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RESEARCH ARTICLE

Development of Hydrogel-based Wound Healing Patch with 6-Gingerol for Animals

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ABSTRACT: Agarose is a material extracted from algae and has a self-gelling ability and flexible mechanical properties. Since it is odorless and edible, agarose can be applied to wound healing patches for animals. To develop an agarose-based hydrogel patch, the concentration of agarose supplemented with glycerol, a non-toxic plasticizer was optimized. After testing the mechanical properties such as flexibility, transparency, stiffness, and tensile strength, a patch with 3% agarose and 10% glycerol and a 0.186 cm thickness was found to be the optimal condition for the hydrogel patch. Then, the hydrogel patch was applied to a live dog's leg and was found to be durable for at least 48 hours. Finally, 6-gingerol, a candidate for an anti-inflammatory substance to be embedded in the hydrogel, was tested. As a result, 6-gingerol effectively decreased the mRNA expression level of Cxcl5, a pro-inflammatory marker, in B16F1 mouse skin cells, *in vitro*. In conclusion, an agarose hydrogel patch can be used as a wound dressing sheet for animal skin, and 6-gingerol may be added into the hydrogel patch as an effective anti-inflammatory agent for epidermal wounds.
KEYWORDS: Cellular and molecular biology; Wound healing; Hydrogel; Inflammation; 6-gingerol.

Introduction

Agarose, a material extracted from algae sources, is a water-soluble substance that has a self-gelling ability and flexible mechanical properties. Initially a white powder, agarose must undergo induction, gelation, and quasi-equilibrium to form the commonly known agarose gel. Its permeability and function can be modified by using different concentrations and blending other substances into the gel. Due to its promising characteristics, agarose has been commonly used and is expected to further expand its application in the field of biomedical research.[1]

Hydrogels are cross-linked 3D networks with hydrophilic structures, which allow them to hold large amounts of water. They have many desirable properties such as biocompatibility, biodegradability, and porous structure. Furthermore, different features of hydrogels can react to different stimuli, such as temperature, pH, and light, which can be used to mimic various *in vivo* environments. Hydrogels can be created from natural resources, such as polysaccharides, and synthetic materials. However, some hydrogels can be very brittle. In order to overcome some of the existing limitations, numerous studies are being carried out to develop novel hydrogels with stronger and more stable characteristics while preserving many of the advantages of conventional hydrogels.[2]

Herbal medicine, also known as botanical medicine, is folk medicine made from plants and plant extracts. It has been traditionally used for about 60,000 years. After the discovery of aspirin and the development of chemical medicine, reliance on herbal medicine had greatly decreased. However, as bacterial resistance to antibiotics has risen and the need for more eco-friendly medications increases, many scientists have turned to plant-based materials for solutions. Much research is being done on the superiority and feasibility of herbal medicine compared to chemical medicine. Thus, public acceptance of herbal medicine has significantly increased, and many scientists believe that herbal products will continue to play a crucial role in the health care system.[3] 6-gingerol is a pharmacologically active component abundantly contained in ginger. It has anti-inflammatory and antibacterial properties, making it an optimal compound for wound healing.[4] It is also able to regulate multiple targets. Furthermore, gingerol suppresses carcinogens in the skin of animals. Due to its favorable qualities and safety, 6-gingerol is likely to be researched further as a therapeutic agent.

Methods

Optimization of agarose-glycerol hydrogel:

Different concentrations of agarose ranging from 0.1 to 5% were added to a glass bottle. Then it was heated in a microwave for 80 seconds. 18 ten-centimeter circular plastic plates were labeled by volume and concentration of agarose. Solutions were then heated and poured into plastic dishes and allowed to cool until the gels became fully solidified. After the gelation, the plastic plates were placed in a 50 °C drying oven for 24 hours. The same procedure was performed for 0, 2, 4, 6, 8, 10% of glycerol in a 3% agarose solution.

Fabrication of hydrogel patches using cellulose fibers:

After the hydrogel patches were synthesized, they were placed on the cellulose fiber. The cellulose fibers and hydrogel patches were microwaved for 1 minute to slightly smear the hydrogel patches onto the cellulose fibers. The hydrogel patches were allowed to cool down to room temperature for 10 min.

B16F1 cell culture and LPS treatment:

5 mg of lipopolysaccharide (LPS) was measured using an electronic densimeter and weight boat. 5 mL of Dulbecco's modified phosphate buffer saline (DPBS) was used to dissolve the 5 mg of LPS. Then, the solution was vortexed for 5 minutes to completely dissolve LPS. 500 microliters of trypsin ethylenediaminetetraacetic acid (EDTA) were used to detach B16F1 cells from the culture plate. After centrifugation, cells

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were resuspended in RPMI-1640 medium. Cells were then treated with different concentrations of LPS and culture for 24 hours in a 37 °C incubator with 5% CO_2.

R condition for Cxcl5:

Polymerase Chain Reaction (PCR) was performed using the Premix PCR kit (Bioneer). 20 μL PCR was prepared to amplify the target cDNA (Cxcl5 and beta-actin). The following PCR condition was used for the amplification of Cxcl15 and beta-actin. Step 1: 95 °C for 3min, step 2: 95 °C for 30 sec, 62 °C for 30 sec, 72 °C for 20 sec, step 2 was repeated for 34 cycles, step 3: 72 °C for 5 min, and step 4: infinite 12 °C.

Results and Discussion

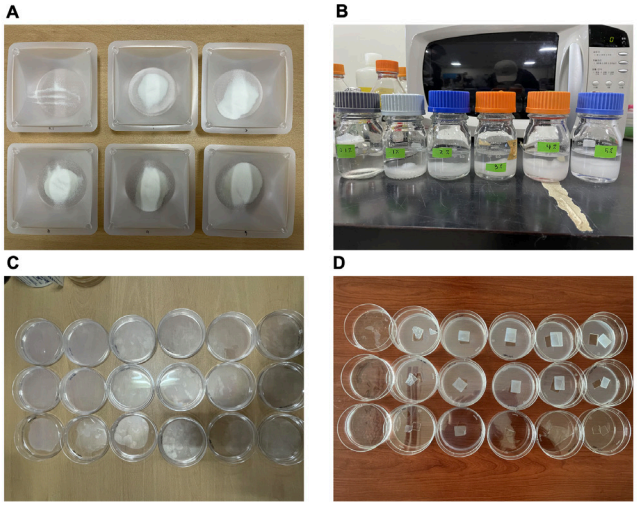

Figure 1: Four steps to prepare agarose hydrogel: A) The weight of the agarose powder was measured by a densitometer. B) Deionized water was added to the agarose powder and microwaved to dissolve completely. C) The dissolved agarose solution was poured into the petri dish. D) The center of the agarose gel was removed to measure tensile strength and plasticity.

The purpose of the first experiment was to find the optimal concentration and thickness of agarose hydrogel for the wound healing patch (Figure 1). 18 samples of agarose hydrogel were prepared to find the optimal conditions for the hydrogel patch. High concentrations and thick agarose hydrogel may increase tensile strength and stiffness. Low concentrations and thin agarose hydrogel may increase the flexibility of the hydrogel.

Table 1: Physical properties of agarose gel with different agarose concentrations.

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

To find out the best concentration of agarose gel, six different concentrations of agarose hydrogel were compared. The agarose gel with 0.1% did not solidify. Although the 1% agarose gel solidified, it significantly lacked tensile strength and stiffness, causing it to rip easily. The 2% agarose gel was more durable, but it still lacked tensile strength and stiffness. 4% and 5% were both highly durable but lacked transparency and flexibility. Thus, the 3% agarose gel was found most optimal because it had balanced characteristics between tensile strength, flexibility, and stiffness. (Table 1)

Table 2: Physical properties of agarose gel with different thicknesses.

Thickness of agarose hydrogel	Gel solidification	Tensile strength	Flexibility	Transparency	Stiffness
0.063 cm	O	Δ	O	O	Δ
0.126 cm	O	O	O	O	O
0.189 cm	O	O	Δ	O	O

To find out the best thickness of the hydrogel, 3 different thicknesses were compared. The 0.063 cm agarose gel lacked tensile strength and stiffness, causing it to rip easily. The 0.189 cm agarose gel lacked flexibility making it hard to apply to the wound area. Thus, the 0.126 cm agarose gel was found optimal due to its well-rounded characteristics (Table 2).

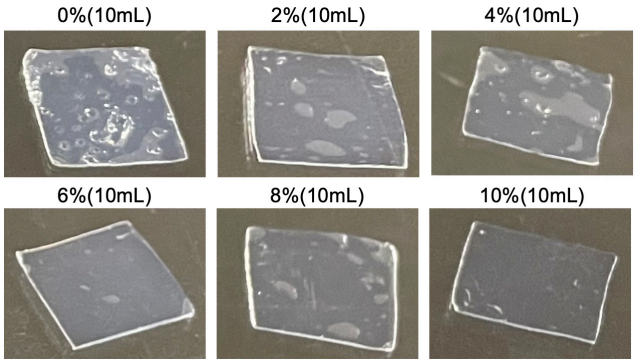

Figure 2: Images of 3% agarose hydrogels with the addition of different concentrations of glycerol.

Table 3: Physical properties of agarose gel with different concentrations of glycerol additive.

Glycerol concentration	Bubble	Flexibility	Surface smoothness	Tensile strength	Adhesiveness
0%	O	X	X	X	X
2%	O	X	X	X	X
4%	O	Δ	X	X	X
6%	Δ	Δ	Δ	X	Δ
8%	Δ	O	Δ	Δ	O
10%	X	O	O	O	O

To find the optimal concentration of glycerol, six different concentrations were tested (Figure 2). Concentrations below 10% formed bubbles and lacked the required physical characteristics. 10% glycerol had no bubbles with the high

est flexibility, surface smoothness, tensile strength, and adhesiveness, making it the optimal concentration (Table 3).

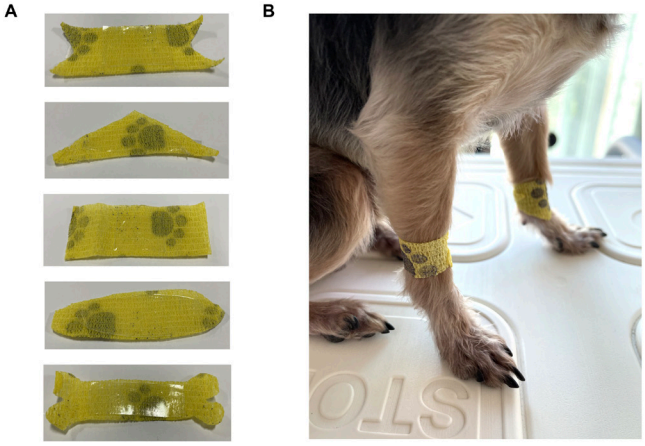

Figure 3: Application of hydrogel using cellulose patches. A) five different conceptual designs of hydrogel patches for the different application areas. B) Application of hydrogel patch on a dog's leg.

After the hydrogel was placed on the cellulose fiber, it was heated by microwave for 1 minute to slightly melt the hydrogel patches on the cellulose fibers. After 10 minutes of cooling at room temperature the hydrogel and cellulose patches were fused together (Figure 3A). These patches were applied to a live dog's leg to test its durability. It was found that the patches remained adhered to for at least 48 hours. (Figure 3B).

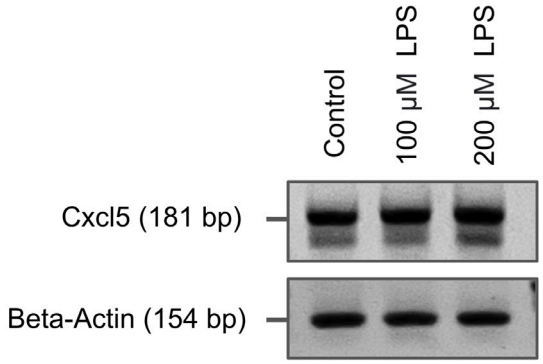

Figure 4: The mRNA expression level of Cxcl5 under different concentrations of LPS treatment.

To mimic an acute inflammation condition, different concentrations of LPS (100 and 200 µM) were treated on B16F1 cells for 24 hours. To confirm the induced inflammatory response, the expression level of C-X-C chemokine (Cxcl5), a neutrophil-activating inflammatory peptide, was measured using PCR. This gene encodes a protein that is a member of the CXC subfamily of chemokines. Cxcl5 chemokines recruit and activate leukocytes. Figure 4 indicates that 200 µM of LPS significantly increased the expression of Cxcl5 in B16F1. Beta-actin was used as the control housekeeping gene.

To test the anti-inflammation effect of 6-gingerol, Cxcl5 expression levels were compared for LPS-induced inflammatory cells. (Figure 5) As expected 20 µM of 6-gingerol decreased the Cxcl5 expression level compared to LPS-induced cells without 6-gingerol treatment. Previous

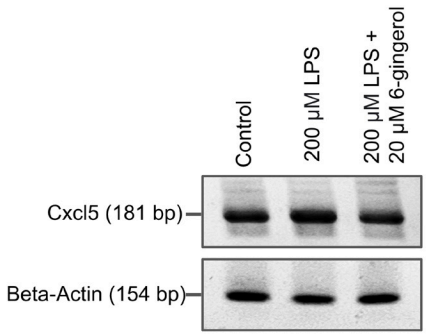

Figure 5: The mRNA expression level of Cxcl5 under LPS and 6-gingerol treatment.

6-gingerol decreased the Cxcl5 expression level compared to LPS-induced cells without 6-gingerol treatment. Previous research indicates that induction of Cxcl5 can be mediated by UVB irradiation in skin. It has been also demonstrated that Cxcl5 causes mechanical pain-related hypersensitivity and induces infiltration of neutrophils and macrophages.[5] Therefore, Cxcl5 may induce the inflammation on skin by UVB irradiated skin cells. Thus 6-gingerol treatment may alleviate inflammation reaction and may reduce pain after skin injury.

Conclusion

An agarose-based hydrogel patch for animal skin wounds was developed in this study. The optimal composition of hydrogel was found to be 3 % agarose with 10 % glycerol. It was also found that 6-gingerol, a natural anti-inflammatory substance, can inhibit Cxcl5-dependent inflammatory pathways. Therefore, the addition of 6-gingerol to agarose hydrogel may reduce inflammation and reduce damage to animal skin tissues. However, more skin cell lines should be tested to verify the anti-inflammatory effect of 6-gingerol. Also, *in vivo* mouse experiments should be performed to check the wound healing effect of this agarose healing patch.

Acknowledgements

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