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Effectivity of *Camellia sinensis* Extract with Nano-chitosan to Fibroblast Amounts of Wistar Rats Gingival Wound Healing Process

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ABSTRACT

Green tea (*Camellia sinensis*) has high level of flavonoids which are proven to have anti-inflammatory activity. Effect of flavonoids can be enhanced by nano-chitosan capsulation as drug carrier. Chitosan is polysaccharide derived from crustacean shells that mostly used as matrix of various drugs and plant extracts. The aim of this study was to determine the effectivity of flavonoids in green tea extract in nano-chitosan capsulation towards the number of fibroblasts on proliferative phase of gingival wound healing process. Green tea was extracted, encapsulated with nano-chitosan and then made into gel. Gingiva labial of 24 male white 3-month-old Wistar rats were wounded by punch biopsy (2 mm diameter), then were treated two times a day, and were divided randomly into four groups of topical gel applications: green tea extract gel encapsulated nano-chitosan, green tea extract gel, base gel as negative control, and NSAIDs gel as positive control, starting at 0 day until 7th day. At 5th and 7th day, three rats from each group were decapitated and the mandibular gingiva was taken in order to make histology slides with hematoxylin eosin staining. Under microscope, the number of fibroblasts were examined. The data were analysed using ANOVA test with 95% confidence level. The results showed that the number of fibroblasts on proliferative phase was significantly higher than control negative ($p < 0.05$) and has no significant differences ($p > 0.05$) with control positive. In conclusion, topical application of green tea extract gel encapsulated nano-chitosan was effective to accelerate rats gingival wound healing process by increasing the fibroblasts.

Keywords: *Fibroblasts; gingival wound healing process; green tea; nano-chitosan*

INTRODUCTION

Trauma is one of aetiologies that can cause oral cavity disorders (Mackay & Miller, 2003). Oral wound healing process is the body's natural process that occurs immediately after an injury happened by replacing damaged and dead tissue with healthy new tissue. Oral wound healing process consists of three overlapping and

dynamic phases, namely the inflammatory phase, the proliferation phase and the maturation phase (de la Torre *et al.*, 2006).

The inflammatory phase occurs when vascular and cellular components react at the injury site and is characterised by the increase of inflammatory cells such as polymorphonuclear leukocytes (Kumar *et al.*, 2014; Murphy & Weaver, 2017). After the inflammatory signs subside, the proliferation phase begins and is characterised by epithelialisation, angiogenesis and fibroblasts proliferation (de la Torre *et al.*, 2006; Kumar *et al.*, 2014). In the maturation phase, the fibroblasts transform into myofibroblasts which generate wound contraction (Ethridge *et al.*, 2008). Sometimes, drugs are used to quicken the wound healing process in the oral cavity such as chlorhexidine or any other substances that works by accelerating the extracellular matrix formation (hyaluronic acid, Alloclair®), Solcoseryl® (increase oxygen and ATP energy intake into cells), or the other anti-inflammatory drugs both steroid and non-steroid type (Hammad *et al.*, 2011). But the use of non-steroidal anti-inflammatory drugs (NSAIDs) has several side effects such as gastrointestinal bleeding, prolongation of bleeding time, and can lead to impaired kidney function, so existence of highly effective substance with minimal side effects is needed (Tripathi, 2003).

Tea contains flavonoids, namely epigallocatechingallate (EGCG) as the main substance that acted as anti-inflammatory agent (Behfarnia *et al.*, 2016). Green tea flavonoids can reduce the number of polymorphonuclear (PMN) leukocytes in gingivitis (Chopra *et al.*, 2016). Innovation of gingivitis therapy can be done with the application of green tea extract with nano-chitosan as the drug carrier, which to the best of authors' knowledge, has never been studied until recently.

Chitosan has been widely used as a drug carrier because it has various advantages including anti-microbial, good biocompatibility, biodegradation and non-toxic properties (Jayakumar *et al.*, 2011). Chitosan is a polymer obtained from thermochemical and enzymatic deacetylation of chitin from the crustacean shell (Casadidio *et al.*, 2019). Its nanoparticle-size make the chemical reactions occurs more and faster as they are easily absorbed by the bloodstream and cells rather than the digestive system. Nano-chitosan capsules are adequate as drug carriers of phenolic compounds because they can maintain the properties and activities of these compounds (Harris *et al.*, 2011).

The purpose of this study was to find out the effect of green tea extract with nano-chitosan capsulation on fibroblasts proliferation in the gingival wound healing process of male white Wistar rats. This study uses green tea and chitosan from crab shells that are easily found in Indonesia. The results of this study are expected to be continued and developed as the Indonesian local wisdom utilisation and perhaps someday it can be applied clinically to humans.

MATERIALS AND METHODS

This research type is a pure experimental laboratory with a post-test only control group design (Fig. 1).

This study had received an ethical eligibility letter from the Research Ethics Commission of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta (Ref. No. 001037/KKEP/FKG-UGM/EC/2017). The research duration was from March to July 2017. Green tea was obtained from PT Pagilaran Yogyakarta. There are three types of green tea, namely Pekoe tea, Gun Powder and Green Powder. These three types of tea were extracted using maceration techniques with 70% ethanol at the Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada (LPPT-UGM).

The three types of green tea extracts were then tested for their flavonoid levels using a UV-vis spectrophotometer with 510 nm wavelength. Tea with the highest levels of

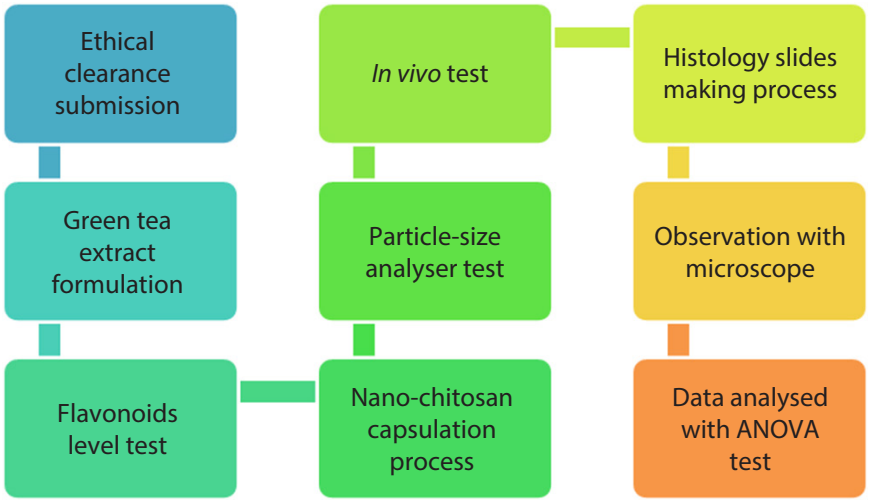

Fig. 1 The research flow chart of the present study.

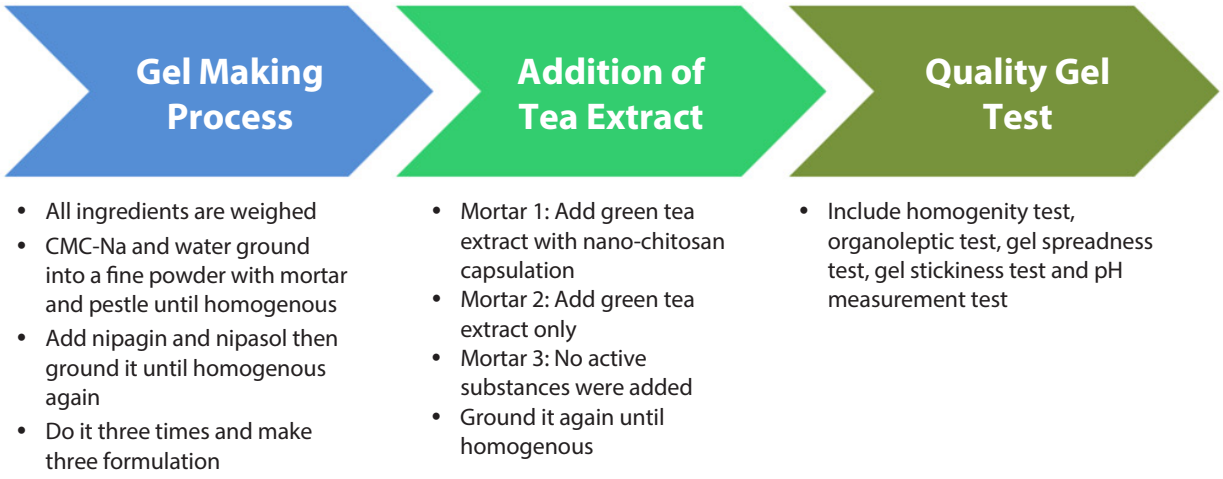

Fig. 2 The gel making process of green tea extract.

flavonoids is then encapsulated with nano-chitosan. Particle size analyser tests are carried out to ensure that chitosan particles are nano-sized which is carried out at Laboratorium Pengujian Obat, Makanan, dan Kosmetik UII. Then the green tea extract was transferred to the gel making process with 20% concentration of active substance (Fig. 2).

The next step of this study was *in vivo* test conducted at Laboratorium Farmakologi, Faculty of Medicine, Universitas Gadjah Mada with 24 white male 3-month-old Wistar rats and body weight of 200 g to 250 g as subjects. Mandibular gingiva of the subjects was injured using 2 mm diameter punch biopsy and then were treated for seven days. They were divided into four gel application groups: (1) green tea extract with nano-chitosan capsulation gel, (2) green tea only extract gel, (3) base gel as negative control, and (4) topical NSAIDs gel, a Difflam mouth gel as positive control. On the 5th and 7th day, three rats from each group were decapitated, the tissues were made into histological slides of mandibular gingiva by using hematoxylin eosin (HE) staining. Histology slides then were observed at Laboratorium Riset Terpadu, Faculty of Dentistry, Universitas Gadjah Mada using a 400× magnification binocular

light microscope in five visual fields. Data were analysed by ANOVA test with 95% confidence level.

RESULTS

Flavonoid level test showed that Pekoe tea had the highest level of 65.211 mg/L, so this type is used in this research to be capsulated in nano-chitosan. Particle size analyser results indicated that 77% of chitosan particle was nano-sized. The four groups histological observation was done on the 7th day (Fig. 3).

Observation of the fibroblast was acquired by counting the number of fibroblasts on the lamina propria in the wound area. Each slide was observed on five visual fields. The mean of total PMN leukocyte on the 5th and 7th day was presented in the Table 1 and Fig. 4.

Table 1 shows that on the 5th and 7th day, the mean of total fibroblasts in green tea extract-nano-chitosan gel and positive control (Difflam mouth gel) group were higher than the negative control group. The fibroblast in green tea extract with nano-chitosan capsulation gel appeared to be higher than green tea extract only gel.

There was an increase in the mean of fibroblast cells in all groups on the 5th and 7th day (Fig. 5). On every observation day, the highest mean was the positive control group (Difflam mouth gel), followed by the green-nano-chitosan tea extract gel group, the green tea extract gel without nano-chitosan group and the lowest mean was the negative control group (base gel). Data analysis was performed to find out the difference in mean of fibroblasts for each treatment group. The data obtained were ratio scale data, which were further

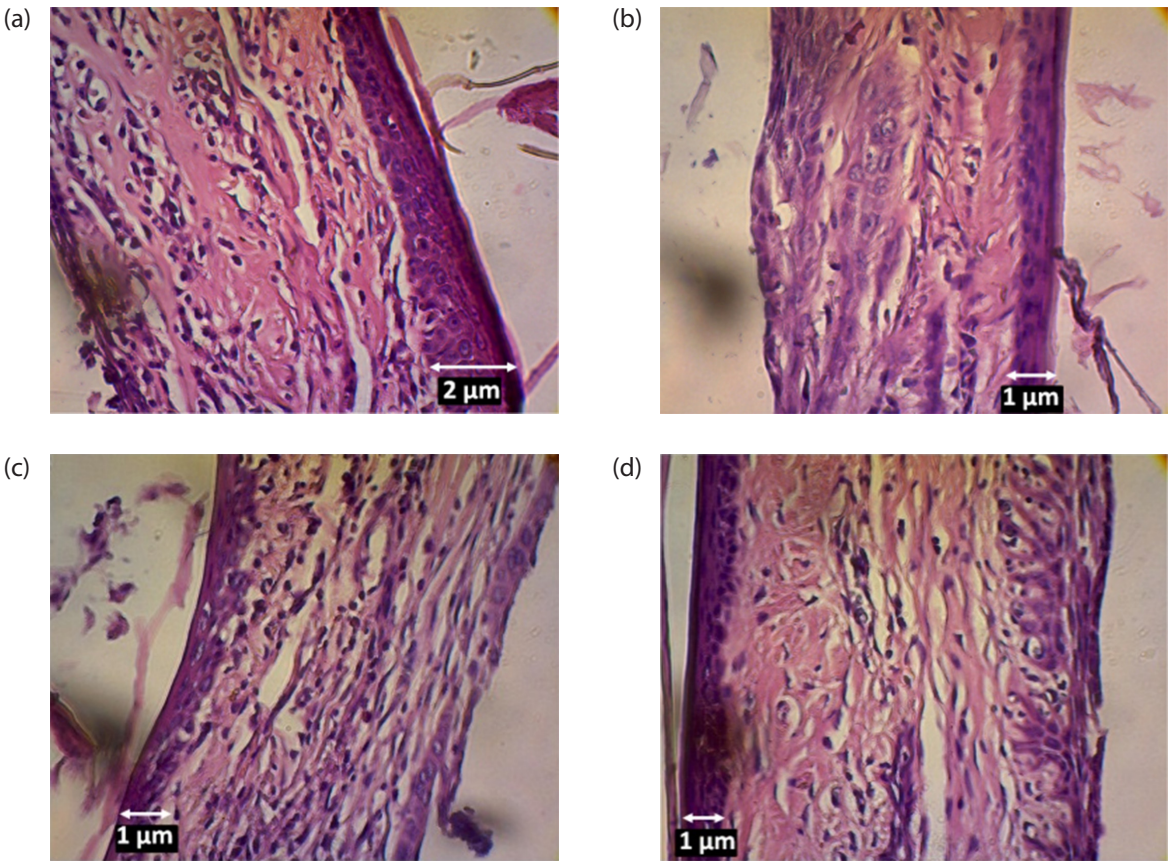

Fig. 3 Histological observation on 7th day using: (a) Chitosan-encapsulated flavonoid (Chicaflo) gel; (b) Green tea extract gel; (c) Base gel; and (d) Difflam mouth gel (Magnification 400×).

Table 1 The mean of fibroblasts on proliferative phase based on treatment group and decapitation periods on gingival wound healing process on white Wistar rats

Period	Green tea extract-nano-chitosan gel	Green tea extract gel	Base gel	Difflam mouth gel
5th day X±SD	46.17±4.16	37.17±1.04	28.00±5.27	47.83±3.40
7th day X±SD	51.00±4.82	46.33±2.02	32.50±0.50	53.50±2.50

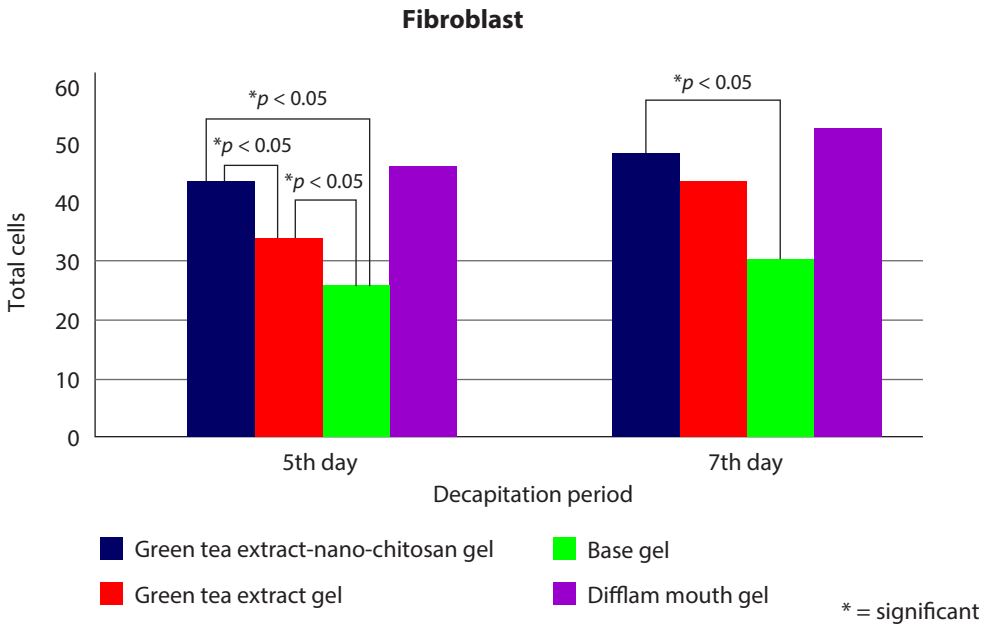

Fig. 4 The mean of fibroblasts on each treatment group on the 5th and 7th day.

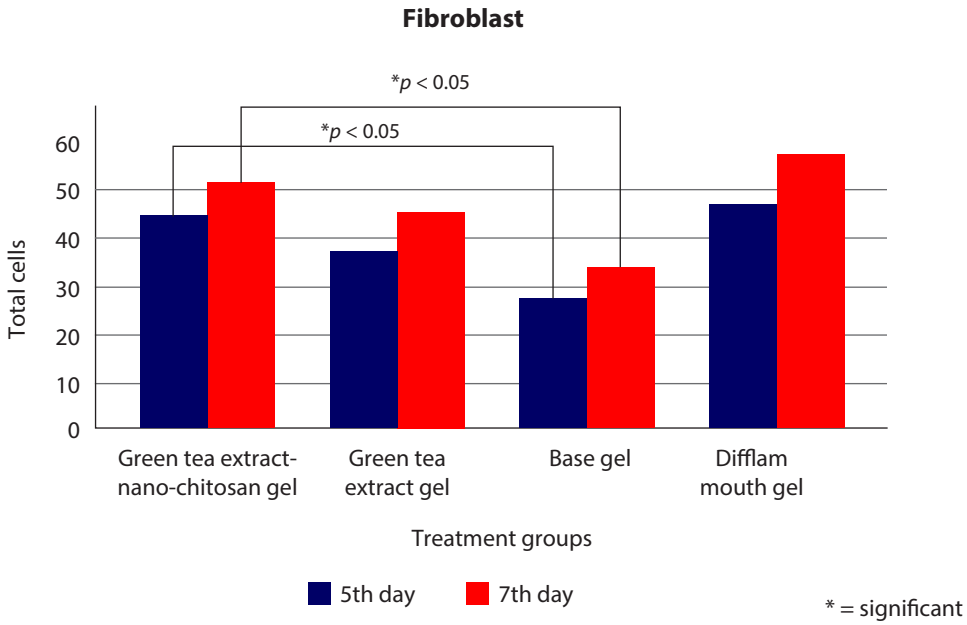

Fig. 5 The increase in the mean of fibroblast cells in all groups on the 5th and 7th day.

tested statistically using the ANOVA test parametric analysis to see the difference in effects between the control group and the treatment group and to see the difference in effects between treatments.

Based on the ANOVA test results, there was significant difference ($p < 0.05$) in the means of fibroblast cells between the treatment groups. A summary of the post-hoc ANOVA test results was presented in Table 2.

Table 2 shows significant difference ($p < 0.05$) on the 5th day, between the green tea-nano-chitosan gel group compared with the green tea extract gel group and the negative control group (gel base) which was indicated by the higher mean of fibroblast cells. There was no significant difference ($p > 0.05$) when green tea-nano-chitosan gel group was compared with the positive control group. On the 7th day observation, there was no significant difference ($p > 0.05$) result between the green tea-nano-chitosan gel group, compared with the positive control group and the green tea extract gel group but indicate significant differences ($p < 0.05$) when compared to the negative control. The comparison between the green tea extract gel groups with positive controls shows a significant difference ($p < 0.05$). This result shows that there is an increased number of fibroblasts in the green tea-nano-chitosan gel group which is faster than green tea gel group without nano-chitosan capsulation.

DISCUSSION

When wounded, the body will initiate the wound healing process and cell regeneration physiologically. Although it is a natural process, certain condition is required to advance the wound healing such as nutrition (Guo & DiPietro, 2010). Fibroblast cells produce collagen and extracellular matrix, and an adequate amount is needed for good wound healing (Aukhil, 2000). Proliferation of fibroblasts begins in the proliferation phase of the wound healing process, approximately five days after the injury occurs (Ethridge *et al.*, 2008), while Morand *et al.* (2017) states that fibroblasts will start to increase in number on the 3rd day and reach a peak on the 7th day.

Table 2 Summary of least significant different (LSD) test of green tea (*Camellia sinensis*) extract effectivity with nano-chitosan as drug carrier on the fibroblast proliferation on gingival wound healing process on white Wistar rat

Treatment group		Sig. (*p*)	
		5th	7th
Chicaflo gel (Green tea-nano-chitosan)	Green tea extract gel	0.022*	0.101
	Base gel	0.000*	0.000*
	Difflam mouth gel	0.654	0.350
Green tea extract gel	Chicaflo gel	0.022*	0.101
	Base gel	0.022*	0.001*
	Difflam mouth gel	0.011*	0.022*
Base gel	Chicaflo gel	0.000*	0.000*
	Green tea extract gel	0.022*	0.001*
	Difflam mouth gel	0.000*	0.000*
Difflam mouth gel	Chicaflo gel	0.654	0.350
	Green tea extract gel	0.011*	0.022*
	Base gel	0.000*	0.000*

Note: *Significant difference $p < 0.05$

Based on the results of the study, application of green tea extract gel with nano-chitosan capsulation was able to increase the number of fibroblasts in the proliferation phase of the gingival wound healing of Wistar white rats, compared with the negative control group and the treatment group without nano-chitosan capsulation. This happens because the green tea extract gel contains flavonoids which have been widely known to have anti-inflammatory and antioxidant effects (Intekhab & Aslam, 2009), so the high flavonoid content can also accelerate the inflammatory phase in the injured area.

Flavonoids are also able to regulate cell function by stimulating the production of TGF-β (transforming growth factor-β) which can increase migration and proliferation of fibroblasts in the injured area and induce vascular endothelial growth factor (VEGF) which plays a role in the formation of new blood vessels (Sabir *et al.*, 2005; Mitchell & Cotran, 2003). The faster and more fibroblasts are in the wound area, the faster the synthesis of collagen, fibronectin and extracellular matrix deposition can begin so that the wound healing period will be shorter (Schultz *et al.*, 2005; Mitchell & Cotran, 2003).

CONCLUSION

The administration of green tea extract gel which is encapsulated by nano-chitosan is effective in accelerating the healing of gingival wounds of male Wistar white rats as demonstrated by the increase in the number of fibroblasts.

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