Effect of Concentration Differences of Snail Mucus Gel (Achatina Fulica) on Collagen Density and Wound Closure Rate in Wistar Rat Skin Punch Biopsy Wounds

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Abstract
Collagen production and deposition is crucial to replace damaged tissue. Glycosaminoglycans in snail mucus has high biological value for wound healing. This study aims to determine the effect of concentration differences of snail mucus gel on collagen density and wound closure rate in skin punch biopsy wounds. Quasi experimental research was done using 6 Wistar rats, each wounded with 4 punch biopsy wound in dorsa skin, and subsequently applied with 24%, 48%, 96% snail mucus gel and 3% CMC-Na gel. Collagen density was measured on 2nd and 7th day using histophotometry analysis. Wound closure rate was measured from wound area on the 4th and 7th day. Data analysis using 2-way Anova showed significant differences in collagen density (p=0.000) and wound closure rate (p=0.000) of all treatment groups and observation period, but there is no interaction between them. Post hoc LSD showed significant difference in wound closure rate between control groups and all treatment groups (p=0.000), but there is no significant difference between treatment groups (p>0.05). Significant differences of collagen density was found between control groups and all treatment groups (p>0.05). Significant difference of collagen density between treatment groups only found on snail mucus gel 96% (p<0.05). In conclusion, topical application of snail mucus gel can increase collagen density and wound closure rate in Wistar rat skin punch biopsy wound healing. Wound closure rate was not influenced by concentration differences of snail mucus gel, although the collagen density proved to be much higher in the 96% snail mucus gel group.

Keywords: Gel, snail mucus, collagen density, wound closure rate.

Received date: 08 December 2020
Accept date: 21 January 2021

Introduction
Management of soft tissue injuries with satisfactory results functionally and aesthetically is a challenge for Oral and Maxillofacial Surgeon¹,². Various attempts were made to be able to get optimal wound healing, including the application of dressing material, organic material, medicines, to environmental manipulations such as temperature and oxygenation³.

Snail mucus has been extensively researched to help accelerate wound healing⁴, containing at least four essential natural elements that are good for the skin, namely alantoin, antimicrobial peptides, enzymes, and glycoproteins⁵,⁶,⁷. Utilization of snail mucus requires further research to scientifically prove its efficacy claim before it can be used widely⁸.

Snail mucus has been heavily integrated with various drug carrier such as alginat-carboxymethyl cellulose (alginate-CMC)⁹, carboxymethyl cellulose sodium salt (CMC-Na)¹⁰, plasticizer polyethylene glycol 400 (PEG 400)¹¹, and cream¹². Existing research suggests snail mucus can work more effectively when integrated with drug carrier and there is a relationship between snail mucus concentration and its wound healing effect⁹,¹⁰,¹¹,¹². The minimum concentration of an animal product with optimal working effect needs to be revealed to maintain its ecological aspect¹³.
Wound healing process rely on correct collagen metabolic process starting from the regulation of collagen production, deposition, and maturation. Evaluation of collagen formation becomes an important parameter to evaluate wound healing. Collagen analysis can be performed biochemically, histologically, and clinically. Histological staining is able to provide clearer information regarding the organizational structure of the formed collagen fibers. Along with the deposition of collagen replacing damaged skin structure, the wound will get smaller and smaller until it disappears clinically. Wound closure rate can be observed macroscopically and quantitatively by measuring the wound area on each observation day. Wound closure rate can be a clinical indicator to assess product potency in accelerating wound healing.

This study discusses the effect of concentration differences of snail mucus gel application on collagen density and wound closure rate in skin punch biopsy wound healing. Interaction between concentration and length of observation days will also be observed.

### Materials and methods

This study had obtained ethical clearance from Ethics Commission of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta (No. 00273/KKEP/FKG-UGM/EC/2019). Six Wistar rats that met the inclusion criteria were adapted and four punch biopsy wounds with diameter of 5mm were created on the dorsal region of each rat. Wound sample on the dorsal region were divided into 4 groups, namely 3% CMC-Na gel, 24% snail mucus gel, 48% snail mucus gel, and 96% snail mucus gel. The amount of 0.1 ml of gel was applied to the wound surface every 24 hours. Six rats were divided into 2 groups based on decapitation day, namely 3 rats on the 2nd day and 3 rats in the 7th day. Collagen density was measured using slide from day 2 and 7. The rat decapitated on day 7 were observed clinically on day 4 and 7 to measure the wound closure rate.

Collagen density was observed using Mallory stained slide. Observation was done using light microscope with 40x magnification first to identify wound area. Five fields of view were selected with a magnification of 400x from each slide, provided that all five fields of view were located in the reticular layer of the dermis in the wound area. Photographs were taken from all five fields of view. The image is then processed with imageJ v1.52a application to isolate the blue area using color deconvolution feature. The blue spectrum image is then processed using threshold feature to mark the collagen fiber. The process produce a binary image that can be used to quantify percentage of observed area. Collagen density is expressed as the percentage of the blue area in the observation field that was processed using imageJ application. Collagen fiber density data is a mean of 5 different fields of view.

Wound closure rate was observed clinically on day 4 and 7 after punch biopsy procedure. Measuring tool using millimeter block paper with a hole is placed over the wound, then the wound surface is photographed with a digital camera. The image is then processed using imageJ application. Draw a straight line connecting two points in the image whis is known to be 5mm long. Set the measurement scale (set scale) by comparing the size of the pixel length of the straight line with the actual known length of 5mm. The wound edge is traced using freehand tool and wound area is measured in cubic millimeters (mm²). Wound closure rate is expressed as percentage of wound area compared with that on postoperative day according to the following formula:

\[
\text{Wound closure rate} = \left( \frac{\text{wound area at each visit}}{\text{initial wound area}} \right) \times 100\%
\]

### Results

All rats were in good health and physically active during the study. No rats died and there was no sign of wound infection. Rats body weight were stable between 250-300 grams and met the inclusion criteria.
5mm was observed using a measuring grid (B).

1. **Collagen Density**

Observation initiated with low magnification of 40x to find punch biopsy wound area (Figure 1). The overall wound area was well observed, the wound margins with healthy tissue were clearly visible, the scab was obvious, and the papillary and reticular stratum of the wound was clearly visible (Figure 2 and 3).

The wound on day 2 showed a thick scab (Figure 2A). Collagen fibers in the wound area have begun to form with multidirectional fiber orientation and appear sparse (Figure 2C). Collagen fibers formation appears to be more dominant in reticular stratum (Figure 2C) than in the papillary stratum (Figure 2B). The wound on day 7 showed a thinning scab (Figure 3A). More collagen fibers can be observed in the reticular stratum with higher density than day 2 (Figure 3C).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Snail mucus gel (48%)</th>
<th>Snail mucus gel (96%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snail mucus</td>
<td>48%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snail mucus</td>
<td>96%</td>
<td></td>
<td></td>
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</tbody>
</table>

**Figure 4.** Microscopic image of collagen density with Mallory staining (400x magnification) on Wistar rat skin punch biopsy wound healing.

Comparison of collagen density at 400x magnification showed an increase in collagen density from day 2 to 7 in all treatment groups (Figure 4). The image was processed using imageJ application (Figure 5) to obtain collagen density data (Table 1).

**Figure 5.** Image processing using imageJ to obtain collagen density data.

<table>
<thead>
<tr>
<th>Observation day</th>
<th>Control (N=5)</th>
<th>Snail mucus gel 24% (N=5)</th>
<th>Snail mucus gel 48% (N=5)</th>
<th>Snail mucus gel 96% (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>16.47 ± 4.92</td>
<td>27.06 ± 1.76</td>
<td>31.34 ± 2.20</td>
<td>35.71 ± 2.89</td>
</tr>
<tr>
<td>Day 7</td>
<td>38.39 ± 2.42</td>
<td>40.18 ± 2.49</td>
<td>42.88 ± 0.40</td>
<td>48.81 ± 3.29</td>
</tr>
</tbody>
</table>

**Table 1.** Mean and standard deviation (SD) of collagen density (%) on each treatment group.
Collagen Density and Wound Closure Rate

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Table 2. Mean and standard deviation (SD) of wound closure rate (%) on each treatment group based on observation day.

<table>
<thead>
<tr>
<th>Observation day</th>
<th>Control</th>
<th>Snail mucus gel 24%</th>
<th>Snail mucus gel 48%</th>
<th>Snail mucus gel 96%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>14.51 ± 1.47</td>
<td>34.89 ± 3.29</td>
<td>35.06 ± 4.46</td>
<td>41.46 ± 1.26</td>
</tr>
<tr>
<td>Day 7</td>
<td>61.93 ± 5.10</td>
<td>71.21 ± 6.67</td>
<td>72.86 ± 9.97</td>
<td>74.07 ± 6.08</td>
</tr>
</tbody>
</table>

The data were normally distributed (p = 0.200) and homogeneous (p = 0.100). The 2-way ANOVA test for the treatment group showed a significant difference (p = 0.000). The observation day group also showed a significant difference (p = 0.000), but no interaction was found between them (p = 0.169). The post hoc LSD test (Table 2) showed that all treatment groups had a significantly higher collagen density than the control group. The highest collagen density was found on 96% concentration which differed significantly with 24% and 48% concentrations.

2. Wound closure rate

Wounds in all groups were reduced in size from day 4 to day 7, clean, covered with scabs, and none had infection (Figure 6). Wound area was measured using ImageJ and processed to obtain wound closure rate data (Table 2).

![Clinical photographs depict wound closure rate on Wistar rat skin punch biopsy wound healing.](image)

The data were normally distributed (p = 0.357) and homogeneous (p = 0.056). The 2-way ANOVA test for the treatment group showed significant differences (p = 0.000). The observation day group also showed a significant difference (p = 0.000), but there was no interaction between them (p = 0.158). Post hoc LSD test showed that all treatment groups had significantly higher wound closure rates than the control group, but there was no significant difference in wound closure rates between treatment groups (24%, 48%, and 96%).

Discussion

This study aims to determine the effect of concentration differences of snail mucus gel on collagen density and wound closure rate in skin punch biopsy wound healing. Secondary dermal wound healing involves a complex physiopathological process due to significant loss of tissue substance, so the analysis of secondary healing processes in response to various forms of dermal substitution is important to study efficient therapeutic products to stimulate wound healing.

Snail mucus has been extensively researched and proven to be beneficial for wound healing. The use of pharmaceutical materials as a drug delivery system for snail mucus has also been widely studied and proven to increase the effectiveness of snail mucus in helping accelerate wound healing. This study used CMC-Na 3% as a gelling agent for snail mucus. CMC-Na binds the snail mucus into its structure, keeps it stable, does not sediment, and helps the substance adhere to the wound surface. The optimal adhesion of snail mucus gel to the wound surface allows the active ingredients of the snail mucus to be absorbed longer, delivered consistently, and work more effectively in helping the wound healing process.

Measurement of new collagen formation and its quality is important in animal skin wound healing studies. Collagen density in this study was calculated using histophotometric analysis. This method is quite simple, fast, inexpensive, and can provide satisfactory and realistic results.

All groups showed a significant difference in collagen density between day 2 and day 7 (p = 0.000). This result is in accordance with the results of research by Caetano et al regarding normal physiology of Wistar rats skin wound healing, which showed that collagen was observed on day 2 and its deposition continued to increase until a higher collagen density was obtained on day 7.

Significant differences were found between the control group and the snail mucus gel group of 24% (p = 0.022), 48% (p = 0.001), and 96% (p = 0.000).
can act as a mitogen of heparin binding growth factor (HBGF) fluid within 24 hours. Heparan sulfate and heparin are found in acute wound phase of wound healing. Naturally, heparan sulfate plays an important role in the initial activitation of angiogenesis through endothelial cell proliferation and influences role in regulating fibroblast, endothelial and epithelial cell proliferation. Heparin is able to increase the stability of fibroblast growth factor 1 (FGF1) and determine the active complex of fibroblast growth factor 1-fibroblast growth factor receptor (FGF1-FGFR). FGF1 plays an important role in regulating fibroblast, endothelial and epithelial cell proliferation and influences angiogenesis through endothelial cell activation.

Increasing amount of heparan sulfate and heparin plays an important role in the initial phase of wound healing. Naturally, heparan sulfate and heparin are found in acute wound fluid within 24-72 hours after injury, bind to heparin binding growth factor (HB-EGF), which can act as a mitogenic agent for fibroblasts, smooth muscle cells, and epithelial cells. The higher the snail mucus content, the higher the additional heparan sulfate and heparin concentrations that will be absorbed by the tissue. The addition of heparan sulfate and heparin will increase the activation and proliferation of fibroblasts, which leads to an increase in collagen density.

Increased collagen deposition in the wound area is generally related to the faster wound healing process. Wound healing can be observed clinically by closure of the wound area. The faster the wound closes, the faster the wound healing process occurs. This study showed a significant difference in wound closure rates in the snail mucus gel group of 24% (p = 0.000), 48% (p = 0.000), and 96% (p = 0.000) compared to the control, although there was no significant difference between the snail mucus gel 24% with 48% (p = 0.778), 48% with 96% (p = 0.248), and 24% with 96% (p = 0.157).

The mean of wound closure rate of 96% snail mucus gel group was 41.46% ± 1.26 in day 4 and 74.07% ± 6.08 in day 7. This mean was higher than the 24% snail mucus gel group (34.89% ± 3.29 in day 4 and 71.21% ± 6.67 in day 7) and 48% snail mucus gel group (35.06% ± 4.46 in day 4 and 72.86% ± 9.97 in day 7), although thus this difference was not statistically significant. The significantly higher collagen deposition in 96% snail mucus gel group did not necessarily make the wound closure rate faster than the 48% and 24% snail mucus gel groups. The insignificant difference between groups of 24%, 48%, and 96% showed that the three concentrations had the same ability to increase the clinical wound closure rate, although the 96% snail mucus gel concentration had the advantage of stimulating higher collagen production significantly.

Conclusions

Topical application of snail mucus gel can increase collagen density and wound closure rate in Wistar rat skin punch biopsy wound healing. Wound closure rate was not influenced by concentration differences of snail mucus gel, although the collagen density proved to be much higher in the 96% snail mucus gel group.

Acknowledgements
The authors would like to deliver gratitude for all of our colleagues who provided support and expertise that greatly assisted this research. All authors have made significant contribution to this study prior to its submission. No potential conflict of interest relevant to this article was reported.

Declaration of Interest
The authors report no conflict of interest.

References