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ANTI-INFLAMMATORY EFFECT OF BETEL QUID ON MUCOSAL WOUND OF MALE WISTAR (*RATTUS NOVERGICUS*) RATS

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ABSTRACT

Objective: True experimental *in vivo* with pre-test and post-test control group design was conducted at Animal House of Medical Faculty of Sriwijaya University Palembang and Biomedical Science Laboratory of Palembang, South Sumatera.

Methods: Thirty rats were divided into five groups; three groups were treated with betel quid ointments at concentration of 5%, 10% and 20%, one group was positive control (hyaluronic acid) and last group was negative control (placebo). One-mm diameter of wound was made on lower lip mucosa of rats with cylinder diamond bur. Wound was induced with carrageenan. The number of neutrophils was counted on the first day and third day after treatment by using hematoanalyzer.

Results: There was a significant decrease of the number of neutrophils for respective groups ($p<0.05$). Betel quid ointment of 10% and 20% concentration had similar effect to 0.2% hyaluronic acid ($p>0.05$).

Conclusion: Betel quid has an anti-inflammatory effect on the mucosal wound of rats in a dose-dependent manner.

Keywords: Anti-inflammatory, Betel quid, Mucosal wound

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INTRODUCTION

Inflammation is a biological response to tissue damage, caused by pathogens, chemicals, or physics. The main function of the inflammation process is to resist tissue damaged and keep it from spreading, eliminate the cause of the wound, and stimulate tissue repair [1]. Inflammation is characterized by heat (calor), redness (rubor), swelling (tumor), pain (dolor) and loss of function (functiolaesa) [2]. The sensation of heat is due to the increased movement of more blood in enlarged vessel on the wound area. The redness is caused by the increased number of erytrocytes to the affected area. Swelling is due to a buildup of fluid on permeable and dilated vessel into the surrounding tissue. Pain is because the stimulated nerve endings are released. Loss of function is related to immobility joint in the inflamed tissue and scar tissue replacement on the wound. This process is happened an hour after injury [3, 4].

Inflammation commonly found in oral mucosa is caused by a number of physical injury (i.e., bitten, collide, or instrumentation) or an infection [5]. Those injuries trigger the immune system as response, and show inflammation signs. The main processes occur during the inflammation are the increased supplying blood to the injury and lead increased blood movement in dilated arteries. The vessels become more permeable and easier for fluids and proteins to infiltrate [6]. After a few hours, neutrophils are released on wound area. The highest number of neutrophils are on first and second days after injury [7]. Neutrophils express as the primary defence against patoghens. On the site of injury, where it assembly activating signals to trigger bacterial killing, neutrophils are mobilized, then secrete antimicrobial agents such as cytokines, chemokines, and proteases. The rapid neutrophil migration from the circulation on to injury is regulated by vascular endothelium [8, 9]. Activation of neutrophils is initiated by cytokines, such as interleukins (IL-1β, IL-8), tumor necrosis factor (TNFα), leukotrienes, pathogens/bacterial products and by hit stimulus [10]. Neutrophils eliminate microorganisms and dead cells through phagocytosis with different cytotoxic mechanism, the release of free radicals and deliver of proteolytic enzymes into phagosome [11]. When neutrophils confront with foreign particles, the cells will recognize the particles, swallow or phagocyte, then destroy the foreign particles. Neutrophils perform the process of marginization, adhesion and migration before being ready for the function. The neutrophil's life cycle is regulated by granulocyte colony-stimulating factor (G-CSF) [7]. Along with the presence of neutrophils, macrophages are formed. Macrophages are formed by chemotaxis and migration. By day 3, the neutrophils are phagocytized by macrophages. Peripheral blood monocytes on the wound site differentiate to be macrophages [12]. Macrophages are formed by chemotaxis and migration. The number of macrophages reaches a maximum after 4-5 d. Macrophages play a role in the process of phagocytosis. Macrophages kill pathogenic micro-organisms and initiate tissue regeneration processes [13].

Anti-inflammation agents are used to reduce the symptom and accelerate wound healing process. One of them is hyaluronic acid. Hyaluronic acid is considered one of anti-inflammatory agents [14]. Previous study mentioned that topical hyaluronic acid of 0.2% concentration is effective on the healing of gingivitis [15]. But hyaluronic acid has side effects, such as allergic or hypersensitivity reaction, edema, ecchymoses, hypercorrection, bluish discoloration, and indurated nodules [16]. Alternative medicine from natural product as anti-inflammatory agent with minimum side effect is needed to find. One of them is betel quid.

Betel quid is combination of betel (*Piper betle* Linn.), areca nut (*Areca catechu* Linn), gambier (*Uncaria gambir* Roxb.), and mineral slaked lime (calcium hydroxide) [17]. All components of betel quid have been proven to have anti-inflammatory effect. Betel has been used as anti-inflammation in oral mucosa for long time. Alam *et al.* reported that *Piper betle* leaves had antioxidant, analgesic and anti-inflammatory activities [18]. The anti-inflammatory activities of *Piper betle* is due to its chemical constituents, such as eugenol, hydroxychavicol, quercetin, β-caryophyllene [19].

The betel nut is the dark red seed of Areca catechu. This plant has several therapeutic properties, including analgesics, anti-inflammatory and antioxidants [20]. Bhandare *et al.* revealed that hydroalcohol extract from *Areca catechu* nut inhibited extravasation of plasma protein and inflammatory progression in durameter [21].

The areca nut extract contains procyanidins, major condensed tannins that widely disseminates its phenolic compound and have pharmacological effect [22]. Procyanidin is effective in inhibiting the expression of pro inflammatory mediators and cytokines [23]. Katoh *et al.* reported that procyanidin suppressed TPA-induced inflammation of mouse, and its activity was stronger than indomethacin and glycyrrhetinic acid [24].

Gambier (*Uncaria gambier* Roxb.) plays important role as an anti-inflammatory agent. Seto *et al.* mentioned that gambier extract was effective in reducing neutrophil cells in inflammation phase of male Wistar's injury [25]. The anti-inflammatory activity of gambier is due to its chemical compound, catechins [26]. Catechins are polyphenol, and group of flavonoids that exhibit water soluble characteristics [27]. Trekli *et al.* reported that catechins suppressed cytokine-induced-IL6 and IL8 and showed anti-inflammatory activity [28].

Mineral slaked lime contained in betel quid is commonly used in dentistry. Mineral slakeed lime has a formula of $Ca(OH)_2$ or calcium hydroxyde. Calcium hydroxyde has been proved has an antibacterial effect and anti-inflammation in the periapical lesion, so that it mostly used in root canal treatment [29]. Anti-inflammatory effect of calcium hydroxyde is rely on alkalinity and calcium ion release. The high pH of calcium hydroxide encourages antibacterial effect, inflammatory reduction, and stimulate tissue repair [30]. Dixit *et al.* stated that calcium hydroxyde was succesful resolution in treating periapical lesions [31].

The study about the anti-inflammatory effect of betel quid in male Wistar rats has not been done. The aim of this study was to investigate the anti-inflammatory effect of betel quid on the oral mucosal wound of male Wistar (*Rattus norvegicus* L.) rats.

MATERIALS AND METHODS

Material

This study was true experimental *in vivo* with pretest-postest control group design. The research was conducted at Animal House of Medical Faculty of Sriwijaya University Palembang and Biomedical Science Laboratory of Palembang, South Sumatera. The protocol had been approved by Research Ethical Commission of Mohammad Hoesin General Hospital (RSMH) Palembang and Medical Faculty of Sriwijaya University with ethical certificate No. 391/kepkrsmhfkunsri/2017.

Animals

Thirty male white Wistar (*Rattusnorvegicus* L.) rats (obtained from Pharmacy School of Bandung Institute of Technology) were divided into 5 groups. Group I was treated with 5% betel quid ointment, Group II was treated with 10% betel quid ointment, Group III was treated with 5% betel quid ointment, Group IV was positive control, treated with 0.2% hyaluronic acid ointment (purchased from Ricefarma Pharm. Co, Surabaya, Indonesia), and Group V was negative control, treated with placebo ointment. Rats (weighing 150-200 mg, aged 8-12 w old) were acclimated for 8 d at room temperature of 20-25 °C under 12:12 h light-dark cycle, prior to the experimental period [32]. Samples were fed with standard pellet diet and water *ad libitum*.

Preparation of betel quid oinment

Betel quid components consisted of betel leaf (*Piper betle* L.), areca nut (*Areca catechu* L.), gambier (*Uncaria gambir* Roxb.) and mineral slaked lime (calcium hydroxide) were collected from Babatoman Village, Sekayu Subdistrict, Musi Banyuasin District, South Sumatra Province, Indonesia. All the constituents and material were identified and authenticated by the Faculty of Agriculture, Sriwijaya University, Indonesia. Betel quid was made in ointment preparats of 5 g. The composition of 5% betel quid ointment was as follows 250 mg betel quid mixture, 4.04 g vaseline album, 712.5 mg adeps lanae; while 10% betel quid was as follows 500 mg betel quid mixture, 3.82 g vaselin album, 675 mg adeps lanae; 20% betel quid was as follows 1 g betel quid mixture, 3.4 g vaselin album, 600 mg adeps lanae; and placebo ointment was as follows 4.25 g vaselin album and 750 mg adeps lanae. The ingredients of betel quid mixture were 8 g betel leaves, 2.5 g areca nut, 3.5 g gambier, and 2 g mineral slaked lime mixed homogeneously [33].

Wound induction and treatment

Prior to wound induction, rats were anesthetized with 0.2 ml ketamine by i. m injection. Mandibular labial gingiva and lower lip mucosa of rats were swabbed with cotton wetted with 10% povidone iodine for sterilization. The lower lip of rats was withdrawn by using tweezer (Fisher brand™, Thermo fisher Co, UK) and labial gingiva was induced with 1% carrageenan by using spuit. One-mm cylinder diamond bur (Microdont, USA) was used to create wound at the depth of 1 mm. Blood was cleaned with wetted cotton sterile and dried. After 5 h, blood samples were performed on orbital sinus to count the number of segmented neutrophil. The blood samples were put in the pots filled with ethylene diamine tetra acid (EDTA) anti-coagulant.

After blood sample was taken on the first day, mucosal wound was treated with the ointments twice daily based on the groups. The treatment was done for 3 d. On the third day, orbital sinus blood samplings were taken to count the neutrophils after treatment. Segmented neutrophils were counted by using hematology analyzer (Mindray BC-2800, Shenzhen Mindray Bio-Medical Electronic Co., Ltd, China).

Statistical analysis

Data were analyzed using SPSS ver. 22 (IBM® inc. pvt ltd, US) and Microsoft Excel (Microsoft inc®, Redmond). Shapiro Wilk's test and Levene's test were used to know the normality and homogeneity of samples, with $p>0.05$ as significance. Paired t–test was used to analyze the significant lowering number of neutrophils before and after treatment. The test was followed with one way Anova and Post-Hoc LSD. P value of<0.05 was indicated as significant statistically.

RESULTS

The number of neutrophils was evaluated. Saphiro-Wilk and Levene's test showed $p>0.05$, so that meant that data were normal and homogen. All groups showed the reduction of neutrophils before and after treatment significantly table 1. The lowest reduction number of neutrophils was showed on hyaluronic acid of 0.2%, followed by betel quid ointment of 20%, 10%, 5% concentration and placebo ointment.

The result of one-way Anova test exhibited $p=0.00$. It meant that there was a significant difference in reducing neutrophils between and within groups after treatment. Data analysis was continued to Post-hoc LSD test fig. 2. The result showed that there was no significant difference between betel quid ointment at 10% and 20% concentration and positive control in reducing neutrophils. All groups presented significant effects to the negative control, except betel quid of 5% concentration. It meant that 10% and 20% betel quid ointment had a similar anti-inflammatory effect to 0.2% hyaluronic acid ointment.

Table 1: The number of neutrophils before and after treatment

	Means+SD		P value
	Before	After	
5% betel quid oinment	28.00+2.28	16.00+1.79	0.00*
10% betel quid ointment	28.50+4.89	11.17+2.04	0.00*
20% betel quid ointment	28.50+2.04	9.67+1.63	0.00*
0.2% hyaluronic acid	28.33+2.73	9.33+1.03	0.00*
Placebo	28.33+5.68	18.33+6.28	0.00*

*significance= $p<0.05$, paired t-test

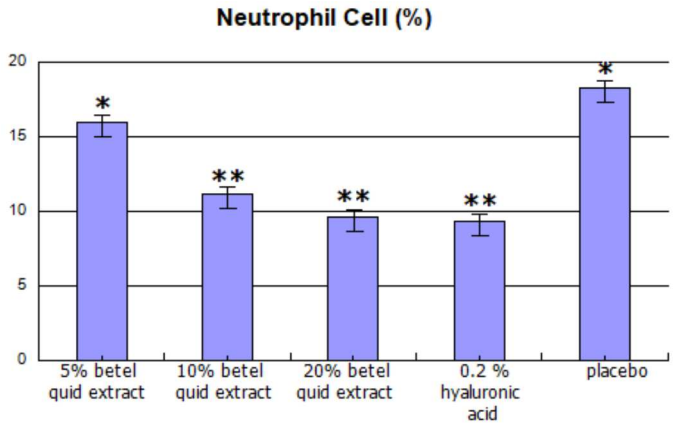

Fig. 1: The number of neutrophils. Betel quid extract of 10% and 20% concentration displayed anti-inflammatory effect significantly and data were expresses as mean+SD, *p<0.05 versus positive control group; **p<0.05 versus the negative control group

DISCUSSION

Chewing betel quid is done for many people all over the world since very ancient times. In Indonesia, the betel leaves, along with areca nut, gambier leaves and mineral slaked lime in wrapped package, are chewed together [34]. All of those components in betel quid consist of active compounds. The effectivity of anti–inflammatory is due to the synergic combination of pharmacological properties contained in betel quid. All constituents of the ingredients has been documented to have an anti-inflammatory effect. Eugenol, hydroxychavicol, terpenes (contained in betel leaf), procyanidin, alkaloids/*arecoline* (contained in areca nut), catechin, tannin (contained in gambier leaf), and calcium hydroxide have been showed in alleviating inflammation. Reddy *et al.* reported that *Piper betle* leaves had anti-inflammatory activity in experimental animals due to its active compounds [35]. Sarpangala *et al.* stated that *Areca catechu* L reduced paw edema in rats and its anti-inflammatory activity was similar to indomethacin [36]. Yimam *et al.* revealed that UP3005 (containing *Uncaria gambir* and *Morus* extract) inhibit inflammatory enzymatic activities of cyclooxygenase-1 (COX-1), COX-2 and lipoxygenase (5-LOX), respectively [37]. Louwakul and Lertchirakarn exhibited that calcium hydroxide was effective in repairing and treating inflamed dental pulp tissue [38].

Eugenol suppresses the expression of cyclooxygenase and decreases the production of proinflammatory cytokines [39, 40]. Hydroxychavicol has the ability in inhibiting platelet aggregation. The previous study reported that hydroxychavicol is effective to hamper COX-1 and COX-2 enzymes, ROS scavenger and platelet calcium signaling [41]. Eugenol and hydroxychavicol inhibit xanthine oxidase (XOD) and lipoxygenase (LOX) [42]. XOD plays an important role in neutrophil mediation. The activation of XOD is a response to proinflammatory and growth factor stimulation [43]. While LOX, group of oxidative enzymes, involves in the metabolism of pro-inflammatory mediators, i.e. prostaglandins and leukotrienes [44]. Terpenes block the oxidation of arachidonic acid and the release of inflammatory mediators [45].

Procyanidins modulate nitric oxide generation that plays an important role in acute and chronic inflammation and reduces COX-2 expression [46]. Alkaloid (*Arecoline*) contained in areca nut is capable to reduce pro-inflammatory mediators, such as prostaglandin E2 an interleukine-6 through the inhibition of COX and LOX metabolisms [47].

Catechin has been proved to have anti-inflammatory activity by inhibiting XO, IL-6, IL-8 production, COX-1/COX-2 expression, lipoxygenase (LOX) and phospholipase (PL) enzyme. The inhibition of COX and LOX disturbs synthesis of inflammatory mediators, such as prostaglandin and leukotriene. Inactivation of PL enzyme causes the inhibition of the release of arachidonic acid. As a result, the number of inflammatory mediators produced will decrease [48, 49]. Another anti-inflammatory mechanism of catechin is to eliminate the expression of pro-inflammatory cytokines, neutralize free radical, inhibit NF-Kb activation so that inflammation reaction will be decreased [50].

Calcium hydroxide has ability in eliminating some bacteria in oral mucosa. The high alkality of calcium hydroxide promotes antimicrobial activities [51]. The presence of calcium hydroxide modify the environmental pH in inflammation areas, regulate inflammatory cell migration and proliferation, control the irritating agents, stimulate tissue mineralization [52]. The effect is based on the dissociation of calcium hydroxide into calcium and hydroxide ions [38, 53].

CONCLUSION

In conclusion, the study shows that betel quid ointment has an anti-inflammatory effect on the mucosal wound of Wistar rats in a dose-dependent manner. The anti-inflammatory activities are due to the synergic pharmacological properties contained in betel quid.

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AUTHORS CONTRIBUTIONS

All authors have made substantial contribution to the work reported in the manuscript. Rindit Pambayun, Budi Santoso, Triwardhani developed the idea and designed study. Rafika Putri conducted the actual study, Siti Rusdiana Puspa Dewi involved in the designed study, administration process, the statistical analysis, and write up of the manuscript.

CONFLICT OF INTERESTS

There are no conflict of interest in this study

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