Chitosan green tea polyphenol complex as a released control compound for wound healing

QIN Yao, WANG Hong-wei, Thirupathi Karuppanapandian and Wook Kim*

【Abstract】Objective: In recent years, oxidative stress has been implicated in a variety of degenerative process and diseases, including acute and chronic inflammatory conditions such as wound healing. Green tea polyphenols have shown anti-oxidant property. The present study discussed the application of chitosan green tea polyphenol complex on the wound healing.

Methods: The wound healing effect of chitosan green tea polyphenol complex was studied in ten-week-old healthy male Sherman rats weighing 150-180 g by two wound models. The rats were randomly chosen and divided into four groups (n=5), administered with distilled water in Group A as control group, epigallocatechin-3-gallate (EGCG) in Group B, chitosan-EGCG complex in Group C and chitosan-green tea polyphenols complex in Group D, respectively. In rats‘ incision wound model, two straight paravertebral incisions were made and skin tensile strength was measured using continuous water flow technology on the 10th day. In rats‘ excision wound model, wound contraction and period of epithelization were measured. The polyphenols release from the complex was continuously monitored by an elution technique in aqueous solution at different pH values (pH=4, 5, 6, 7).

Results: The treatment groups showed significantly enhanced the breaking strength in incision wound (328±14.5) g and (421±18.5) g compared with control (264±16.7) g. In the excision wound model, the wound contraction percentage in treatment groups was relatively increased during the recovery period. Respectively, the percentage of wound contraction ranged from 47.60%±2.15% on day 4 to 107.98%±1.26% on day 16 compared with control group (8.46%±5.42% to 59.80%±4.47%). The complex demonstrated a gradual increase in the release rate from the initial stage and slow increase at different pH values. The release rate approximated 0.6-0.7 in the complex and remained stable 6 hours after injury, which may be the end of the release process.

Conclusions: In our study, chitosan polyphenol complex has enhanced the healing of incision wounds by increasing the breaking strength of the wounds. In excision wound model, the complex hastens the period of epithelialization. The study on the optimal release of complex among various pH values could be applied in the wound test, which can lead to a gradually active substance (polyphenols) release and efficient coverage of epithelial layers found in the healing of incision and excision wound.

Key words: Chitosan; Polyphenols; Wound healing

Green tea originates from China and is associated with many cultures in Asia from Japan to the Middle East. It has been subjected to many scientific and medical studies to determine the extent of its long-purported health benefits. Generally, green tea contains polyphenols, which are thought to improve health condition, particularly epigallocatechin-3-gallate (EGCG) can reduce the risk of cardiovascular disease and cancer, and beneficially impact bone density, cognitive function, dental cavities and kidney stones.1 Chitosan is produced commercially by deacetylation of chitin, which is a structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. Chitosan’s property allows it to rapidly clot blood, and has recently gained approval for use in bandages and other hemostatic agents. Chitosan hemostatic products can quickly stop bleeding and reduce blood loss in comparison to gauze dressings in a variety of tests and are used as bandages on the battlefields.2

The natural polyphenols have the ability to precipitate some alkaloids, polysaccharides, gelatin and other
proteins from solution due to their chemical structures, and they have a wide-ranging practical application. It is also shown that plant polyphenols are responsible for the stimulatory effect on the fibroblasts, provoking a research on medical properties. In view of their utilization to induce differentiation and decrease cell proliferation in epidermal keratinocytes, the study of polyphenols’ interactions with some complex agents have special significance. Chitosan, that is linear polymer of hydroglucosamin, is reported to have irreversible reaction with polyphenols to form complex. The complex layer resulted from its structural arrangement constitutes a chitosan matrix suitable for drug controlled release so as to stabilize collagens of human and bacterial collagenases in a concentration dependent manner.

Our study has investigated a chitosan green tea polyphenol complex treatment in incision and excision wound models of Sherman rats. A further study of the polyphenols release from the complex under various pH values can be performed in the drug release system. The awareness of wound contraction and period of recovery in wound models indicated that the complex could be used to enhance the healing of wounds.

METHODS

Chemicals and animals
EGCG, HCl, NaOH and acetone were purchased from Sigma-Aldrich (Mississauga, Canada). The food-grade ethanol (95%) was purchased from Fisher Scientific (Mississauga, Canada). Chitosan was obtained from the department of applied bioscience and food technology, Kyungpook National University (Daegu, Korea), with the N-deacetylation degree of 90% and the average molecular weight of 150 000.

The healthy male Sherman rats of ten-week-old bred in the animal house of Kyungpook National University of Medicine, weighing 150-180 g were selected for the study. The rats were housed individually in cages and bred under controlled temperature (24°C±1°C). The study was undertaken under the approval of institutional animal ethical committee.

Green tea polyphenol extraction
Crude green tea polyphenol extraction was performed according to Slinkard et al’s7 experiment with some modifications. Green tea leaves were mixed with 9°C water, which was 4-fold heavier than the leaves, for the first extraction. Then, the treated leaves (10 g) were soaked with aqueous 95% food-grade ethanol in a Brunswick incubator shaker (Edison, NJ, USA) at room temperature for 2 hours. The mixture was centrifuged at 3000 r/min for 15 minutes and the supernatant was collected. The residue was extracted twice under the same condition. Supernatants from extraction were stored in a freezer at -30°C for analysis. Because polyphenols were extremely sensitive to light, all procedures were conducted under dim light.

Chitosan green tea polyphenol complex
Chitosan green tea polyphenol (CGP) complex was obtained by interaction between the reactants of polyphenols (crude polyphenol and EGCG) and chitosan prepared by the method adopted from Popa et al.7 The dissolution of chitosan involved the stirring of solid chitosan in 1 mol/L HCl solution, followed by the neutralization of 1 mol/L NaOH solution, up to pH 7.5. Afterwards, the solution of 1% aqueous polyphenols was added to chitosan and the system was stirred continuously at room temperature for 6 hours. After the reaction, the precipitate was separated through centrifugation at 4000 r/min for 10 minutes. The unreacted polyphenols were removed by repeated washing with distilled water. Finally, the product was washed with acetone and dried in vacuum at room temperature.

Wound model
Incision wound was made according to Lee and Tong’s study.8 On the depilated back of rats, two 6 cm-long paravertebral incisions were made by cutting through the full-thickness skin. Interrupted sutures, 1 cm apart, were made for the cut edges of skin. The sutures were removed 7 days after injury and skin tensile strength was measured on the 10th day by continuous water flow technique.

Excision wound was made according to the study of Mukherjee et al9 by cutting away a predetermined area of 4 mm×4 mm full-thickness skin on the depilated back of rats. Wound contraction rate was monitored by planimetric measurement of wound area on alternate days without damaging wound area. The wound area was calculated and expressed as the percentage reduction of original wound size. The period of epithelization of the wound was recorded as the days from injury to complete epithelization.
Animal treatment

The rats were randomly chosen and divided into four groups (n=5) in two wound models. Group A received distilled water as control; Group B received EGCG (1 mg/100 g body weight); Group C received chitosan-EGCG complex (1 mg/100 g body weight); and Group D received chitosan-green tea polyphenols complex (1 mg/100 g body weight). All compounds were taken orally. In incision wound model, all compounds were given when 10 days after injury. In excision wound model, compounds were administered until the wound was completely healed for about 20 days.

Polyphenols release from the complex

The researches found that the polyphenols are antioxidants characterized by the presence of several phenol functional groups and can be easily inhibited by oxidation. The investigation of green tea polyphenols' release from the complex would support the development of some drug controlled systems for application.

The continuous monitoring of intragastric acidity indicated pH ranging from 4-7 after human dietary. The release process was carried out by an elution technique in aqueous medium at pH values of 4, 5, 6 and 7 under stirring at room temperature according to Korsmayer et al. The complex were introduced into the eluent and stirred continuously for 6 hours. Then, samples were extracted from the solution and their weights (Wt) were measured at different time points. In the assay, the weight of the complex sample at initial time was defined as 0.1 g for an eluent volume of 100 ml (Wo). The release rate was calculated as Wt/Wo.

Statistical analysis

Statistical analysis was done by one way of variance (ANOVA) followed by LSD test using SPSS (version 15.0 for Windows) package. P< 0.05 was considered as significant difference.

RESULTS

Incision wound model

Wound breaking strength (WBS) is shown in Figure 1. The mean breaking strength in Group A (control) was (264±16.7) g. Group B showed statistically significant improvement in the wound breaking strength (328±14.5) g as compared with control (P<0.01). The breaking strength has significantly increased to (421±18.5) g in Group C, (387±12.4) g in Group D. The result showed that the complex could be used considerably for incised wounds.

Excision wound model

The percentage of wound contraction was (8.46±5.42)% (47.64±2.15)%, (8.76±2.96)% and (96.87±1.84)% on days 4, 8, 12 and 16 after injury in control group. All the treatment groups demonstrated statistically significant epithelization on days 4, 8, 12 and 16 after injury as compared with control group (Table 1). The result showed that both the complex and polyphenols were effective in healing excision wound. The percentage of wound contraction in all the treatment groups was significantly different as compared with control on the 4th day (P<0.01). The percentage of wound contraction on day 4 was (52.48 ±6.4)% in Group B and (64.52±5.86)% in Group C. As the consequence, the treatment groups showed statistical significance from day 8 to day 12 (P<0.01). However, wound contraction was not significantly different (84.04 ±3.42) in Group B on day 12 compared with control group. Afterwards, the wound contraction rate was significantly increased in treatment groups on the 16th day (P<0.01). The mean period of epithelialization in the control group was (19.76±0.35) days. It was significantly (P<0.01) reduced to (18.42±0.51) days in Group B. The mean period of epithelialization in Group C was (16.98±0.29) days which was significantly (P<0.01) reduced to (16.75±0.34) days in Group D (Table 1).

Polyphenol release from complex

The general aspect of polyphenols' release from the complex is presented in Figures 2-3. The release occurred easily in the initial stage and increased with the increase of pH value of the elution medium. A release rate of the chitosan-EGCG complex was about 0.4 after 2 hours at pH 7, which was two-folds of the value at pH 4 (Figure 2). The total release rate at pH 7, 6 hours after injury, was about 0.7, which perhaps represented the optimal release condition. Similarly, the release amount of the chitosan-green tea polyphenols complex at an alkaline medium (Figure 3) indicated the optimal released condition 6 hours after injury (0.6). Apart from the samples eluted at pH 7 which exhibited the highest release rate, all the other samples showed a strong increase of the release rate from the initial stage and slow increase in the period at different pH values. The release remained stable 6 hours after injury, which may be the end of the release process.
### Table 1. Percentage of wound contraction at different time intervals and period of epithelization in excision wound model (mean±SD, n=5)

<table>
<thead>
<tr>
<th>Groups</th>
<th>14th day</th>
<th>18th day</th>
<th>12th day</th>
<th>16th day</th>
<th>Epithelization period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>8.46±5.42</td>
<td>47.60±2.15</td>
<td>78.76±2.96</td>
<td>96.87±1.84</td>
<td>19.76±0.35</td>
</tr>
<tr>
<td>Group B</td>
<td>52.48±3.64*</td>
<td>68.80±2.72*</td>
<td>84.04±3.42</td>
<td>103.46±2.27*</td>
<td>18.42±0.51*</td>
</tr>
<tr>
<td>Group C</td>
<td>64.52±5.86*</td>
<td>74.20±3.24*</td>
<td>2.75±3.49*</td>
<td>107.98±1.26*</td>
<td>16.98±0.29*</td>
</tr>
<tr>
<td>Group D</td>
<td>59.80±4.47*</td>
<td>73.00±3.82*</td>
<td>94.28±6.75*</td>
<td>103.59±1.24*</td>
<td>16.75±0.34*</td>
</tr>
</tbody>
</table>

**F** values: 81.927, 49.704, 28.280, 21.583, 40.356

**P** values: 0.000, 0.000, 0.008, 0.000, 0.000

*P<0.01, compared with Group A.

### DISCUSSION

In recent years, oxidative stress has been implicated in a variety of degenerative process and disease. These include acute and chronic inflammatory condition such as wound healing. Many plant extracts and medicinal herbs have shown potent antioxidant activity. Research on the role of antioxidants from plant extracts in wound healing has been published widely. Green tea polyphenols have shown anti-oxidant property. Protection of cells against destruction by inflammatory mediators may be one of the ways in which extracts from the plant contribute to wound healing. Chitosan is a hemostat, which helps in natural blood clotting and blocks nerve endings to reduce pain. Chitosan provides a non-protein matrix for tissue growth and activates macrophages for tumoricidal activity. It stimulates cell proliferation and histo-architectural tissue organization.

The present study showed the application of chitosan green tea polyphenol complex on the wound healing by two different wound models. Due to the wound healing process, it consists of different phases such as granulation, collagenation maturation and scar maturation. In our study, the complex enhanced the healing of incision wound by increasing the breaking strength (Figure 1). In excision wound model, the complex hastened the period of epithelization. The results exhibited similar but more efficient wound healing effect compared with the previous studies. It seems more effective in chitosan-EGCG group and the treatment also demonstrated a relatively high breaking strength (Table 1). The result may be in accordance with the gradually depolymerization to release N-acetyl-D glucosamine and antibacterial activity by herb extracts.
The amount of released polyphenols and the release rate show several important properties in wound-healing, which makes it available as an agent for wound treatment. Furthermore, the optimal release condition of complex at various pH values could be also applied in the wound test. Since the complex can play an important role in wound healing, it suggests scientific basis for its utility in medical treatment. However, further studies are required to detect the possible mechanism of action, including the activity of proteolytic enzymes and antimicrobial activity.

REFERENCES


(Received September 21, 2009)
Edited by LIU Jun-lan