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RESEARCH ARTICLE

Development of Herb-based Moist Patch for Animal Wound Healing using Arginate Biofilm

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ABSTRACT: Inflammation is the biggest hindrance to wound healing, especially for animals. To solve this problem, we used widely known herb extracts that have significantly outstanding efficacy towards preventing wound healing inflammation: Hibiscus (H), Ginger (G), and Yarrow (Y) extracts. We directly extracted the herbs (H, G, and Y) and tested if these combined extractions prevented inflammation. Additionally, we produced alginate hydrogel mixed with the herb extracts to create wound-healing patches for animals. Next, we found that less than 0.5% of individual treatments of all three herb extracts showed low skin cell cytotoxicity. Next, we discovered that H and G + H + Y extracts decreased LPS-induced inflammation in mouse skin cells. Additionally, we used 2% alginate gel since the balance of tensile strength, flexibility, and stiffness was optimal. Finally, we created an alginate-based wound healing patch containing herbal extracts with a sticky bandage for animals. We concluded that our healing patches were appropriate for the animal because the patches were non-toxic and did not induce allergic reactions. Our novel wound healing patch may reduce animal suffering from wound inflammation. These novel patches will significantly progress toward new strategies for curing animal wound inflammation.

KEYWORDS: Biomedical and Health Sciences, Wound healing; Inflammation; Herb extracts; Alginate; Wound healing patch.

Introduction

Wounds are injuries that break the skin cell or other parts of the body tissues—identified as one of the most common damages for both animals and humans.[1] Wound healing is a complex process supported by myriad cells to repair damaged tissue. Wound healing is restoring the normal anatomic continuity to an injured area of tissue.[2] The level of contamination, blood supply, and the causation of the wound contribute to the development of the health conditions for infection.[3]

Inflammation is the primary stage of wound healing, beginning immediately after the injury when the injured blood vessels leak transudate causing swelling.[4] The main goal of inflammation is to infuse monocytes and lymphocytes, essential to wound healing, from white blood cells to the wound bed to prevent infections and control bleeding.[4] Inflammation is classified into three phases: acute, subacute, and proliferative. Each phase tends to show the body's different clinical signs. For instance, during the acute phase, heat, redness, and swelling are representative signs.[5] Alternatively, after the subacute inflammatory phase, tissue can repair and be strengthened during the remodeling phase.[5]

During the inflammation process, bacterial infection may lead to the progression of a chronic wound.[6] Furthermore, the increased inflammation in the skin and intrinsic causes of chronic wounds may lead to bacteria colonization worsening the chance of healing. Bacterial infection is the major factor that substantially influences the formation of a chronic wound injury.[6]

Since ancient times, Hibiscus has been traditionally used for medical treatment in various ways.[7] Hibiscus offers a long list of physical benefits for many different systems in the body, for instance, maintaining the healthy function of organs and supporting the reproductive system.[7] Above all, Hibiscus tends to protrude in maintaining beneficial cholesterol concentration and healthy blood pressure.[8] The wound-healing activity of Hibiscus was studied using three different models: excision, incision, and dead space wound. The Hibiscus extract has increased cellular proliferation and collagen synthesis at the wound site, regenerating skin tissue's total protein and collagen content.[9] Predominantly, the extract-treated wounds were found to heal significantly faster, as indicated by enhanced rates of wound contraction.[9]

Traditionally, ginger has been used for many different conditions, including treatment for colds, fevers, digestive abnormalities, and nausea.[10] Ginger contains many active substances, such as triterpenoids, flavonoids and saponins, and 6-gingerol.[11] Gingerol and shogaol are phenolic components that have anti-inflammatory effects. A previous study also showed that a combination of curcumin and ginger extract improves wound healing in rat skin.[12] This study found that 6-gingerol is the most abundant in the ginger extract. Also, pretreatment of 6-gingerol increased the matrix metalloproteinase-1 (MMP-1) protein level, which is associated with skin vascular growth into collagen.[13] Therefore, we hypothesized ginger extract might support skin wound healing with strong antibacterial effects.

Yarrow has been used for centuries for its outstanding potential health benefits, identified as a popular medicinal herb.[14] People usually consume this herb by brewing it into tea. Based on recent research, it has been found that yarrow herb extracts present anti-inflammatory and antioxidant properties, both of which aid wound healing.[15] Specifically, yarrow extracts tend to increase the body cell's fibroblast concentration, the cell's main component responsible for regenerating injured tissues.[16] Besides, yarrow tea reduces skin and liver inflammation, giving huge support in treating skin infections.[17]

Since animal wounds are easily exposed to bacteria, which can enter wounds and release chemicals that prevent immune cells from eliminating bacteria.[17] This process delays wound healing.[18] Therefore, we hypothesized that creating a wound healing patch using an alginate biofilm mixed with the natural anti-inflammatory extracts from hibiscus, ginger, and yarrow may inhibit the bacteria-induced inflammatory response.

We successfully created alginate biofilm mixed with hibiscus, ginger, and yarrow extracts in this study. We also found that a 5% volume-to-volume (v/v) mixture of each hibiscus, ginger, and yarrow showed the most effective anti-inflammatory effect. This study may support the animals that suffer from severe inflammation caused by bacterial infection in their wound.

Methods

Herb extraction

Dried powder of ginger, hibiscus, and yarrow was purchased in the nearby supermarket. 10 g of each dried herb was boiled with 200 ml of distilled water for 2 hours. Then the extracted solution was filtered using filter paper (Whatman). The filtrate was stored at 4 °C.

Herb extract treatment in mouse skin cells

B16-F1 mouse skin cells were purchased from Korea Cell Line Bank. B16-F1 mouse skin cells were maintained in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). Trypsin-EDTA was used to detach the cells every three or four days to maintain the cells in healthy condition. The combined quantity of all substances, RPMI1640 and the herb extract, was set to 1000 μL. For instance, at 0.5% hibiscus concentration, 995 μL of RPMI-1640 and 5 μL of hibiscus extract were combined.

Cell confluency quantification using cell image

The cell confluency was quantified using the EVOS M5000 imaging system (Thermofisher). Cell confluence measurement calculated the percentage confluence of cultured cells based on selected reference objects and background. The Confluence tool measures the percent area covered by cells in the image, representing the quality of live cells.

Optimizing concentration of alginate gel

100 mL of distilled water and 0.1 to 5 g of sodium alginate were mixed to generate a 0.1 - 5% sodium alginate solution. The properties of gel solidification, tensile strength, flexibility, transparency, and stiffness were analyzed.

Results and Discussion

We aim to test the cytotoxicity of ginger, hibiscus, and yarrow extracts in B16-F1 mouse skin cells. Since the previous experiment, concentrations over 5% treatment (all three herb extracts) caused the most cell death. Therefore, to determine the most appropriate concentration of herbs for inhibiting skin inflammation, we tested the concentrations within minimal gaps, having a maximum of 5% concentration. The cell confluency (vertical axis) refers to the proportion of the flask covered by adherent cells. Simply put, the higher the cell confluency, the more cells are alive, indicating cell viability (Figure 1). All three herbs tend to have alive cells in the 0.5% concentration, starting from around 75% of cell confluency (Figure 1). Herb extract shows outstanding cell viability with all six concentrations above 50% of cell confluency (Figure 1). On the other hand, hibiscus and yarrow tend to show higher cytotoxicity as the concentration increases, having almost annihilated cells at 5% concentration (Figure 1). We concluded that 0.5% of all three herb extracts show the most optimized concentration for the downstream experiments.

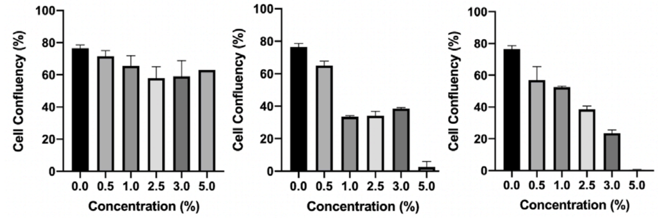

Figure 1: Ginger, hibiscus, and yarrow herbal extracts decreased the cell confluency of B16-F1 mouse skin cells in culture plates. For cells that grow as a monolayer, cell confluency is defined as the percentage of the culture vessel surface area that appears covered by a layer of cells when observed by microscopy. The cell confluency represents cell viability.

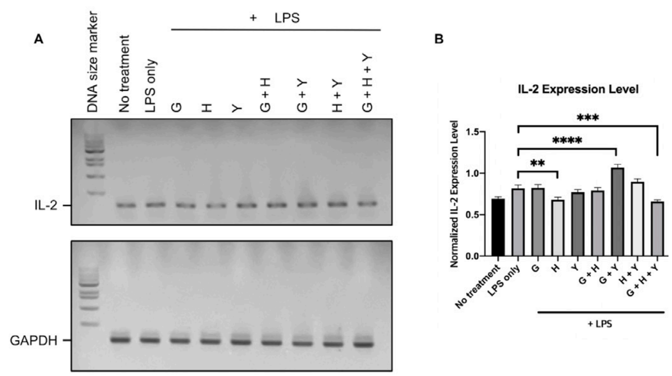

Figure 2: The combination of ginger (G), hibiscus (H), and yarrow (Y) treatment significantly decreased the lipopolysaccharides (LPS)-induced inflammation marker interleukin-2 (*IL-2*) expression level. (A) Agarose gel image representing the amplified *IL-2* and *GAPDH* cDNA products. (B) Ba graph representing the mean and standard deviation of normalized IL-2 expression level. One-way ANOVA with Tukey's post hoc test, ** < 0.01, *** < 0.001, **** < 0.0001.

Next, we tested whether herbal extracts have an anti-inflammatory effect on mouse skin cells. We quantified interleukin-2 (*IL-2*) expression, a bacteria-induced inflammation marker. Hence, increased *IL-2* quantity means an increase in inflammation. We used lipopolysaccharides (LPS), a lipid and a polysaccharide produced from bacteria, to induce inflammation in mouse skin cells. In this study, with the presence of LPS, we treated 0.5% of herbal extracts in the mouse skin cells to find the effect of herbal extracts on inhibiting inflammation. We tested herb extract of ginger (G), hibiscus (H), and yarrow (Y) individual treatment and two or three combination treatments. As shown in Figure 2, configured the experiment with a total of nine treatments: no treatment, LPS only, G only, H only, Y only, G + H, G + Y, H + Y, and G + H + Y. We incubated these nine treatments for

24 hours, then we analyzed the expression level of *IL-2* and GAPDH by RT-PCR. The figure shown on the above shows the expression of *IL-2* in all six samples. We compared the herb-treated samples with the 'LPS only' control sample. The amplified band of *IL-2* and GAPDH cDNA is presented (Figure 2A). Unexpectedly, compared to the 'LPS only' sample, the G + Y sample significantly increased the expression level of *IL-2* (Figure 2B). Inversely, compared to the control sample, the H sample, and G + H + Y sample significantly decreased the expression of *IL-2* (Figure 2B). There were no significant differences from the control sample for the other herb extraction treated samples (Figure 2B). We conclude that H only sample and G + H + Y combination treatment decreased LPS-induced inflammation in mouse skin cells.

Table 1: Physical properties of alginate film with different alginate concentrations.

Alginate (%)	Gel solidification	Tensile strength	Flexibility	Transparency	Stiffness
0.1	No	X	N/A	O	X
0.5	Yes	X	O	O	X
1	Yes	Δ	Δ	Δ	Δ
2	Yes	O	O	O	O
4	Yes	O	Δ	Δ	O
5	Yes	O	Δ	Δ	O

To find out the best concentration of alginate film, we compared six different concentrations of alginate hydrogel. The agarose gel with 0.1 and 0.5 % did not solidify. Although the 0.5 % alginate gel solidified, it significantly lacked tensile strength and stiffness, causing it to rip easily (Table 1). The 1 % alginate gel was more durable, but it still lacked tensile strength and stiffness (Table 1). 4 % and 5 % were both highly durable but lacked transparency and flexibility (Table 1). Thus, we found that the 2 % alginate gel to be the most optimal because it had balanced characteristics between tensile strength, flexibility, and stiffness.

Figure 3: Successfully developed the herb-based moist patch with the alginate film.

Finally, to produce the most appropriate wound healing patch for inflammation, we concluded that alginate biofilm (nontoxic and transparent) is the best fit. After soaking the alginate biofilm into the herbal extraction solution, we successfully created the wound healing patch (Figure 3). To begin with, we used 2% concentrated alginate gel to produce alginate biofilm and dried it for 24 hours to remove all the water solution from the gel. At last, biofilm was successfully created and stored at 4 ^{o}C. Each wound healing patch was then cut into a rectangular shape and soaked in an herbal extracted solution for 1 hour. After all, we produced the biofilm successfully (Figure 3). Since alginate is hydrophilic, the alginate wound healing patch contained the herbal extract solutions for more than 24 hours. The wound healing patch cannot be attached to the animal's skin alone. Therefore, we attached the biofilm to the sticky bandage, making the final patch very flexible and easily attached to the animal's skin (Figure 3). We created an alginate-based wound healing patch containing herbal extracts with a sticky bandage for animals.

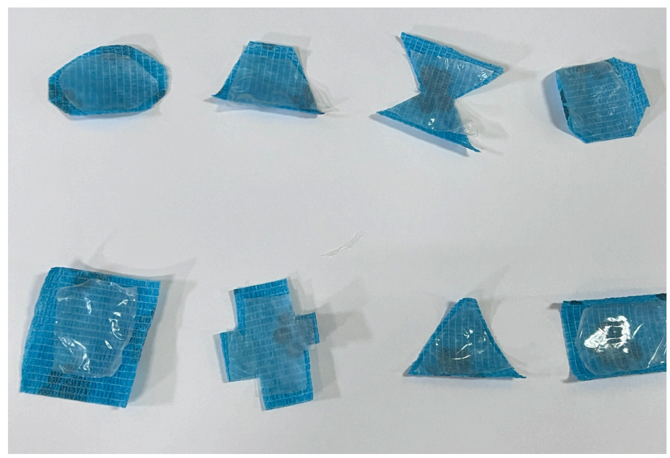

Figure 4: Alginate-based wound healing patches were created of different sizes and shapes, indicating that these patches can be customized to the best-optimized shape based on the animal's wound size and position.

The position of the animal wounds varies depending on the type of injury; hence, the shape of the band is essential to cover the whole wound position. We created Alginate-based wound healing patches of different sizes and shapes. Cutting it into pieces using scissors allowed us to create different shapes easily. When cutting, we had to make sure the shape of the biofilm and bandage had equal in size. We produced circular, triangle, square, rectangle, plus shape, and other shape polygons (Figure 4). This advantage allows our band to shape into any formation depending on the animal's wound position and shape. In case of wound inflammation, depending on the animal's wound position, this patch provides the best-optimized shape based on the wound size and position (Figure 4).

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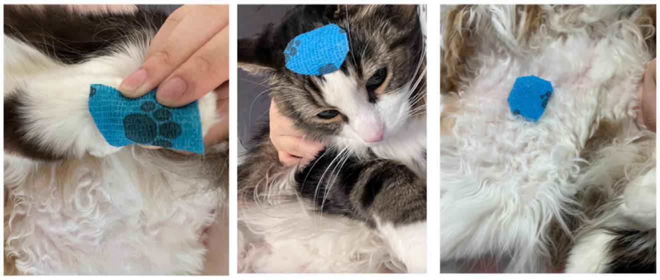

Figure 5: A non-toxic animal wound healing patch is firmly attached to the animal without allergic reactions.

Since we designed healing patches for animals, we had to make sure they were appropriate for animals by attaching the patches to them. We attached the healing patches to our pet's body parts, such as the leg, head, and breast. We also attached these above their fur to ensure the patch's adhesive strength. After securing the healing patches for about a day, we concluded that our healing patches were appropriate for the animals. The patches were non-toxic and did not occur any allergic reactions. Even though they were attached to the animal's fur, we found that the patch was affixed for a long time (Figure 5). Since our patches could be produced in different formations, we were able to stick these patches to various parts of the animal's body (Figure 4). Moreover, the animals did not tend to find these patches encumbered, allowing the patches to be long-lasting.

Conclusion

Based on our first experiment, we found that 0.5% of all three herb extracts show the most optimized concentration for downstream experiments. (Figure 1) Also, we found that the herbal extract of the H-only sample and G + H + Y combination treatment decreased the LPS-induced inflammation in mouse skin cells after several tests. (Figure 2) After figuring out the best alternative concentration and type of wound healing patch for inflammation, we successfully created an alginate-based wound healing patch containing herbal extracts with a sticky bandage for animals. (Figure 3) By this, we could present our band's advantage: the band could be formed in any shape. Depending on the animal's injured position, we customized the patch to be the best-optimized shape based on the wound size and position in case of inflammation. Therefore, we could stick these patches to various parts of the animal's body (Figure 4). Moreover, the animals did not tend to find these patches encumbered, allowing the patches to be long-lasting.

Considering that our experiments used tiny volumes of herbal extracts and LPS, a slight error in the injected concentration of extracts may cause some errors in our results. Likewise, while distributing the experimental cells in equal proportions, small contact with the atmosphere or cell culture dish may affect the viability of mouse skin cells. Additionally, to examine the stabilized results, this experiment required much patience and a long time. For example, to inspect the effect of combined herbal extracts on the LPS-induced cells by the *IL-2* expression level, it required about a day for cells to be completely affected by the extracts.

Alginate film is a highly effective wound-healing patch that offers a range of benefits for patients and healthcare providers. One of the main advantages of using alginate film is its ability to retain moisture in the wound, which helps to promote healing and reduce the risk of infection. Alginate film creates a physical barrier that helps prevent bacterial contamination, making it an ideal option for patients with open or infected wounds. Another benefit of alginate film is its conformability, which is used on many wound types, including irregular or hard-to-reach wounds. In addition, alginate film is biocompatible with human and animal tissue and easy to remove, causing minimal pain or discomfort to the patient. Furthermore, alginate is a natural material derived from seaweed and is biodegradable, making it an environmentally friendly option for wound healing and cost-effective.

Ecofriendly wound healing patches are important because they reduce the environmental impact of traditional wound care products, which often contain plastic and other non-biodegradable materials. We made eco-friendly patches from natural materials, which are biodegradable and compostable. Additionally, we develop these patches by using natural ingredients for wound healing. Using eco-friendly wound healing patches can help reduce waste and pollution and promote more sustainable and natural healing practices.

In conclusion, the final wound healing patches produced were non-toxic and did not occur any allergic reactions toward the animal specimens. We also conclude that our healing patches can be engaged with other strategies to develop various animal medical treatments further.

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