ORIGINAL ARTICLE

Rambutan Peel (*Nephelium Lappaceum L.*) Extract in Chitosan Mucoadhesive Patch to Decrease the Number of Neutrophil Cells After *Porphyromonas Gingivalis* Induction

Trining Widodorini[1], Rahma Syamila Hanif[2]

[1] Department of Community and Preventive Dentistry, Faculty of Dentistry, Universitas Brawijaya, 65145, Malang, Indonesia
[2] Bachelor of Dentistry Program, Faculty of Dentistry, Universitas Brawijaya, 65145, Malang, Indonesia

ABSTRACT

Introduction: Gingivitis is histologically characterized by an increase in the number of neutrophil cells in the tissue due to gingival inflammation. The chitosan-based mucoadhesive patches combination of rambutan peel extract has the potential to optimize the gingival local drug distribution system due to the presence of phenolic compounds in rambutan peel, with minimum side effects. his study aimed to determine the effect of chitosan-based mucoadhesive patches combination of rambutan peel extract as an additional therapy for gingivitis on the number of neutrophil cells in Wistar rats. **Methods:** The study was conducted In vivo using 25 Wistar rats in 5 groups of samples with 2 control groups and 3 treatment groups. The control group consisted of a negative control group (K(-)) and a positive control group (K(+)). The treatment groups were treatment group 1 (KP1), treatment group 2 (KP2), and treatment group 3 (KP3). The rat's gingiva was induced with P.gingivalis LPS then treated using a patch with a concentration of 5%, 10%, and 15% active ingredients. Histological preparations were made with HE staining to evaluate the number of neutrophil cells in the rat's gingival tissue using optical microscope with 400x magnification. **Results:** The results of the Kruskal-Wallis and Mann-Whitney U statistical test showed a significant decrease ($p<0.05$) between the positive control group and the treatment group. **Conclusion:** The preparation of a chitosan mucoadhesive patch combination of rambutan peel extract is able to reduce the number of neutrophil cells.

Keywords: Chitosan; Gingivitis; Mucoadhesive patch; Neutrophil; Rambutan peel

Corresponding Author:
Trining Widodorini., drg., M.Kes
Email: trining.fk@ub.ac.id
Tel: +62 8123249166

INTRODUCTION

RISKESDAS data in 2018 shows a high prevalence of gingivitis in Indonesia reaching 74%, which makes gingivitis the second most common periodontal disease in Indonesia. Gingivitis is an inflammatory condition of the gingiva with clinical characteristics of a redness appearance on the gingival surface, softer consistency, and bleeding on probing (1). The accumulation of plaque-causing microorganisms along the gingival margin area is a fundamental etiology of gingivitis (2). The influence of predisposing local and systemic factors such as caries, foreign object impaction on the gingiva, systemic diseases, hormonal changes, nutritional deficiencies, and blood disorders also play a role in the gingival inflammation process (2,3).

Microorganisms invasion that occurs in the periodontal tissue triggers an inflammatory response. When infection or inflammation occurs, neutrophils as white blood cells that act as innate immune cells, are recruited into the tissues, especially in the gingival sulcus area, as much as >95% by continuous IL-8 gradient to destroy microorganisms through phagocytosis, the release of antimicrobial substances, or with NETosis (4). Through a series of those mechanisms, proinflammatory cytokines are reduced along with a decrease in the number of neutrophil cells. The decrease of neutrophil activity in the area of inflammation can indicate a reduced inflammatory response (5).

Scaling and root planning is the main treatment for gingivitis to eliminate plaque and calculus as local factors, which are the site of bacterial accumulation (6). After the main treatment is done, a Chlorhexidine mouthwash is commonly recommended to maintain oral health (7). Regular use of 0.2% Chlorhexidine gluconate mouthwash, along with inflammation

on the gingiva, might be a concern because of several side effects reported after the mouthwash use. Some side effects commonly complained by patients include pain and discomfort in the oral mucosa, dry and burning sensation, loss of the sense of taste, and tooth stain (8).

Therefore, an alternative gingivitis adjunct therapy with minimum side effects can be considered. The preparation of a gingival patch with chitosan as a base material is one of the alternative options. The natural properties of chitosan, which are biodegradable, biocompatible, bioadhesive, anti-inflammatory, and antibacterial, make chitosan a polymer that can be well utilized in dentistry as a drug delivery agent in periodontal tissues (9). Chitin is a chitosan source from the exoskeletal parts of crustaceans such as shrimp, lobster, crabs, or clams. With gingival patches, the integrity of the preparation on the oral mucosa can be well maintained due to continuous salivary activity and mechanical pressure in the oral cavity so that the duration of drug exposure located in the oral cavity can be well controlled (10). An ideal mucoadhesive patch consists of three layers: an adhesive layer that supports the attachment and opening of epithelial bonds, a layer containing the active ingredient or drug, and an impermeable layer as a backing (10). The patch works using an adhesion system, which is the formation of an attachment between a pressure-sensitive adhesive layer and mucosal surface (11).

Rambutan fruit peel (Nephelium lappaceum L.) extract was added to the patch preparations that worked as an active material. The rambutan peel contains minerals such as zinc, calcium, manganese, copper, and potassium; chemical fibers such as cellulose, hemicellulose, and lignin were also found (12). Further research stated that rambutan skin has high phenolic content and antioxidant activity. The phenolic components in rambutan peel are generally in the form of polyphenols, including geraniin, corilagin, and ellagic acid. Geraniin, which belongs to the ellagitannin group, has various biological properties, including antioxidant, antibacterial, and anti-inflammatory (12). This study was conducted to determine the effect of chitosan-based mucoadhesive patches combined with rambutan peel extract (Nephelium lappaceum L.) as an additional therapy for gingivitis to decrease the number of neutrophil cells in Wistar rats.

MATERIALS AND METHODS

Research Design and Sample

This research is true experimental research with Post Test Only Randomized Control Group Design in an In vivo laboratory to determine the effect of chitosan mucoadhesive patch combination of rambutan peel extract (Nephelium lappaceum L.) on the number of gingival neutrophil cells of Wistar rats (Rattus norvegicus) induced with P.gingivalis LPS.

The samples used in this study were white rats of the Wistar strain (Rattus norvegicus) with the criteria of male, an age range of 2-3 months, a body weight of 100-200 grams, and in a good physical condition. Sample selection was made using a simple random sampling technique. Based on Frederer's formula calculations, 25 white rats were used as test animals divided into five groups with two control groups and three treatment groups. The control group consisted of a negative control group (K(-)) and a positive control group (K(+)). The treatment groups were treatment group 1 (KP1), treatment group 2 (KP2), and treatment group 3 (KP3). This research has obtained an ethical statement from Brawijaya University with the number 073-KEP-UB-2021.

Preparation of Chitosan Mucoadhesive Patches Combined with Rambutan Peel Extract

Three kilograms of rambutan peel were cut into small pieces and then dried in an oven at 40-50˚C. The dried rambutan skin was mashed and then macerated using 96% ethanol for three days. Followed by drying using a rotary evaporator for 2.5 hours to produce rambutan peel extract (13).

The mucoadhesive patches preparation was done using the solvent casting method. Three patch formulations were made in three types of active ingredient concentrations; 5%, 10%, and 15%. The formulation of the mucoadhesive patch preparation was as described in table 1. The first step of making the patch was dissolving chitosan from blood clam shells in acetic buffer pH 4, while rambutan peel extract was dissolved using distilled water. HPMC was dissolved with 96% ethanol added with PVP. The mixture was then added to the rambutan peel extract solution, and the previously prepared chitosan solution was stirred until homogeneous and formed a mucilage. After homogenization, glycerin, tween 80, and peppermint oil were added to the mixture. Glycerin is used as a plasticizer, tween 80 is used as an emulsifier/surfactant, and peppermint oil is used as a flavoring agent in patch preparations. All the ingredients that have been made are mixed and dried using an oven to form a film using aluminum foil as a coating. After forming a film layer, make a top layer of the patch based on HPMC and PVP in a ratio of 3:1, stir until homogeneous, unite and dry in the oven with the previous layer (15).

Animal Treatments

In the five sample groups that have been divided, the negative control group (K(-)) is a group of rats that were not given any treatment. In contrast,

Table I : Formulation of mucoadhesive patches

Ingredients	Formulation (%)		
	1	2	3
Rambutan peel extract (% v/v)	5	10	15
HPMC (% b/v)	38,2	35,4	32,6
PVP (% b/v)	19	17,8	16,6
Chitosan (% b/v)	12,8	11,8	10,8
Tween 80 (% b/v)	2,8	2,8	2,8
Glycerin (% v/v)	19,4	19,4	19,4
Peppermint oil (% v/v)	2,8	2,8	2,8

the positive control group (K(+)) is a group of rats induced by P.gingivalis LPS without being given a chitosan mucoadhesive patch combination of rambutan peel extract. The treatment group (KP) was a group of rats induced with LPS P.gingivalis and treated using a mucoadhesive patch of chitosan combined with rambutan peel extract in a concentration of 5% in treatment group 1 (KP1), 10% in treatment group 2 (KP2), and 15% in treatment group 3 (KP3).

The rats went through seven days of acclimatization, then 10 µL of LPS P.gingivalis was induced in the gingival area of the mandibular central incisor. Induction was done once a day for two days. On the third day post-induction, the rat's gingiva was clinically observed with signs of gingivitis; the gingiva looked reddish, softer, and bled easily when given a light touch. The rat's gingiva was then treated using a chitosan mucoadhesive patch with a combination of rambutan peel extract. The patch was applied by attaching it to the gingival area of the lower central incisor of the rat. Patch application is done twice daily, routinely in the morning or evening for six days. On the seventh day after patch application, five rats in each group were sacrificed, and the gingival tissue was taken for histological preparations. The remaining organs of the test mice that are not used are properly buried in the ground with the help of laboratory experts.

Histological Preparation

The research data were obtained by counting the number of neutrophil cells in the preparations using a light microscope equipped with an Optilab Plus digital camera and Optilab Viewer image processing software. The preparations were observed in five visual fields with 400x magnification to systematically count the number of neutrophil cells starting from the left corner to the right, then upwards, and so on until the entire visual field was read. The number of neutrophil cells in each sample was recorded and counted in the five visual fields, then the average number was calculated.

RESULTS

Observation of Neutrophil Cell Count

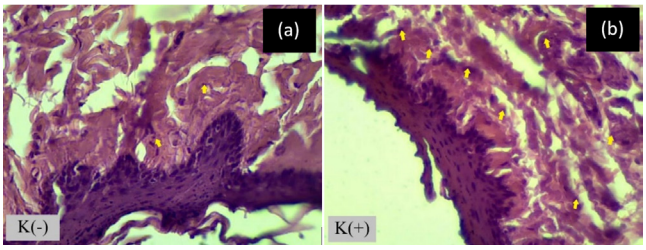

Figure 1 : Neutrophil Cell Number on Group (a) K(-) and (b) K(+).

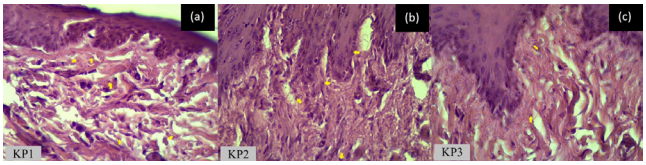

Figure 2 : Neutrophil Cell Number on Group KP1, (b) KP2, and (c) KP3.

Figure. 1 and 2 show histological images of the number of gingival neutrophil cells in Wistar rats with 400x magnification using HE staining indicated by yellow arrows. The images attached are one of the calculation result images from each group picked randomly. In figure 1 (a), two neutrophil cells can be identified, while in figure 1 (b), seven neutrophil cells can be identified. In figure 2 (a), five neutrophil cells are identified, figure 2 (b) shows four neutrophil cells, and figure 2 (c) shows two neutrophil cells. The positive control group (K(+)) shows the highest number of neutrophil cells. While the lowest number of neutrophil cells were in the negative control group (K(-)) and treatment group 3 (KP3).

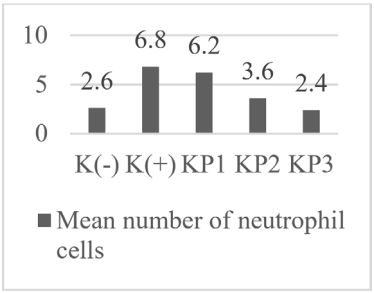

Figure 2 : Diagram of average neutrophil cell count.

Based on the diagram shown in Figure 3, it can be seen that the positive control group (K(+)) had the highest average number of neutrophil cells compared to the other four groups, which was 2.6. Meanwhile,

the lowest average number of neutrophil cells was found in treatment group 3 (KP3), which was 2.4. The negative control group (K(-)) had a slightly higher mean neutrophil cell count than the KP3 group, which was 2.6. Treatment group 1 (KP1) had an average neutrophil cell count slightly lower than group K(+), which was 6.2. Meanwhile, treatment group 2 (KP2) showed the average number of neutrophil cells between groups KP1 and KP3, which was 3.6.

Table II : Results of Kruskal-Wallis Test

Ranks			
	Groups	**N**	**Mean Rank**
Neutrophil cell count	K(-)	5	6.60
	K(+)	5	21.60
	KP1	5	19.40
	KP2	5	12.00
	KP3	5	5.40
	Total	25	

Test Statiscs	
	Neutrophil cell count
Chi-Square	20.763
Df	4
Asymp. Sig.	.000

Table III : Results of Mann-Whitney Test

	Comparison		p
Neutrophil cell count	K(-)	K(+)	0.006
		KP1	0.008
		KP2	0.031
		KP3	0.549
	K(+)	KP1	0.189
		KP2	0.006
		KP3	0.006
	KP1	KP2	0.008
		KP3	0.008
	KP2	KP3	0.020

Results of Statistical Analysis

The Kruskal-Wallis test was carried out because there were groups with data that were not normally distributed in the normality test. The results of the Kruskal-Wallis test in the study of the number of neutrophil cells were obtained as follows:

In table II, obtained Asymp. Sig. (p-value) is 0.000 (p=0.000), which means that the significance value obtained is less than or equal to 0.05 ($p \leq 0.05$), so it can be concluded that there is a significant difference in the results of the calculation of the number of neutrophil cells between K(-), K(+), KP1, KP2, and KP3 group.

Based on the results of the Mann-Whitney test described in table 3, it can be concluded that there is a significant difference between the positive control group (K(+)) with treatment group 2 (KP2) and treatment group 3 (KP3), but in treatment group 1 (KP1) the differences obtained not significant. There was also a non-significant difference between the KP3 group and the negative control group (K(-)). The result indicates that the number of neutrophil cells in the gingival tissue of Wistar rats will decrease significantly along with the high concentration of active ingredients contained in the chitosan mucoadhesive patch combined with rambutan peel extract. In the patch with an active ingredient concentration of 5%, the decrease in the number of neutrophil cells occurred was insignificant. However, in patches with active ingredients concentrations of 10% and 15%, there is a significant decrease in the number of neutrophil cells. The insignificant difference in the patch with a concentration of 15% against the positive control group (K(+)) showed that the patch worked optimally in reducing the number of neutrophil cells at a concentration of 15%.

DISCUSSION

The preparation of mucoadhesive patches works as a local drug delivery agent on the inflamed gingiva through the mucoadhesion system, so that exposure to the active ingredients can be well controlled on the gingival mucosa even in the presence of saliva in the oral cavity. Chitosan as a patch base material can open the epithelial tight junction in the gingival tissue so that it can support the patch's performance in delivering the active drug ingredients well at the site of inflammation.(16). In addition, chitosan also has biodegradable and biocompatible properties so it does not have a toxic effect when the patch is dissolved in the oral cavity by saliva and enters the digestive system. (16).

Rambutan peel extract has an anti-inflammatory

and antibacterial function because of its active substance in flavonoids and phenolic components such as geraniin, saponins, ellagic acid, and tannins. An antibacterial mechanism is done by damaging bacterial cell walls and polysaccharide chains. Pro-inflammatory cytokines such as TNF-α and IL-8 in the tissues are activated as a tissue response to pathogens (12). The reduction of proinflammatory cytokines impacts the decrease in the number of white blood cells, one of which is neutrophils as the dominant cells recruited from the circulatory system to protect tissues when a bacterial invasion occurs as an inflammatory response and works through phagocyte mechanisms or by NETosis (4). The decreased number of neutrophil cells indicates that the inflammatory response of the tissue has been reduced because the activity of neutrophils in reducing pathogens has decreased, and the gingival tissue has returned to its normal condition. (17). The average number of neutrophil cells decreases along with the high concentration of the active ingredient in the patch preparation.

Based on the results of the Mann-Whitney test, which was carried out to determine the difference in significance between groups, it was found that the active patch materials in a concentration of 5% had not been able to significantly reduce the number of neutrophil cells when compared to the positive control group due to the low amount of active materials. It is because the concentration of phenolic compounds in rambutan skin is not sufficient to reduce the inflammatory response in the gingiva either by an antibacterial mechanism (18) through the destruction of the bacterial wall induced in the gingiva or by reducing pro-inflammatory cytokines so that the inflammatory response is still relatively high and a high number of neutrophil cells is still found.

At a concentration of 10% and 15% when compared with the positive control group, the active substance in the patch preparation significantly reduced the number of neutrophil cells so that it could be used as an effective additional therapy for gingivitis. There was also a non-significant difference between the 15% concentration and the negative control group. This shows that the chitosan mucoadhesive patch combined with rambutan peel extract at a concentration of 15% can reduce the number of neutrophil cells. It is an optimal formulation to reduce the inflammatory response to gingival tissue so that there is no significant difference between the gingiva of the negative control group and the group of rats induced by P.gingivalis LPS treated using a patch at a concentration of 15% (19).

Some factors that influence this research process include the variation of salivary flow from the test animals that allows the delivery of different doses of the active ingredient from the patch to the inflamed gingival area. In addition, rats as test animals had a higher oral activity when eating or gnawing, which could cause the patch preparation to come off more easily after being applied. As explained, variations in salivary flow and mechanical stress in the oral cavity can affect the intramucosal drug dose delivery, especially in applying mucoadhesive patches in the gingival area (20). Uniformity and size, weight, and pH adjustment were carried out before the patch was applied to the rat's gingiva to support the consistency and stability of each formulation in delivering the active materials. Evaluation of the patch uniformity is needed to ensure the stability of the preparation before application (21).

CONCLUSION

The application of chitosan mucoadhesive patch combined with rambutan peel extract (Nephelium lappaceum L.) was able to effectively reduce the number of neutrophil cells in the gingival tissue of white rats (Rattus norvegicus) induced by gingivitis, so it can be used as an alternative therapy for gingivitis.

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