	6,2 ± 0,84	5± 0,71	

Table 5 shows the significant differences between each group and time. This was evidenced by the increased expression of MMP-8 in K compared to P1 and P2 groups, characterized by reducing values. Therefore, Nigella sativa extract potentially has the capacity to reduce MMP-8.

Figure 4 shows increased output in group K. Meanwhile, steadily decreasing values were observed in P1 and P2 although P1 specifically showed similar results on day 7 and 21.

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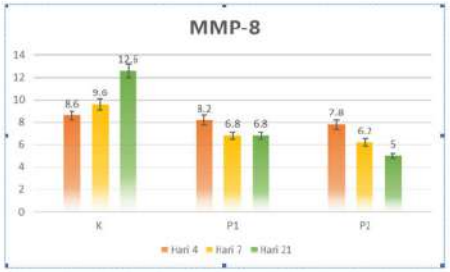

Figure 4. Mean MMP-8 Expression based on Time.

Discussion

The administration of Nigella sativa extract in this research was proven to increase BD-2 expression in the gingival epithelium (Table 5.12 and Figure 5.8). In addition, there was an increased mean outcome in the P1 and P2 groups at each evaluation time compared to group K. The gingival epithelium is known to occur in the stratified squamous form, and serves as a barrier against pathogenic bacteria. This component of the oral cavity is constantly in contact with various microorganisms, and most individuals are ought to maintained in a healthy balance [23]. In addition, oral epithelial tissues are estimated to confer protection on the host by physical defense and also through innate immune responses by means of antimicrobial peptides. This particularly includes the small-sized beta defensins manifested in the form of cations produced by epithelial cells. Moreover, these compounds play a crucial role in mucosal and skin protection. According to a research by Huang EC (2016), the mRNA from BD-1 and BD-2 is located in the suprabasal stratified epithelium of the gingival tissue, while BD-2 peptide was detected in the upper epithelial layers [24].

The gingiva, hard palate, and dorsal part of the tongue are characterized by keratinized epithelium, while the mouth base and buccal area are non-keratinized. In addition, the immunohistochemical examination was performed on normal oral epithelium to evaluate the BD-2 expression. The findings showed weak positive outcome with the keratinized epithelium (including gingiva), while negative results were observed in the other areas. This phenomenon indicates the significant role played by the keratinization process in peptide retention. [25].

Yong X, *et al* (2014) reported on the induction and expression of BD-2 in most tissues, particularly in the inflamed forms. However, BD-2 is expressed under normal conditions (without inflammation) resulting from the continuous exposure of oral cavity mucosal to various bacteria [26].

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Table 5.12 and Figure 5.8 showed the lowest BD-2 expressions in group K. This phenomenon was attributed to the poor induction ability of LPS-induced Porphyromonasgingivalis alone at the gingival epithelium.

Furthermore, matrix metalloproteinase (MMP) is considered a proteolytic enzyme type in the matrixin subfamily and zinc metalloproteinase family, However, humans are characterized by at least 25 distinct types, but MMP is closely involved in pathological conditions. These include rheumatoid arthritis, tumor invasion and metastasis, chronic respiratory infections, periodontal disease, and eye disease [27]. In addition, this specific enzyme is possibly divided into collagenase, gelatinase, stromelysin, matrilysin, and membrane-type matrix metalloproteinases, based on the structural description. Specifically, collagenase has the unique ability to break down naive fibrillar collagen types I, II, as well as III [28] and is subdivided into collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13).

The Matrix Metalloproteinase-8 (MMP-8) is a known collagen-breaking enzyme recognized in the connective tissue of most mammals. This protein is encoded by the MMP-8 gene in humans, and are generally secreted in the form of a proprotein. Therefore, the yield is consequently activated by a structural break down through extracellular proteinase activities. In addition, the product is then stored in the secondary granular of neutrophils after activation by the autolytic breakdown, to further function as a collagen types I, II, and III degrading agent [29].

Table 5.10 and Figure 5.7 shows the potential for Nigella sativa extract to reduce MMP-8 expression, in both P1 and P2 groups, especially from day 7. This outcome suggests the ability to inhibit LPS Porphyromonasgingivalis and degrade collagen. In addition lipopolysaccharides and lipoteichoic acid is assumed to interact with toll-like receptors on epithelial cells, leukocytes, and fibroblasts, and consequently stimulate the production of cytokines, including IL-1 beta, TNF-alpha, IL-6, IL-8, and prostaglandins E2 (PGE2). Moreover, leukocyte infiltration is facilitated by fibroblast stimulation, achieved through the degradation activity of MMP secreted by IL-1 beta and TNF-alpha against ECM molecules, including collagen. Also, patients with periodontitis were estimated to possess increased MMP-8 levels in the GCF.

Conclusion

Based on the data analysis results and discussion, topical Nigella sativa extract on gingival epithelial cells exposed to the LPS toxin P. gingivalis bacteria are confirmed to potentially cause an increase and decline in BD-2 and MMP-8 expression, respectively.

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