WOUND HEALING PROPERTIES OF GREEN TEA EXTRACT IN EXCISION-WOUNDED RATS

BY
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Abstract

The wound healing potential of green tea extract (GTE) was determined in two concentrations (0.6 and 6% in Vaseline base) for topical treatment of excision wounds in rats. Treatments were applied for 14 days. GTE, in both concentrations, caused a significant increase in wound contraction rate and glutathione level, as well as a significant decrease in lipid peroxidation and transforming growth factor-β expression. Moreover, histological examination showed a marked improvement in the skin condition.

This study concludes that GTE was effective in enhancing the healing process in excision wounds.

Key words: Green tea; Wound healing; Oxidative stress

1. Introduction:

Wound healing is a complicated process involving different tissues and cell lineages (Martin, 1997). The fundamentals of a most favorable wound healing are; to minimize tissue damage, to provide adequate tissue perfusion, oxygenation and nutrition; and to restore the anatomical continuity and function of the affected tissue (Pierce and Mustoe, 1995). Wound healing can be divided into 4 phases; hemostasis, inflammation, proliferation (granulation and contraction) and remodeling (maturation) (Gosain and DiPietro, 2004). Timing is important to wound healing, where any interruptions, aberrancies, or prolongation in this process can lead to delayed wound healing or a non-healing chronic wound (Guo and DiPietro, 2010).

Herbal medicines based on plant extracts have been used to accelerate the wound healing process since ancient time (Mantle et al., 2001; Jadhav et al., 2015). Green tea, a product of dried leaves of Camellia sinensis, has been consumed by East Asian people for health promotion since 3000 B.C. (Jankun et al., 1997; Kim et al., 2008a). Many evidences indicate that this plant, with anti-oxidant, anti-cancer, anti-aging, and anti-inflammatory effects, can affect immune responses, as well as collagen production and accumulation (Kim et al., 2008b; Park et al., 2008). Green tea consumption may be an adjuvant in the treatment of diabetes mellitus (Gomes et al., 1995), hypertension and hypercholesterolemia (Dreosti, 1996). Epicatechin, epicatechingallate, epigallocatechin, and epigallocatechingallate are among the key anti-oxidant compounds of green tea, which could affect collagen volume and hence wound healing (Kim et al., 2008b). It was proved that epigallocatechingallate could increase keratinocytes reproduction and distinction (Hsu et al., 2003). Also, its anti-fibrogenic effect has been confirmed in some animal models (Nakamuta et al., 2005).
Therefore the aim of our study was to investigate the potential wound healing activity of methanol extract of green tea in excision-wounded rats, and to determine the possible underlying mechanisms.

2. Materials and Methods

2.1. Plant materials

The leaves of Green tea *Camellia sinensis* family Theaceae were purchased from a local drug shop Ahmad Tea, Batch FG00737, Packing date 28/1/2013, Expiry date 28/1/2016. A voucher specimen (PHG-P-CS-251) has been deposited in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University.

2.2. Extraction of plant materials and ointment preparation

Hundred grams of dried leaves of green tea were soaked in one liter of methanol for one week at room temperature (30 ± 2 °C). It was shacked from time to time. The extract was filtrated concentrated under reduced pressure in a rotary evaporator at (45°C) till complete dryness. The semisolid mass of green tea extracts (methanol free) was homogeneously mixed with pure Vaseline to make a Vaseline-based (0.6%-6%) ointment.

2.3. Experimental Animals

Adult male albino rats weighing 150-200g were used in the present study. They were obtained from the breeding colony maintained at the animal house of the National Organization for Drug Control and Research (NODCAR, Cairo, Egypt). Animals had free access to food and water *ad libitum*. They were maintained at (25 ± 2) °C and 40-60% relative humidity with 12-h light–dark cycle. Animals were subjected to an adaptation period of one week in the animal house before experimentation. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee of Faculty of Pharmacy, Ain Shams University, Egypt and comply with the Guide for the Care and Use of Laboratory Animals.

2.4. Experimental Design

A total of forty eight albino rats were randomly divided into four groups, 12 animals in each. First group was considered as the control non-wounded group. In order to perform excision wound in the 3 other groups, rats were anaesthetized with 300 mg/kg chloral hydrate via intraperitoneal injection. The dorsal surface of rat was shaved, cleaned with 70% ethanol. Excision wounds were made by cutting out predetermined dorsal area (approximately 22 mm diameter) of skin from the shaved area using toothed forceps and pointed scissors. The entire wound was left open (Esimone *et al.*, 2006). The second group was considered as the control wounded group, treated with vaseline base only. The third and fourth groups were treated with 0.6% and 6% green tea extract in vaseline based ointment, respectively.

Treatments were applied for 14 days and the rate of wound contraction was determined every other day. On day 7, half groups were anesthetized and skin tissues were dissected out and washed with ice-cold saline. Portions of the skin tissue were homogenized in 0.1 M phosphate buffer (pH 7.4) producing 10% homogenate. The
homogenates were centrifuged at 4000 rpm at 4°C for 15 min then aliquots of supernatants were separated and used for biochemical analyses. Additionally, specimens from each group were fixed in 10 % formalin for histopathological and immunohistochemical analyses. On 14th day, the rest of the animals were sacrificed and tissue samples were collected for homogenization for biochemical analyses.

2.5. Evaluation of Wound Healing

The rate of wound contraction was measured as percentage reduction of wound size at every other day from each rat wound until wound closure. Progressive decrease in the wound size was monitored periodically using transparency paper and a marker, and the wound area was assessed graphically to monitor the percentage of wound closure, which indicates the formation of new epithelial tissue to cover the wound. Wound contraction was expressed as reduction in percentage of the original wound size on days 1, 3, 5, 7, 9, 11 and 13. The wound healing rate was calculated with a formula as following:

\[
\text{The Percentage (\%) wound contraction} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \times 100
\]

(Esimone et al., 2006).

2.6. Assessment of Oxidative Stress Parameters

2.6.1. Estimation of reduced glutathione (GSH) content

The level of glutathione was determined in skin homogenate using Biodiagnostic kit (Cairo, Egypt) according to the method described by Beutler et al. (1963). It depends on the precipitation of protein SH-group then GSH react with Ellman’s reagent to form stable yellow color, measured colorimetrically at 405nm.

2.6.2. Estimation of malondialdehyde (MDA) content

The level of malondialdehyde was determined in skin homogenate using Biodiagnostic kit (Cairo, Egypt) according to the method described by Kei, (1978). Where thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form pink color its absorbance measured colorimetrically at 534nm.

2.7. Tumor growth factor beta1 (TGF-β1) immunohistochemistry analysis

The skin specimens for immunohistochemistry were fixed in 10% formaldehyde and embedded in paraffin blocks. The sections were deparaffinized and placed in 100 mM phosphate buffer saline (PBS) for 10 min before starting the staining procedure. The tissue sections were deparaffinized, rehydrated in graded alcohols, and submerged in the peroxidase quenching solution (1 volume H2O2 in 9 absolute pure methanol) to eliminate the endogenous peroxidase activity for 10 min, followed by rinsing in PBS in three steps for two min. Sections were blocked in 10% non-immune goat serum for 10 min and incubated at 37°C for 1 h in 1:200 primary antibody. The primary antibody used was TGF-β rabbit polyclonal IgG (Santa Cruz Biotechnology, CA). This was followed by washing in PBS and reacting with streptavidin peroxidase conjugate for 10 min. The peroxidase activity was detected with the peroxidase substrate diaminobezidine tetrahydrochloride (DAB). After counterstaining with hematoxylin, the slides were dehydrated with grade series of alcohol, cleared in xylene, mounted with a cover slip and examined under light microscope.
2.8. Skin histopathological examination

The skin of rats in different groups fixed in 10% formalin was embedded in paraffin wax and tissue blocks were prepared for sectioning at 4 microns thickness. Skin sections were stained by hematoxylin & eosin (H&E) for general histological analysis and were examined by light electric microscopy.

2.10. Statistical Analysis

The results obtained were expressed as mean±SD. Statistical analysis was performed using ANOVA followed by Tukey-Kramer as post hoc test. Wound healing contraction rats were performed with repeated Two Way ANOVA followed by bonferroni as post hoc test. The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using Instat software package (version 3). Graphs were sketched using GraphPad Prism software (version 5).

3. Results

3.1. Wound healing rates

Wound healing rates were measured at days 1, 3, 5, 7, 9, 11, and 13 after wounding. In all groups, wound healing rates increased with time. A significant increase in the wound-healing activity was observed in the animals treated with (0.6 and 6%) green tea extract, compared to the control group while there is no significance between the two treated groups. (Fig.1).

![Fig.1. Effect of green tea on the percentage of wound healing (wound contraction) on excision-induced wound in rats. Data are presented as mean ± SD. b: significantly different from the corresponding control (wounded) at P < 0.05 using repeated Two way ANOVA followed by Bonferroni as post hoc test.](image-url)
3.2. Oxidative stress parameters

3.2.1. Glutathione (GSH) content

Topical application of green tea extract significantly increased the (GSH) value on day 7 and 14 respectively, as compared to control non-wounded and wounded rats. GSH content in skin specimens of the group treated with 6% GTE was significantly lower than the group treated with the small dose 6% GTE after 7 days (Table 1). While GSH content in skin specimens of the group treated with 6% GTE was higher than the group treated with the small dose 0.6% GTE after 14 days (Table 1).

**Table 1** Effect of green tea on the content of glutathione (GSH) in tissue homogenates of excision wounded rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment</th>
<th>Skin GSH Day 7 (μmol / g tissue)</th>
<th>Skin GSH Day 14 (μmol / g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Non-wounded Control</td>
<td>4.74 ± 0.2639</td>
<td>4.651 ± 0.3148</td>
</tr>
<tr>
<td></td>
<td>Wounded CONT-Vaseline</td>
<td>2.985 ± 0.4508 ( ^a )</td>
<td>3.567 ± 0.3445 ( ^a )</td>
</tr>
<tr>
<td></td>
<td>Green tea (0.6g / 1g V)</td>
<td>8.726 ± 0.8304 ( ^a^b )</td>
<td>6.755 ± 0.1853 ( ^a^b )</td>
</tr>
<tr>
<td></td>
<td>Green tea (6g / 1g V)</td>
<td>6.324 ± 1.364 ( ^a^b^c )</td>
<td>7.372 ± 1.182 ( ^a^b )</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD (n=6) \( ^a, b, c \): significantly different from the corresponding control (non-wounded) or control (wounded) or \( ^a \), 0.6% green tea extract treated group, respectively, at \( P < 0.05 \) using one way ANOVA followed by Tukey-Kramer as post hoc test.

3.2.2. Malondialdehyde (MDA) content

Topical application of green tea extract significantly decreased the (MDA) value on day 7 and 14 respectively, as compared to wounded control rats. There was no significant difference in MDA content in skin specimens of both groups treated with different doses 0.6% and 6% GTE (Table 2).
Table 2 Effect of green tea on the content of malondialdehyde (MDA) in tissue homogenates of excision wound in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Skin MDA Day 7 (nmol /g tissue)</th>
<th>Skin MDA Day 14 (nmol /g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-wounded Control</td>
<td>27.65 ± 5.586</td>
<td>26.99 ± 5.806</td>
</tr>
<tr>
<td>Wounded CONT-Vaseline</td>
<td>69.89 ± 4.645&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.16 ± 1.378&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green tea (0.6g/1g V)</td>
<td>18.29 ± 4.363&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>32.23 ± 1.994&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Green tea (6g/1g V)</td>
<td>24.4 ± 1.365&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.12 ± 2.973&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD (n=6). a or b: significantly different from the corresponding control (non wounded) or control (wounded) groups, respectively, at P < 0.05 using one way ANOVA followed by Tukey-Kramer as post hoc test.

3.3. Histology

Histological examination of the skin specimen of the control non wounded group showed normal skin structure with normal epidermis and dermis, many hair follicles and sebaceous glands (fig 2a). Section of wound tissue in control group, treated with vaseline-based ointment, showed complete non separated eschar, severe loss of collagen bundles and edema in dermis, after 7 days of excision wounding (fig 2b). Sections of skin tissue from groups treated with both concentration of green tea extract, showed well-organized bands of collagen, more fibroblasts and less inflammatory cells. No significant difference was observed between treatments with low and high doses. (Fig. 2c,d).
Fig. 2. Histological findings of wound tissue after 7 days of excision wound (H&E stain). (A) Skin of non-wounded control animal showing apparently normal epidermis and dermis with many hair follicles and sebaceous glands are seen in the adjacent control skin. (B) Skin tissue of wounded control is showing complete absence of the epidermis, skin appendages, papillary dermis with congested blood vessels and inflammatory infiltrate complete escher, non separated, severe loss of collagen bundles. (C) Skin of wounded animals treated with 0.6g green tea is showing complete eschar separation, moderate dense collagen bundles and dilated blood vessels. (D) Skin of wounded animals treated with 6g Green tea is showing partial separation of eschar and moderate collagen fibers.

3.4. Immunohistochemistry

The expression of TGF-β was determined immunohistochemically at day 7 after wounding. The expression of TGF-β was estimated using immunohistochemical staining. The control non wounded group showed minimal immunostaining. Control wounded group showed increased expression of TGF-β, as shown by the intense brown staining. Topical application of green teas extract in both concentrations mitigated the wound-induced expression of TGF-β. Also the number of TGF-β1 positive cells in GTE-treated groups was significantly lower than that of the control group (Fig. 3c,b).
Fig. 3a. The expression of TGF-β1 was determined by immunohistochemically at day 7 after wounding. (A) Skin of non-wounded control animal shows minimal expression of TGF-β (X100). (B) Skin of wounded control animal is showing strong positive expression of TGF-β (strong immunopositivity for TGF-β) (brown colour) (X100). (C) Skin of wounded animal treated with 0.6g green tea is showing moderate positive expression of TGF-β (moderate immunopositivity for TGF) (brown colour). (D) Skin of wounded animal treated with 6g Green tea is showing minimal expression of TGF-β (X100).

4. Discussion

Wound healing is a physiological response to injury, required for reconstruction of damaged tissue (Ji et al., 2016). It depends on precise coordination of connective tissue repair, re-epithelialization, and angiogenesis (Reinke and Sorg, 2012). The aim of these processes is to regenerate and reconstitute the disrupted anatomical continuity and functional status of the skin (Geethalakshmi et al., 2013). Several beneficial effects of green tea have been already examined, indicating that this plant, with anti-oxidant, anti-cancer, anti-aging, and anti-inflammatory effects, could also affect collagen production and accumulation (Park et al., 2008).

In the present study, we examined the application of GTE with two different concentrations (0.6% and 6% in 1g Vaseline) on the wound healing in excision-wounded rats, for 14 days. Topical application of GTE, in both concentrations, significantly enhanced the rate of wound healing. This was in accordance with previous study which demonstrated the significant effect of 0.6% green tea extract on surgical wounds’ recovery acceleration (Asadi et al., 2013). This effect was attributed to the anti-oxidant effect of epigallocatechin on speeding up the vessel formation of the skin, as well as anti-inflammatory properties.
Oxidative stress and accumulation of reactive oxygen species (ROS) are associated with impaired wound repair in chronic, non-healing wounds (Schäfer and Werner, 2008). The use of antioxidants in crude extracts may enhance the healing process (Janda et al., 2016).

The results of the present study regarding antioxidant capacity of green tea groups showed a significant increase in GSH level in addition to a significant decrease in MDA level in excision wound. These results are in line with the findings reported by Ali Akbar Abolfathi et al. (2012) who observed that daily treatment with green tea extract markedly improved antioxidant status of liver tissue of rats with streptozotocin induced diabetes. Moreover in a burn model Hosnuter et al. (2015), epigallocatechingallate (EGCG), exerted a potent antioxidant effect. In the EGCG-treated burn group, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were significantly higher than in the burn wounded control group. Additionally, MDA and tumor necrosis factor α (TNF-α) level were significantly lower in the EGCG-treated burn group. Green tea extract contains polyphenols (e.g., catechin, epicatechin, epigallocatechin, and their gallates), tannin, and caffeine. The extract also includes pyrroloquinolinequinone, a discovered vitamin (Kasahara and Kato., 1993). It has been suggested that catechins, antioxidant compounds present in green tea, may improve the defense system as demonstrated in several in vitro and in vivo models (Salah et al., 1995). Green tea extracts are more stable than pure epigallocatechingallate, the major constituents of green tea, because of the presence of other antioxidant constituents in the extract (Kaszkin et al., 2004). In general, herbal medicines are complex mixtures of different compounds that often act in a synergistic fashion and exert their full beneficial effect as total extracts (Loew and Kaszkin, 2002).

Many types of cytokines and growth factors are responsible for inflammation, re-epithelialization, the formation of granulation tissue, and neovascularization during the wound healing process (Lim and Yoo, 2010). TGF-β1 is a multifunctional cytokine and enhances granulation tissue formation and collagen formation in wound healing (Lee et al., 2004). TGF-β1 is a powerful cytokine that can promote fibroblast proliferation and maturation, thereby accelerating the development of fibrosis (Martin et al., 2000).

The inflammatory phase is an essential component of the tissue repair process. Inflammation is normal and necessary prerequisite to healing (Dakin et al., 2014). In recent years, the identification of numerous cytokines and ‘growth factors’ had led to several important discoveries and potential new treatment lines (e.g. Wagner et al., 2003; Leung et al., 2006).

In our study, GTE has decreased TGF-β in wound tissue compared with the control wounded. It was reported that the anti-fibrotic effect of GTE might be through the inhibition of the signal transduction of TGF-β by binding to transforming growth factor receptor II (TGFRII) and attenuation of α-smooth muscle actin (α-SMA) expression (Salem et al., 2014). A study performed by Klass et al. (2010) clarified that EGCG has potential effects on wound contraction and healing through the modification of transforming growth factor-β (TGF-β) signaling which suppresses TGF-β receptors.
Moreover the histological examination in this study indicated a greater degree of organization of the collagen orientation in the treated lesions and a more normal alignment of new collagen. It is possible that this was brought about by a modification of the inflammatory reaction or organization of the fibrin network in the tissue spaces at early stages of inflammatory phase of healing by the green tea extract, which may act as a ‘scaffold’ or template for fibroblast activity. All these mechanisms make GTE a potential anti-scarring and wound healing extract.

In conclusion, the current study revealed that wounds treated with green tea extract, as topical application of wounds, can significantly accelerate the wound healing process. Also, there was no big difference on both green tea concentrations. It’s recommended to use the 0.6g green tea extract to enhance the wound healing process in excision wound.

References


الخصائص العلاجية لمستخلص الشاي الأخضر في التئم الجروح المستصلة بالجرذان

للسادة الدكتوراه

آلاء شاهين، وداد حسن، أميمة الدهشان، سارة عبد الرحمان، هناء منصور، ابتهال الدمرداش.

الجلد هو أكبر عضو في الجسم، يعمل كعزل للماء، وكرع وافي. ويعمل على حراسة الجسم ضد التطرف في درجة الحرارة وأشعة الشمس الضارة. الهدف من هذا العمل هو دراسة الدور المحتمل للشاي الأخضر في تعزيز التنام الجروح الجلدية في الجرذان، تم تحديد إمكانات التنام الجروح من مستخلص الشاي الأخضر من تركيزين (0.2 و 0.6% في قاعدة الفازلين) كعلاج موضوعي للجروح تم تطبيق العلاجات لمدة 14 يوماً. وقد أوضحت الدراسة أن الشاي الأخضر في كلا التركيزين، سبب زيادة كبيرة في معدل انكماش الجروح الجلوباثيون المختلط، فضلاً عن انخفاض كبير في بروكسيم الدهون وعامل تحلل النمو بيتا. وعلاوة على ذلك، أظهرت الفحص النسيجي تحسناً ملحوظاً في حالة الجلد.

وخلصت هذه الدراسة أن مستخلص الشاي الأخضر كان فعالاً في تعزيز عملية الشفاء في الجروح المستتصلة في الجرذان.

الكلمات المفتاحية : الشاي الأخضر، التنام الجروح، القسط الأوكسيدي.