

ACCELERATED WOUND HEALING ABILITY OF SACRAN HYDROGEL FILM BY KERATINOCYTE GROWTH FACTOR IN ALLOXAN-INDUCED DIABETIC MICE

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ABSTRACT

Objective: The main objective of the research work was to fabricate sacran hydrogel film containing keratinocyte growth factor (Sacran/KGF-HGF), and to evaluate their wound healing ability in alloxan-induced diabetic mice model.

Methods: The physicochemical characterization of Sacran/KGF-HGF were investigated by thickness, tensile strength, swelling ratio, x-ray diffractometer (XRD), scanning electron microscope (SEM), and biodegradability. The wound healing ability was investigated by creating two full-thickness excisional wounds in alloxan-induced diabetic mice.

Results: The thickness, tensile strength, and swelling ratio results showed that KGF in the Sacran/KGF-HGF improved not only the thickness of sacran hydrogel film (Sacran-HGF), but also the tensile strength and swelling ability of Sacran-HGF. The XRD and SEM results confirmed that the Sacran/KGF-HGF were amorphous and similar morphology to Sacran-HGF, respectively. The biodegradability results revealed that the Sacran/KGF-HGF degraded for about 41.29% in trichloroacetic acid (TCA) and 22.92% in TrypLE™ (recombinant enzyme) solutions. In addition, KGF improved the degradability of Sacran/KGF-HGF in both solutions. Interestingly, the Sacran/KGF-HGF, which was applied on wound site, considerably improved the wound healing ability of Sacran-HGF at 6, 9 and 12 d in alloxan-induced diabetic mice model, compared to control (non-treated).

Conclusion: These results suggest that KGF has the potential to promote the chronic wound healing ability of Sacran-HGF.

Keywords: Sacran, Hydrogel film, Keratinocyte growth factor, Wound healing

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INTRODUCTION

The skin, a layered organ, covers the entire human body with the main function as a protector for preventing skin dehydration and microorganism's penetration from outside the body. If the skin is injured, it provides complex natural biological reactions and complicated wound healing processes [1]. The wound healing process basically consists of several stages, namely hemostasis, inflammation, proliferation, vascularization, production and restoration of the extracellular matrix (ECM)[2]. However, the healing process in chronic wounds, especially in diabetic condition, will be disrupted with persistent inflammation, slow migration of keratinocytes and fibroblast, abnormal regulation of chemokines and production of growth factor, the irregular response of inflammatory cells, and inhibition of angiogenesis [3-5]. Recently, a number of studies using new therapy for diabetic wound infections were investigated [6, 7]. Regenerative therapy using growth factors can be used to accelerate the chronic wounds healing [8, 9]. One of the essential growth factors for impaired healing therapy is Keratinocyte Growth Factor (KGF). KGF is a major cellular component of the epidermis, not only important for the maintenance of the barrier, but also for the restoration of the wound through a process known as epithelization [10]. KGF also acts specifically on epithelial cells and stimulates cell proliferation and migration [11].

Nowadays, the concept of wound healing by maintaining a moist environment at the wound site has been established as a standard therapy for chronic wounds [12, 13]. The moist wound environments can accelerate the wound healing process by enhance re-epithelization, stimulate collagen synthesis, increase growth factor activity, and accelerate the healing process [14].

Recently, the modern wound dressings have been developed to preserve and protect a moist environment in the both acute and chronic wounds [15]. The dressings are available in the forms of

films, foam, hydrogel and hydrocolloids, which can be incorporated with a bioactive agent to promote wound healing [16]. Among other forms, the thin hydrogel films (HGFs) consisting of water-swollen polymer networks have attracted a lot of attention for the last few decades due to their excellent properties such as adhesive, porous, semi-permeable, flexible and easily conformable, for the treatment of acute and chronic wounds [17].

During last two decades, natural polymers are widely used in the regenerative medicine field, outstandingly for wounds dressing because of their biocompatibility, biodegradability and similarity to the extracellular matrix [18]. Natural polymers are involved in the repair of damaged tissues and consequently in skin regeneration, inducing and stimulating the wound healing process [19]. In addition, the swollen ability of natural polymer-based hydrogels has some functions to absorb the overage of wound exudate and accelerate autolysis process for wound debridement [20].

Sacran, a giant molecular polysaccharide, was newly isolated from cyanobacterium *Aphanothece sacrum*. Sacran has the unique properties to be developed as a new biomaterial in HGFs for wound dressing application, such as a very high molecular weight (2.9×10^7 Da), a safe biomaterial and a high moisturizing effect [21]. In the previous study, we successfully developed a physically crosslinked Sacran-HGF and revealed its potential as a novel wound dressing material [22]. Furthermore, we confirmed that Sacran-HGF has the potential to deliver water-soluble complex of curcumin and 2-hydroxypropyl- γ -cyclodextrin at the wound site and promote the acute wound healing ability [23, 24]. Until now, there is no study regarding the combination of KGF with sacran HGF (Sacran/KGF-HGF) for chronic wound therapy. Therefore, we fabricated Sacran/KGF-HGF, then evaluated their physicochemical properties and wound healing ability in diabetic mice model.

MATERIALS AND METHODS

Materials

The sacran was kindly provided by Green Science Material (Kumamoto, Japan). Keratocyte growth factor (KGF) was purchased from Skin Actives Scientific (Arizona, USA). Trichloroacetic acid (TCA) and TrypLE™ (recombinant enzyme) were bought from Wako Chemical (Tokyo, Japan). All the reagents were analytical grade and used without further purification.

Preparation of sacran/KGF-HGF

The preparation method of the Sacran-HGF was briefly described in our previous study using a solvent evaporation method [22]. The Sacran-HGF (1 × 1 cm²) was placed in Tissue-TekCryomold® (Sakura Finetek, Tokyo, Japan). Then, 10 μL of KGFs (100 ng) in ammonium sulfate solution were placed and casted on the surface of the Sacran-HGF. The Sacran-HGF was swollen for 24 h at 4 °C with light protection and dried for 4 h at 37 °C to obtain the Sacran/KGF-HGFs.

Thickness test of sacran/KGF-HGF

A dial thickness gauge (Teclock Corp., Nagano, Japan) was used to measure the thickness of Sacran-HGFs.

Tensile strength of sacran/KGF-HGF

The sacran/KGF-HGFs were clipped with 2 holding grips and pulled by the top clip at a rate of 0.5 mm/s in Textechnofavigraph (Moenchengladbach, Germany). Tensile strength was measured from the breakpoint of the HGFs for 3 times, then, the average value was calculated by tensile strength equation.

$$\text{MPa} = \frac{p}{b \times d}$$

(p = breakpoint, b = width, d = thickness)

Swollen ratio of sacran/KGF-HGF

The sacran/KGF-HGFs (1 × 1 cm²) were immersed in PBS (pH 7.4) for 24 h and weighted (W_t) at room temperature. The gravimetric method was used to determine a swollen ratio of the sacran/KGF-HGFs [25]. It was measured by comparing W_t and initial weight (W_0), and calculated from the following equation [26].

$$q = \frac{(W_t - W_0)}{W_0}$$

Scanning electron microscopy analysis

The sacran/KGF-HGFs were coated with gold for 10 s using the JFC-1200 Fine Coater (JEOL, Tokyo, Japan). The surface of the HGFs was analyzed using scanning electron microscopy (SEM) with a JSM-6510LA (JEOL, Tokyo, Japan) in 5 μL (x3000).

X-ray diffractometric analysis

The x-ray diffraction (XRD) patterns were acquired by a Powder X-ray diffractometer (Rigaku Ultima IV, Tokyo, Japan). Briefly, the Sacran/KGF-HGFs were kept on a sample holder. The XRD settings were as follows: a Ni-filtered Cu-Kα radiation, the voltage of 40 kV, a current of 20 mA, divergent slit of 10 mm (0.5 °), a scanning speed of 5 °/min, opened scattering and receiving slits.

Biodegradability of sacran/KGF-HGF

The sacran/KGF-HGFs (1 × 1 cm²) were weighed (W_0) and added with 2 ml TCA and TrypLE™ (20%) solutions for 24h. Then, HGFs were weighed as the final weight (W_t) and calculated the percent change in weight by the formula, Percent in Weight = $100 \cdot (W_t/W_0 - 1)$.

Alloxan-induced diabetic mice

Male albino mice (strain: BALB/c, age: 6-8 w, body weight: 30-35 g, source: laboratory of pharmacology, teaching hospital, Universitas Padjadjaran) were housed under standard conditions of control temperature and humidity (25 °C ± 1, 40%) and a light/dark cycle

(12/12 h). Before diabetic induction by alloxan, the mice fasted for 8 h. Alloxan monohydrate (186.9 mg/kg body weight) was injected by intraperitoneal injection. 1 h after induction, 5% of glucose solution was applied to the mice for 24 h, then, replaced by distilled water. The blood glucose level of mice was measured using glucometer after 3 d of induction. Alloxan-induced diabetic mice had blood glucose levels at 150-250 mg/dl on the 3rd day after alloxan induction.

Wound closure study of sacran/KGF-HGF

The *in vivo* wound closure studies were performed as reported previous [22]. Briefly, two full-thickness excisional wounds were made on the dorsal side of hairless mice using 8-mm biopsy punch tools. Three mouse groups were prepared as Sacran-HGF, Sacran/KGF-HGF and control (without treatment). The HGFs were applied precisely on the wound site, after 6, 9 and 12 d, the wound areas were digitally photographed and quantified by Image J software. The wound sizes were expressed as a percentage of the respective initial wounds. All the animal research studies were approved by the Ethics Committee for Animal Care and Use of Kumamoto University (Ethical approval number: 25-203).

Statistical analysis

The quantitative data were expressed as the mean ± standard error of the mean (SEM) while the statistical comparisons were made using the Scheffe's test. A *p*-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Preparation of sacran/KGF-HGF

In the previous study, we successfully prepared a physically crosslinked Sacran-HGF using the solvent-evaporation method and considerably improved acute wound healing ability [22]. In this study, we next examined whether Sacran-HGFs can be formed in the presence of KGF for chronic wound therapy, and evaluated their physicochemical properties. Sacran/KGF-HGF was fabricated with the thickness of the film was higher than that of sacran-HGF (fig. 1a-b). These results suggest that the KGF was effectively incorporated in the sacran-HGF.

Physicochemical properties of sacran/KGF-HGF

The HGFs need an excellent mechanical property for wound dressing application [27]. Therefore, we examined the tensile strength of sacran/KGF-HGF compared to sacran-HGF without KGF. The tensile strength of sacran/KGF-HGFs was determined from the breakpoint of the HGFs in the Textechnofavigraph apparatus. Fig. 1c showed that the addition of KGF in sacran-HGF significantly increased the tensile strength of sacran/KGF-HGF for 1.5 fold of Sacran-HGF (4 Mpa), indicating that sacran/KGF-HGF stronger than that of sacran-HGF.

One of the ideal properties of the HGFs is a high swelling ability which is connected to the moisturizing effect of HGFs [28]. Therefore, we next evaluated the swelling ability of the sacran/KGF-HGF. The gravimetric method was used to calculate the swelling ratio of the HGFs after immersing in PBS for 24 h, comparing to the initial weight. As shown in fig. 1d, the sacran/KGF-HGF was swollen about 12-fold compared to the initial weight. Meanwhile, sacran-HGF was swollen about 10-fold compared to the initial weight. In the previous study, the dried film of cross-linked hyaluronic acid hydrogel films swelled sevenfold in volume [29]. These results suggest that the sacran/KGF-HGF improved the swelling ability of sacran-HGF and it is higher than that of cross-linked hyaluronic acid hydrogel films.

To understand the morphology of the sacran/KGF-HGF, we investigated the HGFs by SEM. The surface of the HGFs was analyzed in 5 μL (x3000). It can be seen in fig. 2, the appearances of KGF could not be detected on the surface of Sacran-HGF, suggesting the KGF penetrate inside the HGFs. Further investigation is needed to clarify these findings.

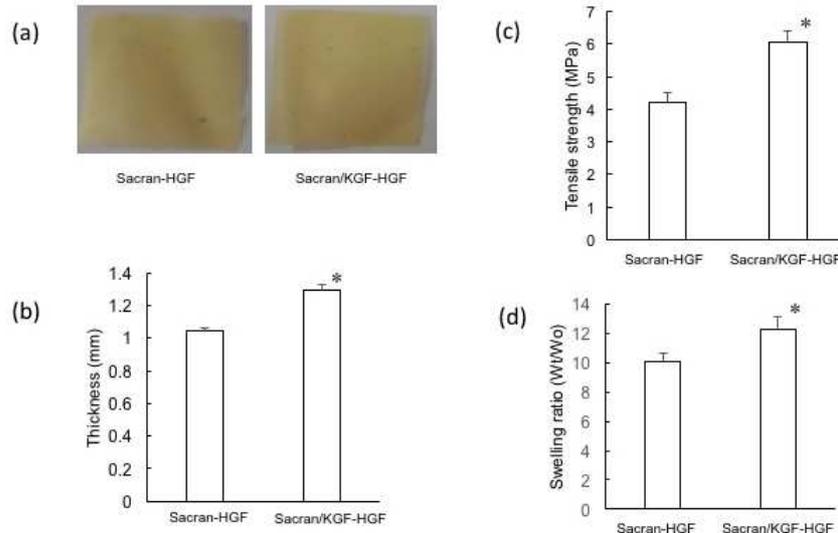


Fig. 1: Physicochemical properties of sacran/KGF-HGF (a) Appearances (b) Thickness (c) Tensile strength (d) Swelling ratio. Each value represents the mean±S. E. of 3 experiments. * $p < 0.05$ compared to sacran-HGF

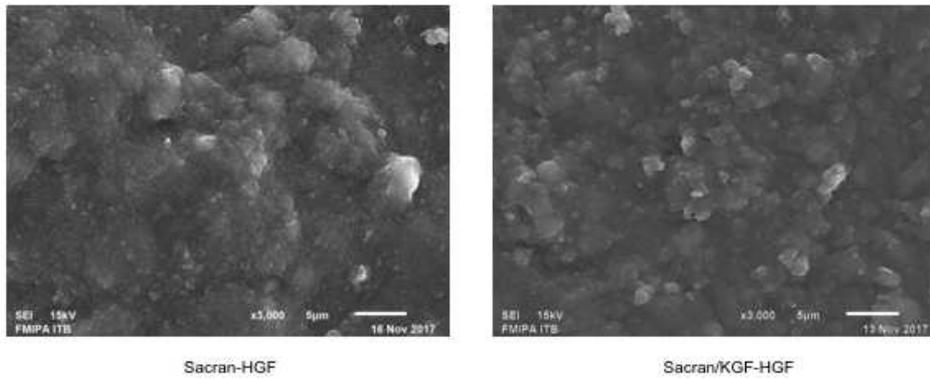


Fig. 2: Scanning electron microscopy analysis of sacran/KGF-HGF, an amorphous HGFs is important due to a high thermodynamic system and a high solubility [24]. To clarify the amorphous form in the sacran/KGF-HGF, we used an x-ray diffractometer. The results showed that the sacran/KGF-HGF had hallow pattern as same as sacran-HGF, indicating a high thermodynamic activities (fig. 3)

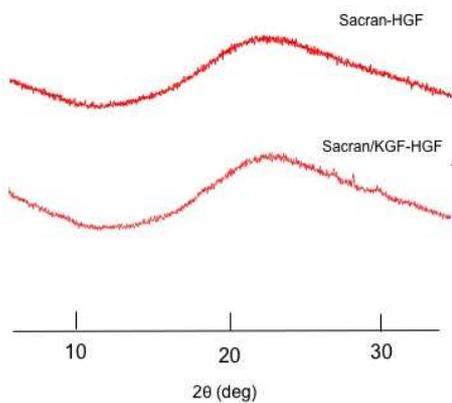


Fig. 3: X-Ray diffractometer pattern of sacran/KGF-HGF

Biodegradability of sacran/KGF-HGF

HGFs for wound dressing application should be biodegradable and presents a suitable environment for the tissue repair [30, 31]. Therefore, we next evaluated the biodegradability of sacran/KGF-HGF in chemical reagent (TCA) and enzyme (TrypLE™) solutions.

Fig. 4 showed that sacran/KGF-HGF degraded for about 41.29% in TCA and 22.92% in the enzyme. In addition, KGF improved the degradability of sacran/KGF-HGF in both solutions. These results suggest that KGF has a synergistic effect with Sacran-HGF for biodegradability of HGF.

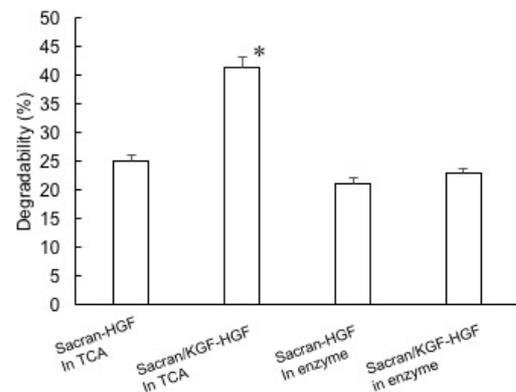


Fig. 4: Biodegradability of sacran/KGF-HGF. Each value represents the mean±SE of 3 experiments. * $p < 0.05$ compared to sacran-HGF in TCA

Wound closure study

Finally, to clarify the effect of KGF in sacran-HGF for chronic wound healing, we created an alloxan-induced diabetic model for wound closure study. A higher dose of alloxan (180 mg/kg body weight) in mice can be used to create severe atrophy of pancreatic islets and induced pancreatic β cell apoptosis [32]. We prepared three mouse groups of sacran-HGF, Sacran/KGF-HGF and control (without treatment). Then, the HGFs were applied precisely on the wound site at 0 d. After 6, 9 and 12 d, the wound areas were digitally

photographed and quantified by Image J software (fig. 5a-b). The sacran/KGF-HGF significantly accelerated the wound healing ability of sacran-HGF at 6, 9 and 12 d, indicating there is a positive effect between KGF and sacran-HGF in wound healing ability. Jimenez and Rampy confirmed that treatment of KGF resulted in an improvement in incisional wound healing due to an increase in breaking strength, collagen content, and epidermal thickness [33]. In addition, our previous study revealed that sacran-HGF increased wound healing ability, probably due to not only the moisturizing effect but also the anti-inflammatory effect of sacran [22].

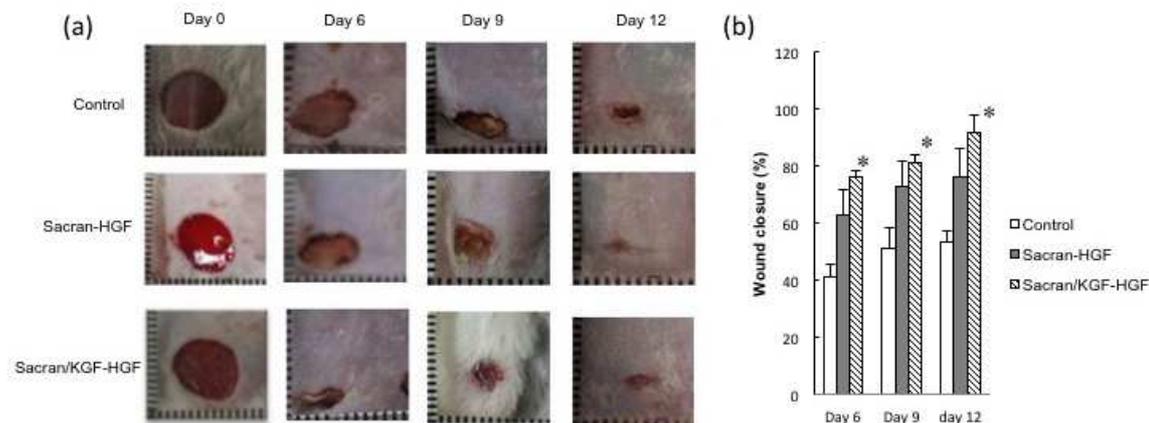


Fig. 5: Wound healing of sacran/KGF-HGF (a) Appearances (b) Wound Closure. Each value represents the mean \pm S. E. of 3 experiments. * $p < 0.05$ compared to control

CONCLUSION

In this study, we successfully fabricated sacran/KGF-HGF for chronic wound healing with excellent physicochemical properties. The existence of KGF in sacran-HGF improved the thickness, swelling ability, and tensile strength of sacran-HGF. In addition, sacran/KGF-HGF had an amorphous form and similar morphology to sacran-HGF. Moreover, KGF improved the degradability of sacran/KGF-HGF. Importantly, the sacran/KGF-HGF significantly accelerated the wound healing ability of sacran-HGF at 6, 9 and 12 d in alloxan-induced diabetic mice. These results suggest that KGF has the potential to promote the chronic wound healing ability of sacran-HGF.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

The authors have no conflict of interest directly relevant to the content of this article.

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