ORIGINAL ARTICLE

# Mixed tea leaves extract gel with chitosan application increase the fibroblasts in wound healing after tooth extraction of Wistar rats

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## ABSTRACT

**Introduction:** Tooth extraction can cause the damage of hard and soft tissue. When an injury occurs, fibroblast will respond in the wound healing process. Herbal medicines such as green tea and chitosan can be used in wound healing. The polyphenol content in green tea, namely EGCG, has an anti-inflammatory effect, increasing wound healing. In wound healing, chitosan promotes hemostasis and tissue recovery. Based on several previous studies, the use of 1.2% green tea extract was effective for improving wound healing in rat open wound models, chitosan 1% could trigger the proliferation of fibroblasts in the wound healing process after tooth extraction. The aim of this study is to analyze the effect of gel mixture of extract *Camellia sinensis* 1,2% with chitosan 1% on post-tooth extraction wound of Wistar rats. **Methods:** This was a randomized post-test only control group design using 40 Wistar rats which were randomly divided into 2 groups. Each group underwent intramuscular anesthesia on the rat's thigh and tooth extraction of mandibular left incisor. The treatment group was applied mixed gel of extract *Camellia sinensis* 1.2% and 1% chitosan as much as 0.01 ml and the control group was not given any treatment, after that decapitated on days 1, 3, 5, and 7. **Result:** The mean number of treated fibroblasts was higher than the control group on days 1, 3, 5, 7, respectively, 75.00; 176.00; 349.00 and 427.00 cells. The mean difference in the number of fibroblasts was significant with p-value 0.001 (p>0.05). **Conclusions:** Mixed extract gel of *Camellia sinensis* and chitosan increased fibroblasts in wound healing process after tooth extraction of Wistar rats with the highest mean of fibroblast on the 7$^{th}$ day.

## KEYWORDS

mixed tea leaves,chitosan, fibroblast,wound healing

## INTRODUCTION

Extraction is a common procedure in dental practice. According to Basic Health Research (Riskesdas) 2018, the average tooth extraction was 7.9% and filling ratio was 4.9%. These data show the high rate of tooth extraction in Indonesia.[2] Extraction may cause injury. Wounds caused by tooth extraction are different from incision wounds as it creates bigger loss of soft tissue. The wound healing phase of extraction and incision are the same.[3,4] Wound healing is a complex biological process that links the coordination of many different cells and molecules to promote tissue integration.[5] After the tissue is damaged, fibroblasts move rapidly to the wound, where they proliferate and create a collagen matrix, and replace damaged tissue. Fibroblasts play an important role during the proliferative phase, which is to produce extracellular matrix to fill the wound.[6]

Recently, herbal medicines such as green tea have frequently been used in Indonesia.[7] The content of green tea, namely flavonoids, plays an important role in the healing process such as anti-inflammatory and increasing the number of fibroblasts.[8] Natural ingredients derived from shrimp shells, namely chitosan, in the wound healing process can stimulate tissue regeneration, activate inflammatory cells such as fibroblasts, and remodeling process during the wound healing process.[9]

In vivo research conducted by Qin et al, chitosan provides a non-protein matrix during tissue growth, assists in natural clotting, and blocks nerve endings to reduce pain. Green tea polyphenols can increase growth factors so that the wound healing process becomes faster.[10] A similar study conducted by Violetta, showed a concentration of 1.2% in green tea stimulated the formation of new tissue through the proliferation of fibroblasts.[11] Other studies conducted by Rahmitasari, chitosan with a concentration of 1% with high and low molecular weight applied after tooth extraction, is a growth factor that optimizes the production of cytokines, TGF-α, TGF-ß1, IL-6, and IL-8 that stimulate growth and trigger fibroblast cell proliferation throughout the wound treatment.[12] Both chitosan and green tea have a potent ability in accelerating wound healing in different mechanism. However, studies regarding the effect of a mixture of both chitosan and green tea extract is still limited, although this mixture has the potential of being an excellent supportive treatment to ease recovery, reduce post-operative pain, induce angiogenesis, and stimulate fibroblast in post-extraction wound sites. This study aimed to analyze the effect of a gel mixture of *Camellia sinensis* extract and chitosan on the number of fibroblasts after tooth extraction of Wistar rats.

## METHODS

The design of this research is a randomized post-test only control group design, performed on selected Wistar rats in laboratory. The subjects were selected according to previously-set inclusion and exclusion criteria. Minimum sampling size was calculated using the Federer formula technique, which resulted in a minimum of 40 animal subjects, with a 10% addition of the minimum sample in case of drop out samples. Healthy male Wistar rats, aged 2-3 months old, with average weight of 150-250 grams were randomly divided into two groups, *i.e.* the treatment group and the control group. The treatment group was given a mixed gel extract of *Camellia sinensis* 1,2 and 1% chitosan no anesthesia, and the control group was not given any treatment.

*Camellia sinensis* extract was independently produced by drying 400g of *Camellia sinensis* leaves and crushed to a size of 60 mesh, then maceration was carried out.[11] The first step was deproteination, demineralization, and finally deacetylation.[13] Preparation of the gel mixture extract of 1.2% *Camellia sinensis* and 1% chitosan was obtained by adding 1.2 g of *Camellia sinensis* extract then mixed with 2% CMC-Na gel base and 1% chitosan concentration. The polyphenols in green tea, namely catechins, have anti-inflammatory, antioxidant, antibacterial and antiviral properties. The anti-inflammatory activity of catechins limits the number of leukocyte migration to injured tissue and the expression of TGF-β1 is not inhibited. The role of TGF-β1 in the wound healing process can trigger fibroblast proliferation where fibroblasts are involved in collagen synthesis. At the inflammatory stage, chitosan regulates inflammatory cell activity and the release of proinflammatory factors, while providing a good microenvironment for wound healing. During the healing process, chitosan helps provide a non-protein substrate for tissue growth.


**Figure 1.** A. 1.2% *Camellia sinensis* extract; B**.** 1% chitosan extract; C. mixed gel extract of 1.2% *Camellia sinensis* and 1% chitosan

Before doing the treatment, the previous research sample was adapted in the Laboratory of the Faculty of Veterinary Medicine, Udayana University for 7 days by giving them food in the form of pellets and drinks, namely distilled water and placed in a cage with sawdust. On the day of the treatment, the experimental animals were anesthetized by intramuscular injection with a mixture of ketamine 75 mg/kgBW and xylazine 5 mg/kgBW.[14] The mandibular left incisor was luxated using an excavator and sterile arterial clamp. Application of gel mixture of 1.2% *Camellia sinensis* extract and 1% chitosan in the treatment group was carried out topically as much as 0.01 ml using a syringe and cannula in the post-extraction socket. Gel application was done twice a day in the morning (10.00) and in the evening (15.00). The application was performed approximately one hour before meal to allow optimal absorption of the extract.

Euthanasia in experimental animals was carried out on days 1, 3, 5, and 7 using the cervical dislocation method. This method was chosen with consideration of the simple and quick procedure to minimize pain for the subject, as recommended after consultation with a veterinarian. The mandibles of the experimental animals were stored in closed plastic pots containing 10% formalin buffer and the cadaver of the experimental animals was then buried properly followed by the making of histological preparations and Haematoxylin Eosin staining. Calculation and observation of fibroblasts using a light microscope and Optilab digital camera with 400x magnification in 5 fields of view. Fibroblasts were observed in which the nucleus was oval or elongated and covered with a smooth nuclear membrane with one or 2 nucleoli. The calculated data was processed using one of the statistical software, SPSS version 20 using a parametric test, namely Two Way ANOVA and continued with the Least Significance Different (LSD) test to test the significant difference in days and which groups affected the number of fibroblasts during wound healing. The data of fibroblasts were tested using the Shapiro Wilk normality test and the homogeneity test using the Levene test.[11]

**Figure 2.** Sample was adapted in the Laboratory of the Faculty of Veterinary Medicine, Udayana University.

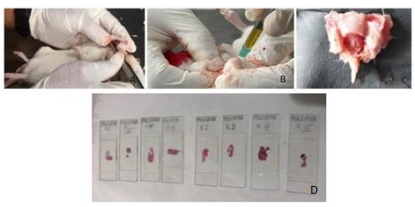

**Figure 3.** A. experimental animals were anesthetized; B. Application of gel mixture of 1.2% Camellia sinensis extract and 1% chitosan in the treatment group; C. Mandibles of samples; D. Dissected tissues of samples to be sent for histological examination

The research design used was experimental laboratory and randomized post-test only control group design research carried out at the Phytochemical Laboratory, Faculty of Mathematics and Natural Sciences, Udayana University and the Laboratory of the Faculty of Veterinary Medicine, Udayana University.

## RESULTS

Socket samples after extraction of the mandibular left incisor of Wistar rats were then made into histological preparations for further investigation using a binocular microscope with a magnification of 40 times in five fields of view. The observed variable was the development of histological wound healing by identifying an increase in the number of fibroblasts. The histological staining was performed with Hematoxylin and Eosin (HE) stain. Microscopic examination was carried out on days 1, 3, 5, and 7. The results obtained by calculating the average number of fibroblasts in each group are as follows:

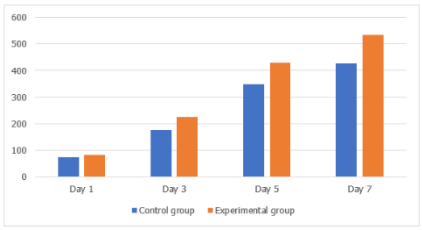


**Figure 4**. The mean and standard deviation of the number of fibroblasts in each group

The results of this study show the mean number of fibroblasts (Figure 4) in the treatment group on days 1, 3, 5, 7, respectively, 80.25; 223.25; 430.50 and 534.25 cells. The fibroblasts are emphasized with red and green arrows (Figure 5). The mean number of treated fibroblasts was higher than the control group on days 1, 3, 5, 7, respectively, 75.00; 176.00; 349.00 and 427.00 cells

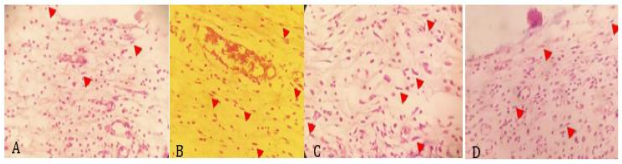


**Figure 5.** Histological image of the control group on: A) Day-1; B) Day-3; C) Day-5; D) Day-7.

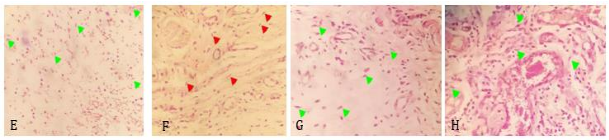


**Figure 6.** Histological image of the treatment group on: E) Day-1; F) Day-3; G) Day-5; H) Day-7

The data of fibroblasts were tested using the Shapiro Wilk normality test and the homogeneity test using the Levene test. The test results were obtained with data p>0.05 indicating normal distribution and homogeneous. The parametric Two-Way ANOVA test was performed, which resulted in p=0.001 (p<0.05), which means that there is a difference between the groups. Then proceeded with the LSD test.

**Table 1.** Test results of differences in observation days in Wistar rats after tooth extraction between control and treatment groups

| | K1 | K3 | K5 | K7 | P1 | P3 | P5 | P7 |
|---|---|---|---|---|---|---|---|---|
| **K1** | | | | | | | | |
| **K3** | 0.001 | | | | | | | |
| **K5** | 0.001 | 0.001 | | | | | | |
| **K7** | 0.001 | 0.001 | 0.001 | | | | | |
| **P1** | 0.389 | 0.001 | 0.001 | 0.001 | | | | |
| **P3** | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | | | |
| **P5** | 0.001 | 0.001 | 0.001 | 0.564 | 0.001 | 0.001 | | |
| **P7** | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | |

**Information:** K1: Day 1 Decapitation of Control Group; K3: Decapitation of Control Group Day 3; K5: Decapitation of Control Group Day 5; K7: Decapitation of Control Group Day 7; P1: Day 1 Decapitation of Treatment Group; P3: 3rd Day Decapitation of Treatment Group; P5: 5th Day Decapitation of Treatment Group; P7: 7th Day Decapitation of Treatment Group

The results of the LSD test (Table 2) showed a significant difference in the mean number of fibroblasts between K1 and K3, K5, K7, P3, P5 and P7 (p>0.05). There was a significant difference in the mean number of fibroblasts between K3 and K5, K7, P1, P3, P5 and P7 (p>0.05). The mean difference in the number of fibroblasts was significant (p>0.05) between K5 and K7, P1, P3, P5 and P7. There was a significant difference in the mean number of fibroblasts between K7 and P1, P3, and P7 (p>0.05). There was a significant difference in the mean number of fibroblasts between P1 and P5 and P7 (p>0.05). There was a significant difference in the mean number of fibroblasts between P5 and P7 (p>0.05). There was no significant difference in the mean number of fibroblasts between K1 and P1 and K7 and P5.

## DISCUSSION

The results of the study from sampling the mandibles of Wistar rats that had been decapitated as many as 36 samples, 3 Wistar rats in the control group died after tooth extraction, possibly caused by inadequate anesthetic doses, causing shock with pain to cause sudden death. In accordance to that, a previous study has suggested that intense pain could cause shock and extreme stress which might lead to the death of the animal subjects. Proper anesthetic administration should be carefully performed to avoid such incident.[15] One sample of Wistar rats in the control group experienced an abnormality in the form of enlargement of the mandibular area suspected of having an abscess so that the sample was eliminated from the group.

In the treatment group, whereas samples received application of gel mixture of *Camellia sinensis* 1.2% and chitosan 1%, experienced an overall higher fibroblasts proliferation compared to the control group. Number of fibroblasts in the treatment group had increased due to the effect of *Camellia sinensis* and chitosan. This study is in line with previous research which discovered that chitosan helps provide a non-protein substrate for tissue growth during the healing process.[16] On the other hand, the terpenoid in *Camellia sinensis* acts as anti-inflammatory and antioxidant in the process of wound healing.[16,17] The polyphenol content in *Camellia sinensis* has anti-inflammatory, antioxidant effects improve wound healing and scar tissue formation by increasing TGF-β1 wound activity. Chitosan acts as an anti-inflammatory agent and stimulates cell proliferation and remodeling during the wound healing process. Chitosan has also been shown to activate inflammatory cells such as fibroblasts.[16]

Treatment group showed the highest number of fibroblasts in the 7$^{th}$ day post-operative (534.25 cells). Meanwhile, the control group also showed its highest number of fibroblast proliferation on day 7, however with less amount of fibroblasts compared to the treatment group (427.00 cells). Similarly, a previous research showed that chitosan 1% with high and low molecular weight, applied to extraction wounds can increase cytokines which are growth factors that stimulate the proliferation of fibroblasts on wound healing.[12] Several recent studies reported that chitosan membranes are widely used in the medical field as wound care because it has benefits in the wound healing process such as proliferating human skin fibroblasts and keratinocyte cells in vitro that Wounds treated with Electro-spun chitosan/gelatin/PEO (CGP) displayed more epithelial tissue recovery than the control group.[9,10,12] Histological observations showed that CGP was found to be effective in accelerating the reformation of granulation tissue in the wound healing process. Histopathological studies confirm the effectiveness of treatment using CGP causing accelerated tissue granulation phase, collagen maturation, and maturation phase.[9,10,12] The LSD test on the control group and the treatment group on the first day showed no significant differences. Qin *et al* found similar results in their research, where there is a lack of significant difference between treatment and control group on the first day observation.[10] This finding is presumably caused by the fact that both groups had only just entered the initial phase of wound healing. Early phases of the wound healing process include the release of macrophages, neutrophils, and lymphocytes are the first cells to reach the wound site.[5,9] Neutrophils begin to accumulate in the first 6-8 hours after injury and the highest number occurs within 24-48 hours after injury. The main function of these cells is to fight infection, sterilize cellular matrix debris and foreign bodies. Thus, during the first to second days the inflammatory phase is still ongoing, and fibroblast proliferation has not occurred yet, causing a stagnation of the fibroblast cell count in both treatment and control group.[5,9,10]

Interestingly, the amount of fibroblasts proliferation of day-5 treatment group is already more than the amount of fibroblasts of the day-7 control group, indicating the accelerating effect of fibroblast proliferation due to the application of gel mixture of *Camellia sinensis* 1.2% and chitosan 1%. This research is in line with research by Bramanti *et al*., who found that *Camellia sinensis* extract gel can stimulate the creation of new tissue through the proliferation of fibroblasts and stimulate the differentiation of fibroblasts into myofibroblasts which are responsible for the contraction of injury.[11] Various compounds in *Camellia sinensis* extract have many benefits in supporting the wound healing process to be faster, one of which is saponin, which is useful as an anti-inflammatory antioxidant and antimicrobial effect. The terpenoid in *Camellia sinensis* acts as an antioxidant and anti-inflammatory. The phenolic in *Camellia sinensis* has an important role as an antioxidant through the scavenging of hydroxyl radicals.[11,18,19]

## CONCLUSION

Based on the research, it can be concluded that the use of a mixture of 1.2% *Camellia sinensis* gel with 1% chitosan had a significant effect on the increase in the number of fibroblasts compared to the control group during wound healing activity after tooth extraction of Wistar rats. Mixed extract gel of *Camellia sinensis* and chitosan increased fibroblasts in wound healing process after tooth extraction of Wistar rats with the highest mean of fibroblast on the 7th day.

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