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Effect of green tea (*Camellia sinensis*) extract on healing process of surgical wounds in rat

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ABSTRACT

Green tea (*Camellia sinensis*) has anti-oxidant and anti-inflammatory properties and may enhance wound healing process. The present study, therefore, was aimed to examine the effect of green tea ethanolic extract on wound healing process.

For this experimental study, 36 healthy male Wistar rats were randomly designated to three groups of A, B, and C which, respectively treated with, Vaseline + 0.6% green tea extract, Vaseline and normal saline for 21 days. Wounds' length and area were measured by caliper every other day and specimens were taken at 3rd, 12th, and 21st day for microscopical examinations. Data were analyzed by SPSS 16 using survival analysis (*Breslow* test), repeated measured ANOVA, one-way ANOVA and Mann–Whitney. $P < 0.05$ was considered as statistically significant.

The mean healing duration of surgical wounds in groups A and B was 14.66 and 20.66 ($P = 0.018$), respectively. Decrease in healing duration in the group A was significantly higher within the first two weeks compared with control groups ($P = 0.05$). Microscopic examinations also indicated a significant difference in wound healing process between groups A and C throughout the whole study duration as well as groups A and B during the 3rd week of the study ($P < 0.05$).

Green tea extract could help wound healing process, probably effective on surgical wounds healing.
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1. Introduction

Wound healing comprises a complex pathophysiological process including several cellular and biochemical sub processes, e.g. inflammation, angiogenesis, and collagen deposition.[1] Inflammation maintenance and inadequate vessel formation comprise the most noticeable causes of delayed wound healing.[2] On the other hand wound fibrosis or abnormal accumulation of collagen in the wound could lead to an unpleasant scar.[3] Recent research has shown that many of the compounds that are used for wound healing such as Acetic acid, Hydrogen peroxide, and etc., is toxic to cells needed for healing.[4]

The majority of plant extracts, e.g. Grape seed, Lemon, Rosemary, and Jojoba, have been employed for wound healing and longevity increase. All of these plants have a common property, i.e., producing compounds with phenolic structure.[5] These phytochemicals ordinarily react with some compounds such as Oxygen free radicals and other macromolecules in order to neutralize free radicals and/or initiate biological effects.[5]

Green tea (*Camellia sinensis*) which is a product of dried leaves has been consumed by East Asian people for health promotion since 3000 B.C.[6,7] Abundantly found in Asia, green tea is also one of the most prevalent drinks worldwide.[6,8,9]

Ample evidence indicates that this plant, with anti-oxidant, anti-cancer, anti-aging, and anti-inflammatory effects, could also prevent exaggerate collagen production and accumulation and induce changes in immune responses, as well[5,6,8]; the majority of

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Table 1
System for scoring the histological features of wound tissue samples.

Score	Epithelial regeneration	Granulation tissue thickness	Angiogenesis
1	Little epithelial organization	Thin granular layer	Altered angiogenesis (1–2 vessels per site)
2	Moderate epithelial organization	Moderate granular layer	Few newly formed capillary vessels (3–6 vessels per site)
3	Complete epithelial organization	Thick granular layer	Newly formed capillary vessels (7–10 vessels per site)
4	Complete epithelial organization	Very thick granular layer	Newly formed and well-structured capillary vessels (>10 vessels per site)

these properties could be attributed to the plant's polyphenolic, in particular, catechin compounds.[10–14]

Epicatechin, Epicatechin gallate, Epigallocatechin, and Epigallocatechin gallate are among the key anti-oxidant compounds of green tea, little of which could increase collagen volume and hence heal the wounds.[15] These compounds (e.g. epigallocatechin gallate) have also been used as an agent for keratinocytes reproduction and distinction.[16] Also, its anti-fibrogen effects have been confirmed in some animal models.[17]

Regarding the above mentioned and easy accessibility, we investigated the effect of ethanolic extract of green tea on post-surgical wound healing process.

2. Materials and methods

After obtaining approval of Shahrekord University of Medical Sciences (SKUMS) Ethics Committee, this preclinical study was conducted in Medical Plants Research Center of SKUMS. Throughout the experiments, we tried to follow all ethical principles of working on laboratory animals so as to impose the lowest possible stress on them.

2.1. Extract preparation

Maceration method was employed to prepare the extract. For this purpose, 100 g green tea (Herbarium No. 304, Medical Plants Research Center, SKUMS) was transported into an Erlenmeyer, 1 L ethanol 70% (*Nasr Co*. Iran) was added and the solution was left at laboratory temperature. Forty eight hours later, the extract was filtrated through a filter paper and the pulp was squeezed to discharge. Then, the extract was concentrated by a rotary evaporator,[18] dried, and mixed with pure Vaseline (*Ehsan Chemi, Iran*) to make a Vaseline-based 0.6% ointment.[5]

2.2. Measurement of phenolic compounds

The phenolic compounds were evaluated equivalent to gallic acid using Folin-Ciocalteu colorimetry method.[19] Different concentrations of standard gallic acid, i.e., 12.5, 25, 50, 62.5, 100, and 125 ppm in methanol 60%, were prepared. Then, 0.1 ml from each sample was transferred into a test tube and 0.5 ml Folin-Ciocalteu 10% was added as reactive agent. The solutions were left for 8 min at room temperature and then 0.4 ml carbonate 7.5% was added. The tubes were maintained for 30 min at room temperature and then assayed in three intervals by a spectrophotometer (Unico UV 2010) at 765 nm wavelength. To measure the overall phenol in the extracts, 0.01–0.02 μg of the extracts was solved in methanol 60%, reaching 10 ml and then, using Folin-Ciocalteu method, the overall level of phenol was measured. However, instead of using the standard solution, 0.1 ml extract solution was added. Finally, the overall phenol level was obtained in mg/g extract in gallic acid equivalent.

2.3. Measurement of flavonoid compounds

Total flavonoids were evaluated equivalent to Rutin, using chloride aluminum colorimetry.[20] First, different concentrations of standard Rutin (25, 50, 100, 250 and 500 ppm) were prepared in methanol 60%. Then, from each solution, 1 ml was transferred into test tubes and mixed with 1 ml of chloride aluminum 2%. Afterwards, 6 ml potassium acetate 5% was added and the optical density level was read after 40 min at 415 nm wavelength. The concentration levels of the standard solutions were assayed in three intervals. In order to measure the overall level of flavonoids in the extracts, 0.01–0.02 g of the extracts was dissolved in methanol 60%, reaching 10 ml. Then, the total level of flavonoids was measured using chloride aluminum colorimetry. However, instead of using the standard solution, 1 ml the extract was added. The total flavonoid level was calculated in mg/g extract, equivalent to Rutin.

2.4. Measurement of flavonol compounds

The total flavonol was measured using chloride aluminum colorimetry and Rutin procedure, however the optical density level reading was obtained after 2.5 h at 440 nm wavelength.[21]

2.5. Measurement of anti-oxidant activity

β-carotene model was employed to measure the anti-oxidant activity of the extract.[22] Half ml chloroform, 5 ml β-carotene (0.2 mg), 20 ml linoleic acid (20 mg), and 0.2 ml Tween 40 were mixed in a suitable container and incubated at 50 °C for 10 min in order to remove the solvent. The solution was diluted with distilled water and mixed with 4 ml aliquots in the following manner. The control solution was prepared including 0.2 ml ethanol, 0.2 ml the extract sample, and 0.15 ml ethanol. The optical density of the control group was recorded at t_0 and t_{90} at 470 nm, in a manner similar to the standard. The samples were incubated in a bain-marie at 50 °C. The anti-oxidant activity was measured on the basis of the ability of the samples in preventing β-carotene washing, calculated through $AA = 100[1-(A_o-A_t)/(Ao_o-Ao_t)]$; where, A_o is the optical density at t_0, A_t is optical density of the sample at t_{90}, and Ao_o and Ao_t are optical density values in the control samples at t_0 and t_{90} respectively.

2.6. Animals and study design

36 healthy Wistar male rats weighing 200–250 g were randomly assigned to three groups of A, B, and C and treated respectively, with Vaseline + 0.6% green tea extract, Vaseline and normal saline for 21 days. The rats had no history of surgery and other medical interventions, were kept in at most 3-member cages, at 23 ± 2 °C temperature, and on nutritionally similar and standard pelleted diet (*Razi* Co. Karaj, Iran).

2.7. Surgical wounds

Incisions were made by one person when the rats were anaesthetized. Rats, in *all* groups, were anaesthetized by a combination of 20 mg/kg Ketamine 10% (Alfasan Co., Netherlands) and 2 mg/kg Xylazine 2% (Alfasan Co., Netherlands), administered intramuscularly. Then, the cases while anaesthetized were positioned prone on a surgical table, their back skin was disinfected with Betadine 10% (*Tolid Daru* Co., Iran), and the hairs of an area of the skin preselected using a caliper were completely shaved with a razor to make a 4 cm incision by means of a No. 24 scalpel; the depth of incision included both dermis and hypodermis, i.e., the thickness was full. Then, 4 stitches at 1 cm intervals were made by means of 3.0 nylon string (*Kamran Teb* Co.,

Table 2
The mean decrease in wound length (cm) during 1st, 2nd, 3rd week and total.

Treatment	1st Week	2nd Week	3rd Week	Total weeks	Result of repeated measures test in total weeks
Vaseline + 0.6% green tea extract (A)	2.07 ± 0.72	1.83 ± 0.69	0.05 ± 0.12	3.95 ± 0.12	<0.001
Vaseline (B)	−1.50 ± 0.35	2.13 ± 0.39	0.30 ± 0.09	3.90 ± 0.08	<0.001
Normal saline (C)	1.12 ± 0.22	2.07 ± 0.21	0.65 ± 0.13	3.85 ± 0.06	0.009
Result of one-way ANOVA test	<0.001	0.001	0.003	0.274	

Tukey test showed statistically significant differences in these cases: between groups A and B in the third week ($P = 0.005$) between groups A and C in the first week ($P < 0.001$), second week ($P = 0.001$) and third week ($P < 0.001$); between Group B and C in the first week ($P = 0.001$), second week ($P = 0.004$) and third week ($P = 0.001$).

Photograph 1. Improvement process of wound healing in group A.

Iran), the given area was again disinfected, and the rats were kept at suitable temperature until consciousness. At 7th day, the stitches were removed.[23–26]

2.8. Treatment

Treatment was performed every day at the same time by one person and continued for 21 days, beginning from the day of making incisions. For this, 1 g Vaseline ointment containing green tea extract was topically applied on a 2 cm^2 (4 cm × 0.5 cm) area of the wound in group A; the same was performed on group B and C using Vaseline ointment and normal saline (*Daru-Pakhsh*, Iran), respectively.[26]

2.9. Microscopic examinations

For purpose of data collection, the animals were numbered and a checklist was prepared to record data individually. Since the 2nd day of the study, the length of the remaining wound was measured by a predetermined person using a caliper, every other day; the case's data in the given days was separately recorded.[23–26]

For the purpose of comparison, photographs of all animals' wounds were taken at the 2nd day and, since the 7th day, every other day until the study completion.

For this, 3 rats were randomly recruited from each group at 3rd, 12th, and 21st days; on 21st day, the day of study completion, the recruited rats were euthanized after measuring of the remaining wound length. After euthanizing them through ether inhalation in a closed space, the wound tissue histological specimens in full thickness accompanied with neighboring healthy skin were taken and placed into 10% Neutral Buffered Formalin fixator. Tissue processing was done by paraffin and wax. Transverse incisions, 5 μm thick, were made by means of Microtome fixed blade. Lesions were stained by hematoxylin and eosin.[27,28] Histopathological examinations, in view of epithelial regeneration, granulation tissue thickness, and angiogenesis (Table 1), were separately done by a pathologist who was blinded in grouping.

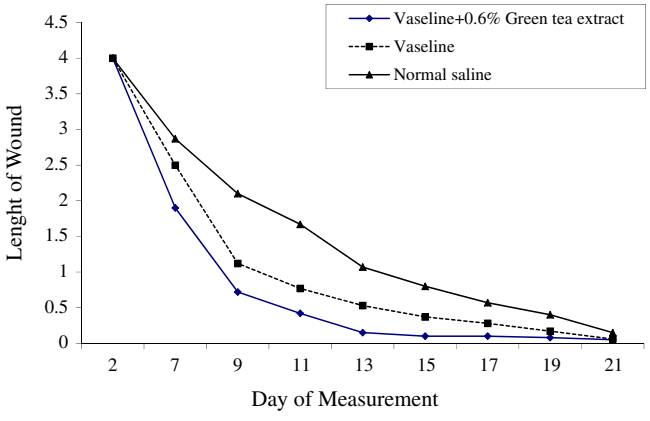


Fig. 1. The size of the wound incision length (cm) in the three treatment groups during different days.

2.10. Data analysis

Data analysis was performed by SPSS 16 using Survival analysis (*Breslow* test), Repeated measures ANOVA and One-way ANOVA (for macroscopic analysis), and Mann–Whitney (for histopathology analysis). $P < 0.05$ was considered as statistically significant.

3. Results

Totally, 5 of 6 (83%) rats in group A (Vaseline + 0.6% green tea extract) and 3 of 6 (50%) rats in group B (Vaseline) recovered by 21st day. No inflammation, eczema and/or infection were observed.

Regarding *Breslow* test, the mean healing duration in group A was shorter compared to group B [$P = 0.018$, 14.66 days (SE = 1.94) vs. 20.66 days (SE = 0.373)].

The wound length in group A, based on repeated measures ANOVA, was significantly shorter compared to group B. Fig. 1 indicates wound lengths during the study. Table 2 indicates the

Table 3
The mean and median scores for the histological features of the wound tissue samples (mean ± SD and median [min–max]).

Wound healing' scaling group	Epithelial regeneration	Granulation tissue thickness	Angiogenesis
Vaseline + 0.6% green tea extract (A)	1.333 ± 0.942 (median = 2, min = 0, max = 2)	1.444 ± 0.831 (median = 1, min = 0, max = 3)	1 ± 1.118 (median = 1, min = 0, max = 3)
Vaseline (B)	1 ± 0.816 (median = 1, min = 0, max = 2)	0.777 ± 0.416 (median = 1, min = 0, max = 1)	0.222 ± 0.441 (median = 0, min = 0, max = 1)
Normal saline (C)	0.222 ± 0.415 (median = 0, min = 0, max = 1)	0.333 ± 0.471 (median = 0, min = 0, max = 1)	0.222 ± 0.441 (median = 0, min = 0, max = 1)

mean decrease in wound length during 1st, 2nd, and 3rd week as well as the whole study duration.

Based on one-way ANOVA, there was no significant difference in the mean decrease in wound length between groups A and B during 1st and 2nd weeks ($P = 0.094$ and $P = 0.536$, respectively); during the 3rd week, the difference between the two groups was significant ($P = 0.005$). Also, the decrease in wound length in group A was significantly higher compared to group B during the first 14 days ($P = 0.043$). The Photograph 1 shows improving in group A in macroscopic study.

Table 3 shows degrees of histopathological indices of wound healing in different groups throughout the whole study. Regarding Mann–Whitney test, comparing groups A and C during the whole duration, indicated statistically significant differences in histopathological indices of epithelial regeneration, granulation tissue thickness, and angiogenesis ($P = 0.006$, $P = 0.007$, and $P = 0.016$, respectively).

In addition, this comparison at 21st day of the study indicated statistically significant differences in histopathological indices of epithelial regeneration, granulation tissue thickness, and angiogenesis ($P = 0.006$, $P = 0.007$, and $P = 0.016$, respectively); no significant difference in *all* indices was observed throughout the whole study duration.

The Photographs 2 and 3 illustrate a significant difference between group A and the other two (control) groups.

4. Discussion

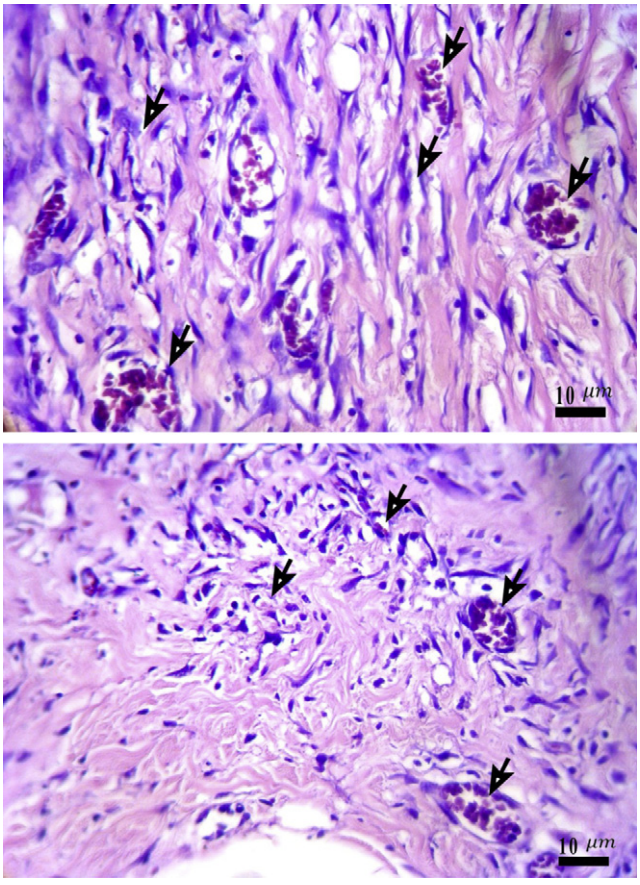
Wound healing is a complex process, which interruption could lead to a delayed healing or excessive fibrosis.[29] Delay in wound healing increases the possibility of getting infected, inappropriate recovery, and unpleasant scar. Several effects of green tea and its compounds have been already examined, indicating that this plant, with anti-oxidant, anti-cancer, anti-aging, and anti-inflammatory effects, could also prevent collagen production and accumulation[8,14]; the majority of these properties could be attributed to the plant's polyphenolic compound, i.e., catechin in the leaves.[11–13] Consistent with our findings, Safari and Sadrzadeh's study indicated anti-oxidant effects of epigallocatechin, one of the green tea's compounds.[30]

In addition, the beneficial effect of epicatechin gallate on wound healing quality and hence leaving more pleasant scar has been shown, which probably confirm its effect on increased level of vascular endothelial growth factor, accelerated vessel formation, and enhanced nitric oxide and cyclooxygenase.[31] Consistently, the superiority of group A over the other groups in view of vessel formation, in the present study, is indicative of this effect. A favorable vascular expansion around the wound could lead to the cells' appropriate nutrition and improved wound healing process.[32]

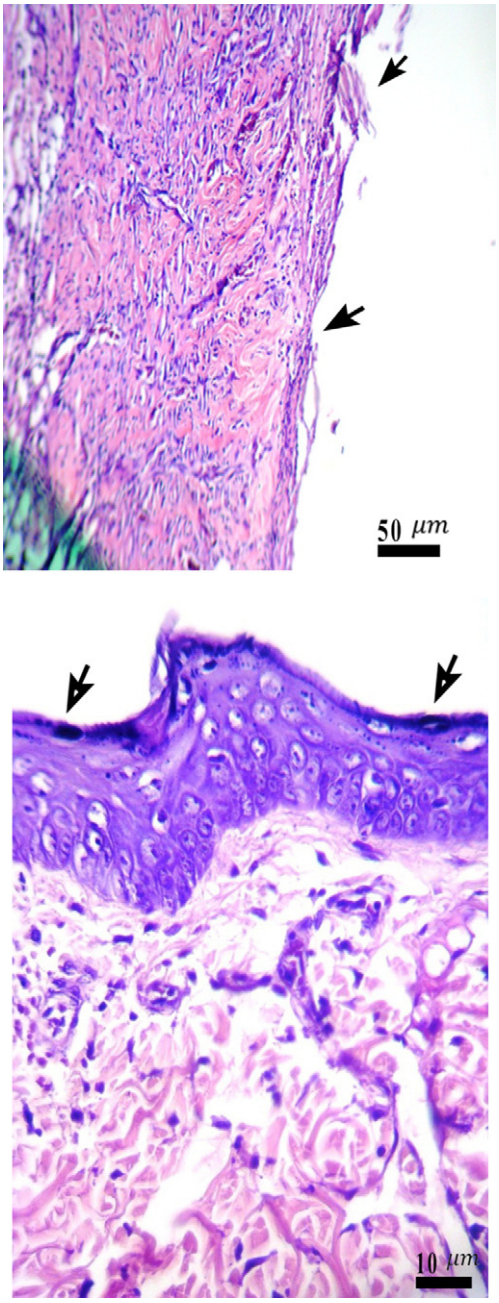
Monitoring surgical wounds during the study demonstrated the significant effect of Vaseline + 0.6% green tea extract in contrast to Vaseline on the wounds' recovery acceleration. The recovery acceleration could be due to the anti-oxidant effect of the present epigalocatechin on speeding up vessel formation of the skin as well as anti-inflammatory properties.

Also, the effects of polyphenolic compounds (e.g. epigalocatechin gallate) as the expressive agent for connective tissue growth factor gene and inhibitory regulator of collagen gene expression have been proposed and their effects on myofibroblasts' production and distinction, connective tissue growth, and regular classification of collagens have been proved[33]; the significant difference in granulation tissue thickness and epithelial regeneration between group A and control groups could result from these properties. One of the limitation of this study is lack of immunohistological staining method to proper assessment of angiogenesis.

Despite significant decrease in healing duration among those treated with Vaseline + 0.6% green tea extract, no significant difference in mean wound length was seen between groups A and B within the whole duration ($P > 0.05$), probably because of our small sample size. Within the first two weeks, however, decrease in



Photograph 2. Tissue samples taken from Group A; Proper and complete angiogenesis in different parts of the broad bands of collagen in tissue and its context is quite apparent that the proper formation of granulation tissue (H&E staining) (×100,400).



Photograph 3. In the first picture lack of skin epithelial tissue layers in group C, while second one shows the complete formation of the epidermis and dermis layers is clearly visible (H&E staining) (×100).

wound length was significantly higher in group A compared to group B ($P = 0.043$).

5. Conclusion

Regarding the properties mentioned for components present in the green tea, it could help wound healing duration decrease considerably; therefore, further research on green tea-derived medications could lead to ensuring its application as a treatment for post-surgical wounds.

Ethical approval

All experiments were performed under supervision of Dr. Shahriyar Adibi and Prof. Mahmoud Rafieian-kopaei, in accordance to the guidelines of the Animal Ethics Committee of Shahrekord University of Medical Sciences by the number of: 91-5-2.

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Author contribution

Sayyed Yazdan Asadi: Study design, Experimental works (Extract Preparation and determination their components, Animal study), Data collecting, Article writing.

Pouya Parsaei: Experimental works (Animal study), Data collecting and some parts of article writing.

Mehrdad Karimi: Determination of surgical and anesthesia procedure and its methods.

Sareh Ezzati: Data Analysing, Article writing.

Alaleh Zamiri: Data Analysing, Article writing.

Fereshteh Mohammadizadeh: Pathologic evaluation.

Mahmoud Rafieian-kopaei: Supervisor of research plan, Study design, Determination of Antioxidant Activity, Article writing.

Conflict of interest

There are not any conflicts of interest in this manuscript.

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