

# Comparison of Wound Healing Effect of Skin Micrograft Impregnated into Two Kinds of Artificial Dermis in a Murine Wound Model

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**Background:** A micrograft (MG) suspension produced by the Rigenera protocol has been used to stimulate tissue regeneration. Recently, a combination therapy of an artificial dermis and skin MG has been used to promote angiogenesis and granulation tissue formation in the artificial dermis. There are no reports comparing the differences in MG impregnation efficiency between different artificial dermis products. Therefore, we compared the impregnation of skin MG in Pelnac Gplus and Integra.

**Methods:** Skin MG was prepared from the skin of C57BL/6J mice using Rigeneracons and administered onto Pelnac Gplus and Integra sheets. The amount of MG suspension impregnated in Pelnac Gplus and Integra was evaluated. Pelnac Gplus and Integra sheets combined with MG were applied to murine defects, and wound area, neopithelium length, granulation tissue formation, and newly formed capillaries were compared with the control groups on days 7 and 14.

**Results:** The weight percentage of the MG absorbed by Pelnac Gplus and Integra was  $88.8\% \pm 3.5\%$  and  $28.2\% \pm 7.0\%$ , respectively ( $P < 0.05$ ). In the in vivo experiment, the area of newly formed granulation tissue and both the number and area of newly formed capillaries in the PelnacG + MG group were significantly larger than those in the control group at 14 days after implantation ( $P < 0.05$ ).

**Conclusions:** Skin MG was successfully impregnated into Pelnac Gplus by simple administration but not into Integra. Administration of skin MG into the Pelnac Gplus promoted granulation formation and angiogenesis. Pelnac Gplus was more suitable than Integra in the combination therapy. (*Plast Reconstr Surg Glob Open* 2022;10:e4636; doi: 10.1097/GOX.0000000000004636; Published online 3 November 2022.)

## INTRODUCTION

In 1980, Yannas and Burke<sup>1</sup> reported artificial dermis as a skin substitute with a bilayer structure combining a collagen sponge inner layer and a silicone membrane outer layer.<sup>2</sup> After an artificial dermis is applied to skin defects, the collagen sponge is infiltrated with fibroblasts and new capillaries and replaced by granulation tissue. Various soft tissue defects can be satisfactorily treated using artificial dermis.<sup>2-4</sup> However, it takes 2-3 weeks from applying artificial dermis to the wounds until sufficient granulation tissue is ready for skin grafting. This delay results in various

complications, such as surgical site infections, particularly in the treatment of chronic skin ulcers.<sup>2,5</sup>

The micrograft (MG) technique using autologous tissue was first established in 1869,<sup>6</sup> and a modified method called the Rigenera protocol was reported.<sup>7,8</sup> In the Rigenera protocol, a subject's tissues are cut into microtissues approximately less than or equal to 50  $\mu\text{m}$  in size using a specific disposable medical device, named Rigeneracons (Human Brain Wave S.R.L., Italy).<sup>8,9</sup> Skin MG generated by the Rigenera protocol was proven to be enriched in both extracellular matrix and specific cell populations, which initiate biological processes of regeneration and enhance wound healing.<sup>9-12</sup> The Rigenera protocol technology has been used for clinical applications, such as in the treatment of chronic ulcers,<sup>13,14</sup> exaggerated scars,<sup>15</sup> and wound openings,<sup>16-18</sup> with favorable therapeutic results.

Recently, a combination therapy of an artificial dermis and skin MG has been used to promote angiogenesis and granulation tissue formation. Combined with scaffolds, the MG can be delivered consistently to the wound

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surface, improving the wound-healing-promoting effect of the MG. Although the clinical results of combination therapy using this artificial dermis and MG suspension have been reported,<sup>13,16-18</sup> no studies have compared the differences in MG impregnation efficiency between different artificial dermis products. Therefore, we compared the impregnation of the skin MG suspension in two kinds of conventional artificial dermis: Pelnac Gplus and Integra.

## MATERIALS AND METHODS

### Animals

All animal experiments were conducted at Kyoto University in accordance with the Guidelines for Animal Experimentation of Kyoto University, Japan. The number of experimental animals used was kept to a minimum, and the protocol was approved by the Animal Research Committee of the Kyoto University Graduate School of Medicine (permit number: Med Kyo 20515). The experimental design of animal groups is presented in Table 1, with details of time points and the usage of artificial dermis and MG.

### Preparation of MG Suspension

Small pieces of murine skin were harvested to prepare MG suspension. C57BL/6J mice (male, 8–9 weeks old) (CLEA Japan, Inc., Osaka, Japan) were shaved with an electric shaver (Thrive; Daito Electric Machine Ind. Co., Ltd., Tokyo, Japan) and depilated using depilation cream (Kracie, Tokyo, Japan). The skin of the mice was obtained using an 8mm punch (Kai Industries Co., Ltd., Tokyo, Japan) after euthanasia by carbon dioxide gas inhalation. Two skin sheets of 8mm in diameter (total area: approximately 100mm<sup>2</sup>) were cut into small pieces using scissors and placed into Rigeneracons with 1mL saline solution (Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan). The skin was minced using a rotating blade in Rigeneracons for 2 minutes and passed through the blade holes (50 μm in diameter) to generate the MG suspension.

### Preparation of Pelnac Gplus and Integra Impregnated with MG Suspension

Pelnac Gplus and Integra sheets, 8mm in diameter, were placed in a 48-well plate (Thermo Fisher Scientific Inc., Tokyo, Japan) with the silicon layer downward. MG suspension (0.1mL) was added to each sheet of Pelnac Gplus and Integra, allowed to settle for 60 minutes at room temperature, and then immediately used for experiments.

**Table 1. Experimental Design of Animal Study**

Group	Time Point (d)	n	Integra	PelnacG	MG
Control	7	6			
	14	6			
Integra	7	6	•		
	14	6	•		
Integra + MG	7	6	•		•
	14	6	•		•
PelnacG	7	6		•	
	14	6		•	
PelnacG + MG	7	6		•	•
	14	6		•	•

Dots indicate procedures performed for each group. PelnacG, Pelnac Gplus.

### Takeaways

**Question:** Of Pelnac Gplus and Integra, which one is more suitable in the combination therapy of an artificial dermis and skin micrograft (MG)?

**Findings:** Pelnac Gplus absorbed more skin MG than Integra. The combination therapy using Pelnac Gplus and skin MG promoted granulation formation and angiogenesis. Pelnac Gplus was more suitable than Integra in the combination therapy.

**Meaning:** The combination therapy of Pelnac Gplus and skin MG is promising to be an effective and convenient treatment in clinical practice.

### Detection of MG Impregnated into Pelnac Gplus and Integra with Hoechst Staining

A stock solution of Hoechst 33342 (Thermo Fisher Scientific Inc., Tokyo, Japan) was prepared at a concentration of 10 μg/mL in phosphate buffered saline (PBS). Then, 0.1mL of Hoechst staining solution (10 μg/mL) was added to each Pelnac Gplus and Integra sheet impregnated with MG suspension in a 48-well plate, and the plate was shaken on a shaker for 30 minutes. Afterward, the samples were cut in half, and the cross-sections were observed using a fluorescence microscope (BZ-X810; Keyence Corp., Osaka, Japan).

### Evaluation of the Amount of MG Suspension Impregnated into Pelnac Gplus and Integra

Pelnac Gplus and Integra sheets (8mm in diameter) were put in a 48-well plate with the silicon layer downward, loaded with 0.1mL of MG suspension, and allowed to stand for 60 minutes. The weights of the samples before and after impregnation were measured, and the increase in weight was expressed as a percentage of the weight of the MG suspension administered (n = 5).

### Application of MG-impregnated Pelnac Gplus and Integra on Murine Skin Defects

Sixty C57BL/6J mice (male, 8–9 weeks old) were individually housed and maintained on a 12-hour light/dark cycle in a temperature-controlled animal facility. The day before surgery, the hair on the back of each mouse was shaved and depilated using a depilation cream. Spontaneously breathing mice were anesthetized using isoflurane (Pfizer Inc., Tokyo, Japan) during all painful procedures. The concentration of isoflurane was kept at 1.5%–2% to provide an appropriate level of anesthesia.

The mice were allocated to five groups (control, Integra, Integra + MG, PelnacG, and PelnacG + MG). A full-thickness skin defect, 8mm in diameter, was created on the back of each mouse using an 8mm biopsy punch and scissors. Neither artificial dermis nor skin MG was applied in the control groups, and the effect of adding artificial dermis and skin MG was evaluated by comparing the control and treated groups. Sheets of Integra, Integra impregnated with MG suspension, Pelnac Gplus, and Pelnac Gplus impregnated with MG suspension were applied to the skin defects with the silicon layer upward in the Integra, Integra + MG,

PelnacG, and PelnacG + MG groups. The sheets were fixed by suturing the surrounding skin with 5-0 nylon (Bear Corporation, Osaka, Japan). The wounds were covered with a silicone-faced wound dressing (SI Aid-Mesh, ALCARE Co., Ltd., Tokyo, Japan), including the control groups, and then fixed with a bandage using surgical tape (Silkytex, ALCARE Co., Ltd.) to prevent contamination and mechanical stress. After these procedures, mice were placed in individual cages inside the institutional animal facility.

### Evaluation of Wound Healing

The wound healing process was evaluated on days 7 and 14 after surgery. Six mice in each group were killed by carbon dioxide gas inhalation at each time point, and the wounds were photographed with a digital camera and harvested with the surrounding tissue. The specimens were fixed in 10% formalin buffer solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), paraffin embedded, and sectioned axially in the central area of each wound. The sections were subsequently subjected to hematoxylin and eosin (HE) staining, Azan staining, and immunohistochemical staining for anti-CD31 antibody.

For anti-CD31 staining, rabbit monoclonal antibody (ab182981; Abcam, Cambridge, UK) at a 1:10,000 dilution was used as the primary antibody, and polymer reagent (simple stain mouse MAX PO; Nichirei Co., Japan) was used as a labeled polymer with a secondary antibody. Sections were exposed to DAB (3-3'-diaminobenzidine-4HCl) (Nichirei Co., Tokyo, Japan) and counterstained with hematoxylin.

### Assessment of the Remaining Wound Area

The wound area was evaluated via gross photographs using ImageJ software (National Institutes of Health, Bethesda, M.d.) on days 7 and 14. The remaining wound areas on days 7 and 14 were compared between the control, Integra, Integra + MG, PelnacG, and PelnacG + MG groups.

### Assessment of Neoepithelialization

Neoepithelium length was measured on HE-stained sections on days 7 and 14. Neoepithelial length was defined as the length from the nearest hair follicle at the wound edge to the end of the epithelium, and the sum of the lengths on both edges of the wound was calculated.

### Assessment of Newly Formed Granulation Tissue

The area of newly formed granulation tissue in the wound was measured on Azan-stained sections on days 7 and 14. The fibrous connective tissue in granulation was stained in light blue with aniline blue; therefore, it was distinguished from the dermis of the wound edge, which was stained in dark blue. The remaining material was barely stained.

### Assessment of Newly Formed Capillaries and the Capillary Area

The number and total area of newly formed capillaries on days 7 and 14 were measured on anti-CD31-stained sections. Newly formed capillaries were detected in the area of newly formed granulation and in the remaining

material. A threshold was set for the brown tint stained with DAB and the size of individual stained areas, and the regions above this threshold were counted using the BZ-X800 Analyzer software (Keyence Corp., Osaka, Japan). For the capillary area, the area with color density higher than this threshold was measured, and the sum of the areas was calculated.

### Statistical Analysis

All data are presented as mean  $\pm$  SD. We used the *t* test for comparisons between two groups and the one-way analysis of variance with Bonferroni post hoc analysis between multiple groups. All statistical analyses were performed using IBM SPSS Statistics version 28. Statistical significance was indicated by probability (*P*) values less than 0.05.

## RESULTS

### Detection of MG Impregnated into Pelnac Gplus and Integra with Hoechst Staining

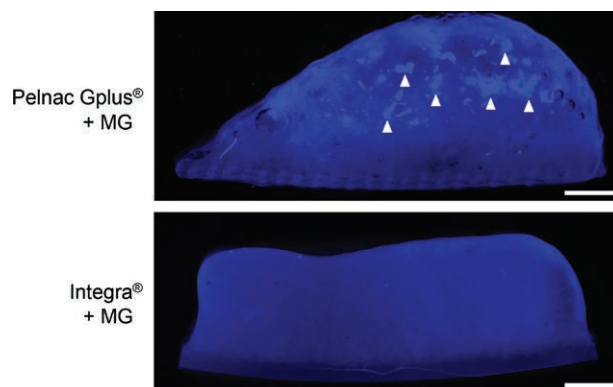
The nuclei of various cells in the MG were stained by Hoechst, resulting in the observation of MG impregnated into the material as blue spots. While a few MG cell nuclei stained with Hoechst were found in Integra, many MG cell nuclei were detected in the upper half in Pelnac Gplus (Fig. 1).

### Evaluation of the Amount of MG Suspension Impregnated into Pelnac Gplus and Integra

Most of the MG suspension applied on the Pelnac Gplus sheets was absorbed in its pores; however, the Integra sheets did not absorb the MG suspension, and most of the suspension spilled outside the samples (Fig. 2A). The weight percentage of the MG suspension absorbed by Pelnac Gplus and Integra was  $88.8\% \pm 3.5\%$  and  $28.2\% \pm 7.0\%$ , respectively ( $n = 5$ ,  $P < 0.05$ ) (Fig. 2B).

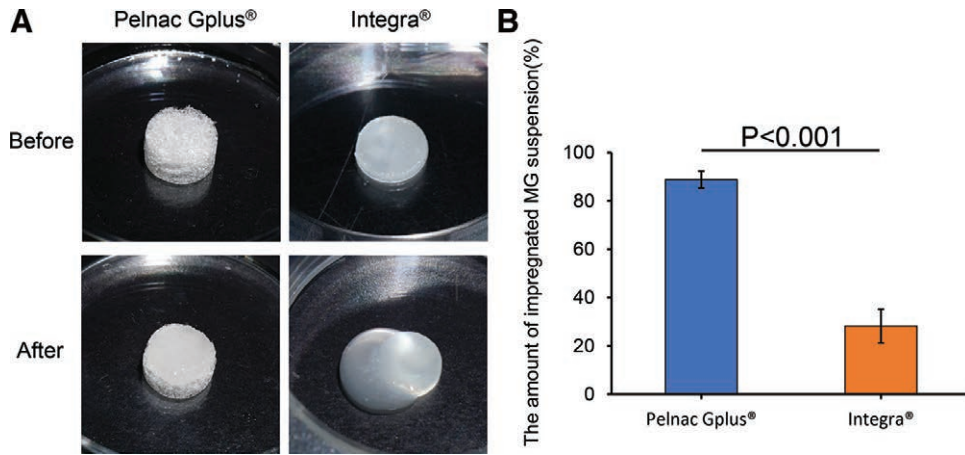
### The Remaining Wound Area

The wounds in the control, Integra, Integra + MG, PelnacG, and PelnacG + MG groups on days 7 and 14 are shown in Figure 3A, and the time course of changes to the wound area is shown in Figure 3B. The remaining wound

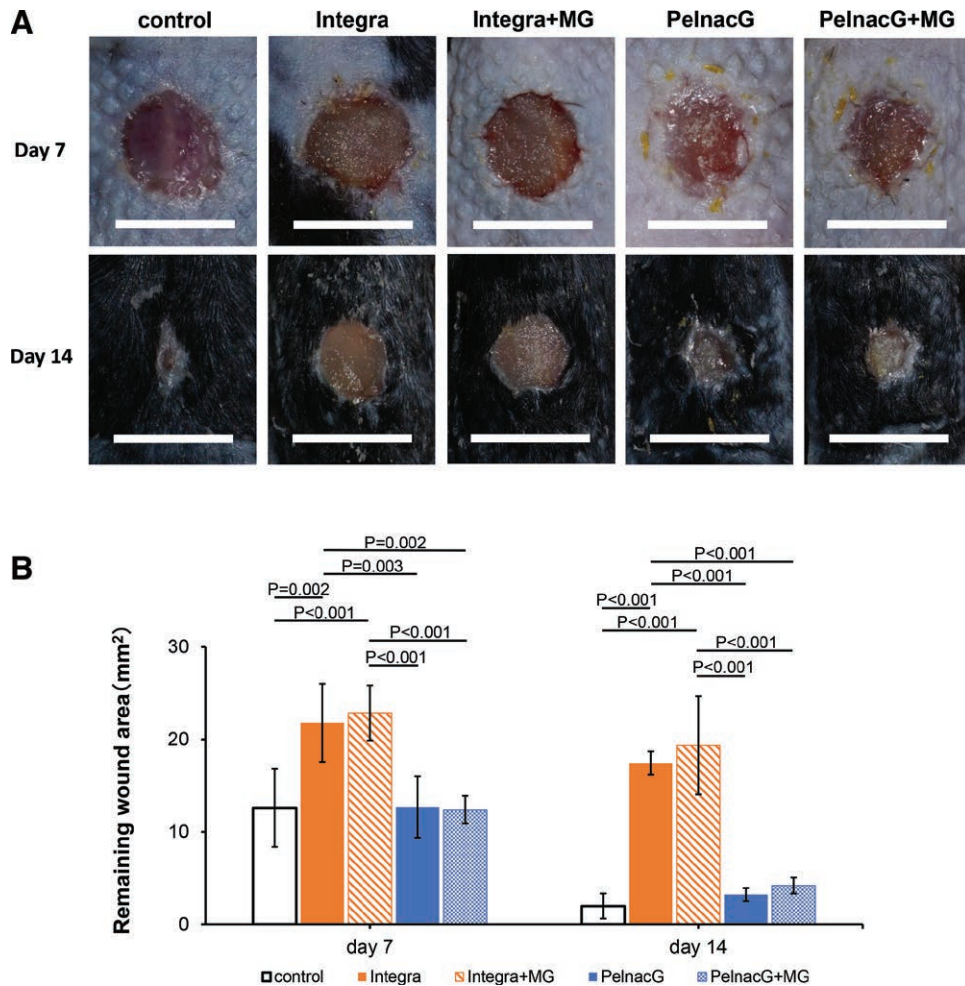


**Fig. 1.** Micrographs of Hoechst-stained sections after adding MG suspension. White arrowheads indicate the stained MG. Scale bar: 1 mm.

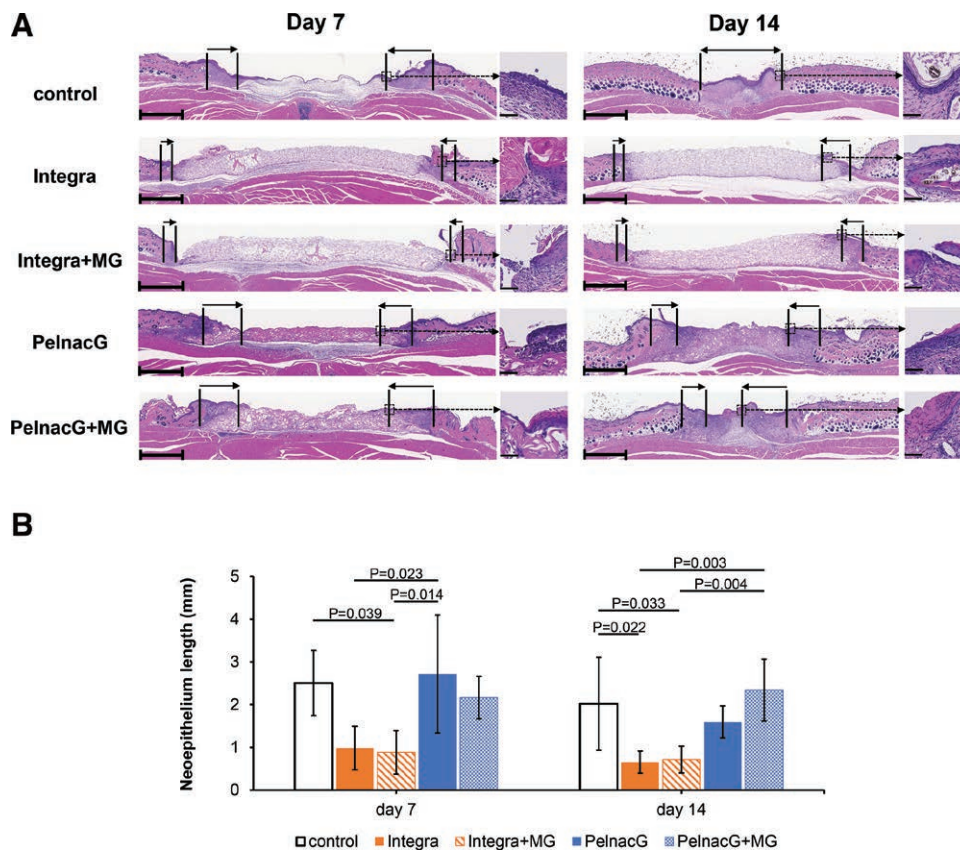




**Fig. 2.** Assessment of impregnation of skin MG suspension on Pelnac Gplus and Integra. A, The Pelnac Gplus and Integra before and after a 60-min incubation with administered MG suspension at room temperature. B, The weight of the samples before and after impregnation was measured, and the increased weight was expressed as a percentage of the weight of the suspension administered.



**Fig. 3.** Assessment of the remaining wound area. A, The wound healing process on days 7 and 14 after applying MG-impregnated Pelnac Gplus and Integra to skin defects in mice. Scale bar: 10mm. B, The time course of the remaining wound area on days 7 and 14. PelnacG: Pelnac Gplus.



**Fig. 4.** Analysis of the neoepithelium. A, Micrographs of HE-stained sections on days 7 and 14. The neoepithelium is indicated by the black arrows. Scale bar: 1 mm (50  $\mu$ m in the high-magnification images). B, The comparison of the length of newly formed epithelium on days 7 and 14. PelnacG: Pelnac Gplus.

areas in the Integra and Integra + MG groups were significantly larger than those in the control, PelnacG, and PelnacG + MG groups on days 7 and 14.

#### Assessment of Neoepithelium Length

HE-staining micrographs of the control, Integra, Integra + MG, PelnacG, and PelnacG + MG groups on days 7 and 14 are shown in Figure 4A, and the neoepithelium length is shown in Figure 4B. On day 7, the neoepithelium length in the PelnacG group was significantly longer than that in the Integra and Integra + MG groups, and the neoepithelium length in the control group was significantly longer than that in the Integra + MG group. On day 14, the neoepithelium length in the control and PelnacG + MG groups was significantly longer than that in the Integra and Integra + MG groups. Although there was no significant difference, the neoepithelium length in the PelnacG + MG group was longer than that in the PelnacG group.

#### Histological Assessment of Granulation Tissue

The area of the newly formed granulation tissue was evaluated in the micrographs of the Azan-stained sections (Fig. 5A). On day 7, no significant differences were observed between groups. On day 14, the area of newly formed granulation tissue in the PelnacG + MG group was significantly larger than that in the control, Integra,

and Integra + MG groups. In addition, the area of newly formed granulation tissue in the PelnacG group was significantly larger than that in the Integra group (Fig. 5B).

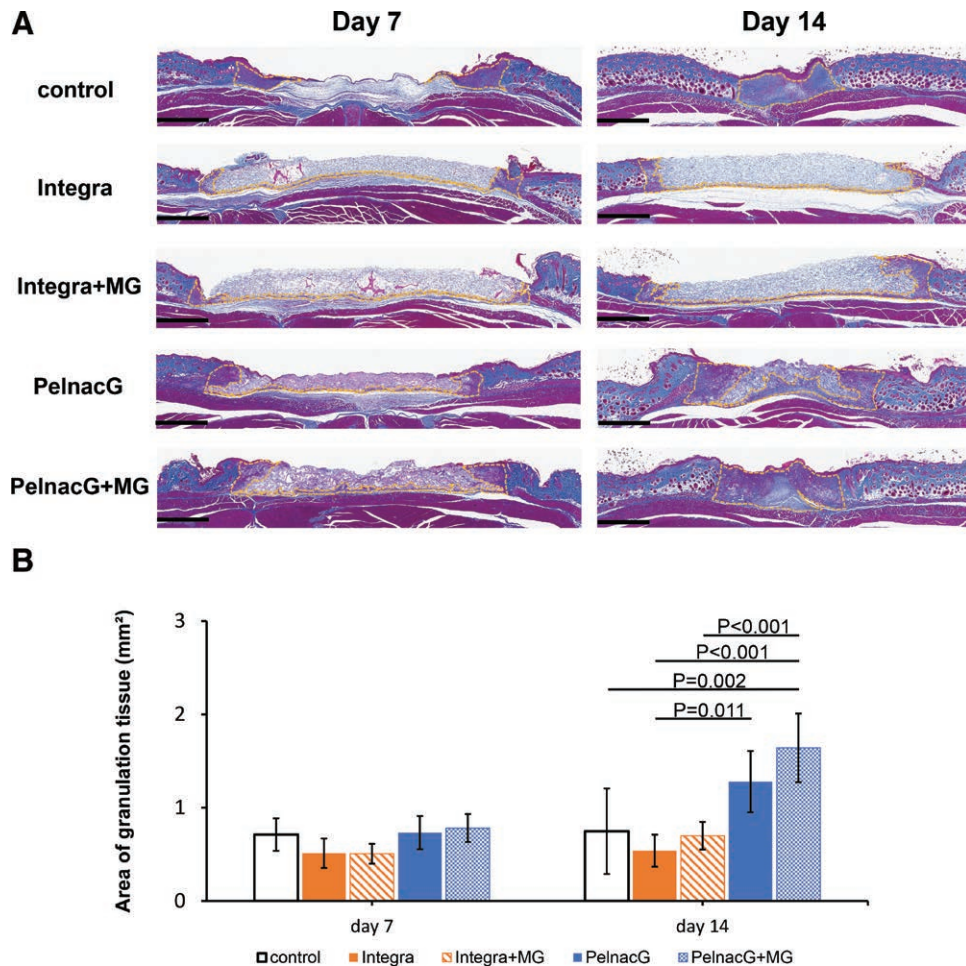
#### Histological Assessment of Newly Formed Capillaries

Micrographs of sections immunostained with anti-CD31 antibody are shown in Figure 6A. On day 7, no significant differences were observed among the groups in either the number or area of newly formed capillaries. On day 14, both the number and area of newly formed capillaries in the PelnacG and PelnacG + MG groups were significantly larger than those in the Integra and Integra + MG groups. In addition, the PelnacG + MG group was significantly larger than the control group (Fig. 6B, C).

## DISCUSSION

In this study, we compared the impregnation of skin MG into two types of conventional artificial dermis in vitro: Pelnac Gplus and Integra. We then explored and compared the ability of Pelnac Gplus and Integra combined with skin MG suspension to promote wound healing.

Bilayered skin substitutes are very useful for the temporary coverage of skin defects and reconstruction of the dermis; however, they are vulnerable to infection until they are infiltrated by recipient cells and blood vessels. Therefore, multiple attempts have been made to improve



**Fig. 5.** Analysis of the formed granulation tissue. A, Micrographs of Azan-stained sections on days 7 and 14. The yellow broken line indicates the newly formed granulation tissue. Scale bar: 1 mm. B, The comparison of the area of formed granulation tissue on days 7 and 14. PelnacG: Pelnac Gplus.

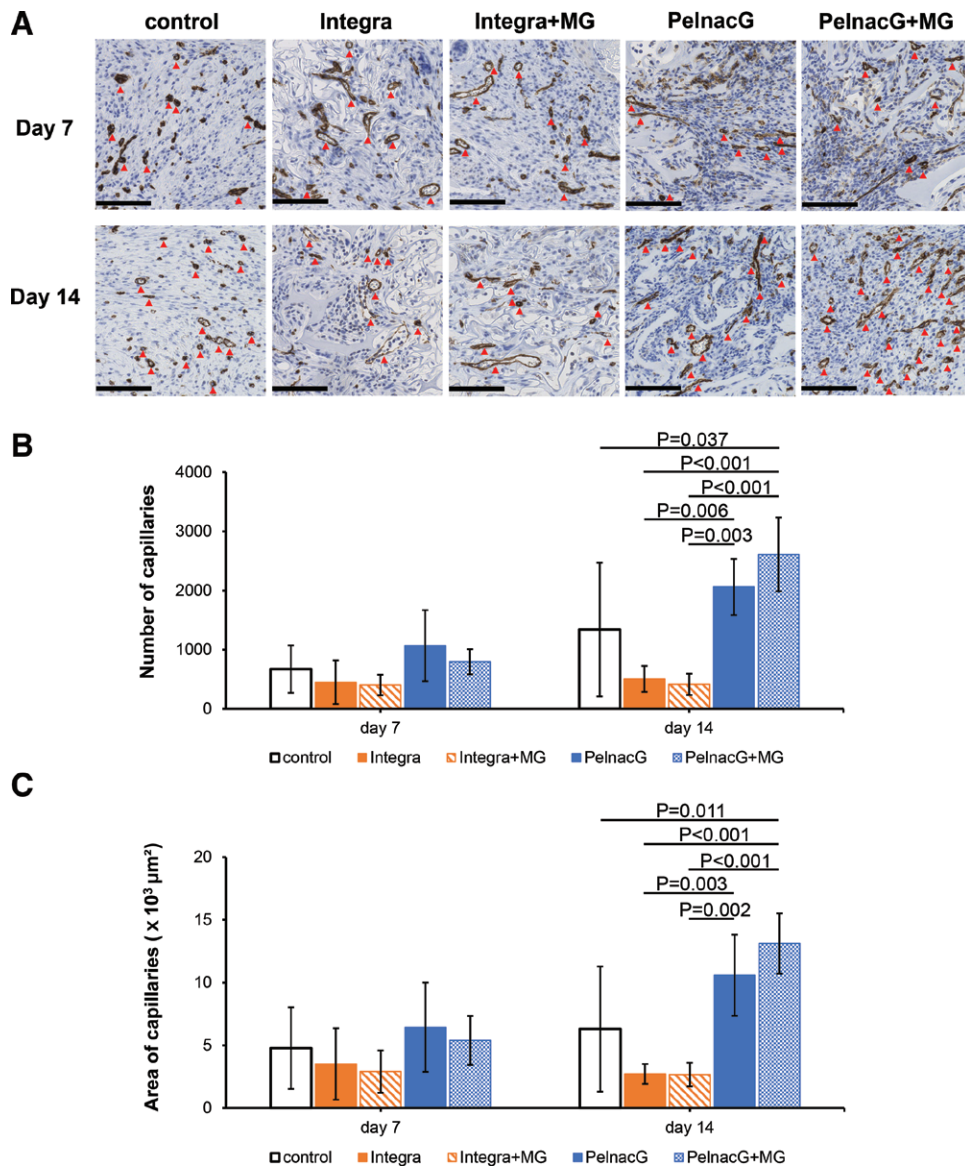
the resistance of artificial dermis to infection.<sup>19–21</sup> We have previously reported a method for creating an antibacterial artificial dermis by impregnating with silver sulfadiazine<sup>19</sup> and setting up a new scaffold of collagen and gelatin for sustained release of basic fibroblast growth factor to accelerate angiogenesis.<sup>20</sup> Recently, several attempts have been made to promote angiogenesis and accelerate wound healing by administering an MG suspension in combination with an artificial dermis.

Reverdin initially introduced the MG technique for wound healing, and many applications of different graft sizes have been utilized.<sup>6,7,8</sup> The Rigenera protocol allows the extraction of 50- $\mu$ m MG from autologous tissue to be directly and immediately used without any cell culture or cell processing.<sup>7</sup> Skin MG produced using the Rigenera protocol contains all skin tissue components, including tissue stem cells, progenitor cells, and various tissue factors.<sup>22</sup> In particular, mesenchymal stem cells as multipotent cells have trophic and support functions, releasing anti-inflammatory cytokines and anti-apoptotic molecules to promote protection and/or repair of damaged tissues.<sup>23–25</sup> In addition, the extracellular components of MG contain cytokines, growth factors, proteinases, and matrix components, which are important candidates as paracrine factors for wound healing.<sup>9,26</sup>

To administer MG to the target site, it is injected directly into the wound or used in combination with the scaffold. Purpura et al<sup>16</sup> prepared biocomplexes by impregnating skin MG prepared by the Rigenera method into a collagen sponge and culturing them for 7 days and confirmed that the viability of the cells in the MG was maintained. Furthermore, the biocomplex was successfully applied to a leg ulcer and promoted epithelialization and tissue softness. Marcarelli et al<sup>18</sup> combined an equine collagen sponge with an autologous skin MG and applied it once or twice to each dehiscence wound in the lower extremities of three patients, and complete remission was achieved after an average of 30 days. In both cases, a collagen sponge was used; however, the details of MG penetration into the sponge have not been investigated. In this combination treatment, a sufficient amount of MG must be introduced into the pores of the sponge; hence, the selection of the collagen sponge is an important factor for effective treatment results.

Pelnac is composed of a porcine atelocollagen sponge with a pore diameter of 60–110  $\mu$ m, made by rapidly freeze-drying a bubbled collagen solution and packaging in a dry state.<sup>5,28</sup> Pelnac Gplus has the same structure as conventional Pelnac; however, by changing 10% of the atelocollagen to acidic gelatin, it has the function of





**Fig. 6.** Analysis of the newly formed capillaries. A, Micrographs of anti-CD31-stained sections on days 7 and 14. Red arrowheads indicate the newly formed capillaries. Scale bar: 100  $\mu\text{m}$ . B, The comparison of the numbers of newly formed capillaries on days 7 and 14. C, The comparison of the area of newly formed capillaries on days 7 and 14. PelnacG: Pelnac Gplus.

sustained release of basic molecules, such as basic fibroblast growth factor.<sup>5,20,27</sup> Integra consists of a porous matrix of cross-linked bovine collagen and glycosaminoglycan, with a mean pore diameter of 80  $\mu\text{m}$ .<sup>2,28,29</sup> In contrast to Pelnac Gplus, Integra is packaged in a phosphate buffer to prevent desiccation. The main properties of the two kinds of artificial dermis are presented in Table 2.<sup>2,5,28</sup>

In the current study, we attempted to impregnate MG suspension into the artificial dermis before application to the wound surface. As the MGs produced with Rigeneracons are less than 50  $\mu\text{m}$ , they can theoretically penetrate the pores of Pelnac Gplus and Integra. When MGs were simply administered on top of the collagen sponges, they were successfully impregnated into Pelnac Gplus but not into Integra. This is because Pelnac Gplus is desiccated, and its pores are empty; therefore, MGs can

penetrate into it as the applied solution is absorbed into the pores by the capillarity phenomenon. However, the pores of Integra were already filled with phosphate buffer, so MGs could not infiltrate when simply administered to the top of the material.

In the in vivo study, the newly formed granulation tissue and the number and area of capillaries in the PelnacG group were significantly larger than those in the control group on day 14. MG administration improved granulation tissue formation and angiogenesis, which can be attributed to the fact that autologous fibroblasts and vascular endothelial cells contained in MG proliferate on-site and that extracellular components promote the proliferation and migration of recipient cells through a paracrine effect.<sup>9,26,30,31</sup>

In the Integra groups, no improvement was observed with the addition of MG. This may be because this simple

**Table 2. Main Properties of Pelnac Gplus and Integra**

Product	Components	Supplied Condition	Pore Diameter	Hardness	Time to Enable Skin Grafting
Pelnac Gplus	90 wt% porcine tendon atelocollagen and 10wt% of porcine dermal gelatin	Dry, spongy, and white	60–110 μm	Supple	In 2 wk
Integra	Bovine tendon type I collagen and shark chondroitin-6-sulfate GAG	Wet, gelatinous, and translucent	80 μm	Hard	In 3 wk

GAG, glycosaminoglycan; wt, weight.

administration method did not impregnate MG into the already filled pores of Integra and did not bring sufficient amounts of MG into the wound. In addition, slow wound reduction and poor epithelialization were observed in both Integra groups compared with the other groups. Hori et al<sup>32</sup> reported that after seeding human fibroblasts on Integra and Pelnac and culturing them for 35 days, Integra maintained its pore size and had less material contracture than Pelnac. The results of our study were attributed to the fact that Integra inhibited wound contraction.

This experiment showed that Pelnac Gplus is more effective than Integra in combination with MG solution. Combination therapy using Pelnac Gplus and skin MG produced using the Rigenera protocol is a highly convenient treatment that can be completed in an operating room without the need for cell culture.

As a limitation, the mechanism by which the cells in the impregnated MGs promote wound healing and the optimal amount of MGs to be administered were not investigated in this study. In addition, comparisons with other scaffolds already available for clinical use should be studied in the future.

### CONCLUSIONS

Impregnation with skin MG suspension was compared in Pelnac Gplus and Integra. The skin MG suspension was successfully impregnated into Pelnac Gplus with simple administration but not into Integra. Administration of skin MG into the Pelnac Gplus promoted granulation formation and angiogenesis. Pelnac Gplus was more suitable than Integra for combination therapy. In the future, the mechanism by which the cells in impregnated MGs promote wound healing and the optimal amount of MGs to be administered should be evaluated.

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