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Preparation of an Oxygen-Releasing Capsule for Large-Sized Tissue Regeneration

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Abstract: Sufficient oxygenation for prevention of cellular damage remains a critical barrier to successful tissue engineering, especially in the construction of a large-sized tissue despite numerous attempts to resolve this issue in recent years. There have been a number of hypothetical solutions to this problem, including the use of artificial oxygen carriers, induction of vascularization, and fabrication of oxygen-generating biomaterials. All of these efforts have improved the efficiency of oxygen supply, but none have been able to support the large tissue mass required for clinical application. Necrosis, which often occurs during hypoxic stress, is one of the most significant limitations in large-sized tissue regeneration. In this study, we developed an oxygen producing capsule using hydrogen peroxide (H_2O_2), PLGA (poly (lactic-co-glycolic acid) and alginate, and also evaluated the capsule as a model of a large-sized tissue. Firstly, H_2O_2 was microencapsulated by PLGA, and subsequently the H_2O_2-PLGA microspheres were embedded into a catalase-immobilized alginate capsule of 5.0 mm in diameter. The alginate capsules of a fairly large size were characterized for their oxygenation capability to cells embedded such as human umbilical vein endothelial cells (HUVECs) by HIF-1α and VEGF expression. The results of this study confirmed that in the oxygen-releasing capsule composed of H_2O_2 polymeric microspheres and catalase-bound alginate, HUVEC cells successfully survived in the hypoxic state. These results demonstrated that our oxygen producing system containing H_2O_2-PLGA microspheres could be a useful oxygenating biomaterial for engineering large-sized tissue.

Keywords: oxygen diffusion; drug delivery system; control release; encapsulation; EDC/NHS

1. Introduction

Necrosis often occurs under hypoxic conditions and is a major limiting factor in tissue engineering, particularly for large tissue sections [1]. One major obstacle to the development of clinically appropriate three-dimensional tissue structures is the lack of adequate nutrients and oxygen supply to these tissues prior to their in vivo vascularization; unlike living tissues in vivo, these grafts are unable to maintain cell viability and function at the core of dense engineered tissues. Diffusion only supplies nutrients and oxygen to cells at the surface of these artificial tissues, resulting in necrotic cell death from a lack of oxygen [2,3]. These results indicate that the limits of oxygen diffusion become increasingly important as the biomass and cell number in these artificially generated tissues increases [4].

In normal human biological systems, tissues are located within close proximity to blood vessels, which ensures their survival via the transportation of nutrients and oxygen. New tissues often require neovascularization which can only extend between 100 and 200 μm from the closest capillary [5,6]. This means that new tissues require sustained angiogenesis to develop beyond 100–200 μm in depth. However, in engineered tissue grafts, the distance between the cells and the blood vessels within the graft is often a few millimeters or even centimeters, as the grafts begin to grow after implantation. Therefore, larger-sized implanted tissues need to undergo rapid vascularization from the new capillary network within the tissue in order to survive. After transplantation, the host's blood vessels typically invade tissues in response to signals secreted by the transplanted cells in response to hypoxia, forming new capillary and blood vessel networks. However, since this spontaneous intravascular growth is often limited to tens of micrometers per day, the time required to fully vascularize a few millimeters of implant is weeks [7–9].

Inadequate vascularization during this period can result in increased malnutrition and hypoxia in these tissues. In addition, hypoxic conditions commonly occur within the deeper regions of these constructs as a result of inadequate oxygen supply in vitro [10]. This means that large scale tissue regeneration is often incomplete and very difficult to accomplish [11,12].

Therefore, it is important to develop novel smart materials that can support the oxygenation of these larger tissues in vitro and in vivo. Previous studies have used hydrogen peroxide as a source of oxygen, but the release of cations into solution following its catalysis could adversely affect some cell types [13–16].

For this reason, our group assessed whether hydrogen peroxide could be used as a source of oxygen and has successfully demonstrated that it is possible to encapsulate hydrogen peroxide (H_2O_2), a small water molecule, in a biocompatible single polymer shell [17–21]. In brief, we used a dual emulsion solvent evaporation method to encapsulate hydrophilic low molecular weight drugs (such as hydrogen peroxide) in PLGA microspheres and tested these microspheres in vitro in a 3D alginate matrix. In order for the hydrogen peroxide to decompose of only oxygen and water, catalase was immobilized to alginate by binding the -NH_2 group of catalase to the -COOH group of alginate by EDC(N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride)/NHS (N-Hydroxysuccinimide) chemistry [22,23]. According to previous studies, cell survival was 60–65% in hypoxic conditions of 1% O_2, but the rate increased up to 100% when oxygen-producing microspheres were added in the same conditions [15,16]. Thus, it was confirmed that oxygen was released in stable condition from polymeric matrix and supported cell survival in hypoxic condition and ultimately resulted in successful tissue regeneration (Figure 1).

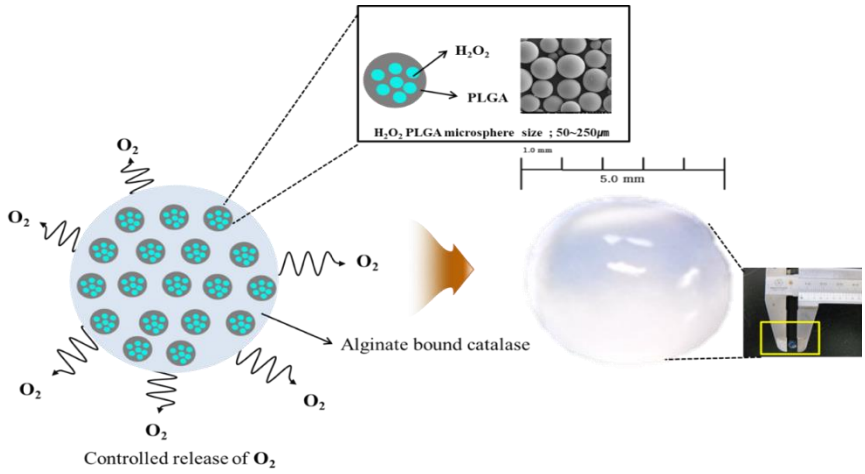

Figure 1. Concept of oxygen-releasing system for large-sized tissue regeneration; enhancement of cell and tissue survival by supply of oxygen.

2. Materials and Methods

2.1. Manufacturing Method

Materials used in this study are poly (lactic-co-glycolic acid) (LA:GA = 50:50, MW = 11,000 g/mol) (PLGA, Boehringer Ingelheim, Germany), polyvinyl alcohol (PVA, MW = 9000–11,000 g/mol), hydrogen peroxide (H_2O_2, 50 wt %, Sigma, MO, USA), sodium alginate (LVG, viscosity: 20–200 mPa·s, Nova Matrix, Norway), catalase (bovine liver, 2950 units/mg solid, Sigma, MO, USA), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, Sigma, MO, USA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, Sigma, MO, USA), N-Hydroxysuccinimide (NHS, Sigma, MO, USA), dichloromethane (DCM, Junsei Chemical, Tokyo, Japan) and deionized water (DW). All chemicals and solvents used in the study were of analytical reagent grade.

The method for manufacturing H_2O_2 PLGA microspheres (HPMs), which is an oxygen producing matrix, was described in a previous paper [17–21]. These HPMs were encapsulated using the $W_1/O/W_2$ double emulsion evaporation method. The HPMs were then coated with an alginate layer using the dripping method. Briefly, oxygen-releasing capsules (OCs) were formed using 1 mL of alginate (alginate concentrations of 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0% (*w*/*v*)) that was mixed with 0.3 mL of 3.0% (*w*/*v*) catalase at 24 h. After, adding 30 mg of HPMs, the mixture was vortexed. Thus, prepared microspheres were then dropped into a 1.0% (*w*/*v*) $CaCl_2$ solution for 5 min to form cross-linked capsules and washed three times with DW (Figure 2).

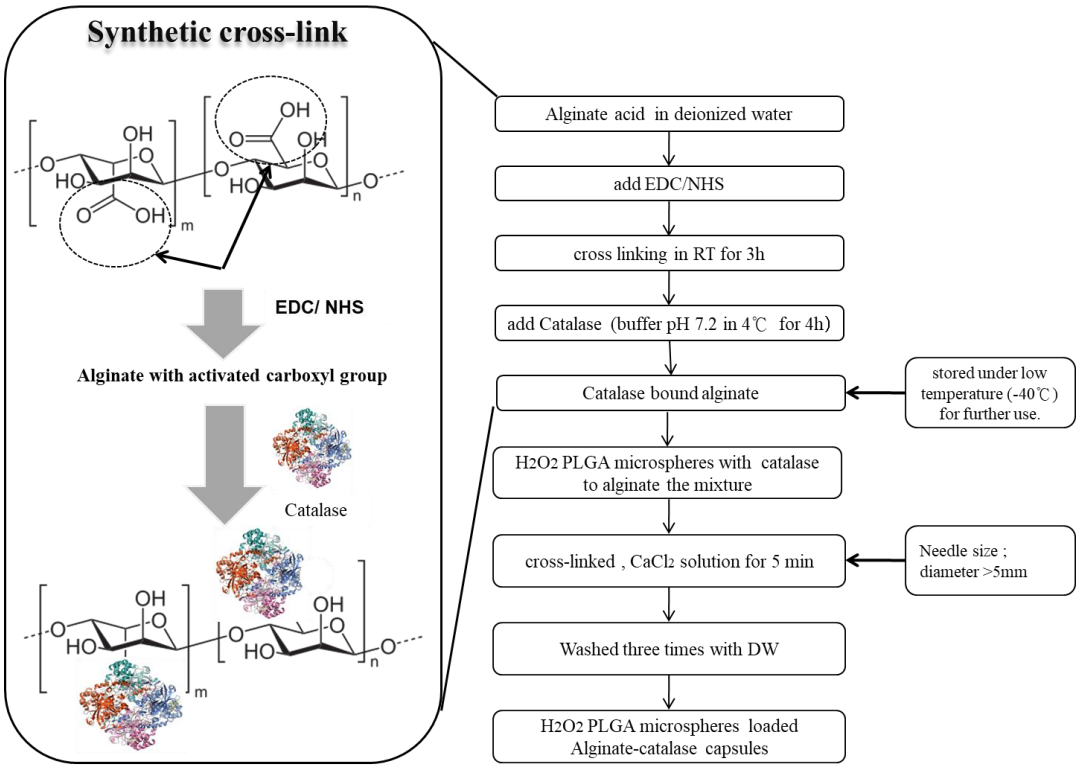

Figure 2. Fabrication process of H_2O_2 PLGA microspheres loaded alginate-catalase capsules.

2.2. Evaluation of Physical Properties

Morphological analysis was undertaken using field emission scanning electron microscopy (FE-SEM, Hitachi_S-4300 and EDX-350, Tokyo, Japan). Observations of cross-sectional and surface views of the OCs were captured using an optical microscope (Nikon ECLIPSE 80i, Tokyo, Japan) and confocal laser scanning microscopy (confocal, Carl Zeiss LSM700, Dublin, CA, USA).

2.3. Analysis of Oxygen Release Profile

A fixed amount of each OC was incubated in 10 mL of standard deionized water solution sealed within a vial and then evaluated for dissolved oxygen (DO). DO was measured over a one-week period using a dissolved oxygen meter (Thermo Scientific Orion Star™, Waltham, MA, USA) and these values were used to calculate the oxygen release profile for each OC.

2.4. Cell Compatibility Analysis

The survival rate of human umbilical vein endothelial cells (HUVECs) under hypoxic condition was also evaluated according to the time spent on the oxygen-releasing capsules. HUVECs were purchased (#C2517A, Lonza, Basel, Switzerland), and cultured in endothelial cell basal Medium-2 (EBM-2, Lonza) containing 10% FBS (Gibco, Waltham, MA, USA), 500 U/mL penicillin (Gibco) and 500 µg/mL streptomycin (Gibco). Cell suspensions were seeded at a density of 1×10^5 and then the OCs were added. Cell proliferation was evaluated using a CCK8 kit according to a manufacturer's instruction (Dojindo Laboratories, Rockville, MD, USA) after 1, 2, 3, 4, 5, 6, and 7 days of growth under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2, MIC-101, Billups-Rothenberg modular incubation chamber, Del Mar, CA, USA). The absorbance of each well was read at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) (n = 3).

2.5. Analysis of DAPI Labeling of Cells

To trace the fate of implanted cells within the scaffold, cells were labeled with DAPI fluorescent dye (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol [24]. Briefly, the cells were incubated in DAPI solution for 20 min at 37 °C and then washed with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen, Carlsbad, CA, USA) to remove any remaining DAPI. The labeled cells were then visualized using an immunofluorescent microscope (Olympus, Tokyo, Japan).

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Study

In order for gene analysis, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 µg of total RNA using a cDNA synthesis kit containing the superscript II RT-PCR, and oligo (dT) priming was performed according to the manufacturer's instructions (AccuPower® RT PreMix, Bioneer Inc., CA, USA). A total of 1 µL of cDNA was then amplified using each of the forward and reverse primers (GoTaqTM DNA polymerase, Promega, Madison, WI, USA). PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light. The relative expression of each gene was then evaluated using the house-keeping gene (GAPDH) (Solg™ Tag DNA polymerase, Daejeon, Korea) using the delta cycle threshold method. The PCR primers for HIF- 1α, VEGF, and GAPDH were designed using the published human gene sequences (Table 1).

Table 1. Primers and cycling conditions for reverse transcription PCR. HIF- 1α: Hypoxia-inducible factor 1-alpha; VEGF: vascular endothelial growth factor.

Gene	Primer Sequence	Product Size (nM)	Denature Temperature (°C)	Annealing Temperature (°C)	Extension Temperature (°C)	Cycles (times)
HIF-1 α	U: CTTCGGTATTTAAACCATTGCAT D: GGACAAACTCCCTAGCCCAA	25 25	95	54.5	72	32
VEGF	U: CTACCTCCACCATGCCAAGT D: GCGAGTCTGTGTTTTTGCAG	25 25	95	53	72	28

2.7. In Vivo Study

The present study followed the ethics criteria of the Institutional Animal Care and Use Committee at Kyungpook National University Hospital (KNUH 2018-08-008-003). Male Jcl:ICR mouse (4 weeks of

age) were given an intramuscular injection of ketamine hydrochloride (100 mg/kg, Yuhan Corporation, Seoul, Korea) and xylazine (10 mg/kg, Bayer Korea, Seoul, Korea) for anesthesia. The epidermis, with a diameter of 5.0 mm × 5.0 mm, made incisions at two places on each side of the back. There were two groups of samples: group 1 was transplanted without OCs, and group 2 was transplanted with Ocs. The top part of the skin was sutured with a blue nylon (3/0) to mark planted spots and fix capsules; at day 7, samples were retrieved for assessment.

2.8. Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) of the mean. Collected data were evaluated using t-tests, and difference type data were evaluated 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. Structural Characterization of Oxygen-Releasing Capsule

The uniformly smooth surfaces of the hydrogen peroxide microsphere alginate capsules are shown in Figure 3. HUVECs were assessed for viability following incubation with these capsules using a confocal and optical microscope. A large number of cells are observed both before and after OC treatment (Figure 3b). Cells are easily necrotic when they are trapped in a large-sized capsule, especially positioned in the vicinity of the center of the capsule where oxygen diffusion from outside is relatively more limited; however, all cells survived well in the capsule when sufficient oxygen was supplied from oxygen producing microspheres embedded in the capsule.

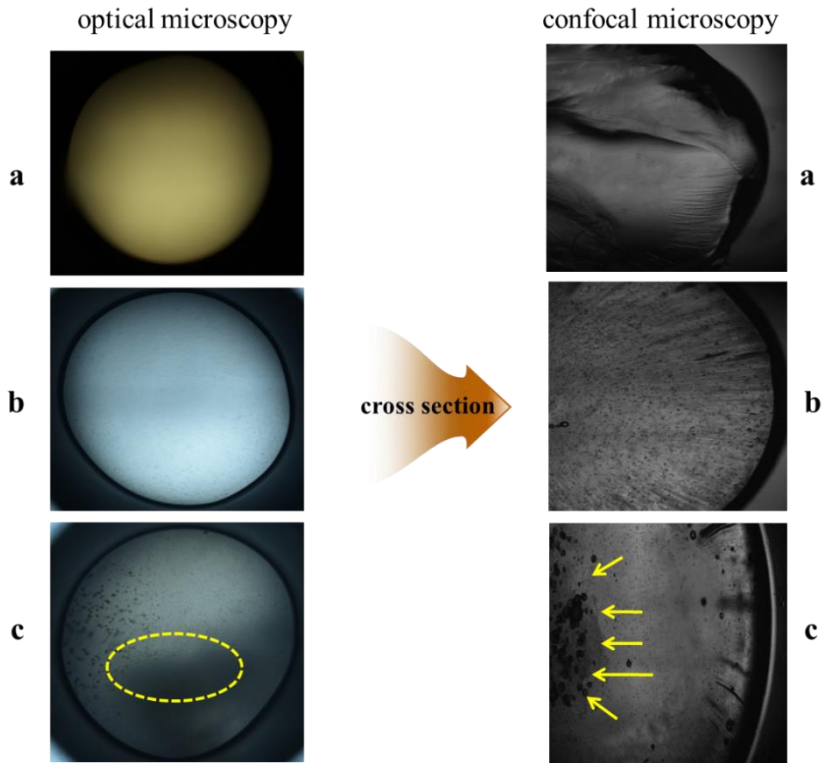

Figure 3. Capsule morphology observed by optical microscopy (left, ×50), and confocal microscopy (right, ×100): (**a**) control (**b**) cells-without Ocs*, (**c**) cells with Ocs. * Ocs: oxygen capsules.

From a study with HUVEC, in the absence of oxygen, cells were only found at the edges of the capsules, while in the capsules embedded with oxygen producing microspheres, cells were viable and distributed homogeneously throughout the capsule.

3.2. Evaluation for Effect of Alginate Ratio

Lower concentrations of alginate showed a higher release rate at the start of the experiment, but the oxygen release dropped after 7 days (Figure 4). This is likely due to the high porosity of the low concentration alginate. Conversely, increasing the concentration of alginate enabled the production of a more tightly packed coating, resulting in slower diffusion of hydrogen peroxide and prolonged oxygen release. In conclusion, the optimal alginate coating criterion was evaluated as 1.6% (*w*/*w*).

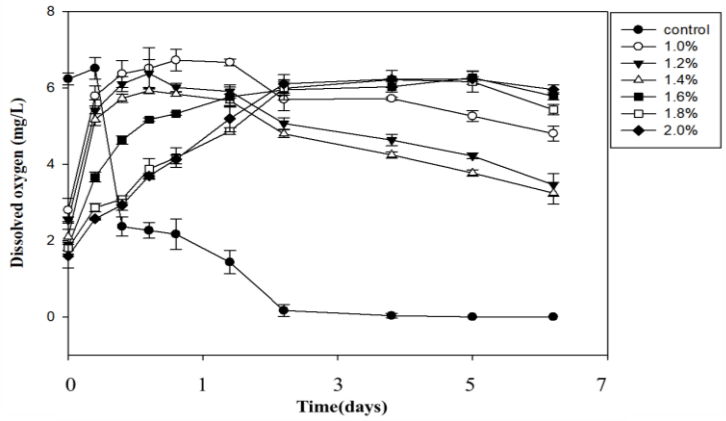

Figure 4. Effect of alginate concentration on the release profile of dissolved oxygen.

3.3. Biocompatibility Evaluation

The CCK-8 assay showed a consistent increase in the proliferation of HUVECs (Figure 5). HUVECs with Ocs showed the greatest viability after 3 days under hypoxic conditions, while HUVECs without oxygen capsules showed the lowest viability. Additionally, when Ocs were present, HUVECs showed a slight increase in cell growth rate for up to 7 days hypoxic conditions. This means that even in hypoxic conditions, cell viability will increase due to oxygen activation in oxygen capsule.

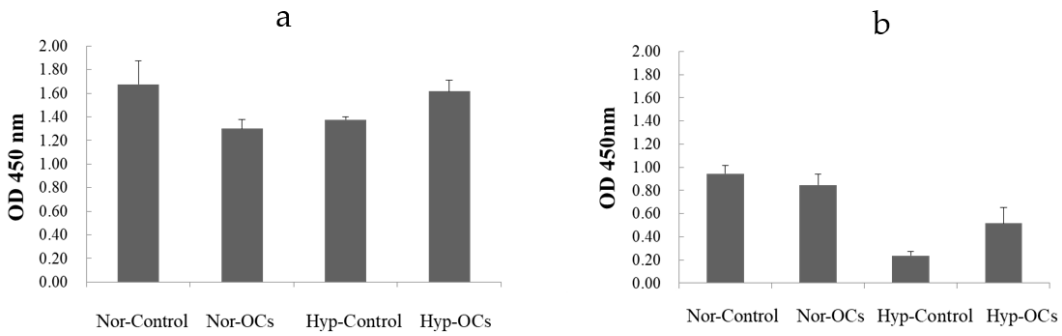

Figure 5. Human umbilical vein endothelial cell (HUVEC) proliferation assay was performed using cell counting kit-8 (CCK-8) for hypoxia condition (n = 3); after 3 days (**a**) and after 7 days (**b**). Nor: normoxia condition (5% O_2), Hyp: hypoxia condition (1% O_2)

3.4. Evaluation of the Oxygen Release Profile

DAPI staining was used to visualize the HUVEC nuclei. The three-dimensional characteristics of the oxygen-releasing capsules and the cellular interactions within these capsules were investigated using fluorescent microscopy (Figure 6). As a result, it was shown that cellular distribution was increased in those capsules that included oxygen-releasing capsules. In particular, the cells were found to be distributed for up to 7 days, indirectly proving that oxygen can be released for up to 7 days.

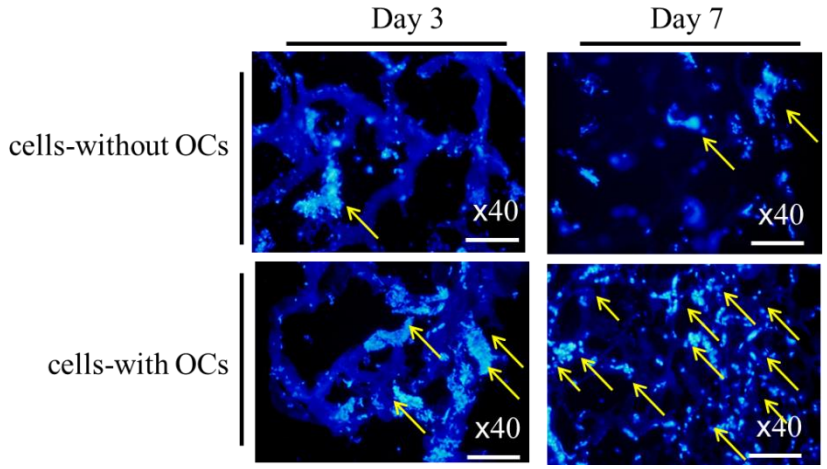

Figure 6. OC-embedded cells were visualized using DAPI staining and 3D fluorescence microscopy.

3.5. Gene Expression

The expression of the angiogenic factor, VEGF, was increased in cells treated with an oxygen-supplying capsule, which was well observed at day 3, and this effect was maintained at day 7. In the same context, the expression of HIF-1α, indicating hypoxic condition, was significantly decreased at day 7 compared to the control group due to oxygen supply by the capsule, even though not evident at the initial stage (Figure 7). Therefore, these genes' expression proved that the oxygen supplying by the capsule can promote angiogenesis, and the release was sustained for several days.

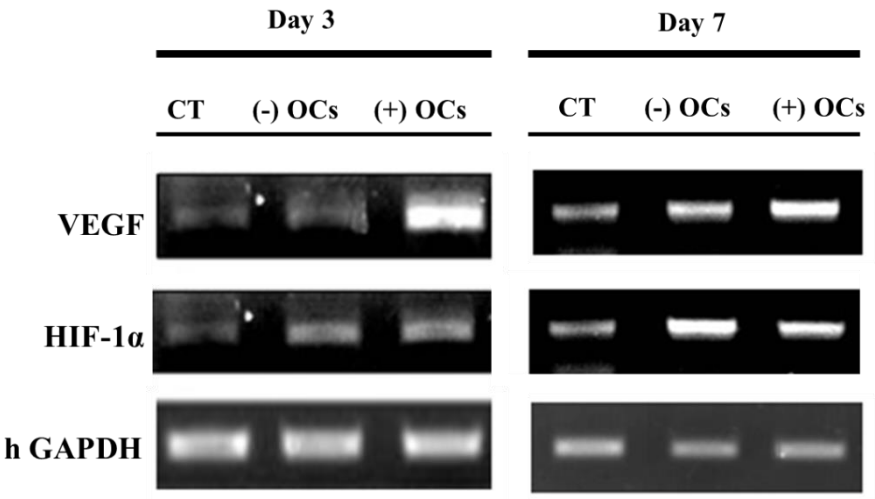

Figure 7. The mRNA expression in HUVECs after cultivation within various scaffolds. CT: control, (−) without, (+) with.

3.6. Evaluation of In Vivo

Each sample was covered with fiber, and it was difficult to extract the transplanted capsules. The oxygen-supplied capsules significantly decomposed when compared to the without Ocs group. Angiogenesis was also frequently found in subcutaneous tissue in the oxygen-supplied capsule group compared to the without Ocs group, suggesting promoted tissue regeneration by oxygen supply (Figure 8).

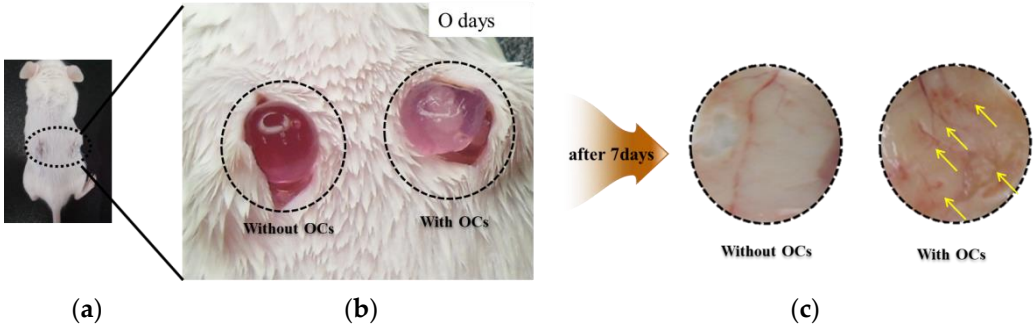

Figure 8. In vivo evaluation of the oxygen-supplied capsule. The transplanted capsules on the back of an Jcl:ICR mouse (**a**), the magnified images of the capsules at day 0 (**b**), and the significantly increased angiogenesis by the oxygen-supplied capsule at day 7 (**c**).

4. Discussion

In this study, we confirmed the efficacy and safety of the newly developed oxygen-releasing polymeric system fabricated by the microencapsulation process of a double emulsion solvent evaporation [25–27]. For optimally safe and efficient release of oxygen from a well-known oxidant H_2O_2, catalase immobilization onto alginate backbone was employed using EDC/NHS chemistry. Consequently, there is direct contact between H_2O_2 and cellular tissues, in which harmful and toxic cellular entities can be reduced, as the H_2O_2 is safely decomposed into water and oxygen in the presence of catalase.

In this study, large-sized alginate capsule of 5 mm in diameter was employed as a model of large-sized tissue. Cell survival in the 3D capsule with and without oxygen-releasing microspheres was evaluated in vitro.

Since oxygen from the atmosphere could also diffuse into the system, the experiments were performed in a hypoxic chamber (1% O_2) to minimize the amount of atmospheric oxygen that could diffuse into the system confounding our results. As expected, the production of oxygen from the OCs appeared to improve cell viability (Figure 4). Samples containing no oxygen-producing capsules experienced a significant reduction in viability (~50%) by day 3. This lack of proliferation followed by a continuous decrease in cell number is likely due to a lack of oxygen. This finding indicates that sustained levels of oxygen can be produced using this dual-layered matrix. Furthermore, these results indicate that oxygen-producing capsules may prove to be useful biomaterials for extending the viability of engineered tissues in vivo, allowing for better vascularization in the host. The ability to produce oxygen over an extended period could potentially enhance our ability to create larger viable 3D tissue grafts containing significantly more cells. With vascularization of tissue scaffolds estimated at 0.5 ~ 1.0 mm/day in tissue, maintaining cell viability during the regeneration process for 5 days could potentially facilitate the use of tissue scaffolds up to half a centimeter in size, significantly expanding the range of conditions that could be treated this way [28–30].

In this study, oxygen-producing capsules were constructed, and demonstrated to enhance cell viability under hypoxic conditions without toxic effects. These results suggest that oxygen-releasing capsules can provide an adequate environment for cells to overcome hypoxic conditions during tissue vascularization. These oxygen-producing capsules may play an important role in the future application of scaffold-based tissue engineering because they provide a solution for oxygen diffusion limitations in the engineering of large tissue implants.

Author Contributions: J.C. and J.O.L. conceived and designed the experiments; J.C. and S.Y.C. performed the experiments; T.G.K. and S.Y.C. analyzed the data; J.O.L. contributed reagents/materials/analysis tools; J.C. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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