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Fabrication of Oxygen Releasing Scaffold by Embedding H_2O_2-PLGA Microspheres into Alginate-Based Hydrogel Sponge and Its Application for Wound Healing

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Abstract: In the regeneration process for new tissues, oxygen promotes re-epithelialization and healing of infected wounds, increases keratinocyte differentiation, proliferation and migration of fibroblast, and induces angiogenesis, collagen synthesis and wound contraction. Therefore, provision of oxygen to cells and tissues at an optimal level is critical for effective tissue regeneration and wound healing. In this study, we developed sustained oxygen-releasing polymeric microspheres and fabricated a sponge type dressing by embedding the microspheres into alginate-based hydrogel that can supply oxygen to wounds. We further investigated the applicability of the microspheres and hydrogel sponge to wound healing *in vitro* and *in vivo*. Oxygen-releasing microspheres (ORM) were made by incorporating hydrogen peroxide (H_2O_2) into poly(lactic-co-glycolic acid) (PLGA) using double emulsion method. H_2O_2-PLGA microspheres were embedded into alginate-based hydrogel to form a porous oxygen-releasing hydrogel sponge (ORHS). Biocompatibility was performed using cell counting kit-8. The oxygen release kinetic study was performed using a hydrogen peroxide assay kit and oxygen meter. The wound healing potential of ORHS was evaluated using the wound scratch model. *In vivo* studies were carried out to investigate the safety and efficacy of the ORHS for wound healing. Experimental results confirmed that oxygen released from ORMand ORHS induced neovascularization and promoted cell proliferation thereby facilitating effective wound healing. It is suggested that the ORM can be used for supplying oxygen to where cells and tissues are deprived of necessary oxygen, and ORHS is an intelligent scaffold to effectively heal wound by enhanced angiogenesis by oxygen. Conclusively, oxygen releasing polymeric microspheres and hydrogel scaffolds have potential for a variety of tissue engineering applications, where require oxygen.

Keywords: oxygen-releasing scaffolds; hydrogen peroxide; controlled release; hydrogel; wound healing; angiogenesis

1. Introduction

The oxygen concentration in the tissues of our body is regulated through biological processes and is important for embryo development, blood vessel formation, bone generation, stem cell function, and wound healing [1,2]. During the regeneration of new tissues, a supply of oxygen is a prerequisite, since it is a key molecule required for maintenance and differentiation of cells and tissues prior to neo-microvessel formation. Oxygen promotes healing by preventing the infection of wounds, enhancing keratinocyte differentiation by inducing angiogenesis, and inducing collagen synthesis by facilitating re-epithelialization through the proliferation and migration of fibroblasts [3].

Unless oxygen is supplied within a certain amount of time, the function of the tissue will be compromised, and tissue neovascularization does not proceed in the correct manner [4]. Several studies have been performed regarding the promotion of the oxygen supply during tissue regeneration. Khattak S.F. et al. [5] reported that perfluorocarbon-loaded alginate spherical capsules that provide an oxygen-carrying matrix that can improve cell metabolism and survival. Selçuk C.T. et al. [6] reported a high-pressure oxygen system, although the method warrants limited use as it requires special equipment, which is inconvenient and difficult to operate. Recently, Oh et al reported oxygen-generating scaffolds where poly(lactic-co-glycolic acid) (PLGA) and calcium peroxide were combined together to overcome the limiting factors in the areas of infection, such as neovascularization, for healing large wounds. This scaffold fosters both cell survival and growth, and solves the problem of limited tissue size through the large surface area and pore structure of the 3D scaffold. However, the byproducts(salts) formed during metabolic processes change the pH of the tissue, cause metabolic reactions that can lead to tissue damage and also reduce the oxygen release rate [7,8]. Therefore, the development of ideal oxygen generating system that can control the oxygen release remains a major focus. To address such limitations, this study developed a microsphere that can be used with a diverse range of pharmacological treatments. Briefly, hydrogen peroxide(H_2O_2) was encapsulated into PLGA (oxygen-releasing microsphere, ORM) allow the induced release of either H_2O_2 as an antiseptic or the release of oxygen [9,10]. Moreover, using this system, we could encapsulate various hydrophilic drugs these results suggest that this method can also be used as drug delivery systems (DDS) [11,12]. H_2O_2, as an oxygen supplier, is environmentally friendly since it can release oxygen over a long period of time without causing toxicity. PLGA microspheres with encapsulated H_2O_2 have been shown to be effective as a therapeutic drug due to the sustained release effect in the body by implementing a DDS, reducing drug side effects, maximizing drug efficacy and efficacy [13]. A diverse array of wound dressings iscurrently being manufactured and commercialized including foam, sheet, and liquid dressings through a variety of development techniques. However, current technology is limited and still requires highly effective remedies. The ideal dressing provides a proper wetting environmentinthe wound area and prevents the invasion of the bacteria. However, traditional wound healing methods use disinfectants until the wound is closed, cover the wound with gauze dressing, and do not address cell function. To overcome the disadvantages of conventional gauze dressings, a number of dressings have been developed and used that can improve cell activity or minimize scarring. However, as wound healing progresses, the condition of the wound changes, and the choice of dressing medication should be changed accordingly [14]. Currently available therapies for wound management increasingly rely on the use of wet dressings to achieve high levels of skin regeneration compared with the conventional gauze forms [15–17]. In this study, based on the idea reported here, a functional oxygen-releasing hydrogel sponge (ORHS) was developed that can enhance the formation of vascular endothelial cells for wound healing and tissue regeneration [18,19]. Traditional wound healing methods use gauze that cover wound (gauze dressing), a disinfectant until the wound is closed, and debridement of immortalized tissue. These conventional gauze dressings do not use any assisting system to enhance host cell function and relay on natural host healing process. Therefore, it takes a long time to complete healing and have a chance to be infected. To overcome these disadvantages of conventional gauze dressings, as well as, enhance cell activity or minimize scarring, a number of dressings have been developed. Numerous wound dressings are currently being manufactured and commercialized such

as foam, sheet and liquid through a variety of techniques. Most of them increasingly rely on the use of wet dressings and still requirehighly effective remedies to achieve high levels of skin regeneration compared with the conventional gauze forms.

In this study, based on the idea reported here, a functional ORHS was developed that can enhance the formation of vascular endothelial cells for wound healing and tissue regeneration. To use the developed dressing as an effective remedy for skin defects, H_2O_2-PLGA microspheres were dispersed in a 3D hydrogel structure (wet dressing) to control the oxygen concentration and cover the wound surface to prevent primary infection.

2. Materials and Methods

2.1. Preparation of the ORHS

To use the developed dressing as an effective remedy for skin defects, H_2O_2 was dispersed intoPLGA microspheres in a 3D hydrogel sponge to control the oxygen concentration and cover the wound surface to prevent primary infection (Figure 1).

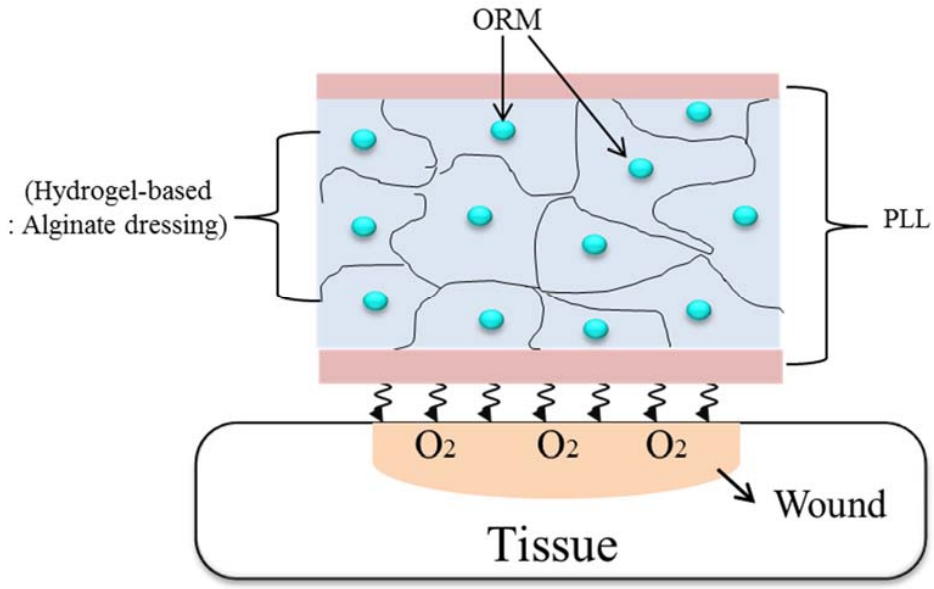

Figure 1. Concept of wound healing using H_2O_2-poly(lactic-co-glycolic acid) (PLGA)microspheres (oxygen-releasing microspheres, ORM) embedded oxygen-releasing hydrogel sponge(ORHS): PLGA poly(lactic-co-glycolic acid), PLL(poly-L-lysine hydrobromide).

H_2O_2 in the PLGA microsphere was produced using the solvent evaporation method ($W_1/O/W_2$) [20]. Briefly, 200 mg of poly(lactic-co-glycolic acid) (PLGA, lactic acid/glycolic acid (LA:GA, 50:50, MW = 11,000 g/mol, Boehringer Ingelheim, Ingelheim am Rhein, Germany) was dissolved in 1.5 mL of dichloromethane (DCM, Junsei Chemical, Tokyo, Japan) (represented as O). Then, 750 µL of 4.0 wt% hydrogen peroxide (H_2O_2, 50 wt%, Sigma, St. Louis, MO, USA) (represented as W_1) was added to the mixture, which was then turned into an emulsion by high-speed stirring [W_1/O]. Then, the [W_1/O] emulsion was slowly poured into 4 mL of 3.5 wt% polyvinyl alcohol solution (PVA, MW = 9000–11,000 g/mol, Sigma, St. Louis, MO, USA) (represented as W_2), followed by 30 s of high-speed stirring. Then, the [$W_1/O/W_2$] emulsion was slowly poured into 40 mL of 3.5 wt% PVA, the speed was adjusted to 800 rpm and the reaction was left at room temperature for 4 h. When the reaction was complete, the H_2O_2-PLGA microspheres (oxygen-release microspheres; ORM) were filtered through a 50–250 µm sieve and rinsed thrice with distilled water to completely remove

any unreacted species. The clean ORM was dried at room temperature for 18 h and stored at −20 °C for subsequent use (Figure 2A).

The ORHS was prepared using the freeze-drying method [21]. Briefly, 20 mg of ORM was added to 20 mL of 1.0 wt% sodium alginate (LVG, viscosity: 20–200 mPa·s, NovaMatrix, Sandvika, Norway) and uniformly mixed. Following this, 500 µL aliquots were distributed into each well (well size: 85.4 × 127.6mm, height 9.75 mm), and the plate was placed inside a lyophilizer (Ilshin Lab, FD5505, Gyeonggi-do, Korea) for 48 h for freeze-0000 drying. Next, 1.0 wt% calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$, Sigma, St. Louis, MO, USA) was added to each well, and the plate was left at room temperature for 24 h to allow for cross-linking to occur, after which the plate was then rinsed five times with distilled water and 95% ethyl alcohol to completely remove the calcium solution. The plate was again placed inside a lyophilized for 48 h for freeze-drying, followed by coating through a 5 min immersion in 0.1 wt% poly-L-lysine hydrobromide (PLL, Sigma, St. Louis, MO, USA). After rinsing three times with distilled water, and complete drying inside the lyophilized, the ORHS plate was stored at−20 °C until subsequent use (Figure 2B).

All chemicals and solvents used in the study were of analytical reagent grade.

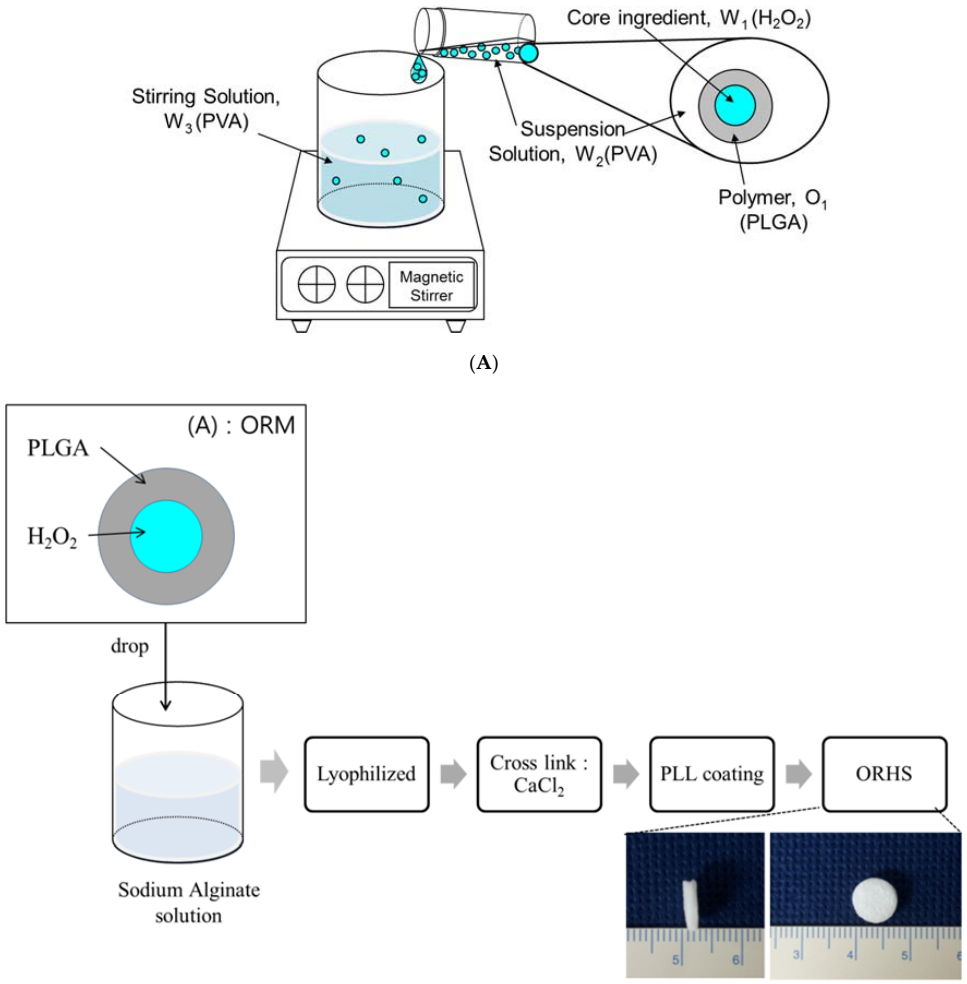

Figure 2. (**A**) Encapsulation process of H_2O_2-PLGA microspheres (oxygen-releasing microspheres, ORM) using the double emulsion solvent evaporation method, (**B**) Schematic diagram showing the fabrication process of the ORHS (oxygen-releasing hydrogel sponge): poly(lactic-co-glycolic acid), PLL(poly-L-lysine hydrobromide), PVA(polyvinyl alcohol).

2.2. Morphological Analysis of the ORHS

A field emission scanning electron microscope (FE-SEM, HitachiS-4300&EDX-350, Hitachi, Tarrytown, NY, USA) was used to morphologically analyze the surface and cross-section of the wound dressing. A porosimeter (MicrometricsAutoPore IV9520, Micrometrics, Norcross, GA, USA) was used to estimate the surface area of the dressing, as well as the size and volume of the pores.

2.3. Analysis of Oxygen Release from the ORHS

Oxygen release from the prepared ORHS was measured using an oxygen meter (Thermo Orion Series, 3 Star, Thermo Fisher, Waltham, MA, USA) [9]. To measure the dissolved oxygen (DO), the experiment was carried out in a sealed space to eliminate any unnecessary interference from the environment, and all measurements were taken at a clean bench. The quantified dressing was sealed inside a 20 mL glass container, immersed in 10 mL of 3 wt% standard catalase solution, and cultured for a specific period of time. Then, the cap of the glass container was replaced with an electrode to measure the DO levels (n = 5).

2.4. Analysis of the Release of H_2O_2 Contained in the ORHS

To measure the levels of H_2O_2 contained in the prepared ORHS, a hydrogen peroxide kit (s-1150, Scinco, Co. Ltd., Seoul, Korea) was used for quantitative analysis, following the protocol approved by Cell Biolabs Inc., San Diego, CA, USA [22]. Briefly, a sample of the suspension was distributed into each well of a 24-well plate. After the addition of 250 µL of lipid working reagent solution (0.250 mL xylenolorange + 0.250 mL AFS + 25 µL BHT + 24.475 mL 90% MeOH), the plate was incubated at 24 °C for 30 min. Next, the optical density (OD) of each well plate was measured at 595 nm and analyzed using an ELISA-reader (n = 5).

2.5. In Vitro Study

2.5.1. Biocompatibility of ORHS on Cell Survival Rate

The survival rate of human umbilical vein endothelial cells (HUVECs) under hypoxic condition was also evaluated according to the time spent on the scaffold. Endothelial cell basal medium-2 (EBM-2, Lonza, Walkersville, MD, USA) containing 10% FBS, 500 U/mL penicillin, and 500 µg/mL streptomycin (Gibco, Taibei, Taiwan) was used as the growth medium and the cells were cultured at 37 °C in a 5% CO_2incubator. To analyze the cell survival rate of the dressing, a cell suspension at a cell density of 1×10^5 cells/well was seeded into wells. We prepared 3 samples which were transplanted by inserting the cells into wells. Group 1 was transplanted with cell only (a control group were used for comparative analysis). Group 2 was transplanted the non- oxygen-releasing hydrogel sponge ('with alginate'). Group 3 was transplanted the oxygen-releasing hydrogel sponge ('with ORHS'). Cells were cultured for 48 h under hypoxic conditions [1% O_2, 5% CO_2, 94% N_2, (Model MIC-101, Billups-Rothenberg)] for 1, 2, 3, 4, 5, 6, or 7 days. After culture, highly water-soluble tetrazolium salt (CCK-8, Dojindo Laboratories, Tokyo, Japan) was added to the cells and they were cultured at 37 °C in a 5% CO_2 incubator for a further 4 h (an orange color signaled the end of reaction). The OD at 450 nm was measured using an ELISA-reader to evaluate the effect on the cell survival rate (n = 5).

2.5.2. Analysis of Cell Migration Using an *In Vitro* Wound Model

We used the human keratinocyte cell line (HaCaT), which is commonly used in studies on skin regeneration, to verify the wound healing effects by analyzing time-dependent cell migration. Modified eagle's medium (MEM, Gibco) containing 10% FBS, 500 U/mL penicillin, and 500 µg/mL streptomycin (Gibco) was used as the growth medium, and the cells were cultured at 37 °C in a 5% CO_2 incubator. For the cell migration experiment, the HaCaT cells were grown in a 6-well plate, and when full growth was attained, wounds were created using 1 mL blue tip. The experiment was

carried out under hypoxic condition [1% O_2, 5% CO_2, 94% N_2, (Model MIC-101, Billups-Rothenberg)] with three different groups of cells: group 1 was transplanted with cell only (a control group was used for comparative analysis). The group 2 was transplanted with the non-oxygen-releasing hydrogel sponge ('with alginate'). The group 3 was transplanted with the oxygen-releasing hydrogel sponge ('with ORHS'). The time-dependent cell migration was observed under a microscope between 0 and 24 h, which represented the time taken for healing.

2.6. Evaluation of In Vivo Efficacy

2.6.1. Animal *In Vivo* Model

The present study followed the ethics criteria of the Institutional Animal Care and Use Committee at Yeungnam University Medical Center (Approval No.: YUMC-AEC2012-023). Male SD-rats (weight 250~300 g, 8 weeks of age) were given an intramuscular injection of ketamine hydrochloride (Ketara, Yuhan Corporation, Seoul, Korea) and xylazine (Rumpum, Bayer Korea, Seoul, Korea) for anesthesia, and the surgical area was shaved. The full thickness of the dermis, with a diameter of 1.0 cm × 1.0 cm, was removed at four places on each side of the back using Metzenbaum tissue scissors. There were 4 groups of samples: the group 1 was transplanted only the thick dermis was removed, group 2 was transplanted with gauze, and a group 3 was transplanted the non-oxygen-releasing hydrogel sponge ('with alginate'). The group 4 was transplanted the oxygen-releasing hydrogel sponge ('with ORHS') (Figure 3). The dressing was soaked in a saline solution and used to cover just the area of the skin defect. The top part of the dressing was covered with a polyurethane film (Tegaderm, 3M, St. Paul, MN, USA) to protect the material within Following this, on days 1, 3, 5 and 7, tissue samples were collected for visual assessment. Visual analysis was calculated by sketching the SD-rat's scratch size over time with an Overhead Projector (OHP) film (3M™ CG6000, 3M Science. Applied to Life™, 3M Korea, Seoul, Korea) and digitizing the area using isolution (image and microscopy techniques).

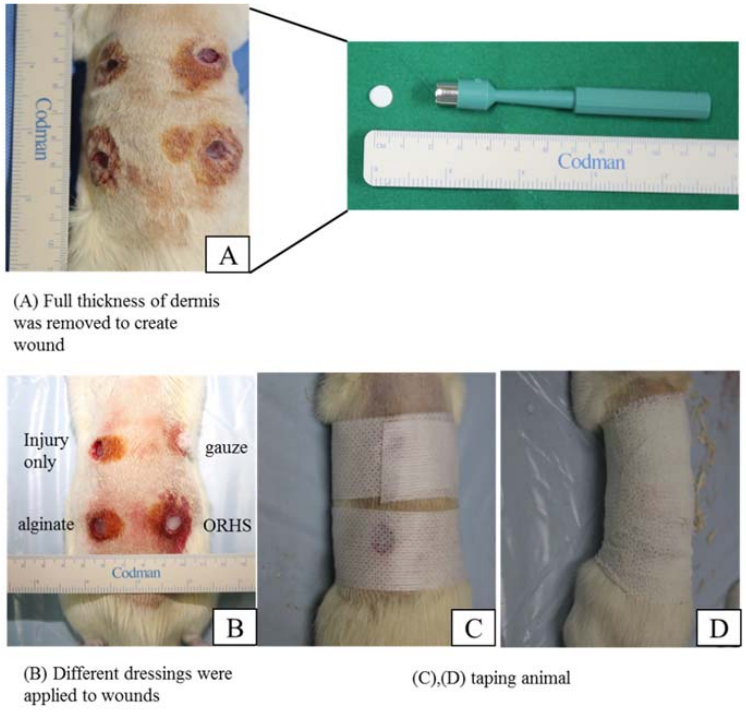

Figure 3. Wound generation on the back of rat. Wound of full thickness skin of 0.8 cm diameter was generated by punching. Four wounds were made on one rat; injury only, gauze, alginate, and oxygen-releasing hydrogel sponge (ORHS).

2.6.2. Immunohistological Analysis

After 7 days, the grafted dressing was collected and fixed in 4% formalin buffer, dehydrated with alcohol, and inserted into a paraffin block to be cut into 7 μm thickness using a microtome (Leica, Washington, DC, USA). Next, using both hematoxylin-eosin (H&E) and Masson's-trichrome staining, an analysis of the tissue immune reaction that had occurred, as well as the extent of neovascularization, was carried out by assessing the extent of cellular influx and the presence of a nucleus inside the cells. In addition, the extent to which epithelialization had occurred (whether the extracellular matrix has formed), was also assessed. Staining for vascular endothelial growth factor (VEGF) was carried out to assess the extent of angiogenesis, after which the staining patterns were observed under by optical microscopy (Olympus, Tokyo, Japan).

2.7. Statistical Analysis

All data are expressed as the mean ± standard error of mean. All collected data were analyzed using a t-test and one-way analysis of variance (ANOVA). The different data were collected and digitized using Sigma Plot software (version 13, Systat Software, Inc., San Jose, CA, USA) and Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

3. Results

3.1. Morphological Analysis

When the surface and cross-section of the ORHS were examined under a scanning electron microscope, the surface of the prepared ORHS was found to be porous, suggesting that it would allow for cell adhesion and promote cell survival (Figure 4B). The porosity of the prepared ORHS was 90.5% with a total pore surface area of 2.1 m^2/g, whereas the porosity of the alginate group in the control group was 96.8% with a total pore area of 7.1 m^2/g. The ORHS had a more irregular porous structure than the control, and both the surface and cross-section were significantly decreased compared to the control. These data indicate that both the total surface area and volume of the pores in the prepared dressing were reduced, giving the ORHS an open structure composed of multiple pores.

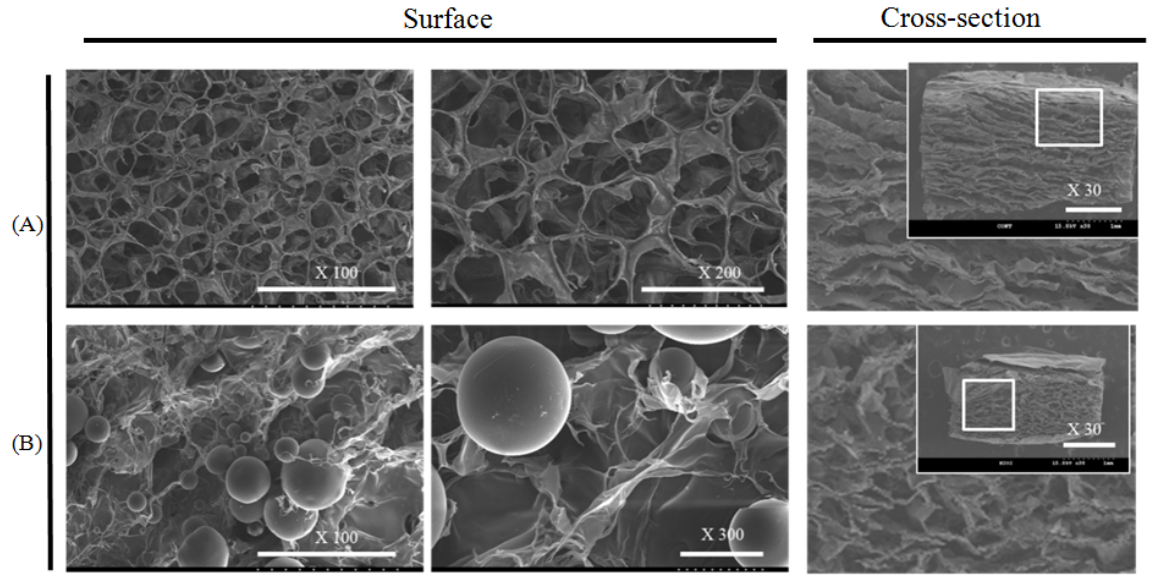

Figure 4. Morphological characterization with field emission scanning electron microscope (FE-SEM); (**A**) alginate group, (**B**) oxygen-releasing hydrogel sponge (ORHS) group. The ORHS showed decrease in porosity, whereas the alginate sponge had higher porosity.

3.2. Release of Oxygen Contained in the ORHS

A three-day long-term oxygen release profilefor the dressing is shown in Figure 5. As a result of evaluating the DO level for analyzing oxygen emissions, it was observed that the DO value decreased sharply after 3 days due to the high surface area despite the initial high oxygen release. Thus, the prepared ORHS was shown to be capable of maintaining the oxygen level until the third day of use.

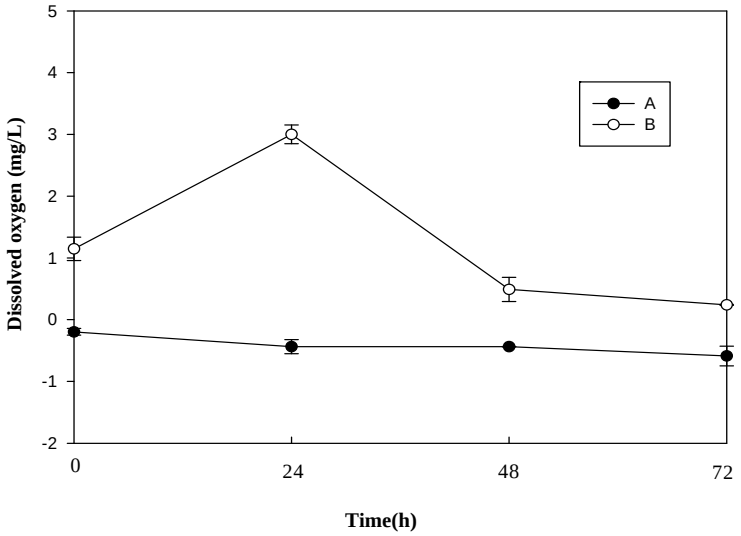

Figure 5. Release profile of dissolved oxygen (n = 5); (A) alginate group, (B) oxygen-releasing hydrogel sponge (ORHS) group. Oxygen released from the sponges for over a period of 3 days.

3.3. Release of H_2O_2 Contained in the ORHS

The H_2O_2 concentration released from the H_2O_2–containing PLGA microsphere was 6.6 μM and that from the ORHS was 5.8 μM (Table 1). The maximum level of tolerance to H_2O_2 exposure in the human is 29.4 μM (1 mg/L), which suggests that the ORHS would be well tolerated and remain stable in humans, since the H_2O_2 levels released were lower than the tolerance limit.

Table 1. Loading efficiency of hydrogen peroxide in the oxygen-releasing hydrogel sponge (ORHS).

H_2O_2 % of Control [c] (n = 5)	Weight	Total H_2O_2 μM/Loading Efficiency (%) [b]
ORHS [a]	10.0 mg	5.822/0.44 ± 0.0568

[a]: [non-treat]–[ORHS]; [b]: (H_2O_2 [a]/4.0% ORHS) × 100%; [c]: y = 0.0048x + 0.1344 R^2 = 0.9946; Control: 4.0 wt% H_2O_2.

3.4. Biocompatibility of the ORHS

To assess how the ORHS influenced cell survival and proliferation, the experiment was allowed to proceed for a total of 7 days. As a result, when the same number of cells were seeded, the rate of survival and proliferation of the cells in the experimental group ('with ORHS') was not significantly different compared to the control group ('with alginate') (Figure 6). However, comparing to the rate of HUVEC cell proliferation over the 7-day period revealed that the cell proliferation improved slightly upon exposure to oxygen release.

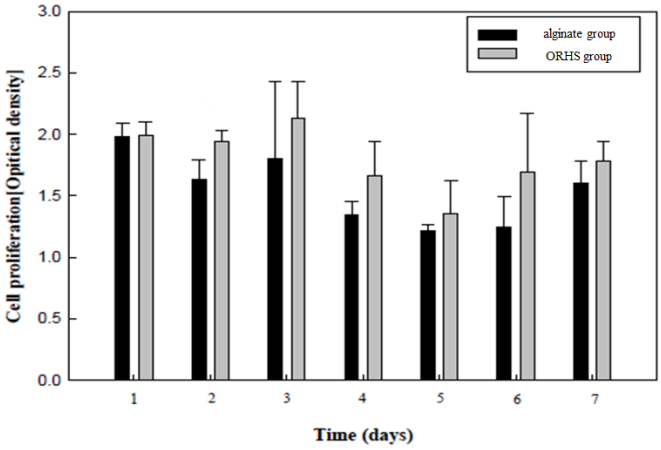

Figure 6. Cell proliferation (CCK-8 assay) in the alginate group and oxygen-releasing hydrogel sponge (ORHS) group. Cell proliferation was observed for 7 days. No toxicity compared to the control was observed. Therefore, the ORHS did not affect cell viability. The assay was performed in hypoxia conditions (1% O_2, 5% CO_2, 94% N_2) (n = 5).

3.5. Evaluation of the Wound Healing Rate Using an in vitro Wound Model

To analyze the wound healing effects of the ORHS, the migration of HaCaT cells was assessed in an in vitro wound-healing assay. The data showed that with ORHS, compared to the only cell and without ORHS groups, produced a far superior cell migration indicating better wound healing (Figure 7). These data provide indirect evidence for the positive effects of the supply of oxygen on wound healing.

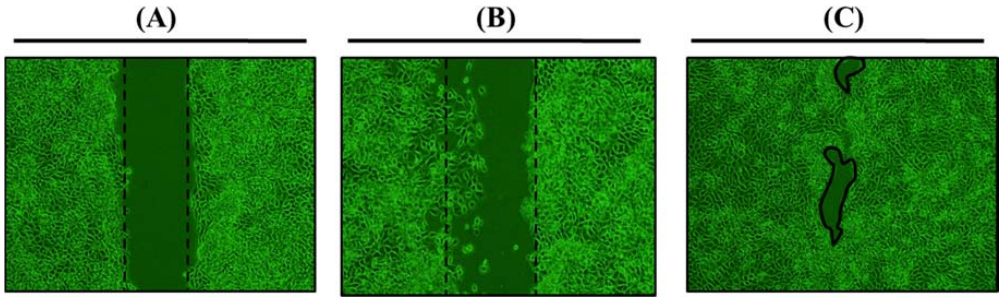

Figure 7. Scratch wound closure model using HaCaT cells; (**A**) Cells only, (**B**) alginate group, (**C**) oxygen-releasing hydrogel sponge (ORHS) group. Wound closure was significantly faster with ORHS. The ORHS group significantly increased epithelial keratinocyte cell migration.

3.6. In Vivo Evaluation

In wound group using ORHS, wound size was smaller than that of gauze group and alginate group, and surface scar was also improved. Especially after 3 days of injuries, the size of the wound covering the ORHS group decreased compared to the gauze group and the suture was maintained from 5th to 7th day (Figure 8). An immunohistological analysis of the wound tissue collected from each group on days 1 and 7 showed that the distribution of inflammatory cells (red) on day 1 became more clearly distinguishable in the 'with ORHS' group than in the control group or in the 'without ORHS' group, and a complete formation of the skin layer was observed on day 7 (Figure 9). Neutrophils were detected in the wound area in all three groups, although on day 7 there was a slight decrease only in the 'with ORHS' group. These data provide indirect proof that the release of oxygen prevents infection at the wounded area and consequently reduces the inflammatory reaction. The results of Masson's Trichrome

staining to examine collagen deposition showed that the healing wound had significantly increased numbers of fibroblasts(blue). The 'with ORHS' group, in particular, showed larger blue stained areas compared to the control, indicating that the release of oxygen allowed for the effective synthesis of collagen in the wounded area. Furthermore, the ORHS was found to improve the wound healing process based on the levels of gene expression of the angiogenesis factor VEGF. The 'with ORHS' group showed enhanced VEGF expression and a facilitation of the wound healing process was also observed.

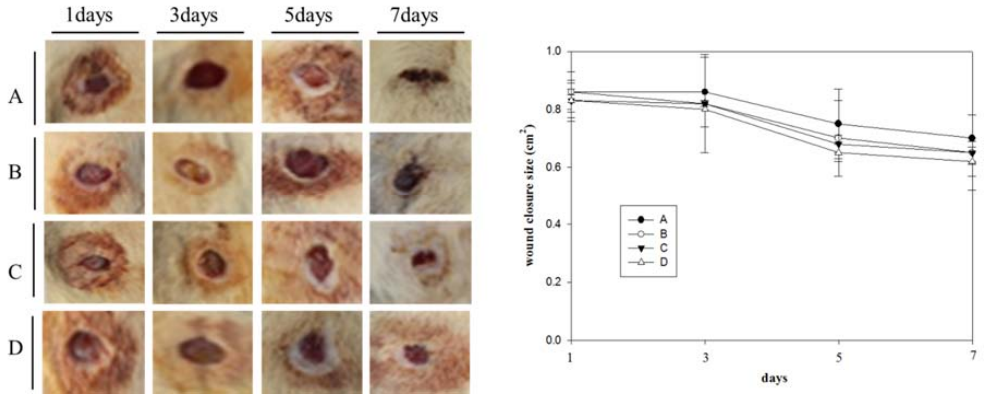

Figure 8. (**Left**) Gross morphology of wounds at different time points after surgery. (**Right**) Wound closures were observed on days 1, 3, 5, and 7 after wound generation via surgery; (A) full thickness skin defect group, (B) gauze group, (C) alginate group, (D) oxygen-releasing hydrogel sponge (ORHS)group.

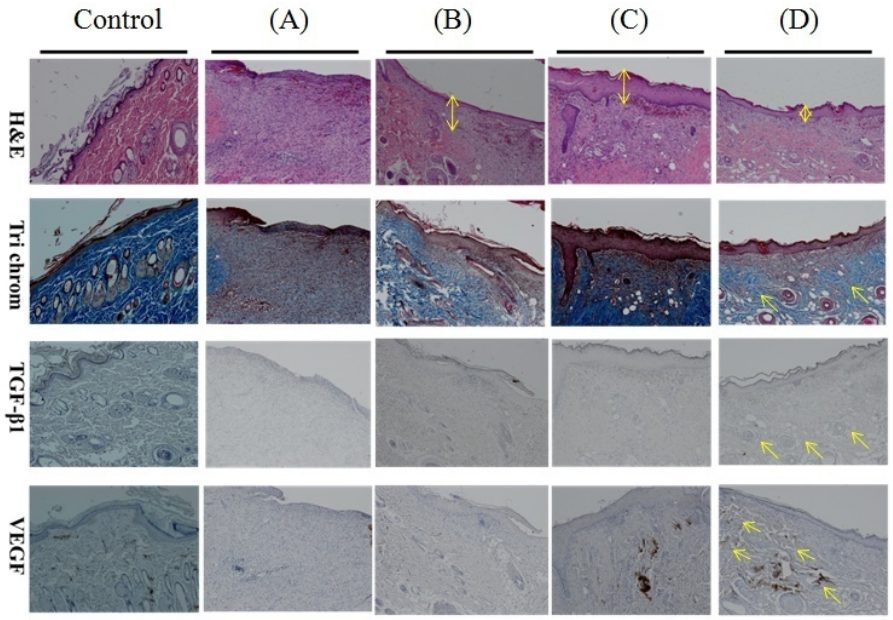

Figure 9. Histologic findings of the wounds at postoperative day 7 of the (**A**) full thickness skin defect group, (**B**) gauze group, (**C**) alginate group, and (**D**) oxygen-releasing hydrogel sponge (ORHS) group. Control groups showed that the dermis layer was uneven and remained patent area. The epidermal layer which was covered by new epithelial keratinocytes was also formed irregular. The alginate group (**C**) showed no patent area of dermis layer. But, epidermal layer was irregular. The ORHS group (**D**) had a uniform dermis layer and hair follicles were present in the wound area. The epidermal layer was regular as layer-by-layer. Control groups showed that dermis layer was uneven and remained patent area (H&E, ×100). On the 7th day, a mature arrangement of collagen fiber was shown in the alginate group (**C**), and ORHS group (**D**) (Tirchrom, ×100). On the 7th day, increased TGF-β1 expression was shown in (**D**) and increase VEGF expression was shown in (**D**) (Immuno-histochemical staining, ×100).

4. Discussion

The term wound refers to the damage to the human body caused by an external force that cuts the skin or other tissues, or leaves a subsequent partial defect in the tissue [23]. The dressings that are used to protect the damaged surface of a wound by preventing any leak of body fluids or the entrance of bacteria are called wound dressings, wound camouflages, or skin substitutes. Wound healing is a complex process that occurs though a series of steps that involve a number of different cellular activities. These steps determine the rate of healing, which is influenced by the release of bioactive materials such as growth factors, enzymes, carbohydrates, and proteins, as well as the inflammatory response to infection. When the skin is wounded, it is essential that the tissue is restored early on in order for the wound to be repaired in such a way that it resembles the original skin structure. Early wound closure is therefore vitally important, as an open wound that remains untreated for a long time may lead to a secondary complication such as infection, or leave a severe scar even after closure, causing functional or cosmetic problems [24]. The general methods for the treatment of wounds involve applying an antiseptic to the wound until closure, using a gauze or band type wound dressing to simply protect the wound, or using a dressing material containing a wound healing promoter. Nevertheless, a limitation of currently available wound dressings is that they encourage passive tissue regeneration that relies on the inherent regenerative capacity of the wounded area. Currently, an effective regenerative therapy is being sought to overcome this limitation. There have been attempts to transplant cells or tissues from another individual, or even from animals, as a way to produce a regenerative therapy; however, such methods are of limited use owing to the problems of immune rejection and an inadequate supply of therapeutic agents. These issues have prompted research toward developing an effective regenerative therapy for damaged skin tissues using tissue-engineering techniques that employ biomaterials [25]. To treat skin defects, studies are underway to deliver genes, growth factors, or drugs that are effective at the regeneration stage. In particular, since they provide more stable and efficient regeneration than the transplant of cells alone, there have been numerous studies using wound dressings, where cells such as fibroblasts, keratinocytes, and the matrix cells of adipose tissue are used for transplantation, as well as wound dressings that incorporate growth factors [26]. However, the integration of transplanted cells with the follicles found in healthy skin and the surrounding tissues has presented a challenge, indicating there is still an urgent need for the development of an efficient treatment method.

When the cells of the body are damaged, VEGF plays a key role in the cellular regeneration process that occurs in response to the insult. On day 1 after wounding, platelets are produced, followed by the proliferation of endothelial cells and recruitment of macrophages by day 2. Following this, on days 3–4, neovascularization occurs via the formation of new capillary vascular endothelial cells. On day 5, new fibroblasts emerge, and granulation tissues are formed, leading to wound healing. VEGF plays a key role in these processes since it induces the proliferation and migration of endothelial cells (vascular and lymphatic cells) leading to the release of other growth factors (PDGF, TGF-β, EGF, and FGF) that facilitate healing, and thus, the various bioactive substances work together to orchestrate the accelerated wound healing process [27]. In the present study a novel oxygen-releasing wound dressing was developed that promotes angiogenesis to allow for rapid wound healing. Angiogenesis generally occurs on days 3–4 after wounding (the angiogenesis zone), and if a stable adequate oxygen level is maintained until that time, angiogenesis is likely to be enhanced. Here, oxygen release was monitored using an oxygen meter, which showed that the release of oxygen was at a maximum 24 h and subsequently gradually decreased up until 72 h. These data prove that a double layer matrix allows for the controlled release of oxygen. During the normal wound healing process, the extracellular superoxide dismutase (SOD) metabolites, such as H_2O_2 and superoxide (O^{2-}), play roles as cell signaling messengers that switch on the key processes of wound healing, such as cell motility, cytokine action, and angiogenesis. Several studies have reported that angiogenesis and wound healing respond differently with respect to H_2O_2 concentration [28,29]. At 10 nM (0.003%) H_2O_2, both angiogenesis and wound healing are facilitated, while at 166 mM (0.5%), wound healing is delayed. Another study has reported that the

adhesion and migration of cells occurs at H_2O_2 concentration up to 100 μM, with 20 μM (0.0006%) being the most effective, suggesting the effects varying depending on the H_2O_2 concentration. This is because, at low H_2O_2 concentrations, MAP kinase activation leads to the increased production of VEGF and MMP-9, which are factors that induce cell proliferation and facilitate the production of MMP-1, a growth factor that activates cell migration [30]. H_2O_2, derived from the action of extracellular SOD, plays a crucial role in facilitating the phosphorylation of the VEGFR2 thereby enhancing cellular growth and differentiation through the proliferation and migration of endothelial cells, leading to an acceleration of angiogenesis in the body. It is true that an excessive amount of reactive oxygen species such as H_2O_2 is toxic to the cells; however, if provided at an appropriate concentration in the place required, H_2O_2 can promote cellular growth and differentiation [31]. In this study, the amount of H_2O_2 released from the newly developed ORHS and the amount of SOD generated were compared. As the data showed, the H_2O_2 concentration in the ORMs was 6.6 μM, whereas that in the ORHS was 5.8 μM, an extremely small amount. These data provide indirect evidence that 0–100 μM H_2O_2 can enhance cell adhesion and migration to activate the growth factors, leading to accelerated angiogenesis. The oxygen generated through the decomposition of H_2O_2 is a key molecule in cellular metabolism and plays a crucial role in most wound healing processes. Although diffusion allows for an oxygen supply at 40–200 μm from capillaries or vascular endothelial cell membranes [32], when the diameter of the tissue to be transplanted exceeds 0.75–1.5 cm [33], the consequent limited diffusion makes the supply of oxygen and nutrients difficult, resulting in possible tissue necrosis [8,34]. Thus, in the present study, the size of the ORMs was restricted to be 250 μm and the ORHS was designed to fall within 1.0 cm, so that oxygen diffusion could be maintained. The structure that allows oxygen diffusion was observed using a scanning electron microscope and a porosimeter. This structure was based on dressings with a large surface area that induce interactions between cells and pore structures. These data lend support to there being stable oxygen diffusion during cell maintenance and regeneration, in concordance with previous studies.

As shown, the ORHS developed in this study using a biocompatible macromolecular scaffold, could maintain the wetness around the wounded area and induce the secretion of various growth factors (TGF-α, VEGF, FGF, EGF, MMP-2, MMP-9, etc.) and cytokines (IL-1 α, etc.) from the skin cells, the result of which improves wound healing [35]. The ORHS creates an appropriate environment around the wounded area for healing and the stimulation of angiogenesis, while enhancing the fibroblast proliferation. Therefore, this study proved that with the supply of an adequate level of oxygen, wound healing may be improved, and that the activation and proliferation of vascular endothelial cells leads to enhanced angiogenesis. Nonetheless, a limitation lies in the difficulty in predicting the actual amount of released oxygen, and in assessing the time frame during which oxygen is released, as there is a diverse range of enzymatic activities and metabolic process that occur in a tissue. Since the present study assessed oxygen generation using indirect methods, such as the expression of growth factors, subsequent studies should verify the effects reported in this study.

We developed a novel dressing for wound healing using an oxygen-generating system by loading an alginate hydrogel of PLGA microspheres with H_2O_2, a major source of oxygen. The newly developed oxygen release wound dressing features; (i) the biomaterial has excellent biocompatibility; (ii) there is an excellent biological stability of the generated oxygen; (iii) no byproducts are created during degradation; (iv) the dressing allows for continuous oxygen release and control; (v) the dressing provides the tissue with oxygen that can enhance cell migration and viability. Furthermore, in a small clinical evaluation, skin-calming effects were also found. Based on these results, the ORHS, which provides a solution for improving wound healing, is expected to be useful in different medical devices that require oxygen and may be useful in tissue engineering for cell or skin regeneration.

In the further studies, we are going to confirm the effect of oxygen supply from hydrogel complex system on neovascularization and wound healing process by gene analysis such as CD32, α-SMA, MMP-2 and MMP-9 and using large animals.

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References

1. Schäfer, M.; Werner, S. Oxidative stress in normal and impaired wound repair. *Pharmacol. Res.* **2008**, *58*, 165–171. [CrossRef] [PubMed]
2. Cai, L.Y.; Xiong, X.; Kong, X.L.; Xie, J. The role of the lysyl oxidases in tissue repair and remodeling: A concise review. *Tissue Eng. Regen. Med.* **2017**, *14*, 15–30. [CrossRef]
3. Kimmel, H.M.; Grant, A.; Ditata, J. The Presence of oxygen in wound healing. *Wounds* **2016**, *28*, 264–270. [PubMed]
4. Sen, C.K. Wound healing essentials: Let there be oxygen. *Wound Repair. Regen.* **2009**, *17*, 1–18. [CrossRef] [PubMed]
5. Khattak, S.F.; Chin, K.S.; Bhatia, S.R.; Roberts, S.C. Enhancing oxygen tension and cellular function in alginate cell encapsulation devices through the use of perfluorocarbons. *Biotechnol. Bioeng.* **2007**, *96*, 156–166. [CrossRef] [PubMed]
6. Selçuk, C.T.; Kuvat, S.V.; Bozkurt, M. The effect of hyperbaric oxygen therapy on the survival of random pattern skin flaps in nicotine treated rats. *J. Plast. Reconstr. Aesthet. Surg.* **2012**, *65*, 489–493. [CrossRef] [PubMed]
7. Harrison, B.S.; Eberli, D.; Lee, S.J.; Atala, A.; Yoo, J.J. Oxygen producing biomaterials for tissue regeneration. *Biomaterials* **2007**, *28*, 4628–4634. [CrossRef] [PubMed]
8. Oh, S.H.; Ward, C.L.; Atala, A.; Yoo, J.J.; Harrison, B.S. Oxygen generating scaffolds for enhancing engineered tissue survival. *Biomaterials* **2009**, *30*, 757–762. [CrossRef] [PubMed]
9. Ng, S.M.; Choi, J.Y.; Han, H.S.; Huh, J.S.; Lim, J.O. Novel microencapsulation of potential drugs with low molecular weight and high hydrophilicity: Hydrogen peroxide as a candidate compound. *Int. J. Pharm.* **2010**, *384*, 120–127. [CrossRef] [PubMed]
10. Abdi, S.I.H.; Ng, S.M.; Lim, J.O. An enzyme-modulated oxygen-producing micro-system for regenerative therapeutics. *Int. J. Pharm.* **2011**, *409*, 203–205. [CrossRef] [PubMed]
11. O'Donnell, P.B.; McGinity, J.W. Preparation of microspheres by the solvent evaporation technique. *Adv. Drug Deliv. Rev.* **1997**, *28*, 25–42. [CrossRef]
12. Bae, Y.J.; Cho, C.H.; Lee, W.J.; Huh, J.S.; Lim, J.O. Optimization of recombinant human platelet-derived growth factor-BB encapsulated in Poly(lactic-co-glycolic acid) microspheres for applications in wound healing. *Tissue Eng. Regen. Med.* **2016**, *13*, 13–20. [CrossRef]
13. Park, J.H.; Kwon, D.Y.; Heo, J.Y.; Park, S.H.; Park, J.Y.; Lee, B.; Kim, J.H.; Kim, S.M. Effect of Drug Carrier Melting Points on Drug Release of Dexamethasone-Loaded Microspheres. *Tissue Eng. Regen. Med.* **2017**, *14*, 743–753. [CrossRef]
14. Takeo, M.; Lee, W.; Ito, M. Wound Healing and Skin Regeneration. *Cold Spring Harb. Perspect. Med.* **2015**, *5*, a023267. [CrossRef] [PubMed]
15. Ng, W.Y.; Migotto, A.; Ferreira, T.S.; Lopes, L.B. Monoolein-alginate beads as a platform to promote adenosine cutaneous localization and wound healing. *Int. J. Biol. Macromol.* **2017**, *102*, 1104–1111. [CrossRef] [PubMed]
16. Faragò, S.; Lucconi, G.; Perteghella, S.; Vigani, B.; Giuseppe, T.; Sorrenti, M.; Catenacci, L.; Boschi, A.; Faustini, M.; Vigo, D.; et al. A dry powder formulation from silk fibroin microspheres as a topical auto-gelling device. *Pharm. Dev. Technol.* **2016**, *21*, 453–462. [CrossRef] [PubMed]
17. Theodora, C.; Sara, P.; Silvio, F.; Alessandra, B.; Giuseppe, T.; Barbara, V.; Barbara, C.; Sabrina, R.; Silvia, D.; Stefania, P.; et al. Platelet lysate and adipose mesenchymal stromal cells on silk fibroin nonwoven mats for wound healing. *J. Appl. Polym. Sci.* **2016**, *133*, 42942. [CrossRef]

18. Shapiro, L.; Smadar, C. Novel alginate sponges for cell culture and transplantation. *Biomaterials* **1997**, *18*, 583–590. [CrossRef]
19. Lai, H.L.; Abu'Khalil, A.; Craig, D.Q.M. The preparation and characterisation of drug-loaded alginate and chitosan sponges. *Int. J. Pharm.* **2003**, *251*, 175–181. [CrossRef]
20. Freitas, S.; Merkle, H.P.; Gander, B. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. *J. Control. Release* **2005**, *102*, 313–332. [CrossRef] [PubMed]
21. Schoof, H.; Apel, J.; Heschel, I.; Rau, G. Control of pore structure and size in freeze-dried collagen sponges. *J. Biomed. Mater. Res.* **2001**, *58*, 352–357. [CrossRef] [PubMed]
22. Hydrogen Peroxide Assays. Available online: https://www.cellbiolabs.com/hydrogen-peroxide-assay (accessed on 31 December 2018).
23. Kirsner, R.S.; Eaglstein, W.H. The wound healing process. *Dermatol. Clin.* **1993**, *11*, 629–640. [CrossRef]
24. Gurtner, G.C.; Werner, S.; Barrandon, Y.; Longaker, M.T. Wound repair and regeneration. *Nature* **2008**, *453*, 314–321. [CrossRef] [PubMed]
25. MacNeil, S. Progress and opportunities for tissue-engineered skin. *Nature* **2007**, *445*, 874–880. [CrossRef] [PubMed]
26. Werner, S.; Grose, R. Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* **2003**, *83*, 835–870. [CrossRef] [PubMed]
27. Um, J.H.; Yu, J.Y.; Dubon, M.J.; Park, K.S. Substance P and thiorphansynergically enhance angiogenesis in wound healing. *Tissue Eng. Regen. Med.* **2016**, *13*, 149–154. [CrossRef]
28. Liu, Z.H.; Li, S.; Cai, Y.C.; Wang, A.X.; He, Q.T.; Zheng, C.X.; Zhao, T.T.; Ding, X.Q.; Zhou, X.F. Manganese superoxide dismutase induces migration and invasion of tongue squamous cell carcinoma via H_2O_2-dependent Snail signaling. *Free Radic. Biol. Med.* **2012**, *53*, 44–50. [CrossRef] [PubMed]
29. Bretón-Romero, R.; Lamas, S. Hydrogen peroxide signaling in vascular endothelial cells. *Redox Biol.* **2014**, *2*, 529–534. [CrossRef] [PubMed]
30. Oshikawa, J.; Urao, N.; Kim, H.W.; Kaplan, N.; Razvi, M.; Mckinney, R.; Poole, L.B.; Fukai, T.; Ushio-Fukai, M. Extracellular SOD-derived H_2O_2 promotes VEGF signaling in caveolae/lipid rafts and post-ischemic angiogenesis in mice. *PLoS ONE* **2010**, *5*, e10189. [CrossRef] [PubMed]
31. Marinho, H.S.; Real, C.; Cyrne, L.; Soares, H.; Autunes, F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol.* **2014**, *2*, 535–562. [CrossRef] [PubMed]
32. Muschler, G.F.; Nakamoto, C.; Griffith, L.G. Engineering principles of clinical cell-based tissue engineering. *J. Bone Jt. Surg. Am.* **2004**, *86*, 1541–1558. [CrossRef]
33. Lewis, D.; Goldztein, H.; Deschler, D. Use of hyperbaric oxygen to enhance auricular composite graft survival in the rabbit model. *Arch. Facial Plast. Surg.* **2006**, *8*, 310–313. [CrossRef] [PubMed]
34. Lim, J.O.; Huh, J.S.; Abdi, S.I.H.; Ng, S.M.; Yoo, J.J. Functionalized biomaterials-oxygen releasing scaffolds. *J. Biotechnol. Biomater.* **2015**, *5*, 2.
35. Chua, L.S.; Kim, H.W.; Lee, J.H. Signaling of extracellular matrices for tissue regeneration and therapeutics. *Tissue Eng. Regen. Med.* **2016**, *13*, 1–12. [CrossRef]