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Original article

The effect of antimicrobial peptide gel RISE-AP12 on decreasing neutrophil and enhancing macrophage in nicotine-periodontitis Wistar rat model

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ABSTRACT

Background: *Periodontitis, an inflammation that causes alveolar bone destruction, is caused by bacteria and aggravated by nicotine exposure and is therefore a disease that many smokers have. Antibacterial agents are essential for the rejuvenation process in periodontitis treatment; antimicrobial peptide (AMP) gel is a broad-spectrum antibacterial agent that is hardly cause bacteria resistance.* ***Purpose:*** *The objective of this study is to determine the effect of AMP gel administration on neutrophil and macrophage counts on periodontitis regeneration in nicotine-exposed rats.* ***Methods:*** *24 Wistar rats were separated into four groups: nicotine-exposed, non-nicotine-exposed, treatment and control. Rats with periodontitis were given AMP in the gingival line on days 1, 3 and 7 after having their mandibular central incisors ligated for 14 days to induce periodontitis. After AMP treatment, two groups of rats were collected randomly. Each group were decapitated, followed by treatment and histological examination with hematoxylin-eosin staining in the pathology laboratory to view neutrophils and macrophages. The asymmetric Kruskal Wallis test was used to analyse the data.* ***Results:*** *In mice treated with AMP, neutrophil counts on day 3 were lower than in distilled water (Aquadest) controls. The number of macrophages on day 3 was higher than that of the Aquadest control. Kruskal Wallis test results for neutrophils were $p = 0.017$ and for macrophages $p = 0.01$, where both test results had $p < 0.05$, there were significant differences between the neutrophil and macrophage groups.* ***Conclusion:*** *The administration of AMP effects on decreasing the number of neutrophils and enhancing macrophages in the periodontitis regeneration. in nicotine-exposed rats.*

Keywords: *antimicrobial peptide; nicotine; neutrophil; macrophage; periodontitis*

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INTRODUCTION

Periodontal disease is a chronic inflammatory condition caused by bacteria on the tooth's surface and supporting tissues.[1] Smoking is associated with severe periodontal disease, as data show that smokers have higher rates of clinical attachment loss and chronic periodontal disease.[2] One of the most harmful components of tobacco is nicotine. A more severe form of the disease is produced by nicotine, which disrupts the immune system and balances bacteria in the gingival biofilm.[3,4]

Numerous studies have established that smoking contributes to periodontal disease, obstructs periodontal therapy and significantly affects the chance of effective periodontal treatment outcomes.[5] An electronic database search comparing the outcomes of periodontal treatment in non-surgical and surgical patients including smokers and non-smokers, as well as supportive periodontal therapy, revealed that the healing response of smokers' tissues to treatment was lower than that of non-smokers and that smokers had a higher risk of periodontal disease recurrence.[2]

Periodontal therapy's primary objective is to restore periodontal supporting tissue that has been lost or destroyed as a result of periodontitis. This happens because the healing of periodontitis accompanied by alveolar bone loss

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is also influenced by the presence of large bone damage and is exacerbated by bacterial activity and smoking, so the treatment needs to be accompanied by therapy that involves an infection control process to obtain satisfactory tissue regeneration. Until now, no satisfactory treatment for periodontitis has been found through conventional therapy, the use of antibacterials or with bone graft as an adjunct therapy.[6]

Microbiological and biochemical analyses were performed in addition to clinical periodontal evaluations. It was shown that smoking and non-smoking patients received equivalent clinical results for non-surgical periodontal therapy. Gram-negative bacteria repopulated far more quickly in smokers, presumably indicating a greater risk of recurrence.[7] *Fusobacterium* and *Porphyromonas gingivalis (P. gingivalis)* bacteria are more commonly found in smokers than non-smokers, which the smokers group need potent antimicrobials.[3] According to research by Panpradit et al.[7], nicotine and *P. gingivalis* had a negative effect on osteogenic differentiation. Bacterial drug resistance is a severe health concern that affects people across the globe. Long-term usage and misuse of antibiotics results in bacteria developing drug resistance.[8] Antimicrobial peptide (AMP) is the first line of defence against invasion and infection caused by bacteria and other pathogens in the oral cavity.[9] There are 45 different kinds of antimicrobial peptides released by the salivary glands, oral epithelial cells and neutrophils that are present in the mouth to inhibit the invasion and infection of microorganisms and bacteria. Antimicrobial peptides' inability to respond appropriately to inflammation results in the formation of periodontal disease.[10] By delivering artificial antimicrobial peptides, various reactions and host circumstances that might result in decreased antimicrobial peptide synthesis can be resolved. Artificial antimicrobial peptides, usually in the form of gels or creams, are natural molecules that do not cause side effects like other antibiotics in the form of gastrointestinal reactions, allergies or the production of antibiotic-resistant bacterial strains. JETTINGCLEAN Antimicrobial Peptide RISE-AP12, a periodontal gel biological agent, exhibits broad-spectrum, bactericidal effects and long-term antimicrobial activities, especially on periodontal inflammation caused by sensitive bacteria, such as *P. gingivalis*. This antimicrobial peptide plays a long-lasting inhibitor bacterium and promotes the repair of periodontal tissue.[11]

JETTINGCLEAN as an antimicrobial peptide is naturally involved in apoptosis, wound healing and immunological regulation. It also possesses a broad-spectrum bactericidal activity, capable of attacking gram-positive and gram-negative bacteria, viruses, fungi and protozoa. Thus, it can be an alternative to anti-infective therapy and immunomodulatory agents.[12]

Many cells contribute to the regeneration of periodontal tissue, including neutrophils, monocytes, macrophages, lymphocytes, dendritic cells, fibroblasts and osteoblasts, each of which play a very important role from the beginning of the healing process, namely from the inflammatory stage to the final stage of readhesion.[13] This is an inflammatory response starting from neutrophils moving from the gingival blood vessels to the junctional epithelial tissue and destroying the bacterial biofilm, turning it into apoptotic tissue. Subsequently, macrophages play a critical role in cleaning apoptotic tissues by phagocytosis, activating lymphocytes that make IL-17 and finally, producing fibroblasts which undertake the remodelling process.[14]

The novelty of this study is the use of peptide gel antimicrobial therapy in smokers with periodontitis. The aim of this study is to analyse the impact of the RISE-AP12 antimicrobial peptide application on rats exposed to nicotine on the regulation of periodontitis in terms of the decreased number of neutrophils and enhanced number of macrophages.

MATERIALS AND METHODS

The research was conducted at the Integrated Research and Testing Laboratory unit 4, Gadjah Mada University (LPPT UGM unit 4). All the procedures of the in vivo experiment were approved by the Ethical Committee of the Faculty of Dentistry, Muhammadiyah University of Yogyakarta, 195/EP-FKIK-UMY/IX/2019. The laboratory experiment was done using JETTINGCLEAN antimicrobial peptide periodontal gel containing antimicrobial peptide RISE-AP12, which has bacteriostatic and bactericidal properties against *P. gingivalis* (Rise Biopharmaceuticals Inc, Beijing, China).[10]

This study used 24 healthy and active Wistar male rats, weighing between 300 and 400 grams and aged 3 to 4 months. The experimental animals were adapted in a clean cage for 7 days. The rats were housed in separate cages and maintained under a 12-hour light/dark cycle at a temperature of 23 °C and relative humidity of 50%, with access to standard rat chow pellets and water ad libitum.[15] The rats were then divided into two groups of nicotine (n = 12) and non-nicotine (n = 12), and each group was further divided into two, namely AMP treatment (n = 6) and Aqua Dest control (n = 6) exposures. In the group of rats with nicotine exposure, nicotine (RTS Vapes, Charlotte, USA) injection was provided at a dose of 16 µg/20 g of rat bodyweight for three consecutive days peritoneally on days 1, 2 and 3.[16] Furthermore, the experimental rats were locally anesthetized by using Ketamine 10% (Kepro BV, Deventer, Netherlands). A 3 mm silk ligature (B. Braun, Rubi, Spain) was then attached as a ligation to the mandibular incisors for 14 days to generate periodontitis, with the description of the gingiva being red, swollen and moving apically away from the ligature.[15]

The ligation was released after 14 days. Both nicotine and non-nicotine groups were then further divided into two subgroups with a total of four groups (n = 6) for anti-microbial peptide gel and control administration as

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follows: nicotine with AMP (Group 1); non-nicotine with AMP (Group 2); nicotine with Aqua Dest (Group 3); and non-nicotine with Aqua Dest (Group 4). All rats were given treatment on day 1, day 3, and day 7. For both Group 1 and Group 2, rats were given an application of a single spread of antimicrobial peptide periodontal gel consisting of Rise AP-12 (Rise Biopharmaceuticals Inc, Beijing, China) using a cotton swab on the margin gingiva of mandibular anterior incisor that had undergone periodontitis. Meanwhile, the negative control groups, Group 3 and Group 4, were given one spread of Aqua Dest.

On days 3, 7, and 14 after the treatment, two rats were decapitated in each group of test animals. The Wistar rats were anesthetized with ketamine (0.1 ml) and xylazine (0.1 ml) by intramuscular injection in the thigh (dose 6, 12 mg/kg).[7,15]

Tissue that had periodontitis was then employed as a specimen to be made into preparations by the Anatomical Pathology Laboratory of AMC Hospital using an automatic tissue processor (Medimeas, Haryana, India) and then placed in a paraffin block and cut using a Finesse 325 microtome (Thermo Fisher Scientific Inc, Massachusetts, United States). The slides were stained using an hematoxylin-eosin (HE) stain and viewed using an Olympus Cx23 microscope (Olympus Corporation, Tokyo, Japan) with a magnification of 400x and captured using an OptiLab microscope camera (PT Miconos, Yogyakarta, Indonesia) in the alveolar crest of the mandible incisor using six visual fields.

The normality test was obtained from the observational data on the number of macrophages and neutrophils using the Kolmogorov-Smirnov test analytical method, and comparative tests were performed using Kruskal Wallis. The data analysis was done using SPSS Statistics 23.0 (IBM Corporation, New York, United States).

RESULTS

The data from the analysis of the number of neutrophils in each of the six fields of view are shown in Table 1. This shows that there are significant differences in the number of neutrophils in the exposure and treatment groups with $p = 0.017$ ($p < 0.05$). The test results show the highest average treatment rating on nicotine on day 3 on AMP and the lowest on day 7 on AMP (Figure 1).

Table 1. The mean and standard deviation of the number of neutrophils in the Kruskal Wallis test in the nicotine + AMP, non-nicotine + AMP, nicotine + Aqua Dest and non-nicotine + Aqua Dest groups based on the day of observation

	AMP			Aqua Dest		
	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
Nicotine	4.17±1.72	1.83±0.75	2.50±0.84	3.00±2.00	3.00±1.79	3.00±1.26
Non-nicotine	1.50±1.22	3.83±1.17	2.83±1.17	6.50±2.95	4.00±1.90	3.33±1.97
Asymp. Sig (p)	0.017*					

Note: *p < 0.05

Table 2. The mean and standard deviation of the number of macrophages in the Kruskal Wallis test in the nicotine + AMP, non-nicotine + AMP, nicotine + Aqua Dest and non-nicotine + Aqua Dest groups based on the day of observation

	AMP			Aqua Dest		
	Day 3	Day 7	Day 14	Day 3	Day 7	Day 14
Nicotine	5.50 ± 1.87	5.67 ± 4.08	8.50 ± 3.62	6.50 ±1.51	5.67±3.55	7.50±2.81
Non-nicotine	5.50 ± 0.81	8.00 ± 5.02	8.50 ± 1.52	0.67±3.23	5.83±3.31	7.00±2.45
Asymp. Sig (p)	0.001*					

Note: *p < 0.05

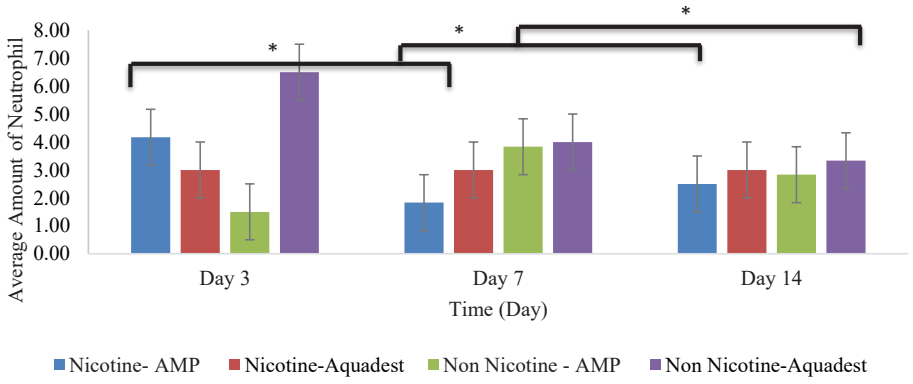

Figure 1. The increasing pattern of the number of neutrophils between the nicotine exposure and treatment groups based on observation day 3, day 7 and day 14 between the neutrophils in nicotine + AMP, nicotine + Aqua Dest, non-nicotine + AMP and non-nicotine + Aqua Dest groups. *p < 0.05

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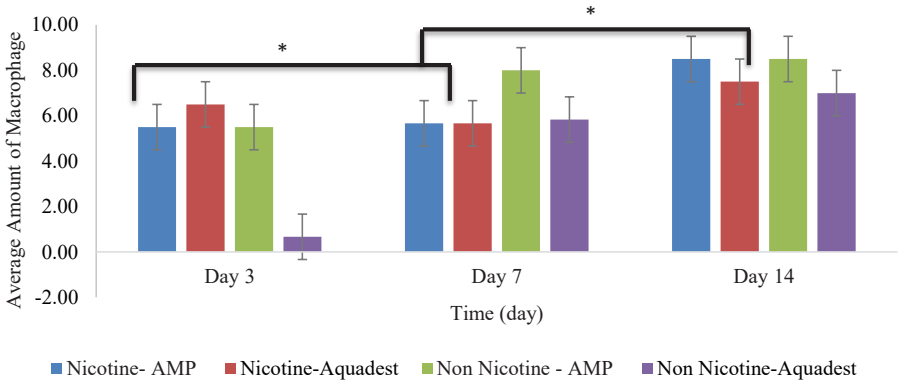

Figure 2. The increasing pattern of the number of macrophages based on observation day 3, day 7 and day 14 between macrophage in nicotine + AMP, nicotine + Aqua Dest, non-nicotine + AMP and non-nicotine + Aqua Dest groups. *p < 0.05

The data in Table 2 shows that there is a significant difference in the number of macrophages between the exposure and treatment groups p = 0.001 (p < 0.05). The test results showed that the average nicotine treatment was highest on day 14 on AMP (Figure 2).

DISCUSSION

Based on the statistical results of the study a greater number of neutrophils and macrophages were observed in the periodontitis area in non-nicotine-exposed subjects compared to the nicotine-exposed. This shows that the inflammatory process is inhibited by nicotine through neutrophil activity and automatically reduces the function of macrophages in response to inflammation. Based on ex vivo studies, it was stated that cigarette extract significantly (p < 0.001) resulted in minimal movement and a decreased neutrophil migration rate to where it could affect signal communication between neutrophil cells, which in turn could inhibit the production of neutrophils and other inflammatory cells. Although neutrophils can still reach the infected area, it takes a longer time.[17]

The neutrophil count of the treatment of nicotine + AMP on day 3 was lower than on day 7, with the possibility on the third day of new neutrophils slightly appearing and reaching their peak on days 5 and 6; unfortunately, on the fifth day of the study, neutrophil counts were not carried out. Moreover, on the seventh day, the number of possible neutrophils started to decrease and was still decreasing on the fourteenth day. Due to minimal movement and a decreased rate, this can cause more severe tissue damage mediated by neutrophils. In general, smoking can affect the success of neutrophils in eliminating pathogenic bacteria in periodontal disease.[17] In their study, Dhall et al. explained that nicotine can weaken monocytes to differentiate into macrophages, thereby interfering with the inflammatory process.[18] Likewise, research by Ertugrul et al. stated that patients who smoked could reduce the bactericidal effect of antimicrobial proteins on periodontal tissue, which then caused damage to periodontal tissue.[19]

The number of neutrophils increased on day 3 compared to day 7, while neutrophil activity in the baseline periodontitis of subjects exposed to nicotine is inhibited because of the effects of nicotine that affect antimicrobial performance on neutrophils. Neutrophils activity can be identified in the number of neutrophil in the subjects exposed to nicotine, which is lower than in the subjects without nicotine exposure, who were not inhibited by neutrophil activity in the inflammatory response. In a previous study, it was explained that one type of antimicrobial peptide, LL-37, which was found in neutrophils and gingival crevicular fluid, would be significantly higher in subjects with periodontitis than in healthy subjects.[20] This is consistent with data on higher neutrophil counts in periodontitis subjects which were not exposed to nicotine. From an in vitro study, after 24 hours of administration of AMP gel, it was identified that the metabolic activity of *P. gingivalis* bacteria decreased in the form of a loss of its ability to transfer resazurin to resorufin in the mitochondrial activity of bacterial cells.[21] Although different responses were seen in subjects without nicotine exposure, the number of neutrophils decreased in both the AMP and control groups. This is because the high neutrophil count with no nicotine exposure is believed to have undergone apoptosis on day 3, leading to a decrease in the number of neutrophils. This is related to a previous study on neutrophils which stated that neutrophils in the tissue will undergo apoptosis after two days, which will then be phagocytized by macrophages.[18] In contrast to the group of rats that were not exposed to nicotine on the third day, there was a decrease in the number of macrophages. It was explained in a study by Reinke and Sorg[22] that on the third day after inflammation occurs, new macrophages move to the previous tissue that had experienced inflammation when neutrophils were found in the inflammatory tissue on day 2.

On the third day of treatment, histological examination showed higher inflammation in macrophages and neutrophils in the AMP treatment group than in the control group. According to the results, neutrophil and macrophage counts increased in nicotine-exposed subjects with AMP

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therapy, indicating that neutrophil activity was restored to normal function in the inflammatory response after three days of topically applied artificial AMP.

On the seventh and fourteenth days of treatment, the AMP treatment group had lower neutrophils than the control group, with or without nicotine exposure. This suggests that artificial AMP improves in the acceleration of the periodontal healing process, which can be seen in the reduced inflammatory process in terms of the number of neutrophils. Likewise, the number of macrophages on the seventh and fourteenth days of the AMP treatment was higher than in the Aqua Dest control. This indicates that the inflammatory process returned to normal function on the seventh day of treatment. The effect of AMP application was proven by Lin et al., who researched wound-healing by employing an AMP dressing mixed with alginate/hyaluronic acid/collagen, wherein application of the AMP dressing on day 7 had a significant effect in that it could reduce the number of *S. Aureus* bacteria and *E. Coli* on wound tissue properly and thereby accelerate the healing process.[23] Research by Wang et al. also asserted that the application of AMP can increase the ability of macrophage cells, as evidenced by the application of AMP Sublacin to inflammatory tissue, which can increase protein and mRNA in IL-1β, IL-6 and TNF-α so that it can increase activity of macrophages in phagocytizing bacteria, dead cells or debris in inflammatory tissue.[24] It is also evidenced by the research of Wang et al. that one of the antimicrobial peptides, including salivary and gingival crevicular fluid (GCF) and known as LL-37, when administered to inflammatory tissue can increase the expression of cytokines in macrophages, which in turn increases the phagocytic ability of macrophages.[25] In conclusion, this study has shown that the administration of AMP, by examining the amount of neutrophils and macrophages, affects the regenerationsof. periodontitis in nicotine-exposed rats.

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