In vivo evaluation of wound healing property of zinc smectite using a rat model

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Zinc, magnesium and silicon ions are known as bioactive ions that promote the hard and soft tissue regeneration. We hypothesized that Zn-smectite consisting of zinc and silicon ions provide a more potential biomaterial in regenerative medicine field. Zn-smectite powder was thus synthesized in this study and used for wound healing. The aim of the present work was to evaluate the effect of Zn-smectite on wound healing in rats. An aqueous solution was prepared by dissolving zinc chloride (ZnCl₂) and water glass in distilled water, and then refluxed at 90°C for 12 h; after which crystalline Zn-smectite powder was obtained. The amount of Zn²⁺ and Si⁴⁺ ions releasing from Zn-smectite in physiological saline was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). It was observed that the amount of the Zn²⁺ ion released from the Zn-smectite powder increased with the soaking time, but the released amount of Si⁴⁺ ion decreased with the soaking time. To evaluate the biofunctional ability, Zn-smectite was applied to a full thickness surgical wound created in the rat’s abdomen. In the results for skin regeneration, there was no significant difference in wound size between the control and Zn-smectite group at 1 and 2 weeks. However, it was observed that Zn-smectite promoted greater vascular regeneration and more skin appendages in comparison with the control. Zn-smectite can be expected to stimulate wound healing and heal the tissue close to the native tissue by releasing Zn²⁺ and Si⁴⁺ ions, suggesting that this newly formed material could offer greater potentiality in the field of regenerative medicine.

Key-words : Zn-smectite, Powder, Ion release, Angiogenesis, Wound healing

1. Introduction

Skin is the largest organ in the human body, which acts as a biological defense against toxic bacteria and harmful UV-rays. In clinical treatment, skin transplantation using autograft, allograft and xenograft has been used to heal full-thickness skin defects. However, skin transplantation has clinical problems, including invasive surgical procedures, intense immune rejection and severe wound contraction. Therefore, development of a bioengineering material for regeneration of lost skin functions is needed.

During the wound healing process, it is important to promote angiogenesis in the early stage of wound healing that helps in the growth of skin appendages. Effective wound treatment material should have the capacity of stimulating angiogenesis, because the blood vessels play a role in nutrient and oxygen transport to the cells of the defective portion. To promote angiogenesis in the wound healing process, the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are widely used in clinical application. However, clinical application of these growth factors has several disadvantages such as high cost and the loss of bioactivity in in vivo environments as well as delivery of those growth factors are difficult with the current tissue engineering techniques. Inorganic factors having angiogenic effects have been attracting attention as bioengineering materials. Biodegradable glasses such as 45S5 and 13-93B3 are known to promote angiogenesis by releasing trace metal ions. Recent studies reported that Si⁴⁺ and Zn²⁺ ions are effective for angiogenesis in vivo and in vitro. Li et al. reported that a concentration of 0.7–1.8 mg L⁻¹ of Si⁴⁺ ion is important for angiogenesis. Zn²⁺ ion is a critical component of many proteins that play an important role in physiological processes. For example, matrix metalloproteinase (MMP) is a family of zinc dependent endopeptidases that works in the wound healing process. MMP-9 is a member of the MMP family that promotes cell growth by hydrolysis of collagen molecules. Hence, materials that release metal ions such as Si⁴⁺ and Zn²⁺ ions could be expected to promote cell growth and wound healing. We propose smectite as one candidate material for this purpose. Smectite has been widely used in industrial fields because of its high specific gravity, cation exchange capacity and gelation property. The structure of smectite is a layered structure composed of two SiO₄ tetrahedral sheets and an Al(OH)₆ octahedral sheet. Smectite has intercalated ions that cause charge neutralization by replacing the position of the Si⁴⁺ and divalent metal ions. Smectite is also known to release silicon ions and interlayer ions when immersed in an aqueous solution containing other metal ions. We hypothesized that angiogenesis at the wound site could be promoted by using a combination of this smectite and Zn. The purpose of this study is to evaluate the wound healing effect of Zn²⁺ and Si⁴⁺ ions released from Zn-smectite in vivo.

2. Experimental procedures

2.1 Synthesis and analysis of Zn-smectite

2.1.1 Synthesis of Zn-smectite

A schematic chart for the method of Zn-smectite preparation is described in Fig. 1(A). Zinc chloride (ZnCl₂, Nacalai tesque, inc, Kyoto, Japan) and water glass solution (Na₂SiO₃, Nacalai tesque, inc) were dissolved in distilled water, at a concentration of 0.01 mol L⁻¹, respectively. ZnCl₂ aqueous solution was added to the Na₂SiO₃ solution with the (ZnCl₂/Na₂SiO₃) at a volume ratio of 0.75 to the mixed solution. The solution was then adjusted to
pH 8.0 by adding sodium hydroxide. Smectite powder containing Zn$^{2+}$ ion was then obtained by refluxing the prepared solution at 90°C for 12 h. The temperature was controlled during this process according to the following procedure. In brief, the temperature of the mixed solution was set at 90°C using a hot magnetic stirrer; afterward, the temperature was maintained by refluxing of the solution at 90°C ± 2°C for 12 h, and cool down the solution up to room temperature. The obtained crystalline smectite (Zn-smectite) was analyzed by scanning electron microscopy (SEM, JCM-5100, JEOL, Tokyo, Japan), powder X-ray diffraction meter (XRD, UltimaIV, Rigaku, Tokyo, Japan) and fourier-transform infrared spectroscopy (FT-IR, FT/IR-6000, Jasco, Tokyo, Japan) to measure the crystal and molecular structure.

2.1.2 Zn$^{2+}$ and Si$^{4+}$ ion release test
Zn$^{2+}$ and Si$^{4+}$ ions released from Zn-smectite were determined as follows: 1.5 g of Zn-smectite powder was dispersed homogeneously in 5.0 mL of physiological saline at 37°C. The physiological saline with the powder containing Zn$^{2+}$ and Si$^{4+}$ ions was collected after 1-7 days and measured by inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRCII, Perkin Elmer Japan co., Ltd., Kanagawa, Japan).

2.1.3 Measurement of slurry pH
Zn-smectite powder slurry was prepared by suspension in sterilized physiological saline using a magnetic stirrer. Changes in the pH value of the powder slurry over time were measured by pH meter (HI2002-01, Hanna Instruments Japan, Chiba, Japan) at 37°C.

2.2 Animal experiments
2.2.1 Preparations for animal surgery
Prior to the surgery, the Zn-smectite powder was sterilized by dry heat sterilizer (STN420DA, Jasco, Tokyo) at 180°C for 2 h. Autoclave sterilization of the surgical instruments was performed at 121°C for 20 min to prevent bacterial infection.

2.2.2 Animal models
The animal research committee of Yamagata University, Yamagata, Japan approved the protocols for the animal experiment described in this study. Two male Jcl:SD-rats (CLEA Japan, inc., Tokyo, Japan), weighting 400 to 420 g were used in this study. The procedures followed in the animal experiments are shown in Fig. 1(B). The animals were given general anesthesia with 3% sevofoane (Marushi Pharmacatal Co., Ltd., Osaka, Japan). Subsequently, each rat was sedated with an intramuscular injection of 10 mg kg$^{-1}$ xylazine hydrochloride (Sedeluck, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan). Abdominal hair was removed with an electric clipper and hair removal cream. After disinfectant with iodine, 2% lidocaine hydrochloride containing 1:80,000 epinephrine (Xylocaine Poly Amp 2%, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was administered locally. Eight skin defects with 10 mm in diameter were made on both side of the abdomen. The control wound groups were covered with commercial wound dressing material (Duo Active ET$, Convatec Inc., USA). The experimental wound groups were first coated with 0.005 g of Zn-smectite powder and then covered with the same commercial wound dressing material as the control wounds. Evaluation was conducted at 1 and 2 weeks interval.

2.2.3 Morphometric analysis
Digital photography of the wounds was taken at each time period. The area of the wounds was measured from the captured image using an image-processing program (Image J, National Institutes of Health, Bethesda, USA). The values measured by the program were used for calculating the residual wound area of each image by the following equation:

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\text{Proportion of the residual wound} (\%) = \frac{W_t}{W_0} \times 100
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Here, $W_0$ and $W_t$ represent the initial wound area and the wound area at 1 or 2 weeks, respectively.
2.2.4 Histological evaluation
After 1 and 2 weeks, an overdose of anesthetic (Pentobarbital sodium salt, Nacalai tesque, Inc., Kyoto, Japan) was delivered intraperitoneally to euthanize the rats. Skin tissue including wounds was collected for histological evaluation. The excised tissues were fixed with 20% phosphate-buffered paraformaldehyde (PFA) for 48 h. The fixed tissues were then embedded in paraffin wax to make a paraffin block. The specimens were sectioned into thicknesses of 6–8 μm by microtome. The sections were stained using hematoxylin and eosin staining (HE staining) and observed with an optical microscope (BX53, OLYMPUS, Tokyo, Japan).

2.3 Statistical analyses
For each parameter mean value ± standard deviation (SD) was calculated, and the proportion of the residual wound was then evaluated by student’s t-test for statistical significance at a significance level of 5% (p value <0.05).

3. Results
3.1 Powder characterizations
The SEM micrographs of the Zn-smectite powder are shown in Fig. 2. The aggregated particles in the range from 10 to 50 μm were observed at low magnification [Fig. 2(A)]. At high magnification [Fig. 2(B)], it was found that the aggregated particles consisted of small particles just a few μm in size.

Figure 3 shows the results of XRD and FT-IR measurements on the Zn-smectite powder. In the X-ray diffraction pattern [Fig. 3(A)], the synthesized material was found to be smectite with its characteristic layered structure. The peaks of d (001) and d (002) are known to represent the interlayer space and the trioctahedral structure of smectite, respectively.20),21) Additionally, these diffraction peaks correspond to a XRD pattern previously reported by Pascual et al.21) On the other hand, the FT-IR spectrum of the synthesized Zn-smectite is shown in Fig. 3(B). The IR absorptions assigned to the binding of Si=O–Zn and Si=O–Si were observed at 663 and 1000 cm⁻¹, respectively.22)-25)

These results showed that replacement of metal ions in the structure, such as Zn²⁺ for Si⁴⁺ ion, has occurred in the tetrahedral layer and the octahedral layer.26) The H–O–H bond observed at 1633 cm⁻¹ indicates water molecules clustered around the metal ions in the interlayer spacing. The range of 2600–3800 cm⁻¹ suggests free O–H stretching and hydrogen bonded O–H groups.25) By dispersing the powder sample into physiological saline, the pH value of physiological saline increased from pH 6.5 to 7.3. This pH value of the powder slurry is an optimal pH value for cells, such as keratinocytes and fibroblasts proliferation during the wound healing process.27)

Change in the concentration of Zn²⁺ and Si⁴⁺ ions released from the Zn-smectite in physiological saline is shown in Fig. 4. During the immersion period, a sustained release of Zn²⁺ and Si⁴⁺ ions was observed, as shown in Figs. 4(A) and 4(B), relative to Zn²⁺ and Si⁴⁺ ions, respectively. The total amounts of Zn²⁺ and Si⁴⁺ ions released from Zn-smectite to the wound site were 60 and 390 μmol L⁻¹, respectively.

3.2 The wound healing effect of Zn-smectite
3.2.1 Macroscopic changes and proportion of the residual wound size
Photos used to calculate the residual wound area are shown in Fig. 5(A). Figure 5(B) shows a comparison of the proportions of residual wound sizes calculated after 1 and 2 weeks. The average wound size at each time point was compared to the original wound size. After 1 week, there was no significant difference in the proportions of residual wound sizes between control and Zn-smectite groups. However, the formation of white tissue was observed in the Zn-smectite group. After 2 weeks, similar to the case after 1 week, although there was still no significant difference in the proportions of the residual wound sizes between control and Zn-smectite groups, the average of residual wound size in the Zn-smectite group was smaller than that in the control group (20.25 ± 10.04%, 12.29 ± 9.90%, respectively).

3.2.2 Histological evaluation
To examine tissue healing with Zn-smectite powder and a
commercial dressing material (Duo active ET®), we performed a histological evaluation of the wound tissue at 1 and 2 weeks. Figure 6(A) shows microscopic images of wounds in the control and Zn-smectite groups after 1 week. A uniform white tissue observed in the Zn-smectite group was recognized as granulation tissue, composed of bundles of collagen and abundant vessel formation. In contrast, in the inflammatory cell stage of infiltration, such as neutrophils (which are expressed in initial wound healing) was observed in the control group. These results were presumed that applying Zn-smectite did promote faster wound healing process by inducing angiogenesis compared to the control group.

Microscopic images of the wounds in the control and Zn-smectite groups after 2 weeks are shown in Fig. 6(B). In the control group, angiogenesis and collagen formation of regular granulation tissue were observed. However, in the Zn-smectite group, regenerated collagen fibers, and regeneration of skin appendages such as hair follicles similar to these in normal tissue were observed after 2 weeks [Fig. 6(C)]. All these results described above indicated that Zn-smectite had an effective wound healing effect compared to commercial wound healing materials and that it can repair and regenerate the tissue defects to resemble normal tissue more closely.

4. Discussion

Wound healing is a complex process involving a number of coordinated events including inflammation, cell migration, cell proliferation, matrix production and angiogenesis. The basic wound healing process is shown in Fig. 7. During the wound healing process, just after the skin is damaged, the healing process including hemostasis, inflammation, proliferation and a remodeling phase is immediately started. The hemostasis phase is characterized by platelet aggregation and fibrin clot deposition.
In the inflammatory phase, harmful bacteria and dead cells present in the wound site are removed by the phagocytosis of neutrophils and macrophages. Angiogenesis, collagen production and re-epithelialization occur in the proliferation phase. Fibroblasts and vascular endothelial cells help in the production of collagen and angiogenesis, respectively. In order to promote neovascularization on the wound site, fibroblasts and vascular endothelial cells release various factors such as vascular endothelial growth factor (VEGF), bFGF and TGF-β. The new tissue (consisting of new vessels and collagen fibers) formed in the proliferation phase is called granulation tissue. Finally, in the remodeling phase, mature scar tissue is formed. In our experiment, systemic arrangement of mature collagen fibers was observed in the wound area at the same time. Our results showed that the total amount of Zn$^{2+}$ ion released from Zn-smectite might be suitable for the migration and proliferation of cells such as fibroblasts and keratinocytes. In addition, the concentration of Si$^{4+}$ ion released from Zn-smectite was similar to the concentration that induces angiogenesis as reported by Li et al. Restoration of tissue integrity and homeostasis following injury to the skin is of vital importance, as the integument provides the first barrier against invading microbes and pathogens. In our experiment, systemic arrangement of mature collagen fibers was observed in the wound area at the same time. Our results showed that the total amount of Zn$^{2+}$ ion released from Zn-smectite might be suitable for the migration and proliferation of cells such as fibroblasts and keratinocytes. In addition, the concentration of Si$^{4+}$ ion released from Zn-smectite was similar to the concentration that induces angiogenesis as reported by Li et al.
bundles was found that the covering the wound area in the Zn-smectite group after 2 weeks, and the appearance of the skin appendages was also detected beneath the scar tissue. These results indicated that healing process had reached the remodeling phase. In contrast, in the control group healing process had reached only the proliferative stage of granulation tissue formation along with abundant inflammatory cells. Zn-smectite releases Zn\(^{2+}\) and Si\(^{4+}\) ions. Zn\(^{2+}\) ion is known to be the main component of many metalloenzymes and plays a critical role in wound healing, biosynthesis, and homeostasis of various connective tissues. Zn\(^{2+}\) ion functions as an essential cofactor in matrix metalloproteinase (MMPs) and pro-collagen C-proteinase, indicating their pivotal physiological role in collagen synthesis. Consequently, Zn\(^{2+}\) ion deficiency leads to impaired healing in various organs.\(^{33}\) In the present study, we found abundant collagen formation in the experimental sample as compared with the control, suggesting that Zn\(^{2+}\) ion released from Zn-smectite works mainly on collagen synthesis and promotes the healing process. The interaction of Zn\(^{2+}\) ion with collagen was reported in the literature.\(^{34}\) In addition, zinc ion has antioxidant and anti-inflammatory activities.\(^{35,36}\) Additionally, zinc ion stimulates neovascularization by activating the growth factors VEGF, Insulin Growth Factor 1 (IGF-1) and Transforming Growth Factor beta-1 (TGF-\(\beta 1\)).\(^{37}\) Thus, Zn-smectite could be considered as an effective material for promoting wound healing.

Although Si\(^{4+}\) ion, not which is identified as essential trace elements for human health, has some very important functions in the human, Si\(^{4+}\) ion can be found in bone and connective tissues in the body.\(^{38}\) Recently, the presence of silicon induced VEGF expression in human dermal fibroblasts, which in turn upregulated nitric oxide synthesis and nitric oxide production in human endothelial cells has been reported.\(^{21}\) Similarly, another study utilizing a calcium silicate in a rabbit femur defect also demonstrated the angiogenic effects of Si\(^{4+}\) ion.\(^{39}\) In our experiment, a large amount of neovascularization was observed after applying Zn-smectite, indicating the angiogenic effect of these molecules. From these results, we conclude that zinc and silicon ions are supplied to the wound site by applying Zn-smectite. This material then reacts with the body fluid exudated from the wound edge to accelerate the healing process.

5. Conclusion

In summary we can conclude that synthesized Zn-smectite powder promotes angiogenesis and collagen formation on wounds. This wound healing effect of Zn-smectite is associated with Si\(^{4+}\) and Zn\(^{2+}\) ions released from the smectite. All these findings indicate that this newly synthesized material could be a candidate dressing in future wound healing therapy.

References