Effects of Snail (*Achatina fulica*) Mucus on the Levels of 8-Hydroxydeoxyguanosine and Nuclear Factor Kappa Beta Biomarkers in Periodontitis-induced Wistar Rats

I Gusti Agung Ayu Putu Swastini[1], I Gusti Agung Ayu Dharmawati[1], Sagung Agung Putri. Dwiastuti[1], Ida Ayu Dewi Kumala Ratih[1], Ida Ayu Made Sri Arjani, Ni Luh Suriani[*]

[1]*Health Polytechnic of Denpasar, Bali, Indonesia*
[2]*Master of Science in sustainable finance and development, postgraduate, Udayana University, Bali, Indonesia*

Corresponding author: E-mail Ni Luh Suriani:niluhsuriani@unud.ac.id

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ABSTRACT

Periodontitis, a common form of dental disease, is a bacterial infection causing inflammation and tooth loss due to the loss of connective tissues and bones. Nuclear factor-kappa B (NF-kB) and 8-hydroxydeoxyguanosine (8-OHdG) regulate inflammatory processes involving the tooth gingival and alveolar bones. Snail mucus, a natural ingredient, has been proposed as a cure for periodontitis. The aim of the current study was to evaluate the impact of snail mucus on the levels of the 8-OHdG and NF-kB biomarkers during the healing process in periodontitis-induced Wistar rats. White Wistar rats were categorized into three groups: treatment, non-treatment, and control. *Aggregatibacter actinomycetemcomitans* bacteria were used to induce periodontitis in the treatment and non-treatment groups. Snail mucus was administered to the treatment group. Blood was collected from the three groups to assess the 8-OHdG and NF-kB biomarker levels. The analysis of 8-OHdG serum levels in rats showed that the highest optical density of 2.523 was observed at a concentration of 0 ng/mL, while the lowest optical density of 0.201 was recorded at a concentration of 100 ng/mL. The treatment group exhibited a significantly lower 8-OHdG level (14.7 ng/mL) compared to the non-treatment group (28.2 ng/mL). A similar observation was made with NF-kB levels, with the treatment group having a significantly lower level (121.3 pg/mL) than the non-treatment group (179.4 pg/mL). The study found that administering snail mucus at 250 mg/kg body weight to rats with periodontitis can decrease 8-OHdG and NF-kB levels, enhancing the healing process.
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Introduction

Periodontal disease, especially periodontitis, is a disease found in the oral cavity and can be suffered by people in developed and developing countries. Awareness of the importance of dental and oral health care has not been fully realized, because it is considered not to cause death, although, it can impact masticatory and aesthetic functions (Deby et al., 2022). Periodontitis is a significant dental problem characterized by an inflammatory disease caused by infection, primarily due to biofilm composition. According to the findings of Basic Health Reseach (2018), the prevalence of periodontitis is 67.8% in people aged ≥15 years old, which indicates that out of ten Indonesian populations, seven people suffer from periodontitis (Pallav.et al 2013). The accumulation of dental plaque at the gingival margin triggers an inflammatory response that further causes microbial changes and can cause drastic risk in the susceptible individuals' periodontium. Periodontitis can originate from chronic inflammation of the gingiva and cause permanent loss of adhesions and alveolar bone. The disease usually appears in the adult age of the population, but young individuals can also experience it and the results are dangerous. Periodontitis is linked to various chronic conditions that impact general health (Eija K, et al., 2019).

The inflammatory response in periodontal tissues caused by bacterial plaques, such as Aggregatibacter actinomycetemcomitans, results in the generation of free radicals like hydrogen peroxide, superoxide anion radicals, hydroxyl radicals, and hypochlorous acid (Maria et a.l, 2021). Oxidative damage is observed in patients with chronic periodontitis, accompanied by the formation of 8-hydroxydeoxyguanosine (8-OHdG). Patients with chronic periodontitis,gingivitis, and healthy people have high salivary levels of 8-OHdG (Sezeret et al., 2012). Inflammation is known as the protective response of the host to infection and tissue damage. Vasodilation and recruitment of immune cells and plasma proteins to the site of infection or tissue injury are part of a reaction series that characterizes an inflammation (Zhang et al., 2015). Inflammation, typically beneficial and healing-oriented, can lead to acute or chronic diseases due to excessive or lasting tissue damage. The central mediator of pro-inflammatory gene induction is nuclear factor kappa Beta (NF-kB), which functions in innate and adaptive immune cells (Ulayya et al., 2015).

Indonesia is a tropical country with many types of snails (*Achatina fulica*). Thepresence of *A. fulica* is underutilized and its mucus can accelerate wound healing (Suriadi, 2020). Previous research has shown that snail mucus contains a glycosaminoglycan that plays an important role in wound healing by forming complexes in the proliferative phase, called acharan sulphate. Calcium content plays a role in haemostasis while achatin in the isolates is an antibacterial and pain reliever (Bagaskara, 2019). . Snail mucus, rich in antibacterial and anti-inflammatory ingredients, can expedite wound healing by accelerating the inflammatory phase (Nuringtyas dan Rini 2010) . Heparan sulphate in snail mucus significantly influences fibroblast proliferation, aiding blood clotting and accelerating wound healing through fibroblast cell proliferation.

The present study examined the effects of snail mucus on the levels of 8-OHdG and NF-kB biomarkers during the healing phase in periodontitis-induced Wistar rats.

1. Methodology

Sources of animals

The snails used in the study were obtained from Uma Anyar, Nyalian Village, District of Banjarangkan, Klungkung Bali, Indonesia. Also, white Wistar rats, weighing between 200-250 g, were acquired from the Histology Laboratory at Udayana University in Denpasar, Bali, Indonesia.

Experimental grouping and treatment

Fifteen (15) Wistar rats were assigned to three groups: the treatment group (P1-P7), the non-treatment group (N1-N7), and the control group (C). The Wistar rats were injected with *Aggregatibacter actinomycetemcomitans* bacteria at the gingival of the lower jaw's incisor teeth to induce periodontitis in both the treatment and non-treatment groups. After periodontitis developed on the seventh day, 1 mL of snail mucus was applied topically three times daily to the treatment group. This was followed by a gingival examination of all the groups on the eleventh day. Blood samples were collected from the rats to measure the levels of 8-OHdG and NF-kB.

Measurement of 8-hydroxydeoxyguanosine levels by the enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was employed to measure the levels of 8-hydroxydeoxyguanosine in blood samples, following the manufacturer’s instructions. The ELISA kit operates on the competitive ELISA principle. Micro-ELISA wells were pre-coated with 8-OHdG. During the procedure, the 8-OHdG in the solid-phase support competed with the 8-OHdG in the sample or standard for detection by biotinylated 8-OHdG-specific antibodies. The conjugated and unbound sample or standard was then washed off the plates, and avidin conjugated to horseradish peroxidase (HRP) was added to each well. The microplate was incubated, and a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was introduced into each well. The enzyme-substrate reaction was terminated by adding a stop solution, and the color change was measured using a spectrophotometer at a wavelength of 450±2 nm.The sample's OD was then compared to a standard curve to measure the concentration of 8-OHdG.

Determination of NF-kB-p65 concentration by the enzyme-linked immunosorbent assay

The ELISA kit (Elabscience; Catalogue Number: E-EL-R0674) utilizes the sandwich-ELISA method. Micro-ELISA plates were coated with antibodies specific to NF-kB-p65 in mice. The sample or standard was then added to the wells of the micro-ELISA plate and mixed with the specific antibodies. Following this, biotinylated detection antibodies targeting mouse NF-kB-p65 and avidin-HRP conjugate were sequentially introduced to each well and incubated. A substrate solution was subsequently added to each well. Only the wells containing NF-kB-p65 serum, biotinylated detection antibodies, and avidin-HRP conjugates turned blue. The enzyme-substrate reaction was halted by adding a stop solution, which resulted in a yellow color change. The optical density (OD) was measured using spectrophotometry at a wavelength of 450±2 nm. The serum concentration of NF-kB-065 in the sample was related to its OD value and calculated by comparing the standard curve with the OD of the sample.

Statistical analysis

The normality test data were analyzed using the Kruskal-Wallis and Spearman rank correlation tests. Kolmogorov-Smirnoff test was performed to examine regularly distributed data with a p-value ≥ 0.005. The Levene's test was conducted to assess data homogeneity between experimental groups at a 5% significance

level. The data were homogeneous if the p-value of Levene's test was ≥ 0.005 for the treatment effect test. If the data were normally distributed, the average difference between groups was analyzed with a one-way analysis of variance (ANOVA) was performed, and the Kruskal-Wallis test was applied for data that did not follow a normal distribution.

2. Result and Discussion

3.1 Result

The highest optical density (2.523) of serum 8-OHdG in rats was observed at 0 ng/mL, while the lowest optical density (0.201) was obtained at 100 ng/mL (Table 1). It was observed that the absorbance values varied with the concentration of 8OH- dG.

Table 1. Absorbance values for measuring the levels of 8-hydroxydeoxyguanosineusing a UV-Vis spectrophotometer

Well	Concentration (ng/mL)	Absorbance (OD)
A1	100	0.201
A2	50	0.340
A3	25	0.553
A4	13	0.902
A5	6	1.342
A6	3,13	1.702
A7	1,56	2.105
A8	0	2.523

Table 2 shows that the highest levels of 8-OHdG in the rat serum (28.2) were obtained in the non-treatment group, while the lowest level (7.3) was in the control group. The average 8-OHdG levels in the treatment and non-treatment groups were 14.70±0.43 and 28.2±0.73, respectively (Table 3), indicating a statistically significant difference ($p < 0.00$).

Table 2. Serum levels of 8-hydroxydeoxyguanosine in the experimental groups

Group	Sample ID	OD	8-OHdG (ng/mL)	8-OHdG/group
Treatment	P1	1.625	11652	14.7
	P2	1.689	9631	
	P3	1.567	13.483	
	P4	1.315	21.442	
	P5	1.425	17.968	
	P6	1.542	14.273	
	P7	1.546	14.147	
	N1	1.094	28.421	
	N2	1.142	26.905	

Non-treatment	N3	1.012	31.011	28.2
	N4	0.892	34.800	
	N5	1.154	26.526	
	N6	1.092	28.484	
	N7	1.332	20.905	

Control	1	1.762	7.325	7.3

Table 3. Statistical analysis of the measurement of 8-hydroxydeoxyguanosine levelsin the topical group

Group	n	Mean	SD	p-value
Treatment	7	14.7	0.427	p= 0,001
Non-treatment	7	28.2	0.728	
Control	1	1.10	0.970	

Treatment group: periodontitis-induced rats treated with snail mucus; Non-treatment group: periodontitis-induced rats that were not treated with snail mucus; Control: Normal mice without treatment; 8-OHdG: 8-hydroxydeoxyguanosine.

As demonstrated in Table 4, the absorbance value of each snail mucus NF-kB concentration had different absorbance values. The study found that snail mucus ata concentration of 5,000 pg/mL had the highest absorbance value among all concentrations

Table 4: Absorbance values for measuring the levels of nuclear factor kappa Beta using a UV-Vis spectrophotometer

Well	Concentration (pg/mL)	Absorbance (OD)
A1	5000	2.456
A2	2500	1.665
A3	1250	0.922
A4	625	0.482
A5	313	0.188
A6	156,25	0.101
A7	78,13	0.055
A8	0	0.000

The results (Table 5) showed that the serum NF-kB level of rats was higher in the non-treatment group (179.4 pg/mL) compared to the control group (81.9pg/mL). The average NF-kB level of the treatment group was 121.30±3.00 pg/mL, while the mean of the non-treatment group was 179.40±0.73 pg/mL, indicating a statistically significant difference ($p > 0.005$).

Table 5: Serum levels of nuclear factor kappa Beta in the experimental groups

Group	Sample ID	OD	NF-kB (pg/mL)	NF-kB/group
	P1	0.142	116.137	
	P2	0.145	121.837	
	P5	0.154	138.937	
	P6	0.155	140.837	
	P7	0.128	89.537	
	N1	0.168	165.537	
	N2	0.188	203.538	

Non-	N3	0.192	211.138	
Treatment	N4	0.157	144.637	179.4
	N5	0.184	195.938	
	N6	0.201	228.238	
	N7	0,137	106,637	
control	C	0,124	81,937	81,9

Treatment group: periodontitis-induced rats treated with snail mucus; Non-treatment group: periodontitis-induced rats that were not treated with snail mucus; Control: Normal mice withouttreatment; NF-kB: nuclear factor kappa Beta

However, there was no statistically significant difference between the NF-kB level in the topical and the control groupsas shown in Table 6.

Table 6: Statistical analysis of the measurement of nuclear factor kappa Beta levelsin the topical group

NF-kB Levels	n	Mean	SD	p-value
Treatment	7	121.3	2.995	
Non-treatment	7	179.4	0.728	0.005
Control	1	81.9	0.970	

3.2 Discussion

In this investigation, mice were induced with the bacterium, *Aggregatibacter actinomycetemcomitans* to develop periodontitis and this was followed withtreatment with snail mucus. The results demonstrated that treatment with snailmucus lowered 8-OHdG and NF-kB levels in white Wistar rats with periodontitis, as evidenced by statistical analysis. Snail mucus contains active chemicals, such as phenols, saponins, and tannins, as well as flavonoid compounds, which influence antioxidant activity. Snail slime has strong antioxidant activity at concentrations of 5 and 10% (83.63±0.57 and 57.93±0.82 ppm, respectively), while snail mucus exhibited strong antioxidant activity at 15% (31.59±0.59 ppm) (Suhesti, et al., 2018) . Previous studies have shown a positive correlation between 8-OHdG levels and periodontal tissue loss. The administration of snail mucus has been shown in studies to treat periodontal lesions and lower 8-OHdG levels. The 8-OHdG content and plasma concentrations of 8-OHdG in the periodontitis group were reduced in response to snail mucus treatment. The number of 8-OHdG-positive fibroblasts correlates with plasma oxidative stress markers. In periodontitis, 8-OHdG is formed via the hydroxylation of deoxyguanosine at the C-8 location in DNA, which generates reactive oxygen species (ROS) in response to lipopolysaccharide (LPS) stimulation. Reactive oxygen species expression (such as H_2O_2) is observed at junctional epithelia (Daisuke, et al 2018). Another study reported the concentrations of the components in snail mucus to include heparan sulphate (16.45/100), acharan sulphate (21.33/100), achatin sulphate (30.06/100), Ca^{+} ions (86.12/100), β-agglutinin (58.22/100), achasin protein (90.2/100), and glycokonyugat (8.86/100) (Swastini, et al., 2022). Also, very strong antibacterials against Gram-negative bacteria have been found in snail mucus. This analysis demonstrates that the high concentration of chain protein in snail mucus can activate gene transcription, immunological response, inflammation, and apoptosis, as observed by

a reduction in NF-kB levels (Backwel et al., 1997).. The transcriptional factor NF-kB regulates innate and adaptive immune activity and plays a crucial role in the inflammatory response. Nuclear factor kappa Beta stimulates the expression of several pro-inflammatory genes, including chemokines and cytokines. Also, it is involved in the regulation of inflammation. Furthermore, NF-kB regulates the survival, activation, and development of innate immune cells, as well as inflammatory T cells. As a result, uncontrolled NF-kB activation contributes to the pathogenic processes of a variety of inflammatory disorders (Liu, et al 2017).

The limitation of this study is in the use of the ELISA method. A more accurate method is the immunohistochemical analysis because it detects antibodies (proteins and carbohydrates) in cells and tissues. The technique is based on the principle of antibody reactions that bind to antigens in tissues, and the results usually reveal more microscopic details. Immunohistochemical analysis is highly recommended in this type of study because it involves bacteria that cause oxidative stress, as well as investigating snail mucus function in apoptosis. The future direction of this study is to prepare snail mucus in gel form for application to the gingival pocket to reduce the chance of materials leaking out of the pocket and promote more effective healing.

3. Conclusion

Snail mucus can decrease 8-OHdG and NF-kB levels in Wistar rats with *Actinobacillus actinomycetemcomitans*-induced periodontitis, promoting the healing process of periodontitis.

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