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Research Article

Spirulina-PCL Nanofiber Wound Dressing to Improve Cutaneous Wound Healing by Enhancing Antioxidative Mechanism

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Skin regeneration is a complex process involving massive proliferation and alignment of cells, where there are obstacles to completion of regeneration, the main one being excessive generation of reactive oxygen species (ROS) from inflammation or infection. *Spirulina*, blue-green algae that has antioxidant and anti-inflammatory activities, has been used to relieve such ROS stress. In this study, *Spirulina* extract loaded PCL (*Spirulina*-PCL) nanofiber was evaluated as a cutaneous wound dressing in view of antioxidative mechanism. In addition to increasing fibroblast viability, the *Spirulina* extract and its dressing modulated intra- and extracellular ROS by enhancing antioxidant mechanism of fibroblast under oxidative stress. Finally, *in vivo* assays confirmed that *Spirulina*-PCL helps regenerate wounds and enhance regeneration. Taken together, the results of this study indicate that *Spirulina* and nanofiber have the potential for application to cutaneous wound to facilitate skin regeneration.

1. Introduction

Skin protects the body from external stresses and pathogens, and tissue regeneration is critical for maintaining body homeostasis when injured [1]. Skin is composed of the dermis and epidermis, and the extent of a cutaneous wound is measured based on how defective the layers are. In the case of full-thickness wounds, it is important to assist and control recovery of fibroblasts, which mainly occupy the dermal layer [2]. However, this takes a prolonged time while exposed to dangers such as infection and inflammation. Infection, fibroblast dysfunction, or ECM excessive synthesis leads to a lag in recovery or permanent scarring [3]. Overresponses of the immune system are a primary reason for slow wound regeneration [4]. Therefore, researchers have investigated methods to relieve the immune system, with a focus on ROS as a main factor to upregulate immune responses [5]. ROS are molecules generated during cellular respiration under normal conditions, but their overexpression at wound sites usually delays tissue regeneration [6]. For example, ROS disrupt DNA structure, oxidize proteins and lipids, and eventually cause cell and tissue death. ROS are synthesized by excessive oxygen supply and accumulation associated with active cellular metabolism during regeneration [7, 8].

Various antioxidants have been investigated for their potential to regulate ROS during tissue regeneration [9]. *Spirulina*, blue-green algae, is known as a bioactive supplement having physiological promoting effect to cell viability or survival [10, 11]. One of the major effects of *Spirulina* is its antioxidant effect. Phycocyanin, a major component, has been reported to suppress effects of oxidant and microbials to cells [12]. In addition, our previous study demonstrated that *Spirulina* extract enhances the viability of rat dermal fibroblasts; accordingly, *Spirulina* extract is expected to be a potential supplement in fibroblast culture [13]. However, in-depth researches are few about antioxidative mechanism of *Spirulina* extract on cutaneous wound.

In addition, to transfer the *Spirulina* effect well, there has been studied scaffold affecting fibroblast-reconstruction, which must pertain to ROS-scavenging effects, the major

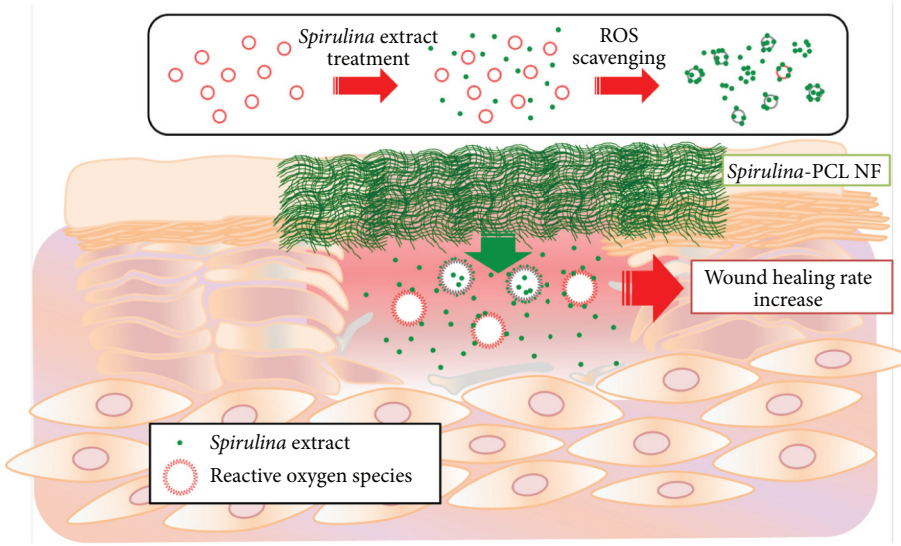


Figure 1: Schematic design of the *Spirulina*-PCL nanofiber application to cutaneous wound.

effect of *Spirulina* extract, without inflammation or cytotoxicity. In various forms of scaffolds that employ synthetic polymer, electrospinning is used as a dressing to cover wounds. Application of nanofibers to wounds can protect exposed tissues from external pathogens or drying of deficit sites [14, 15]. Pores in fibers make it easy to transfer embedded nutrients and air into the sites [16]. This helps produce suitable environments to guide cellular mitosis and migration to fill the deficit. Nanofiber has not only these structures and functions, but also superior mechanical strength to its basic materials and it is easily modified by blending or adsorption (Figure 1) [17]. Recently, some groups reported *Spirulina* loaded nanofibers and their applications to skin injuries [18, 19], but there is still insufficient information about *in vivo* efficacy and antioxidant mechanism of the dressing to cutaneous wound.

In this study, we evaluated *Spirulina* aqueous extract as an antioxidant during wound regeneration. The extract was applied directly to cells with or without hydrogen peroxide, which was found to reduce ROS synthesis and relieve decreased cell viability. Additionally, the activity of scavenging enzymes (catalase, CuZnSOD, MnSOD, and ecSOD) [20] was enhanced by the addition of *Spirulina* extract. These effects of *Spirulina* were also observed after embedding in electrospun nanofiber consisting of PCL, FDA-approved polymer, shows biocompatibility and safety to human. Based on these findings, we evaluated the potential for application of *Spirulina* extract and *Spirulina*-PCL nanofiber as a wound dressing using an *in vivo* wound model. *Spirulina* extract reduced the wound size significantly. Application of the nanofiber did not induce a dramatic change; however, it did show the potential to enhance regeneration as a wound dressing.

2. Materials and Methods

2.1. Rat Dermal Fibroblast Procurement. Primary fibroblasts were isolated from the dermal layer of 1-day old SD rats (Sprague-Dawley rats, Samtaco, Osan, South Korea) according to the manufacturer's instructions, with minor modification. Briefly, a piece of dissected skin tissue was incubated in HBSS with dispase 2 (1 mg/mL) (Gibco, Grand Island, NY, USA) for 24 hr at 4°C, after which the epidermal layer was peeled off and dermal layers were separated. The separated dermis tissue was then incubated with 1 mg/mL collagenase 2 (Gibco, Grand Island, NY, USA) in HBSS media for 24 hr at 4°C. Next, individual cells were harvested without tissue debris using a cell strainer with a 70 μm pore size (BD Biosciences, Franklin Lakes, NJ, USA) and cultured in DMEM high glucose medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (Gibco, Grand Island, NY, USA) and 1% (v/v) antibiotic-antimycotic (Gibco, Grand Island, NY, USA). Four passaged fibroblasts were prepared by subculture for all experiments.

2.2. Spirulina Extract. To obtain *Spirulina* extract, cells were mixed with 20 g/L of distilled water and stirred gently for 24 hr at room temperature. The solution was then centrifuged and filtered with Whatman™ No. 1 filter paper to remove the cell mass, which was subsequently lyophilized and then collected as powder.

2.3. Composition of Spirulina. Carbohydrates, proteins, lipids, nucleic acids, and chlorophyll a of *Spirulina* water extract were quantified. Carbohydrates were measured using the phenol sulfuric acid method. Briefly, 50 μL of *Spirulina* solution in water (2 mg/mL) was reacted with 150 μL of concentrated sulfuric acid (OCI, South Korea). The mixed solution was subsequently stirred at 80 rpm for 30 min, after which 30 μL of phenol (Daejung, South Korea) in water (5% w/v) was added to the solution and heated for 5 min at 90°C. The amount of carbohydrate was subsequently determined based on the absorbance at 490 nm (Tecan, Austria). Total protein was analyzed using a BCA assay, with

bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) as a standard.

Lipids were determined by the sulfo-phospho-vanillin reaction. Briefly, *Spirulina* solution was reacted with sulfuric acid at 90°C for 20 min to form carbonium ion. Vanillin (Sigma Aldrich, St. Louis, MO, USA) solution in phosphoric acid (Sigma Aldrich, St. Louis, MO, USA) was added to the solution, which resulted in formation of a purple color, after which the absorbance was measured at 540 nm using a microplate absorbance reader. Nucleic acid was detected at 260 nm after extraction using perchloric acid (Sigma Aldrich, St. Louis, MO, USA). Chlorophyll a was determined using a microplate absorbance reader based on the absorbance of methanol extracts at 665.2 nm (Sigma Aldrich, USA).

2.4. Fabrication and Characterization of Nanofibers. PCL (15% w/v) was dissolved in THF and DMF solution at a ratio of 7 : 3. *Spirulina* samples were added at 1% (w/v) to 15% (w/v) PCL. The solutions were then electrospun using a distance between the 18-gauge needle and collector of 15 cm and a power of 15 kV. The fabricated nanofiber was stored in vacuum to remove solvent entirely and its removal was checked with FT-IR. The nanofiber mats were subsequently observed using a scanning electron microscope (SEM) (Hitachi S-4300, Japan). From the images, we measured the diameter of each nanofiber mat using an Image J program. We measured over 200 points of nanofiber diameter. To confirm the addition of the extract, the contact angle was measured and its differences were compared. Based on the contact angle, we determined the effects of embedding *Spirulina* extract on the hydrophobicity of the PCL nanofiber. Briefly, a 4 μL droplet of distilled water was poured onto the nanofiber mat through a 22 G needle, after which the contact angle was measured using a Phoenix Touch 300 contact angle analyzer (Surfaceware 7, SEO, South Korea).

2.5. ROS Detection. Reactive oxygen species (ROS), the major stress during long-term culture, were analyzed with the fluorescent reagent, CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA). The fluorescent reagent was reconstituted to stock solution with DMSO shortly before analysis and then sealed until use. In this assay, there were two groups of experiments: 2-dimensional culture using 24-well plate and 3-dimensional culture using 1 cm^2 scaffold. The prepared cells under each condition and on each scaffold were incubated for 11 days. The samples were incubated in a CO_2 incubator with diluted reagent of stock solution in PBS at a concentration of 10 μM CM-H2DCFDA for 10 min. At 2-dimensional culture, the reagent solution was subsequently exchanged for PBS solution, and samples were observed by fluorescent microscopy and quantified. At 3-dimensional culture, the pieces of scaffold were then washed with PBS three times and quantified. To quantify its florescence, cells were lysed with 100 μL of RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), after which the lysate was standardized by BCA assay and measured using a fluorescent reader. The excitation wavelength was 492 nm and the emission wavelength was 525 nm. These measured values were converted to relative values based on the value of the cell culture plate.

2.6. MTT Assay. An MTT assay was performed to evaluate the viability of dermal fibroblasts on the nanofibrous mat. MTT solution was prepared by mixing DMEM-H and MTT at a ratio of 9 : 1. Initially, culture media was eliminated and 500 μL of MTT solution was added and incubated in a CO_2 incubator at 37°C for 1 h. After 1 h, MTT solution was eliminated and DMSO 1 mL was added. The produced crystal formazan was then dissolved in DMSO by stirring for 1 h, after which the optical density of DMSO solution that had dissolved crystal formazan was measured using a microplate reader at 540 nm.

2.7. Enzyme Activity Assay. To assess the potential of *Spirulina* extract to reduce ROS using intracellular antioxidant enzymes, the activities of representative enzymes such as total SOD and catalase were analyzed. Each enzyme activity was observed using an enzyme activity assay kit (Abcam, UK). *Spirulina* extract was applied to confluent cells in a 25T flask at 25 μg/mL concentration or not (control). The cells on each surface were cultured for 4 days. To observe the cellular response to direct stimulation of ROS, 200 mM hydrogen peroxide was applied under the same conditions for 24 hr, after which cells were harvested for detection of enzyme activities.

2.8. Gene Expression Quantification of Antioxidant Enzymes. Enzyme expression levels were measured against oxidative stress using primers specific for catalase, CuZnSOD, ecSOD, and MnSOD (Table 2). The samples to use at the assay were cultured at the same conditions as the sample of enzyme activity assay. The RNA of fibroblast at each condition was extracted using RNeasy Mini Kit (Qiagen, Germany). Total RNA contents were extracted from fibroblasts cultured under the same condition as enzyme activity using RNeasy Mini Kit. The quantity and purity of RNA were measured based on the optical density ratio at 260 nm and 280 nm. The total RNA was reverse-transcribed using QuantiTecT Reverse Transcription kit (Qiagen, Germany). The reverse-transcribed RNA was measured via Rotor-Gene Q PCR (Qiagen, Germany). The cDNA was synthesized using a QuantiTecT Reverse Transcription kit and qRT-PCR was conducted using Rotor-Gene Q PCR system (Qiagen, Germany). PCR was conducted under the following conditions: initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. To compare RNA expression values, threshold values were determined at 0.1 and RNA samples were normalized to β-actin.

2.9. In Vivo Assay. An *in vivo* assay using SD rats was performed to evaluate the effects of *Spirulina* extract or imbedding scaffold on full-thickness wounds. Experimental method in accordance with the ethical issues was carried out as specified by the INHA-IACUC (Inha University-Institutional Animal Care and Use Committee) (Approval Number: INHA 150924-381). All animal experiments were

Table 1: Gross composition of the *Spirulina* aqueous extract.

Components	Composition (%)
Carbohydrates	22.21
Proteins	48.38
Lipids	6.47
Nucleic acids	2.41
Chlorophyll a	0.36
Ashes	20.17

conducted on blinding. For the experiment, 4-week-old SD rats were allowed to acclimatize for 1 week, after which they were anesthetized via inhalation using isofuran (Hana Pharm, South Korea). The back of the anesthetized rat was shaved with an electric razor, sterilized with povidone-iodine (Sungkwang, South Korea), and washed with 100% ethanol. Full-thickness wounds were then created on the left and right of the spine uniformly using a 0.8 mm diameter round punch.

In vivo assays were processed as two groups of experimental sets. First, gauze soaked with PBS and *Spirulina* extract solution (6 mg/mL) was used to evaluate *Spirulina* extract. To evaluate the *Spirulina* extract imbedded in the nanofiber mat, gauze, PCL nanofiber, *Spirulina*-PCL nanofiber, and a commercial product (Duoderm; 3M, St. Paul, MN, USA) were compared in second experimental sets. Each sample was fixed with Opsite Flexifix (3M, USA) and prevented from physically contacting the rats with a compress. At days 3, 7, and 12 after creating wounds, the wound size was observed and the wound tissue was recovered to observe its anatomical morphology and regeneration by cryosection and H&E staining.

2.10. Statistical Analysis. All experiments were analyzed at least three times and data were expressed as the average ± SD (standard deviation). Data were graphed using Sigma plot (Systat Software Inc.). Differences between groups were identified by Student's t-test, with a p value taken to indicate significance ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3. Results and Discussion

3.1. Characterization of Spirulina Extract. The composition of *Spirulina* extract is shown in Table 1. The most abundant compounds were proteins and carbohydrates. *Spirulina*, which is commonly used in dietary supplements, is known to contain many bioactive proteins including antioxidants and anti-inflammatory compounds. As shown in Table 1, the extract contained abundant proteins and was expected to have bioactive materials. Therefore, a ROS assay was performed to evaluate its antioxidant properties.

3.2. ROS Scavenging Effect of Spirulina Extract. Stress intensity was determined based on the production of ROS, a major stress factor, under various conditions [21]. As shown in Figure 2 and Supplementary Material 1 available online at http://dx.doi.org/10.1155/2016/6135727, visualization of ROS by fluorescent microscopy revealed significantly different amounts among samples. In both cases, *Spirulina* extract attenuated cellular ROS synthesis during culture. As shown in Figure 2(a) and Supplementary Material 1a, 25 μg/mL of *Spirulina* extract reduced an intensity of ROS synthesis compared to that obtained using 0 μg/mL of *Spirulina* extract. As shown in Figure 2(a), the lowest ROS intensity was observed in response to treatment with 25 μg/mL of *Spirulina* extract. Additionally, *Spirulina* extract relieved intracellular ROS slightly under normal oxygen concentrations without additional stress. This intracellular ROS was primarily produced from mitochondria [22, 23]. Cells rarely generate ROS as a byproduct of respiration under normal conditions, and the low levels of ROS that are produced are scavenged immediately [24]. However, when ROS accumulate, they are known to have adverse effects on cell mitosis or other functions. Overall, ROS denatures the components of cells and their organelles, and the stress derived from ROS further decreases cellular viability and metabolic activity [25]. However, under these *in vitro* conditions, *Spirulina* extract treated fibroblasts produced less ROS, which implies that it played a role in modulation of ROS stress under wound-like conditions.

In addition to intracellular ROS, there are ROS derived from phagocytosis and other processes. Direct exposure of ROS to cells has negative effects on cell survival and functions. External ROS molecules destroy the cellular membrane directly or infiltrate into cytosol and induce apoptosis [26, 27]. As a result, ROS-exposed cells are more readily denatured than cells in a normal state. At wound sites, pathogens or microbes are able to contact the tissue under the epidermis, dermis, hypodermis, blood vessels, and so forth. These infectious agents cause inflammation and other critical symptoms. To eliminate this danger, phagocytes like macrophages generate ROS for sterilization and phagocytosis; however, the ROS can affect cells in the body such as fibroblasts. To evaluate the effects of *Spirulina* extract, ROS derived from phagocytes were replaced with hydrogen peroxide. As shown in Figure 2(b) and Supplementary Material 1b, *Spirulina* extract mitigated the effects of stress from extracellular ROS. In Figure 2(b), the extract was good at scavenging extracellular ROS and reduced the amount of ROS that entered cells significantly. Additionally, the amount of synthesized ROS was reduced, and we expected that *Spirulina* extract would have a positive effect on defense from intra- and extracellular ROS. The expression of synthesized ROS was lowest in response to 25 μg of *Spirulina*, indicating that *Spirulina* extract has potential for use as a defense against intra- and extracellular ROS.

3.3. Effect of Spirulina Extract on ROS Related Pathways. Enzyme assays performed to define the mechanisms of ROS scavenging showed that *Spirulina* extract affected enzymes (Figure 3). Comparison of nontreated hydrogen peroxide samples and *Spirulina* treated samples revealed that intracellular catalase and extracellular SOD activity were both higher activity in the *Spirulina* treated samples. These results showed that *Spirulina* extract has the potential to enhance cellular activity against ROS. These intracellular enzymes were especially effective against mitochondria-producing ROS [28], which cause apoptotic cell death by loss of DNA

Table 2: Primer information for real time-qPCR.

Gene	Forward ($5' \rightarrow 3'$)	Reverse ($3' \rightarrow 5'$)
Catalase	GGCACAAGCCTCACCAGTAA	CCCTCGGGAAATGCCATCAA
MnSOD	CGGGTTTCTGAGTGAGGTCAG	GTCAGGGAAAGGGTGTCCTTC
CuZnSOD	TCACTTCGAGCAGAAGGCAA	TGAGGTCCTGCAGTGGTACA
ecSOD	GAGAGCTTGTCAGGTGTGGAA	CGGACTCTCCGGTATCTGAC
β-actin	CCGCGAGTACAACCTTCTTG	CATGCCGGAGCCGTTGTC

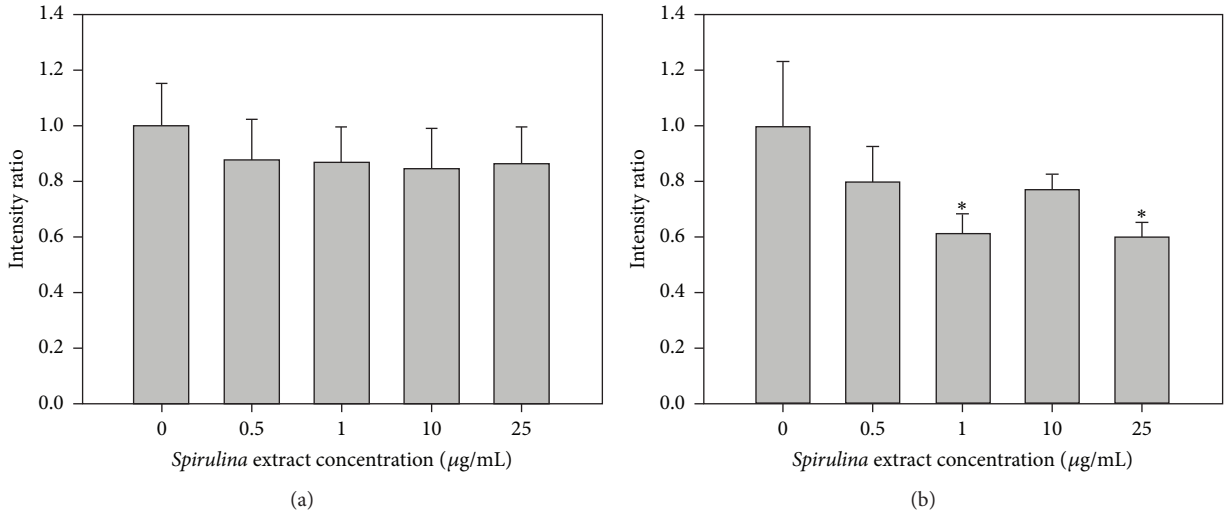


Figure 2: Cellular ROS quantification by serial treatment (0, 0.5, 1.0, 10, and 25 μg/mL) of (a) *Spirulina* extract and (b) the extract with 200 mM of hydrogen peroxide. Error bars represent mean ± SD. $n = 9$; $^{*}p < 0.05$.

denaturation or DNA repair proteins. Furthermore, ROS can directly contact the inner molecules and organelles without cell membrane penetration. In the case of hydrogen peroxide treatment, ROS were present around cells and attacked proteins and lipids of the cellular membrane. This resulted in loss of functions of the cellular membrane (e.g., cell adhesion, maintenance of cell structure, nutrient transport, and byproduct production) [29]. As a result, cell viability decreased via apoptosis or necrosis. However, treatment with *Spirulina* extract resulted in direct removal of the hydrogen peroxide via its action as a ROS scavenger. In addition, the activity of SOD was enhanced by treatment with *Spirulina* extract (Figure 3(b)). Extracellular SOD (ecSOD) was applied to the extracellular region to scavenge ROS and modulate its concentration [30]. Additionally, SOD protects ECM from degradation by ROS and prevents inflammation derived from ECM loss. However, excessive ROS can inactivate ecSOD, limiting its scavenging effects. Previous studies have reported that ecSOD inactivation appeared under hypertension conditions, which cause cells to produce excessive superoxide anions. In wound regions, there are several causes of superoxide anion production, including high oxygen levels and macrophages [31, 32]. This speculation has also been presented in other studies. For these reasons, we suggest that *Spirulina* extract protected ecSOD molecules from hydrogen peroxides and the scavenger led to a synergistic increase in cellular activity on both intra- and extracellular regions. Overall, the enzyme enhanced activity by *Spirulina* extract could cause rapid response to cellular stress and prevent decreased viability and metabolic activity.

3.4. ROS Related Gene Expression following Treatment with Spirulina Extract. Before *in vivo* assay, *Spirulina* extract was embedded in PCL nanofiber, which is widely used as a medical material, and evaluated to determine if the extract was subsequently released and acted on the cells. The safety of the nanofiber was confirmed whether solvent remained or not. Any peak of solvent such as 1676 cm^{-1} was not observed at all nanofiber (Supplementary Material 2). As shown in Figures 4(a) and 4(b), *Spirulina*-PCL nanofiber was well-fabricated with 6.67% *Spirulina* extract in PCL, exhibiting characteristics similar to the PCL nanofiber. Diameter of PCL and *Spirulina*-PCL nanofiber was 0.77 ± 0.21 and 0.90 ± 0.54 μm, respectively. Structures of both nanofibers have pores of the mesh and cells can infiltrate into the pores and attach at threads forming 3-dimensional morphology [33–36]. The cell infiltration was shown at our previous studies [33, 37]. However, the contact angle was different due to the embedded extract. Specifically, *Spirulina*-PCL nanofiber had a larger angle than PCL nanofiber, indicating that the *Spirulina*-PCL nanofiber was more hydrophilic. Scaffolds with the same topology were used in the ROS assay (Figure 4(c)). The results also revealed that both nanofibrous scaffolds reduced intracellular ROS production of cells in

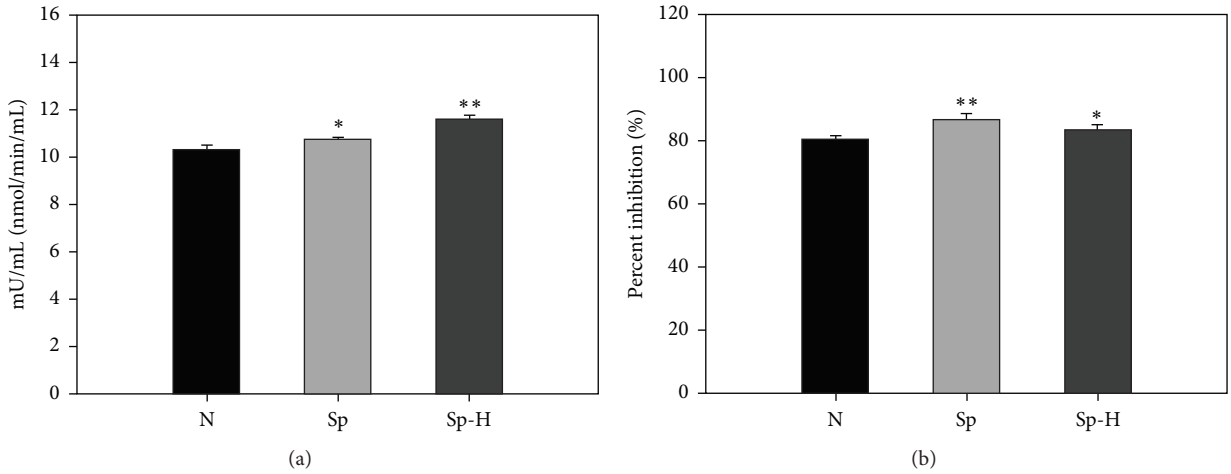


Figure 3: The quantified graphs of ROS related enzyme activities: (a) catalase activity and (b) SOD activity. Error bars represent mean ± SD. $n = 9$; $^{*}p < 0.05$; $^{**}p < 0.01$.

each scaffold. The ROS synthesis was reduced by replacing the surface TCPS by PCL nanofiber. PCL nanofiber did not have any antioxidant compounds but the result showed reduction of ROS level. However, *Spirulina*-PCL nanofiber was more effective than PCL nanofiber. In addition, the nanofiber helps to release *Spirulina* extract in the long term. This control release helps to apply the extract at specific site and increase its treatment time. These findings imply that *Spirulina* extract acts as a ROS scavenger, even though the extract was embedded in PCL nanofiber and has the potential to act as a supplement in tissue engineering and regenerative medicine.

The same effective concentration of *Spirulina* extract at which fibroblasts showed the lowest ROS level in 2-dimensional culture was embedded into the nanofibers. After incubation for days 1, 3, and 5, cell viability was measured (Figure 4(d)). At days 1 and 3, there were no differences in viability on PCL nanofiber and *Spirulina*-PCL nanofiber. However, at day 5, the viability of the *Spirulina*-PCL nanofiber was higher than that of the PCL nanofiber. *Spirulina* is well known to have anti-inflammatory and antioxidant effects. Additionally, previous studies confirmed that cellular viability was enhanced by *Spirulina* in culture media. The effects of the *Spirulina*-PCL nanofiber were similar to those observed in the MTT assay, indicating that *Spirulina* embedded in the PCL nanofiber successfully enhanced cellular viability. This level of *Spirulina* also exerted a positive effect on 3-dimensional culture with nanofibrous mats. Taken together, these findings indicate that *Spirulina* has potential for treatment of tissue.

The antioxidant effects of *Spirulina*-PCL nanofiber were clarified based on the mRNA levels. The major antioxidant enzymes, MnSOD, CuZnSOD, ecSOD, and catalase, were analyzed. It is well known that cell conditions become worse in response to adverse effects, leading to apoptosis. In this situation, antioxidant enzymes protected cells, reducing the damage caused by ROS, and increasing its expression level induced resistance to ROS [38, 39]. However, excessive ROS stimulation worsened the cell condition while decreasing the antioxidant enzyme expression level. Therefore, we identified the expression level of ROS enzymes by RT-PCR, which confirmed the mechanism of ROS resistance.

In 2-dimensional culture, the expression levels of all antioxidant enzymes (excluding catalase) were enhanced, with those of ecSOD showing an especially high increase when *Spirulina* extract was added (Figure 5(a)). The addition of *Spirulina* extract to cultures increased cell viability, while decreasing ROS generation, indicating that *Spirulina* extract was not a ROS inducer or a cytotoxic molecule. Finally, *Spirulina* extract in culture media interacted with the cell and induced ROS resistance. However, when the *Spirulina* extract was treated, the cells did not show dramatic expression of catalase at hydrogen peroxide addition. Under high ROS resistant conditions, it would be hard for ROS to remain in cytosol and then it would affect less mitochondria.

In 3D culture using nanofiber, the addition of *Spirulina* extract led to decreases in most expression levels, except for ecSOD under nonoxidative stress conditions (Figure 5(b)). The presence of MnSOD, CuZnSOD, and catalase as ROS enzymes present in the cell led to variations in expression levels depending on the intracellular concentration of ROS. When the enzyme expression decreases, there would be two contradictory possibilities: decrease of enzyme activity in cells or stable cell state by lack of stress to the enzymes. In this study, the latter was suggested; when the expression levels of the enzymes are reduced, it implied that cells had received less stimulation of ROS. These findings were confirmed when compared to the intracellular production of ROS and viability (Figures 3 and 4(c)). *Spirulina* nanofiber also reduced ROS synthesis with stable viability. The inclusion of *Spirulina* in the nanofiber effectively conveys the antioxidant in the cell, providing a beneficial environment that can maintain stable cell state against ROS [40]. Upon addition of the hydrogen peroxide, cells not treated with *Spirulina* showed lower enzyme expression ratios, while enzyme expression of cells treated with *Spirulina* extract increased. Some of

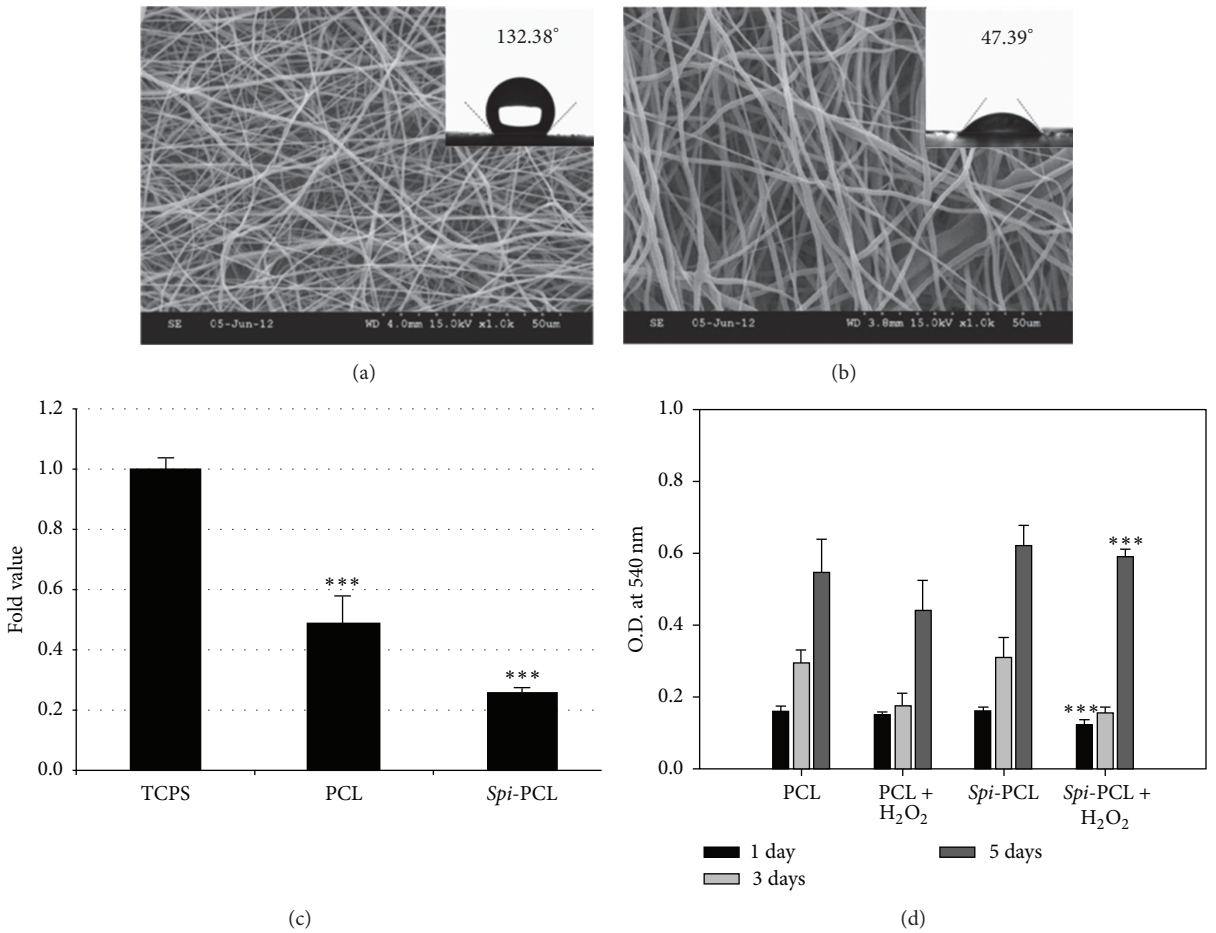


Figure 4: The characterizations and cellular behavior assays with the nanofibrous scaffolds. SEM images and contact angle assay of (a) PCL nanofiber and (b) *Spirulina*-PCL nanofiber. (c) Quantification of cellular ROS synthesis at each scaffold. (d) Cellular viability at each condition measured by MTT assay. Error bars represent mean ± SD. $n = 9$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

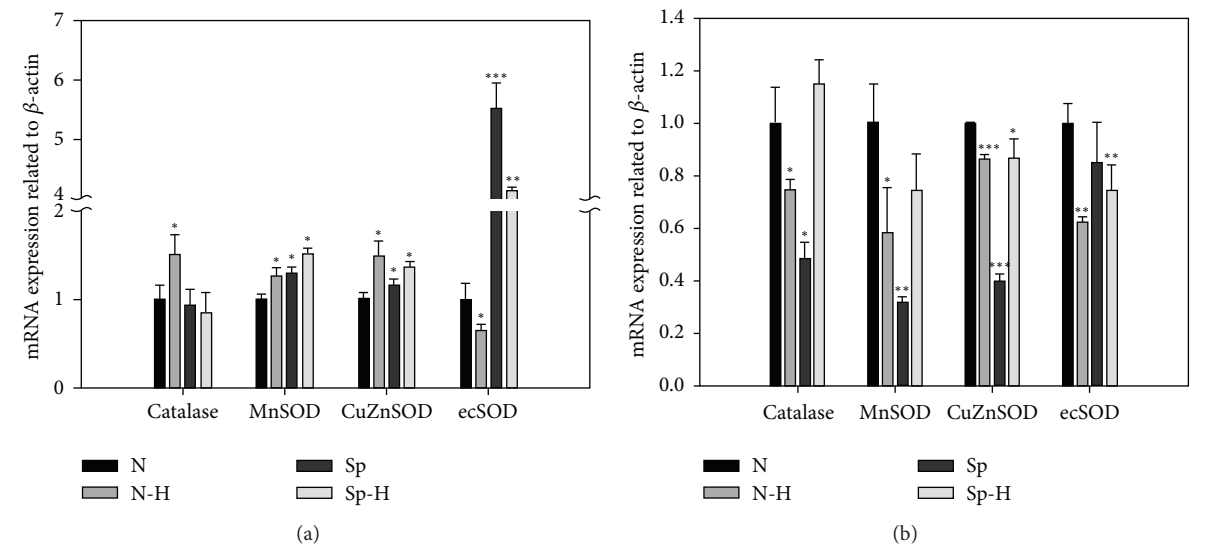
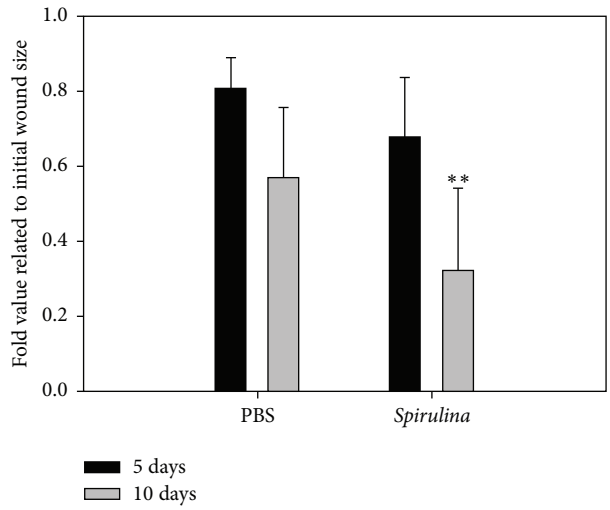
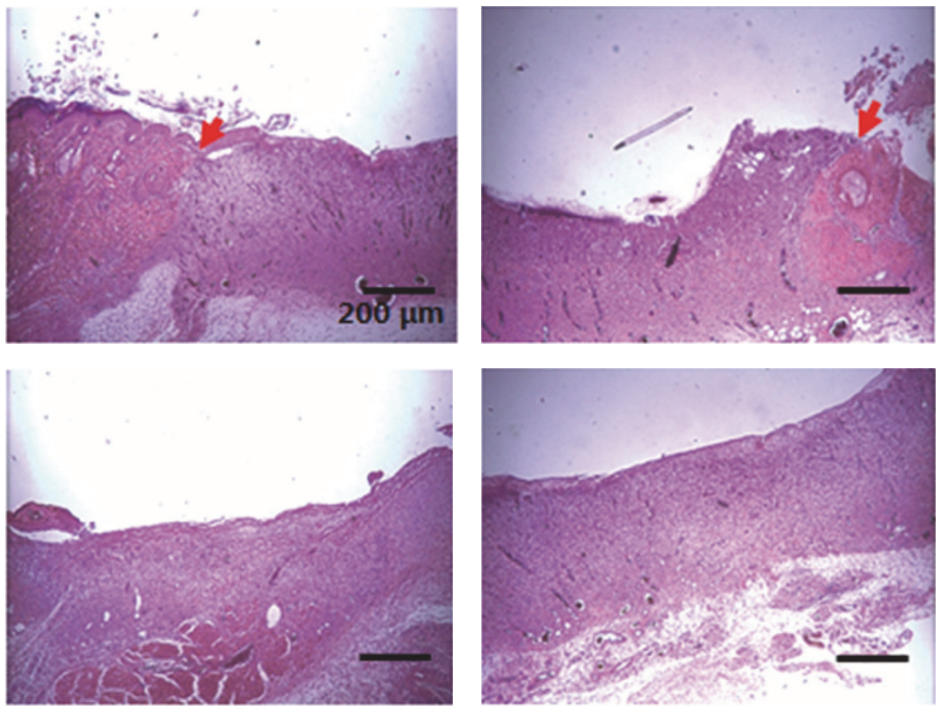


Figure 5: Gene expression assay of ROS related genes at each culture condition: (a) gene expression at 2-dimenstional culture and (b) gene expression at 3-dimenstional culture. Error bars represent mean ± SD. $n = 9$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.



(a)



(b)

Figure 6: *In vivo* assay to determine efficacy of *Spirulina* extract and nanofibrous mat at cutaneous wound healing: (a) wound size changes with PBS and *Spirulina* extract treatment for 10 days and (b) histography of each sample treatment: (1) gauze treated, (2) PCL nanofiber treated, (3) *Spirulina*-PCL nanofiber treated, and (4) commercial product treated. The arrow indicates the boundary of not aligned tissue by wound. Error bars represent mean ± SD. $n = 9$; $^{**}p < 0.01$.

the enzyme catalase and ecSOD levels were higher than those of untreated cells. Cells in the absence of stimulation were stable; however, it showed rapid increases in expression upon addition of stimulus and reacted more effectively when cultured with nanofibers containing the *Spirulina* extract.

3.5. In Vivo Assays for Evaluation of Spirulina Extract. *In vivo* tests demonstrated the effects of the nanofiber on skin, which has complex reactions with various components. First we applied *Spirulina* extract solution itself and evaluated its effect on full-thickness wound, which revealed a rapid decrease in the size of the wound (Figure 6(a)). Specifically, the average size differed between groups from day 5, and this difference continued until day 10. These results demonstrated that the effects of *Spirulina* extract *in vitro* also occurred *in vivo*. *Spirulina* extract enhanced cell viability and reduced stress derived from ROS through several pathways. Many studies have shown that ROS could enhance cell proliferation

[41]; however, many studies also showed that excessive ROS induced apoptosis of cells [41, 42]. Based on these findings, a balance of ROS level is required to enhance the regeneration of wounded skin. As shown in Figure 6, the efficacy of regeneration increased, which implied that the ROS reducing effect of *Spirulina* extract played a role in the balance of ROS levels. To determine if *Spirulina* extract maintains its ability in nanofiber, the nanofiber samples were put on the wound (Figure 6(b)). Histography revealed that the section of *Spirulina* imbedded scaffold and commercial product showed greater arrangement of the dermis layer than gauze and PCL nanofiber. The arrangement of the dermis is a step of skin regeneration that occurs during the final step. In addition, epidermis started to cover the dermis slightly except for gauze and PCL nanofiber treated sample. Furthermore, no additional side effects were observed on *Spirulina* embedded nanofiber compared with commercial product, which confirmed its biocompatibility. Taken together, histography of the dermis and epidermis indicated that the nanofibrous scaffold is not harmful to skin wounds and that *Spirulina* extract is not denatured during scaffold formation. The detailed effects and efficacy should be investigated in a future study.

Overall these findings indicate that *Spirulina* extract functioned to suppress excessive ROS at wound site, leading to lower inflammation and higher tissue regeneration. Almost conventional wound dressing is a patch form having only the function of maintaining humidity of wound site. This maintained humidity prevents scab formation and maintains the activities of complements, but it does not affect ROS stress. However, the *Spirulina* extract imbedded PCL nanofiber has more advantages than other wound dressings in that it enhances the function of ROS suppression, maintaining humidity by structure of nanofiber. The application of *Spirulina* extract to cutaneous wounds has sufficient potential to regenerate wounds via antioxidant mechanisms and could be used as a supplement for nanofibrous patches.

4. Conclusions

Spirulina is typical blue-green algae well known as an antiagent of inflammation and oxidant that is widely used as a food supplement. The bioactive effects of *Spirulina* can positively regenerate skin wounds, especially by exerting antioxidant effects that relieve cells that have been exposed to excessive ROS by inflammation and phagocytosis. In this study, we confirmed that *Spirulina* aqueous extract reduced ROS levels in cells via increased cell resist against ROS. These findings were also observed in 3-dimensional cultures. Even though *Spirulina* extract released from nanofiber reduced enzyme activity to levels observed in the nonstressed state, the enzyme activity was dramatically accelerated in the presence of excessive ROS. This extract also accelerated the regeneration rate of skin wounds. Based on these findings, *Spirulina* embedded nanofibers have the potential for application to skin wounds and to regenerate skin.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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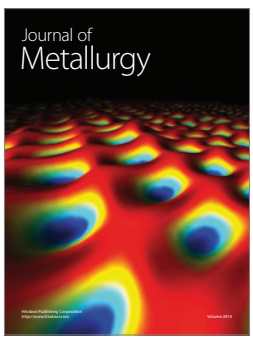
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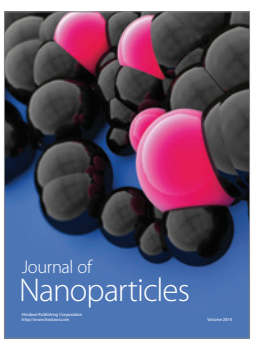
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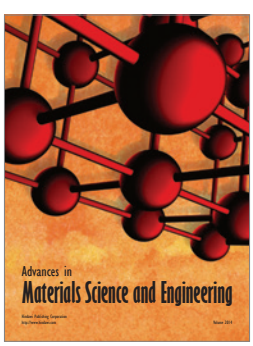
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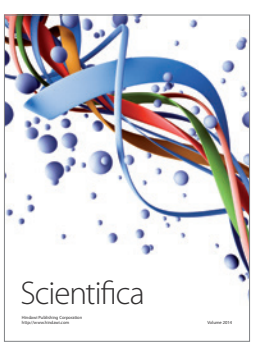
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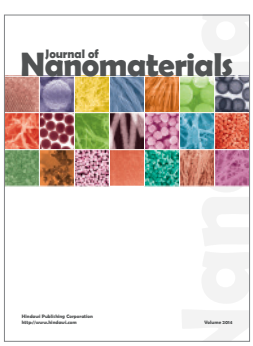
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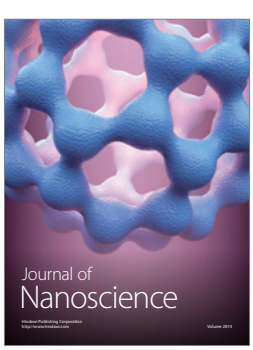
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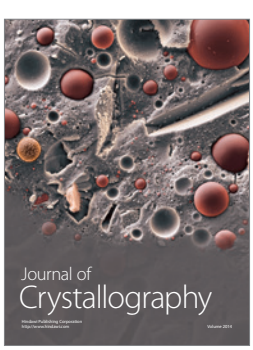
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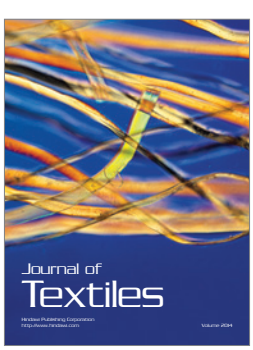
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