In Vivo Evaluation of Kumazasa Extract and Chitosan Films Containing the Extract against Deep Skin Ulcer Model in Rats

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In the modern era of the aging society, the number of bedridden elderly patients is increasing gradually, and many of them are troubled by pressure ulcers (decubitus ulcers), which are often serious, resulting in refractory skin ulcers. Various dosage forms have been developed to treat skin ulcers such as pressure ulcers. We also prepared a patch with a tamarind gum-sucrose hydrogel containing silver sulfadiazine, and demonstrated that the formulation displayed a better effect than Geben cream. Generally, a pressure ulcer goes through the stages of an infection and necrosis, an agglutination period, and a proliferation period until cure. To each stage, many formulations, including ointments, creams, films and dressings, have been developed. Ointments containing minocycline, which exhibited controlled release, were reported recently because minocycline is highly effective against pressure ulcer bacteria. Many formulations applied to pressure ulcers possess an antibacterial function.

Kumazasa extract (KE) and its whole solid component (EXT)-containing chitosan films produced by drying in air and lyophilization, abbreviated to ND and FD films, respectively, were examined for efficacy and healing features using a deep skin ulcer model in rats. Their effects were compared with those of clinically available dosage forms, Beschitin W, Geben cream and U-PASTA. KE alone exhibited a better effect as compared with other preparations, and FD films also more effective than control in the early stage. Histological analysis showed that KE alone reduced necrosis rapidly and accelerated granulation. ND films delayed healing rate as compared with control. FD films showed histological features between control and KE alone, but tended to delay healing rate in the later period. Thus, reduction rate of wound area and histological features suggested that KE alone should be excellent for the promotion of wound healing. Although FD films were less effective than KE alone, they were superior as to usability such as changing the preparation.

Key words Kumazasa extract; wound healing; chitosan film; deep skin ulcer; histological analysis

Materials and Methods

Materials Liquid obtained by hydrothermal extraction of Sasa veitchii (Carr.), named Kumazasa extract (KE), which was composed of water (50%, w/w) and residual components, named EXT (50%, w/w), was supplied by Hoshi Pharmaceutical Co., Ltd. (Japan). Chitosan 1000 (deacetylation degree of 80%; viscosity grade 1200 cP at 0.5% at 20 °C) was purchased from Wako Pure Chemicals Industries, Ltd., and used as chitosan (CS). Beschitin W, chitin non-woven fabric, was obtained from Unichika, Ltd. (Japan). Geben cream, sulfadiazine silver cream, was purchased from Mitsubishi Pharma Corporation (Japan). U-PASTA, a combination of sugar and povidone-iodine, was obtained from Kowa Company, Ltd. All other chemicals were of reagent grade.

Animals Male Wistar rats weighing 200—250 g (6—7 weeks old) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Japan), and soon used for animal experiments. They were kept on the breeding diet MF (Oriental Yeast, Japan) with water ad libitum, at room temperature maintained at 23±1 °C, relative humidity of 60±5%, and a 12 h light–dark cycle. The experimental protocol was approved by the Committee on Animal Research of Hoshi University, Tokyo, Japan, and the animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan.

Preparation and in Vitro Characterization of Chitosan Films
Films Containing Kumazasa Extract  CS (1.2 g) was dissolved in 40 ml of 2% (v/v) acetic acid aqueous solution by stirring at room temperature for 2 h. KE was added to CS solution at the EXT/CS ratio of 1/1, 1/3 and 1/7 (w/w), and stirred until the mixture became homogeneous. The bubbles in the mixture were removed under reduced pressure, and the mixture was poured into a Teflon mold (6×6×0.2 cm) until it was filled up. The mold with mixture was left in air at room temperature for 2 d to obtain a naturally dried film (ND film). ND films with EXT/CS of 100, 50 and 25% (w/w) were named ND50, ND25 and ND12.5, respectively. In addition, the mold with mixture was lyophilized to produce a freeze-dried film (FD film), and FD films with EXT/CS of 100, 50 and 25% (w/w) were abbreviated to FD50, FD25 and FD12.5, respectively.

The films were examined in vitro for absorption of aqueous buffer and tensile strength in the buffered solution. Namely, ND or FD films were cut into rectangular films (4.0 cm×0.5 cm). After the rectangular films were weighed, they were put into phosphate-buffered saline, pH 7.4 (PBS) and incubated at 37 °C for 9 d. At appropriate time points, the films were taken out, and weighed. The amount of the absorbed aqueous buffer was calculated by subtraction of the initial weight from the weight of swelling film. As to tensile strength, the rectangular films (4.0 cm×0.5 cm) were prepared and incubated in the same manner as above. At appropriate time points, both edges of a swelling film were longitudinally fixed to the clamps of a rheology apparatus, FUDOH RHEO METER (FUDOH KOGYO Co., Ltd., Japan), and pulled longitudinally. The minimal force required for breakdown of the film was determined as the tensile strength.

Induction of Deep Skin Ulcer and Wound Treatment  A deep skin ulcer model was created using rats as follows. The rats were anesthetized by i.p. injection of pentobarbital at 25 mg/kg (4 ml/kg in saline), fixed on their front, and their back hair was shaved. The full skin at the point 1.5 cm from the midline to the right axillary region was removed in a circle with 1.5 cm diameter. Next, the circular flat face of the top of the brass cylindrical tube (1.5 cm in diameter, 0.02 cm in wall thickness) containing dry ice–acetone mixture (−79 °C) was put on the defective injury covered with a plastic film for 5 min with the tube weight. After the cylindrical tube and plastic film were detached, the wound was covered with four sheets of gauze and surgical adhesive tape. Each injured rat was bred separately with one animal per cage.

Each formulation was applied 24 h after freezing with a dry ice–acetone mixture. KE was applied to the whole wound with four sheets of gauze, and covered further with other four sheets of gauze. The control was treated with four sheets of gauze alone. The films and Beschitin® W, larger than the wound size, were applied and covered with four sheets of gauze. These preparations were fixed with surgical adhesive tape. Each preparation was replaced with a new one every three days, except for Geben® cream and U-PASTA. Geben® cream and U-PASTA were applied so that the whole wound could be covered with approximately 1 mm thickness of the preparation, and replaced with a new one every two days only for the initial 6 d, and then Beschitin® W was applied and replaced with a new one every three days. Whenever each preparation was replaced, the wound surface was washed gently with saline, and then a new preparation was applied in the same manner.

In Vivo Evaluation  Wound healing was examined by measuring the reduction of the wound surface area. The ratio of the wound surface area to the initial area, named the area ratio, was calculated by the following equation.

\[ \text{area ratio (\%)} = 100 \times \frac{[(\text{wound length} \times \text{wound width}) \text{ (observed time point)}] - [(\text{wound length} \times \text{wound width}) \text{ (immediately before initial application)}]}{[(\text{wound length} \times \text{wound width}) \text{ (immediately before initial application)}]} \]

In addition, wound healing was evaluated histologically for some formulations. Gauze alone, KE alone and FD film were applied to the wound in the same manner as above. At appropriate time points after the treatment started, the animals were euthanized by ether inhalation, and the tissue, including the wound and its surrounding skin and muscle, were excised. After the excised tissue was fixed with formalin, it was cut at the mid-portion of the wound, and each part was embedded in paraffin. Both cross-sections were thinly sliced at 4 μm, and stained with hematoxylin–eosin. Thus, two stained slices were obtained per sample. The histological image of the stained slice was analyzed by a histological expert as follows. The image was digitized by the film scanner Prime Film 1800i (Pacific Image Electronics, Inc., U.S.A.), and the necrosis region and granulation one were traced, and these areas were measured using an NIH Image (1.62) (U.S.A.). The mean of the areas for two slices was used as the area for each sample. Furthermore, the length of epithelium regenerated from the wound edge was measured with a micrometer. The mean of the lengths for two slices was used as the reepithelialization length for each sample.

Statistical Analysis  Statistical analysis was performed with ANOVA, followed by the Scheffe post hoc analysis. Significant difference was set as \( p < 0.05 \).

RESULTS AND DISCUSSION

In Vitro Characterization of ND and FD Films  When both types of films were taken out of the mold, no materials remained outside the films. Therefore, the whole EXT was included. The size and weight of the obtained films are shown in Table 1. The EXT content in KE alone was calculated using the density of 1.2 (w/v). ND films were thin, but FD films had a thickness of 1.8—2.0 mm. All the film weights were approximately 1.5 times greater than expected from the mold size, probably because more KE/CS mixture was poured into the mold when the mold was filled. FD films were a little heavier than ND films, which was presumed to be because FD films, much more porous, might have more moisture than ND films. As shown in Fig. 1A, FD films absorbed PBS much better than ND films. EXT appeared to prevent the swelling of CS films. ND films displayed greater strength than FD films under both dried and swollen conditions (Fig. 1B). Film strength was almost in inverse relation to the absorption of PBS, suggesting that film density would be greatly related to film strength. However, the swollen films did not break down during the incubation, indicating that all films could cover the wound well.

Deep Skin Ulcer and Change in Wound Surface Area  Although various techniques for the creation of pressure ulcer animal models have been reported, induction of the models is not necessarily easy; therefore, in this study,
ND50 could not absorb the exudate sufficiently, while FD50 applied, the wound seemed to be too wet to promote recovery. Similar results to that of the control. When KE0.6 was applied, the wound state in the early period. KE0.3 and FD50 also reduced the body weight, probably because ND50 worsened the wound state in the early period. KE0.3 and FD50 showed the greatest increase in body weight, which was significantly larger than that of the control (p<0.05). Geben® cream and U-P ASTA showed a moderate effect on the increase in body weight, and Beschitin® W exhibited only a slight increase in the body weight. The increase in body weight almost correlated with the healing effect in the early period. It was considered that EXT might improve the whole body condition due to its antibacterial activity, immune-enhancing effect, etc.20,23)

Histological Evaluation For the control, KE0.3 and FD50, histological analyses were conducted 6 and 12 d after the treatment started. Both cross-sections of the midportion of the wound sample were stained with hematoxylin–eosin, and two stained slices per sample were obtained as shown in Fig. 4A. The necrosis and granulation areas were identified by a histological expert, and are shown as red and blue zones in Fig. 4B, respectively. The re-epithelialization was evaluated from the length of the epithelium regenerated from the deep skin ulcer was created by removing the full skin and subsequent freezing of the defective injury.81 The resultant ulcer, reaching the muscle, was handled as a deep skin ulcer. In terms of circulatory deficit, the present model was similar to a pressure ulcer, although the wound was not formed by the usual causes such as pressure and friction of the skin.

KE alone was used at a dose of 0.15, 0.3 and 0.6 ml per rat, which was an adequate volume to apply to the wound. These are named KE0.15, KE0.3 and KE0.6, respectively. Shibata et al. applied a high molecular weight fraction of KE to the incision wound or defective injury in skin of guinea pigs at several mg per wound, suggesting that the present doses would be adequate.24) When the moisture was disregarded, the EXT content in ND and FD films was calculated as KE density of 1.2, and as a half of KE weight.

The change in body weight is shown in Fig. 3. In the control, the body weight slightly decreased in the initial period, which was due to the damage by the serious wound. ND50 also reduced the body weight, probably because ND50 worsened the wound state in the early period. KE0.3 and FD50 showed the greatest increase in body weight, which was significantly larger than that of the control (p<0.05). Geben® cream and U-P ASTA showed a moderate effect on the increase in body weight, and Beschitin® W exhibited only a slight increase in the body weight. The increase in body weight almost correlated with the healing effect in the early period. It was considered that EXT might improve the whole body condition due to its antibacterial activity, immune-enhancing effect, etc.  

Table 1. Characteristics of EXT-Containing Preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>KE volume or EXT/CS ratio</th>
<th>Sheet size</th>
<th>Film weight</th>
<th>EXT content*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Length (cm)</td>
<td>Width (cm)</td>
<td>Thickness (mm)</td>
</tr>
<tr>
<td>KE0.15</td>
<td>0.15 ml</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KE0.3</td>
<td>0.3 ml</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KE0.6</td>
<td>0.6 ml</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ND12.5</td>
<td>12.5/87.5</td>
<td>5.62±0.40</td>
<td>5.48±0.37</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>ND25</td>
<td>25/75</td>
<td>5.42±0.40</td>
<td>5.40±0.16</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>ND50</td>
<td>50/50</td>
<td>5.64±0.15</td>
<td>5.56±0.15</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>FD12.5</td>
<td>12.5/87.5</td>
<td>5.67±0.19</td>
<td>5.67±0.22</td>
<td>1.91±0.16</td>
</tr>
<tr>
<td>FD25</td>
<td>25/75</td>
<td>5.67±0.19</td>
<td>5.67±0.22</td>
<td>1.95±0.27</td>
</tr>
<tr>
<td>FD50</td>
<td>50/50</td>
<td>5.67±0.19</td>
<td>5.67±0.22</td>
<td>1.88±0.15</td>
</tr>
</tbody>
</table>

The results are expressed as the mean±S.D. (n=5). a EXT content was calculated as KE density of 1.2, and as a half of KE weight.

The results are expressed as the mean±S.D. (n=3). 1) p<0.05 vs. FD12.5, 2) p<0.05 vs. FD12.5 and FD25. 3) p<0.05 vs. ND50, 4) p<0.05 vs. ND50, 5) p<0.05 vs. ND12.5, ND25 and ND50.

Fig. 1. Absorption of PBS (A) and Tensile Strength (B) of ND and FD Films before and during Incubation in PBS at 37 °C

The results are expressed as the mean±S.D. (n=3). #1 p<0.05 vs. FD12.5, #2 p<0.05 vs. ND50, ¥1 p<0.05 vs. ND12.5 and ND25. ¥2 p<0.05 vs. ND12.5, ND25 and ND50.
wound edge. The results in their morphometry are shown in Fig. 5. Necrosis was eliminated faster in KE alone and FD50, and it was observed to a fair extent even on 12 d in the control. Granulation appeared to be accelerated with KE0.3 and FD50, but was slower in the control. Regeneration of the epidermis tended to be better with KE0.3. Although there was no significant difference among the preparations, KE0.3 and FD50 tended to promote the elimination of necrosis and granulation process in the early stage, which was considered to be based on EXT biological effects such as antibacterial action, immune-enhancing function, antiulcer property and cell repair function.20,23) FD50 appeared to exhibit an intermediate effect between KE0.3 and the control.

In summary, it was demonstrated that Kumazasa extract (KE) alone tended to improve the healing of deep skin ulcers. As to combination between EXT and CS films, as far as the wound area reduction was concerned, FD films seemed to be better than ND films, especially in the early stage. However, FD films tended to slow the wound area reduction in the later period. This point was examined by changing the period to apply the formulation to the wound with respect to KE0.3 and FD50. That is, in the early application, each formulation, changed every three days, was applied for the first 9 d, and then Beschitin® W, changed every three days, was applied. In the later application, Beschitin® W was applied for the early 9 d while changed every three days, and then each formulation was applied while changed every three days. As a result, KE0.3 exhibited better effect with the early application, but FD50, applied earlier, showed the tendency to slower the healing in the later stage (Fig. 6). KE0.3 appeared to act well in the early application. On the other hand, FD50 might cause hyperplasia of the granulation (Fig. 5B), or CS in FD might influence the wound negatively in the early stage, though the reason could not be explained well. The therapeutic features of FD50 will have to be further studied to make clear its effectiveness. Although these ND and FD films are inferior to KE alone in effectiveness, they were superior in the usability due to changing the formulation easily or suppressing the pain in formulation change. Furthermore, FD films were effective in the early stage and showed a better whole body conditions (Figs. 2, 3). Therefore, further examination of the formulation and application manner such as application periods may be needed to get a better EXT/CS film formulation.

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The results are expressed as the mean±S.E. (n=3).

The results are expressed as the mean±S.E. (n=4).
REFERENCES


