IJPT
1735-2657/13/121-9-14
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 12: 9-14, 2013

ORIGINAL ARTICLE

Study of Efficacy of Aqueous and Methanolic Extract of Green Tea on the Process of Opened Skin Wounds Healing in Male (NMRI) Mice Race

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Received July 7, 2012; Revised October 23, 2012; Accepted November 8, 2012

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

Green tea used for year has a popular cancer preventive activity. Researchers have showed green tea inhibited growth of cancer in the animals. This research has been done with awareness of positives effect of green tea, which is approved by researchers and the importance of treatment of opened skin wound. This work has been done experimentally. There were 56 male mice in 7 different groups. Different dose of water and alcohol such as 50, 150 and 300 μL were injected. After anaesthetizing the mice, skin wound was created on the back of the mice by a 6-mm punch. While the mice in control group were treated by normal saline, water and alcohol extract of green tea was injected around the wound on the back of each mouse. The dimensions of ulcers and the recovery percent of the wound in the 1st , 3rd, 5th, 7th, 10th, 13th and 15th day of study were measured. Furthermore, the needful time for recovery was evaluated. Some histological studies were done as well. Two Specimen of wounds were supplied at 4th, 7th and 15th day of the study. In this way, fibroblasts, inflammation, epitheleum and endothelial cell of blood vessels from the wounds were studied. The results show that there are no significant differences among control, water and alcohol groups in recovery processes ($p > 0.05$.) Evaluation of recovery processes showed there were significant differences among these groups on 7th day of study ($p < 0.01$). Evaluation of recovery processes showed there were significant differences among three injected doses of study ($p < 0.001$). The degree of differences in fibroblasts, inflammation and epithelium distortion in different days for 6 groups ($p < 0.05$) was meaningful. According to these findings, although both water and alcohol extracts of green tea speed up the wound healing, there isn't any difference between the uses of water or alcohol extracts.

Keywords: *Green tea, Wound healing, Water and Alcohol extract, Race NMRI*

Wound healing, or wound repair, is an intricate process in which the skin (or another organ-tissue) repairs itself after injury. The classic model of wound healing is divided into three or four sequential, yet overlapping phases: hemostasis (not considered a phase by some authors), inflammatory, proliferative and remodeling. Upon injury to the skin, a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage [1].

Green tea is made from *Camellia Sinensis* [2]. Leaves of this plant are processed with minimal oxidation. It is mainly used in Asia specifically in China [3-4]. There have been extensive researches on the effects of green tea and results have been surprisingly pleasing. Some of the major potential benefits of green tea include; anti-Cancer properties, increases in metabolic rate, anti-diabetes effect, enhancement of mental alertness, improvement of immune system, improvement of quality of life for HIV-infected

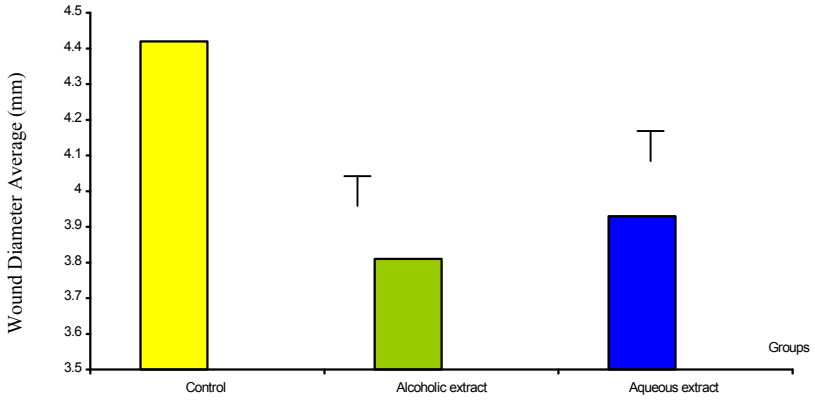

Fig 1. The macroscopic study of wound diameter average between control and treatment group on days 1, 3, 5, 7, 10, 13 and 15. ($p < 0.001$)

patients, cardioprotective effects [5-8]. In this study, green tea extracts has been investigated for their effects on the opened skin wound healing.

MATERIALS AND METHODS

In this experimental research, 56 male mice of NMRI race with average weight of 25-35 grams were studied. The mice were held in 7 cages in Professor Torabi Nejad Research Center in Isfahan with light cycle of 12 hours darkness and 12 hours light in 22 ± 2°C. In this period, sufficient water and food were in hand of animals and they were randomly classified to control and experimental groups.

Green tea extract was prepared using Soxhlet instrument. The green tea leaves were studied by Isfahan University and were transferred into laboratory. Then using electric mill, they were grinded to a powder. Forty grams of green tea powder was placed into filtration paper and were transmitted to a specific container. In order to produce water extract, 400 milliliters of purified water was added and in order to produce alcoholic extract, 400 milliliters of 85% methanol was added. After producing the extract by Soxhlet, it was dried and concentrated in rotary evaporator and then in 48-hour incubation in 70°C Bon marry. In next stage, 2 g of each extract (alcoholic or aqueous) was solved in 100 mL normal saline and therefore, 2% aqueous or alcoholic extract was achieved.

In order to make a wound in animal, first the mouse became comatose with ether and then its back hair was shaved. After immersing the skin with betiding, with 6-millimeter punch and in accordance to surgery principles, a 6-millimeter wound was developed. The wound depth was full skin thickness and the surgery day was named the day zero. After making the wound, in order to prevent potential putrefaction, 0.2 mg penicillin and 0.2 mg gentamicin were injected.

The mice were injected 2% aqueous or alcoholic extract for 7 days, once a day and at 9 am. The amount of 50, 150 or 300 mL of extract were injected in four direction surrounding the wound. All injection were performed by one person. After developing the wound, the mice were classified into 7 groups each 8, as follows:

Group 1 (control): the wound surface of this group was treate d by normal saline;

Groups 2, 3 and 4: the wound surface was treated by 50, 150 and 300 mL of 2% aqueous extract respectively;

Group 5, 6 and 7: the wound surface was treated with 50, 150 and 300 mL of 2% alcoholic extract respectively.

For macroscopic study, on days 1, 3, 5, 7, 10, 13 and 15, the length measurement method of wound and imaging with digital camera was used for all groups. The development of wounds was assessed and the wound stages according to imaging digital camera and size measurement were recorded.

For microscopic evaluation, sampling and tissue study was carried out. On days 4, 7 and 15, the mice were killed by smelling ether in air. Then, two samples were taken from wound tissue and surrounding skin which were placed inside 10% Formalin solution. The tissue processing and molding was done by paraffin and wax and the German microtome with firm blade of LEItz to develop width cuts including skin, bed with the thickness of 4 microns. The cuts were painted by Haematoxylin and Eosin (H&E) coloring methods and edematous cell, fibroblasts and sweating sections were recognized through quality method. The wound improving was determined through rating the pathology parameters as follows:

Rating 1: The tissues with no repeating epithelisation and fibrosis tissue but with the low numbers of vessels and extreme edema.

Published online: January 31, 2013

Table 1. The microscopic study of aqueous and alcoholic extract of green tea on days 4, 7 and 15 based on the inflammation, fibrosis, epithelium and blood vessels.

Parameter	Days	Groups						
		Control	Aqueous extract			Alcoholic extract		
			50 µL	150 µL	300 µL	50 µL	150 µL	300 µL
Inflammation	4	4.50 ± 0.07	0.01 ± 4.10	0.02 ± 3.50	0.05 ± 3.52	0.02 ± 4.0	0.01 ± 3.70	0.001 ± 3.11
	7	3.21 ± 0.05	0.2 ± 2.80	0.02 ± 2.50	0.09 ± 2.10	0.01 ± 2.70	0.01 ± 2.30	0.03 ± 2.0
	15	1.81 ± 0.01	0.03 ± 1.50	0.01 ± 1.2	0.001 ± 0.09	0.01 ± 1.40	1.0 ± 0.01	0.001 ± 0.07
Fibrosis	4	4.81 ± 0.01	0.02 ± 4.51	0.01 ± 4.20	0.05 ± 3.91	0.02 ± 4.52	0.01 ± 4.52	0.0 ± 3.70
	7	1.21 ± 0.01	0.001 ± 1.0	0.081 ± 0.02	0.01 ± 0.06	0.90 ± 0.06	0.001 ± 0.70	0.50 ± 0.002
	15	2.31 ± 0.01	2.0 ± 0.02	0.01 ± 1.62	1.21 ± 0.02	0.05 ± 2.11	0.05 ± 0.70	0.001 ± 1.25
Epithelium	4	5	5	5	5	5	5	5
	7	4.80 ± 0.01	0.01 ± 4.11	0.02 ± 3.80	0.05± 2.52	4.0 ± 0.01	3.20 ± 0.02	0.04 ± 2.32
	15	2.0 ± 0.001	0.02± 1.42	0.01 ±1.0	0.04 ± 0.51	0.02 ± 1.50	0.05 ± 1.0	0.03 ± 0.51
Blood Vascular	4	5.0 ± 1.13	1.10 ±4.92	1.0 ± 4.90	1.0 ± 4.89	1.2 ± 4.93	1.0 ± 4.90	0.01 ± 4.88
	7	4.5 ± 1.10	1.12 ± 4.25	1.12 ± 4.23	0.01 ± 4.210	1.12 ± 4.25	1.10 ± 4.21	1.02 ± 4.22
	15	0.01 ± 3.5	0.01 ± 3.25	1.10 ± 3.0	1.10± 3.01	0.01 ±3.28	0.01 ± 3.01	1.10 ± 3.0

Rating 2: The tissues with repeating epithelisation, low quantity fibrotic tissue, low number of vessels and extreme edema

Rating 3: The tissues with epithelisation and fibroblast in small limit and also low number of vessels and low edema.

Rating 4: The tissues with no edema and the medium number of epithelisation and fibroblast

Rating 5: The tissues with complete epithelisation, complete fibrotic tissue development, high number of vessels and no edema.

All the data were analyzed using one-way ANOVA by SPSS statistical software. The p values < 0.05 were considered significant.

RESULTS

The average wound diameter in control group was 4.42 ± 1.66 mm, in the group which received the alcoholic extract of green tea was 3.81 ± 1.74 mm, and in the group which received aqueous extract of green tea, it was 3.93 ± 1.69 mm. No meaningful difference between 3 groups was observed (not significant). The average of wound diameter among control and treatment group on the days 1, 3, 5, 7, 10, 13, and 15 has been illustrated in Fig 1. There is a meaningful difference between groups ($p < 0.001$).

The microscopic results show that edema, fibroblast and epithelium amount in mice received aqueous or alcoholic extract did not have a meaningful difference. The edema, fibroblast and epithelium amount were significantly different in groups received aqueous or alcoholic extracts when compared with control group ($p < 0.001$). In contrast, the blood vascular amount were not significantly different in groups received aqueous or alcoholic extracts when compared with control group (Table 1).

DISCUSSION

There are 150 reports from *in vitro* and *in vivo* studies in the effects of green tea on skin. The primary focuses of these studies are the chemical carcinogens or photo carcinogens in animals [9]. Generally, The polyphenols which are present in teas are categorized as catechins. Green tea leaves contain six primary catechin compounds: catechin, gallaogatechin, epicatechin, epigallocatechin, epicatechin gallate, as well as

Published online: January 31, 2013

apigallocatechin gallate (also referred to as EGCG). glicoproteins have different biological activities like anti-tumor, anti-edema, anti-virus, anti-ratification, anti-oldness, and lowering the blood sugar [7-10]. Chemical structure of these molecules is the polyphenol of green tea which is the beginner of antioxidant theory [11]. EGCG is the primary combination of green tea polyphenolitic that has properties like antioxidant, anti-tumor, and anti-mutagenic [9]. The biological and epidemiological studies in the past 10 years show that EGCG can be the preventer of tumor growth in chest, lung, liver, sweetbread, stomach, pancreas, skin, cyst, and prostate [11]. EGCG is the preventer of secretion of chymotrypsin, tumor necrosis factor alpha and glucose-6-phosphate dehydrogenase in liver [11-12].

In this study, there is not a meaningful difference between the alcoholic and aqueous extract of green tea in studied groups. This finding is important for two reasons. Firstly, using green tea extract doesn't have any relationship with aqueous or alcoholic treatment. Secondly, in this study, the effect of aqueous and alcoholic variables is excluded. In the current study, on fourth day, as the edema stage indicator is considered as the wound treatment process [13], the excess of edema in treatment group is meaningfully less that of control group ($p < 0.001$). This shows that the green tea makes the edema stage of treatment process faster and therefore the wounds heal faster. In addition, injecting the 2% extract of green tea into mice wound caused meaningful increases in fibrous tissue and reduction in the edema in seventh day of study in comparison to the control group. This meaningful increase of treatment group fibrous in considering their role in following issues are important and indicate the positive effect of green tea on distribution phase of wound treatment process.

1. Fibroblasts are responsible for synthesis of the matrix components of primary outer cell of wound bed including fibronectin and proteoglicans that provide a proper substrate for immigration and propagation of cells [14].

2. The fibroblasts then synthesize the collagens that develop tension power in wound substrate [15].

3. Miofibroblasts that are exclusive fibroblasts participate in wound shrinkage through providing contraction force [14].

During granulation, fibronectin develops a proper substrate for immigration and growth of cells and therefore links with miofibroblasts so that wound contraction is developed influentially. In addition, this fibronectin is a support for fibrilligenesis [16]. Regarding the above-mentioned results, it was indicated that the green tea extract has improved the wound treatment at seventh day that these influences are observed in reduction of wound surface and increase of healing percent and also in reduction of required time for complete healing. Reduction in edema resulted in speeding the wound stage. In 2004, Bayer and colleges show that polyphenols prevent the discharge of gamma-interferon and have anti edema, anti oldness and wound healing [11]. The other researchers showed that polyphenols cause the infusion, contrast and propagation in epidermis Keratinocytes [9]. Catkins are also from polyphenol group that have anti-oxidant and anti-ratification property and have role in prevention of bleeding and reducing thrombosis [9]. From seventh day on, is the propagation stage [17]. On seventh day, in treatment group, the wound surface is reducing in contrast with control group that this shows the reconstruction stage commencement [14] or in other word, the earlier start of revival phase of collagen synthesis take place in this stage and collagen groups with more diameter are constructed and the width link between molecules also change [18]. The collagen yarn causes the wound after healing to look like the tissue before wounding and prevents the white and ugly scar. In addition, increasing blood and oxygen availability to wound location takes place through widening the veins [19]. Researches show that green tea reduces blood sugar, blood lipids, blood pressure, heart disease reduction, heart bit and also vein widening [11,20]. This influences on the practical capacity of fibroblasts, synthesis increase in collagen fibers and increase in wound insistence because of increase in collagen content and because fibroblasts are responsible for developing collagen. So we can conclude that green tea (polyphenol, catechin and EGCG) cause the propagation of fibroblasts and influence the practical capacity of fibroblasts and increase the synthesis of fibro Collagen [20]. The higher the injection dose (300 mL), the higher the meaningful number of fibroblasts [9]. The research of Madham *et el.* show that catechin polyphenol and EGCG prevent the collagenase activity against Collagens [18]. In fact, Catkin and EGCG prevent the action through linking with hydrogen and reaction with hydrophobic with collagens prevent its activity and play a role in collagens registration [18]. Research of Young *et al.* also shows the prevention of collagen destruction and collagenase activity through setting reactions of cellular signal by EGCG [19].

The broad studies during past decades show that the healing process of wound through general and localized different factors is under influence [19]. Many different Neuron and hormonic like cell and vein factors or motion and secretary activities influence the wound location. In this relation, we can point out to study of EGCG and the properties of antibacterial and antivirus of green tea in order to fasten the healing of wound [20]. EGCG causes the propagation, division, and motivation of natural cells growth and does this through cell division and anti apoptosis division. Also, it increases the Keratinocytes survival and influences on the propagation and fixing of fibroblasts [20]. The preventing effect of green tea is related to its anti-oxidant power. Polyphenols and glycoprotein play the role of scavenger in special conditions and thus it implements its preventing effects on bacteria and virus growth. In this regard, preventing effect of green tea (Camellia Sinensis) and black tea on the bacteria growth has been shown [21]. It is possible that green tea

Published online: January 31, 2013

improve the healing speed of wound. It has been reported that antibiotic medicine speeds the healing of wound by infection control [21]. But in this study the exterior symptoms of infections are not observed in control group. Therefore, it seems to be actions other that preventing the wound infection for green tea for fastening the wound improvement. Bayat *et el*. explain the ultrasound treatment effect and gel on healing the wound section and they believe that wet wound is the speeding factor of wound healing process. In current study, the wounds were daily wetted by the alcoholic and aqueous extract.

The experimental studies on animals show that the localized usages of epidermal growth factors have an important influence on speed of epidermal healing in wounds with relative thickness and burnings. The usage of this material on human wounds also has similar effects and its usefulness has been proved [22]. The epidermal healing is a complex phenomena from which the rest epidermal cells are propagated so there will be another healthy epidermis. The molecular actions that set the natural epidermal healing are not completely known, but it seems that the peptide growth factors that act through autocrin or paracrin mechanisms have important role on them [23-25]. In 2003, *Chung et el.* showed that the green tea extract (EGCG) cause the epidemic creationists survival in human. In 2003, Bollag *et el.* proposed cellular propagation and healing of wound through polyphenols of green tea. Many numbers of growth factors are known including the epidermal growth (EGF). This factor is a polypeptide of 53 amino acids that DNA and protein is activated by the mRNA [25]. It has been shown that the peptide growth factors increase significant proliferation of cells in wounds with relative wounds and also increase traction influence on Mesenchyme cells [26]. In fact, the growth factors of exterior peptide will increase other production of growth factors like transforming growth factor which is revealed from plackets and macrophages, indirectly activates the healing and improving the wound [27]. Without considering the structure, immediate facing of cells during healing with growth factors of epidermal, increases the epithelial [28]. Kwon *et al.* stated that EGCG motivates the growth of human hair through proliferation and has Anti-apoptosis effects on DPCs cells [28]. The histology of wound showed that proliferation of cells increase that is probably because of chemical combination of green tea and epidermal growth factors.

In addition, role of vitamins on wound healing process and the relationship of green tea contents with them can be considered. Lack of vitamin C is important in delay of wound healing. In such patients, wound healing in fibroplasis stage is stopped. In this state, even when the number of fibroblasts is natural, they do not produce sufficient collagen. Vitamin C is required for ion link of (OH) with amino acid of proline and lysine and hydroxyl of them inside fibroblast cell. Without hydroxy-lysine, fibrils of collagens will not obtain width links. In extreme Scurvy, not only the new wounds will not heal, but also the previous healed scars will lose their integrity and will open, Because the amount of collagen synthesis will exceed the reconstruction of it [29]. In other hand, vitamin C is required for construction of veins, immigration of macrophages and correct function of nutrofiles [30]. Some studies show that green tea is a rich resource of vitamin C and includes 18 amino acids including lysine and proline [9,12,20]. Lack of vitamin B_6 (pyridoxine) damages this phenomenal link process. Lack of vitamin B_2 (riboflavin) disorders the wound healing process [29]. In other hand, B group vitamins are cofactors for enzyme reactions and are required for correct function of blood cells and construction of antibodies [30]. The results have shown that green tea includes vitamins B_1, B_2 and B_6 [9,12,20]. Therefore probably we can conclude that mentioned issue is one of the factors speeding the healing process in treatment group.

It seems that one of the functions of green tea that helps the healing of wound is the positive effect of polyphenols, Catechin, Glycoproteins, EGCG and vitamins. The increased speed of healing has many effects regarding the economic and hygiene. Higher the speed of wound healing, the less the wound infection and an increased speed in all the process of wound healing. In all of current study for the first time it was shown that green tea extract can speed the wound healing process of male mice NMRI skin.

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Published online: January 31, 2013

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Published online: January 31, 2013