Effectiveness of bone marrow stromal cell-sheets in maintaining random pattern skin flaps in an experimental animal model

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Keywords

bone marrow stromal cell; skin flap; cell-sheet

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Short Running Head

Bone marrow stromal cell-sheets on skin flaps

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[1] The paper has never been presented at any meeting.

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Authors' Roles/Participation in Authorship of Manuscript

Tsutomu Kira designed this study and conducted animal experiments.

Shohei Omokawa designed this study and interpreted the results.

Manabu Akahane conducted biological analysis.

Takamasa Shimizu conducted biological analysis.

Kenichi Nakano conducted data analysis.

Yasuaki Nakanishi conducted data analysis.

Tadanobu Onishi conducted data analysis.

Akira Kido conducted biological analysis.

Yusuke Inagaki jointly interpreted the results.

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All authors have approved the manuscript for submission.

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INTRODUCTION

Flap surgery is a highly effective technique used for various tissue grafting or reconstruction applications. However, donor flaps are limited with respect to the area comprising reliable vascularity and donor-site morbidity. Recent studies have shown that bone marrow stromal cells (BMSCs) are abundant and produce growth factors, including hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF).[1-3] Multipotent stromal cells within the BMSC population are capable of differentiating into endothelial; therefore, application of BMSCs to flap surgery represents a promising approach.[4] However, transplantation of BMSCs is limited owing to their poor survival within ischemic tissues.[5] Consequently, a more effective method of BMSC transplantation is required.

With recent advances in tissue engineering, cell sheets can be produced from BMSCs, using ascorbic acid phosphate (vitamin C).[6] Vitamin C induces biosynthesis of collagen and extracellular matrix (ECM) by changing the physiological environment of immature cells,

contributing to constitution of *in vivo* cell-sheets. The cell sheets can be transplanted without a scaffold because of the plentiful ECM included, which preserves cell-cell junctions and mimics the cellular microenvironment in terms of mechanical, chemical, and biological properties. Because angiogenesis by BMSCs is stimulated under hypoxia, through increased expression of angiogenic factors,[7,8] hypoxic conditions may also increase the angiogenic potential of BMSC sheets.

Cell-sheet engineering has provided an alternative approach to tissue engineering in corneal, [9] myocardial,[10] hepatic,[11] periodontal,[12] and musculoskeletal tissues,[13,14] To date however, there have been no reports regarding the application of cell-sheets to skin flaps. We hypothesized that BMSC sheets cultured under hypoxic conditions may enhance the survival of skin flaps. The purpose of this study was to determine whether application of BMSC-sheets could maintain a greater area of survival and enhance the expression of angiogenic factors in skin flaps.

MATERIALS AND METHODS

Primary culture

BMSCs were obtained by flushing the femur shafts of a 7-week-old male Fischer 344 (F344) rat with 10 ml of culture medium. The released cells were collected in two flasks (T75-cm^2 culture flasks; Falcon BD, Franklin Lakes, NJ, USA) containing 10 ml of standard medium,

comprising minimal essential medium (MEM; Nacalai Tesque Inc., Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS; JRH Bioscience Inc., Lenexa, KS, USA) and 1% antibiotics (10000 U/ml penicillin and 10,000 μg/ml streptomycin; Nacalai Tesque Inc.). The cells were cultured in an incubator under 5% CO_2 at 37°C. On reaching confluence, the primary-cultured cells were detached from the flasks using trypsin/EDTA (Gibco, Invitrogen, Carlsbad, CA, USA). The research protocol was approved by the Institutional Animal Care and Use Committee of Nara Medical University, and followed all appropriate guidelines.

Differentiation assay

A total of three F344 rats (7 weeks old) were used. After the primary culture, BMSCs were seeded at 1×10^4 cells/cm^2 in 6-well plates (9.6 cm^2; Falcon BD) and cultured for 2 weeks in media for osteogenic, adipogenic and chondrogenic differentiation:

1) The osteogenic differentiation (OD) medium included MEM, 15% FBS, 82 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (vitamin C, Wako Pure Chemical Industries, Kyoto, Japan), 10 nM dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO) and 10 mM β-glycerophosphate disodium salt pentahydrate (β-GP, Calbiochem, Boston, MA). OD was identified with Alizarin red S (0.25 g Alizarin red S in 50 ml of 0.1M barbital buffer; Nacalai Tesque) for calcium deposition and fast red violet B (Nacalai

Tesque) for staining of alkaline phosphatase (ALP) activity.

2) Adipogenic differentiation (AD) medium included MEM, 15% FBS, and a Mesenchymal Stem Cell Adipogenic Differentiation Kit (Trevigen, Cat # 5010-024-K, Gaithersburg, MD). AD was identified with oil red O staining (Trevigen).

3) Chondrogenic differentiation (CD) medium included MEM, 15% FBS, 10 nM Dex and 10 ng/ml transforming growth factor-β1 (TGF-β1, Uscn Life Science Inc. Wuhan, China). CD was identified by staining with Alcian blue (ScienCell Research Laboratories, San Diego, CA).

BMSCs were cultured in standard medium in 6-well plates as a control. Staining was compared between differentiated BMSCs and control BMSCs.

BMSC-sheet preparation

To create BMSC sheets, primarily cultured cells harvested from three rats were seeded at 1×10^4 cells/cm^2 in 6-cm dishes (60×15 mm; Falcon BD) containing vitamin C and cultured until they reached confluence (approximately 2 weeks). The BMSC sheets were randomly divided into four different culture groups (n=18 per group): The N-N group was cultured under normoxia for 2 weeks; the H-H group was cultured under hypoxia for 2 weeks; the N-H group was cultured under normoxia for 1 week followed by hypoxia for 1 week; and the H-N group

was cultured under hypoxia for 1 week followed by normoxia for 1 week. For hypoxic conditions, cells were cultured in a gas mixture composed of 90% N_2, 5% CO_2, and 5% O_2 using a special incubator. The BMSC-sheets were rinsed twice with phosphate-buffered saline (PBS; Gibco) and then lifted using a cell scraper (Fig. 1).

Eighteen BMSC sheets in each group were evaluated for their *in vitro* angiogenic potential by quantitative real-time polymerase chain reaction (qRT-PCR) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). RNA was harvested using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol, and converted into cDNA. Target mRNA levels of VEGF-A, Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor B (PDGF-B), and Insulin-like Growth Factor 1 (IGF-1) were compared after normalization to β-actin mRNA levels, which were used to adjust for differences in the efficiency of reverse transcription between samples. The mRNA expression levels were assessed using the respective primers and probe set purchased from Applied Biosystems: VEGF-A: Rn01511602_m1; β-actin: Rn00667869_m1; EGF: Rn00563336_m1; PDGF-B: Rn01502596_m1; and IGF-1: Rn00710306_m1.

In vivo angiogenic potential of BMSC sheets

A total of 20 F344 rats (16 weeks old) were divided into the BMSC-sheet group and control

group (n=10 per group). A caudally-based dorsal skin flap (12×3 cm) model was designed and used. The rats were anesthetized with 2% isoflurane. The operative site was shaved and prepared with ethanol. For the BMSC-sheet group, BMSC sheets showing the highest gene expression levels of angiogenic factors *in vitro* were transplanted into the rat subcutaneous tissue via injection with 1 ml of PBS, 2 days before flap creation. Injection areas were at four points on the middle line along the long axis at 1.5, 4.5, 7.5, and 10.5 cm proximal to the distal flap end (Fig. 2). For the control group, PBS was injected at equivalent points. On day 2 after transplantation, random-pattern flaps were elevated with cauterization of the lateral thoracic, posterior intercostal and deep circumflex iliac vessels. The flaps were then immediately sutured back to their original sites with 4-0 nylon. Postoperative pain was managed by subcutaneous administration of buprenorphine hydrochloride. Postoperative antibiotics were administered by intramuscular injection of penicillin prophylactically. On day 7 after the operation, flaps were harvested and photographed with a digital camera. Flap survival areas were identified using Image-J software (NIH, Bethesda, MD, USA), and percentages of the survival area compared with the original flap size were used for analysis. Three blind examiners (T.K., T.S., and K.N) independently demarcated the area of original flap size in each photograph, and intra-rater reliability of the measurement was assessed by calculating interclass correlation coefficient (ICC) values.

Additionally, 10 skin samples (5×5 mm) from each group were collected from the flaps at 3 cm distal to the flap base. After homogenization, VEGF-A and basic fibroblast growth factor (bFGF) gene expression levels were determined to assess the *in vivo* angiogenic potential of the flaps by qRT-PCR. Target mRNA levels were compared after normalization to β-actin mRNA levels, which were used to adjust for differences in the efficiency of reverse transcription between samples. The mRNA expression level was measured using the respective primers and probe set purchased from Applied Biosystems (USA)): bFGF: Rn00570809_m1.

Immunohistochemical analysis

For histological evaluation, four rats from the each group were deeply anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde in 0.1 M PBS. We harvested a 20 mm length of musculocutaneous specimen at the basal, middle and distal survived region of skin flap. After postfixation of the skin, panniculus carnosus tissues and skeletal muscles with 25% sucrose and cryoprotection, we sectioned segments (15 mm in length) in the longitudinal plane at 20-μm thickness on a cryostat (CM3050 S; Leica, Nussloch, Germany). The frozen sections were mounted on glass slides. The mounted sections were then incubated over night at 4℃ with primary antibodies. The following primary antibodies used were: goat polyclonal antibody against mouse CD31/Platelet Endothelial Cell Adhesion

Molecule-1 (PECAM-1) (R&D Systems, Minneapolis, MN, USA). After rinsing the primary antibody from the sections, we incubated them with secondary antibodies for 2 h at room temperature. The secondary antibody used for staining was: rabbit anti-goat immunoglobulin G labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). The slides were then imaged on a confocal laser-scanning microscope (Fluoview 300, Olympus, Tokyo, Japan). The slides were stained with diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) for nuclear staining.

Statistical analysis

All values are expressed as means±standard deviation. Parametric analysis was performed for comparisons between two experimental groups. Student's *t*-test was used for qRT-PCR comparison, and the Welch test was used for comparison of flap survival area. Multiple comparisons among groups were made using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. All data analyses were conducted using EZR (version 1.24; Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). Probability values of $p<0.05$ were considered to indicate statistical significance.

Regarding ICC values, 0–0.21 was interpreted as slight agreement, 0.21-0.4 as fair, 0.41–0.6

as moderate, 0.61–0.80 as substantial and 0.81–1 as almost perfect based on the criteria of Landis and Koch.[15]

RESULTS

Differentiation assay

Alizarin red S and ALP staining of BMSCs cultured in OD medium and Alcian blue staining of those cultured in CD medium were strongly positive, but very weak in control BMSCs. Oil red O staining revealed lipid droplets in BMSCs cultured in AD medium (Fig. 3), whereas cells grown in control medium were not stained.

In vitro study

Expression levels of mRNAs for all angiogenic factors were high in the H-H group, but low in the N-N group (Fig. 4). The VEGF-A level in the H-H group was significantly higher than those in the other three groups, and was significantly higher in the N-H group than in the N-N and H-N groups. The EGF and PDGF-B levels in the H-H group were significantly higher than those in the N-N and N-H groups, and there was a significant difference between the PDGF-B levels in the H-N and N-N groups. There was no significant difference in EGF and PDGF-B levels between the H-N and H-H groups, but these levels in the H-H group showed a tendency

to be higher than those in the H-N group. There was no significant difference in the expression level of IGF-1 between any two groups. Thus, we conducted the next *in vivo* experiment using BMSC sheets from the H-H group.

***In vivo* study**

The H-H group BMSC sheets were used for *in vivo* studies because they exhibited high levels of all angiogenic factors in the *in vitro* study. On day 7 after implantation, regions of survival and necrosis were clearly demarcated, and neither infection nor wound dehiscence was observed in all flaps.

The mean flap survival percentage was 51.5±3.3% in the control group and 71.6±2.3% in the BMSC-sheet group (Fig. 5), with a significant difference between the two groups ($p<0.001$). The ICC value of three examiners' measurement data was 0.996, indicating almost perfect agreement of the measurement.

VEGF-A and bFGF gene expression levels in tissues from the BMSC-sheet group were significantly higher than those from the control group ($p=0.012$ and $p=0.023$, respectively, Fig. 6).

Immunohistochemical analysis

In immunostaining for PECAM-1/CD31, PECAM-1-positive microvessels (circular blue and green areas) were observed in the panniculus carnosus layer of BMSC sheets, in comparison with controls (Fig. 7). There was no marked difference in the number of PECAM-1-positive microvessels among the basal, middle and distal regions of surviving skin flap.

DISCUSSION

The oxygen concentration in bone marrow is known to be lower (1–7%) than that in the atmosphere, and hypoxia during culture of BMSCs enhances their proliferation, expression of angiogenic factors, and paracrine effects.[16] However, no reports are available on how levels of angiogenic factors in BMSC sheets change under hypoxic conditions. In the current study, *in vitro* experimentation demonstrated that hypoxic conditions in the secondary culture significantly enhanced gene expression of VEGF-A, EGF, and PDGF-B factors in BMSC sheets. The H-H group, which was cultured under hypoxia for 2 weeks, had the highest expression levels. These results indicate that hypoxic conditions may enhance the angiogenic potential during formation of BMSC sheets.

We speculated that BMSC sheets cultured under hypoxia could enhance the angiogenic factors expression, leading to maintenance of a larger area of transplanted tissue. Previous investigators have reported that BMSCs cultured under hypoxia show increased production of

angiogenic factors, and resistance to oxidative stress and cell-adhesion molecules of bone marrow cells.[17-19] Moreover, hypoxic culture has been demonstrated to increase skin-regenerative potential.[20] Most of these studies report viability of BMSCs in culture for only 24 hours. In the current study, hypoxic conditions led to increasing levels of angiogenic factors in BMSC sheets during culture for 2 weeks. We consider that BMSC sheets require more time than BMSCs to be influenced by hypoxic conditions because of the abundant ECM within the BMSC sheets.

Simman et al. transplanted bone marrow stem cells into random-pattern skin flaps in experimental models and investigated the increase in the flap survival area.[21] They concluded that administration of stem cells alone was incapable of improving flap survival, while addition of angiopoietin-1 and VEGF led to a significantly greater area of flap survival. Efficient maintenance of a flap survival area conventionally requires angiogenic factors or a scaffold in addition to stem cells.[22] Our current study demonstrated that BMSC sheets alone could improve the survival of random-pattern skin flaps without a scaffold. This BMSC-sheet transplantation technique represents a promising tool for local ischemic tissues and may accelerate angiogenesis continuously through an abundance of ECM.

VEGF is a heparin-binding glycoprotein and is a potent endogenous stimulator of both angiogenesis and vascular permeability. During skin flap survival, VEGF can be a stimulus for

acute vasodilation and increased capillary permeability in the acute phase. In the sustained phase, VEGF can stimulate the proliferation and migration of endothelial cells and lead to the subsequent formation of new capillary tubes for skin flap survival.[23] The increased levels of VEGF in the transplanted flap area may have contributed to the prolongation of ischemic flap survival in the current experimental model. Furthermore, bFGF was also increased in our *in vivo* study. This result suggests that improved blood circulation may have increased the number of fibroblasts in the skin, vessels, or subcutaneous tissue.

PECAM-1 is expressed in endothelial cells exhibiting adhesive properties and has roles in angiogenesis.[24] The PECAM-1-positive microvessels observed in BMSC sheets may induce angiogenesis and increase the flap survival area.

There are several limitations to this study. The exact locations of the BMSC after transplantation were not clearly demonstrated. Further studies are needed to prove whether neovascularization is enhanced by the transplanted BMSCs, and to purify the cell and ECM components of the BMSC sheets using cell-labeling techniques and collagen analyses.

CONCLUSION

Using the BMSC-sheet transplantation technique under hypoxic condition, enhanced angiogenic potential to local tissue areas was observed in the absence of a scaffold. This

technique may have potential as a minimally invasive treatment to rescue ischemic flaps, for example in hand and plastic surgery practice.

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Figure Legends

Fig.1. Macroscopic appearance of a BMSC sheet. BMSC monolayers cultured in the presence of vitamin C were lifted as a cell sheet using a cell scraper.

Fig. 2. Injection points of BMSC sheets. BMSC sheets were injected into the subcutaneous tissue at each indicated by asterisks.

Fig. 3. Arizalin red S staining was positive in BMSCs cultured in OD medium, but negative in control cells. ALP staining was positive in BMSCs cultured in OD medium, but very weak in control. Alcian blue staining indicated a chondrocyte mass in BMSCs cultured in AD medium, whereas there was no chondrocyte mass in control cells. Lipid droplets stained red were found in BMSCs cultured in AD medium, whereas there were no lipid droplets in controls.

Fig 4. Comparison of VEGF-A, EGF, PDGF-B, and IGF-1 mRNA expression levels in the four groups using qRT-PCR (n=18; $p<0.05$). For clarity, the values shown for VEGF-A in each group are half of the actual values, and those for IGF-1 in each group are one-tenth of the actual values.

Fig. 5. (A) Photographs of a postoperative flap (left), a flap in the control group at day 7 postoperatively (center), and a flap in the BMSC-sheet group at day 7 postoperatively (right). (B) Comparison of percentage of mean flap survival between groups (n=10; $p<0.05$).

Fig 6. Comparison of growth factor gene expression levels in flap tissues from control and BMSC-sheet groups. (A) The mean VEGF mRNA level evaluated by qRT-PCR was higher in the flaps from the BMSC-sheet group than in those from the control group ($n=10$; $p<0.05$). (B) The mean bFGF mRNA level evaluated by qRT-PCR was higher in the flaps from the BMSC-sheet group than in those from the control group ($n=10$; $p<0.05$).

Fig 7. Immunostaining in three regions of the skin flap: (A) basal region, (B) middle region, (C) distal region. Immunostaining of PECAM-1/CD31 revealed PECAM-1-positive microvessels (blue and green circular areas) in the panniculus carnosus layer of BMSC sheets. In contrast, few such vessels were observed in control cells. White arrows depict PECAM-1-positive microvessels, indicating neovascularization labeled by PECAM-1/CD31.

Figure 1
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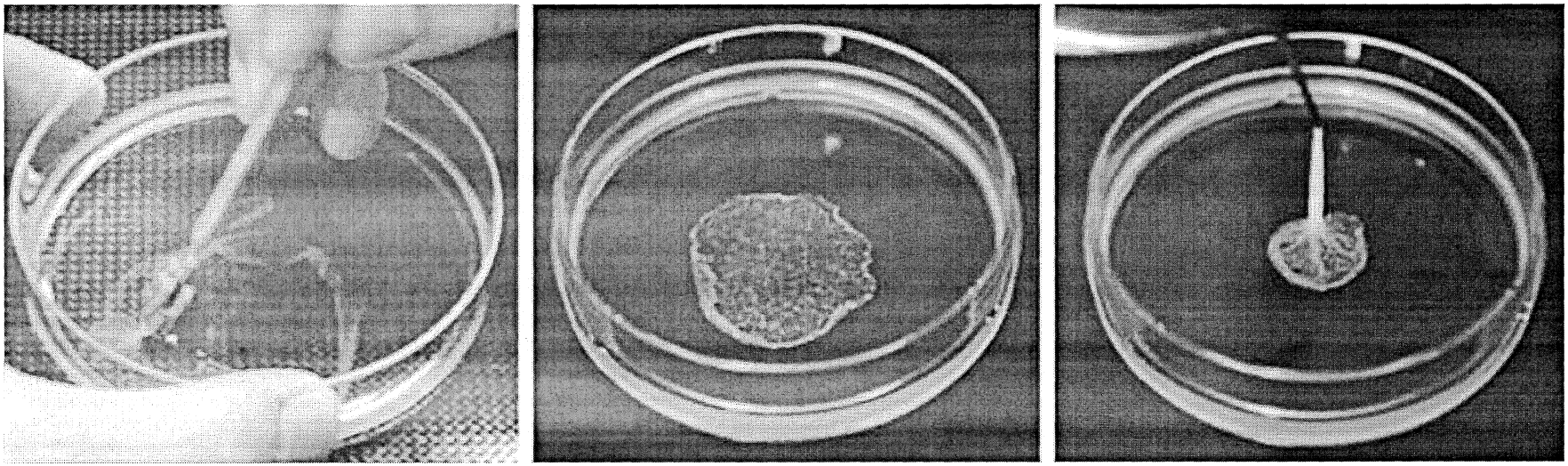

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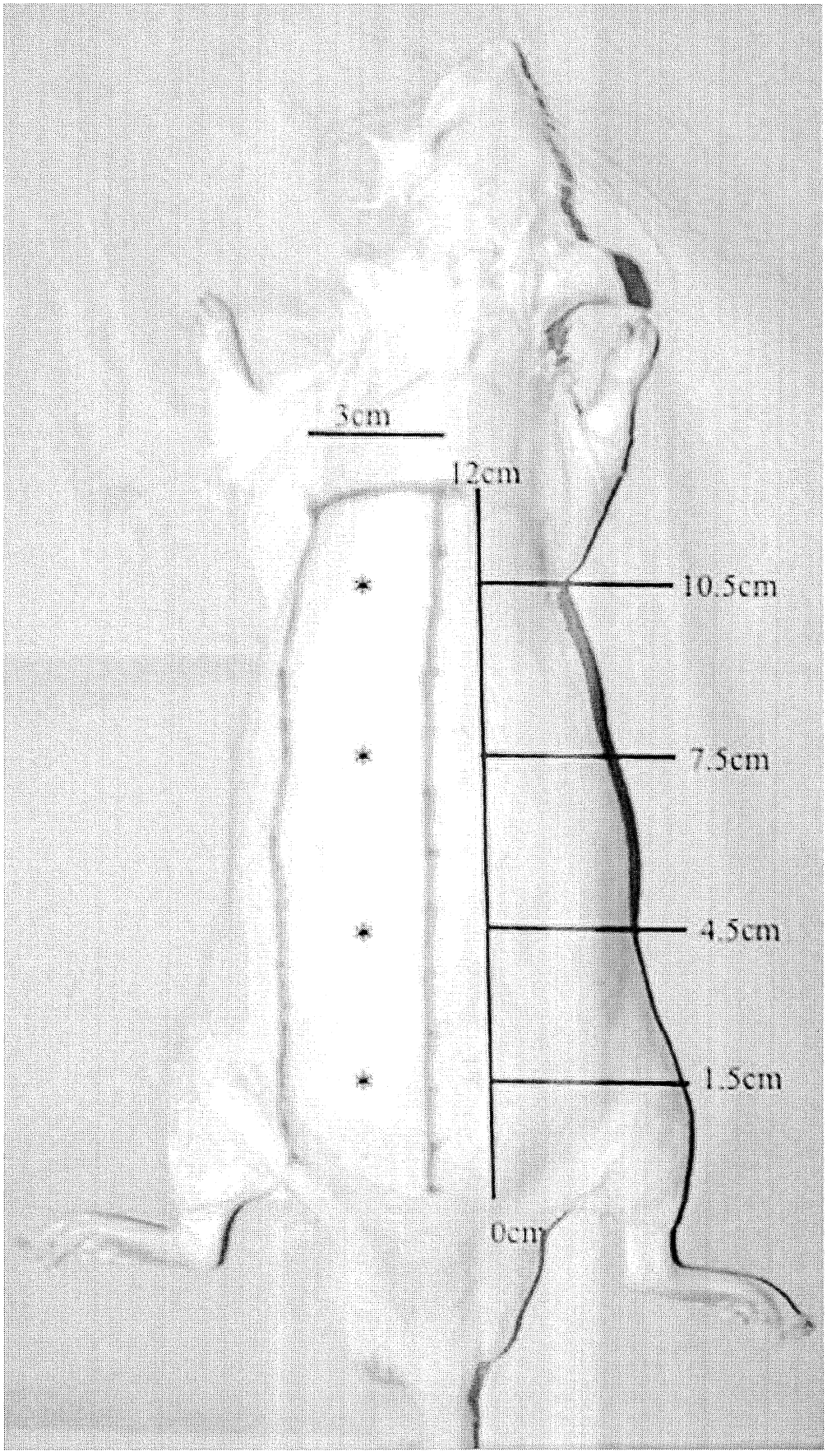

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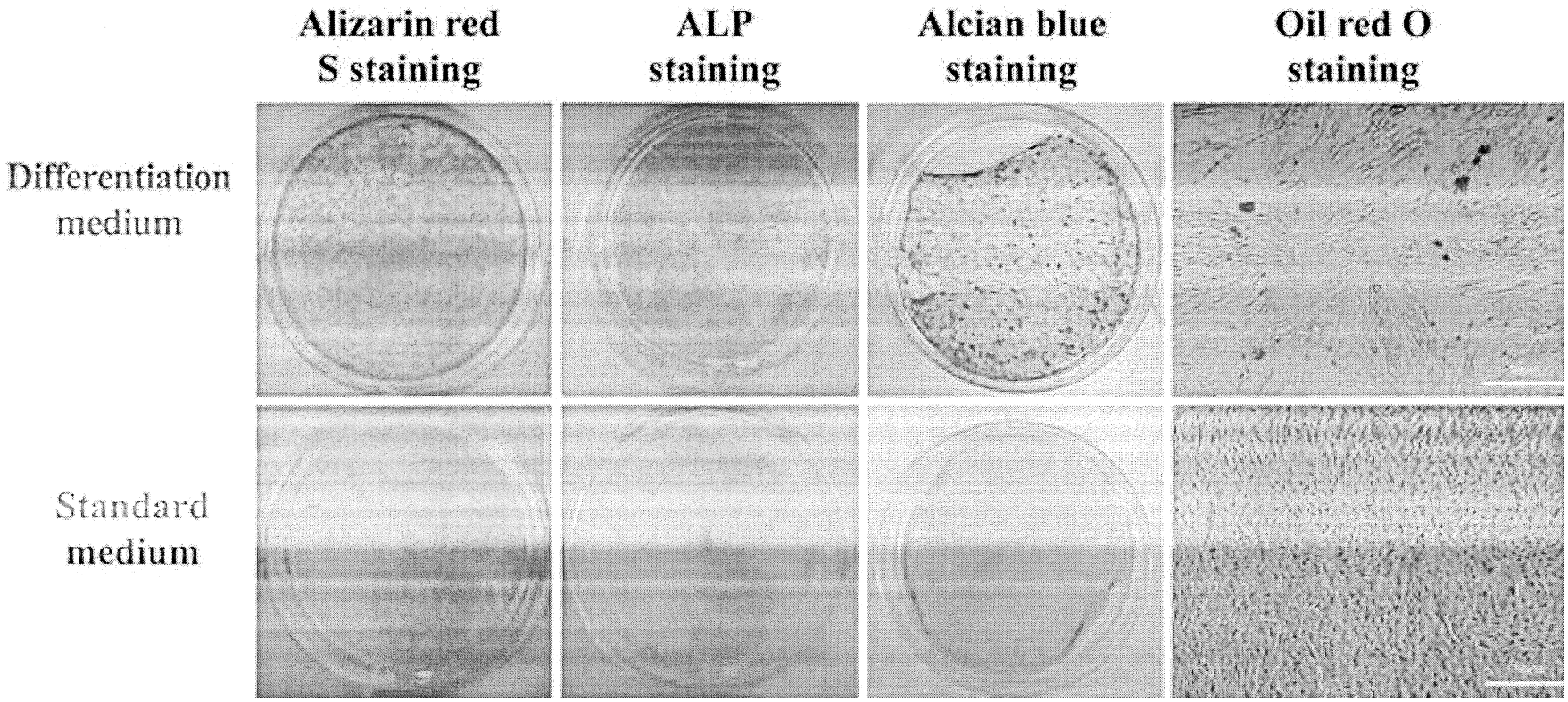

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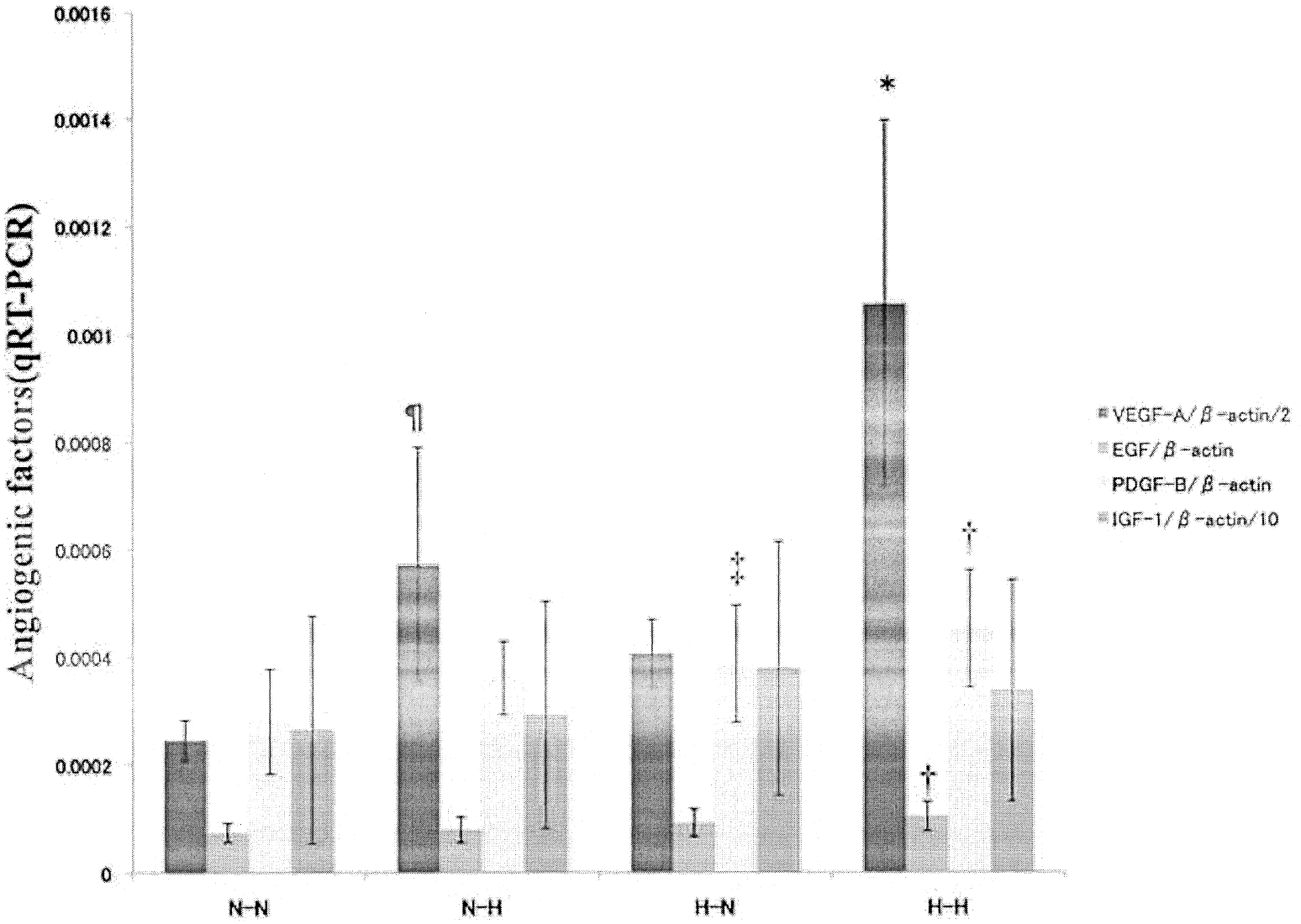

*: indicates significant difference from the other three groups, $p < 0.05$
†: indicates significant difference from the N-N and the N-H groups, $p < 0.05$
¶: indicates significant difference from the N-N and the H-N groups, $p < 0.05$
‡: indicates significant difference from the N-N group, $p < 0.05$

One-way ANOVA

Figure 5
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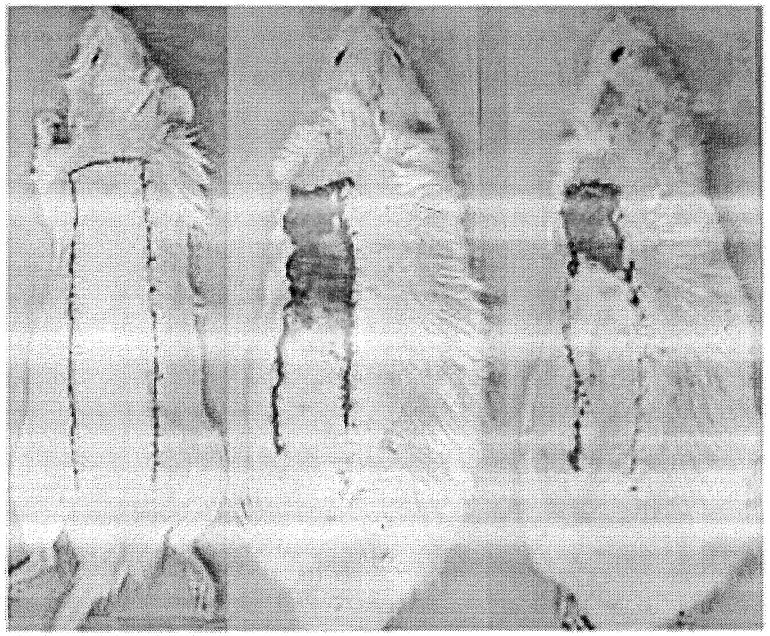

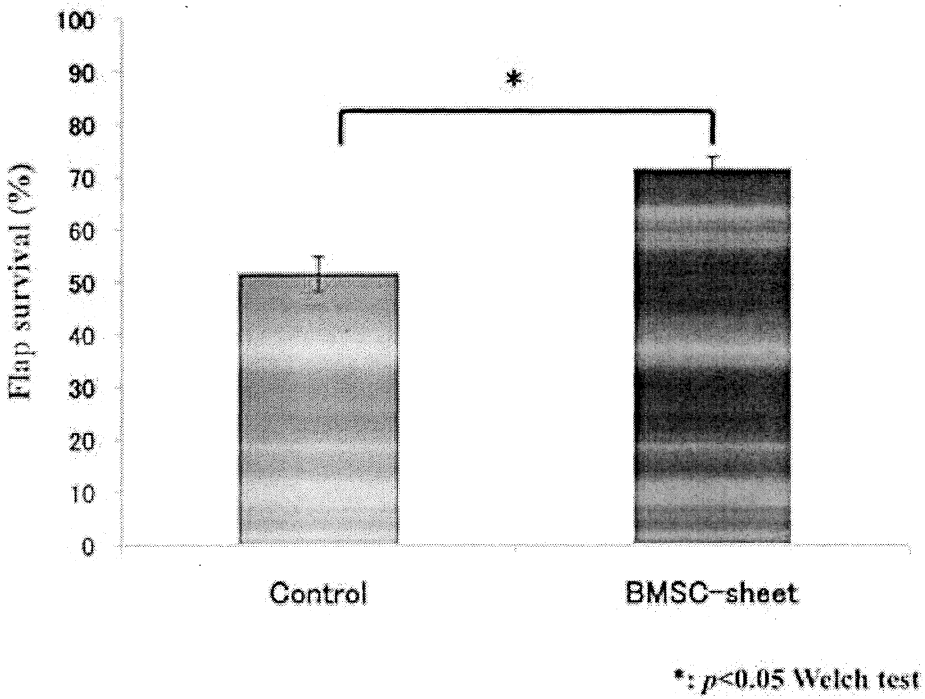

*: $p<0.05$ Welch test

Figure 6
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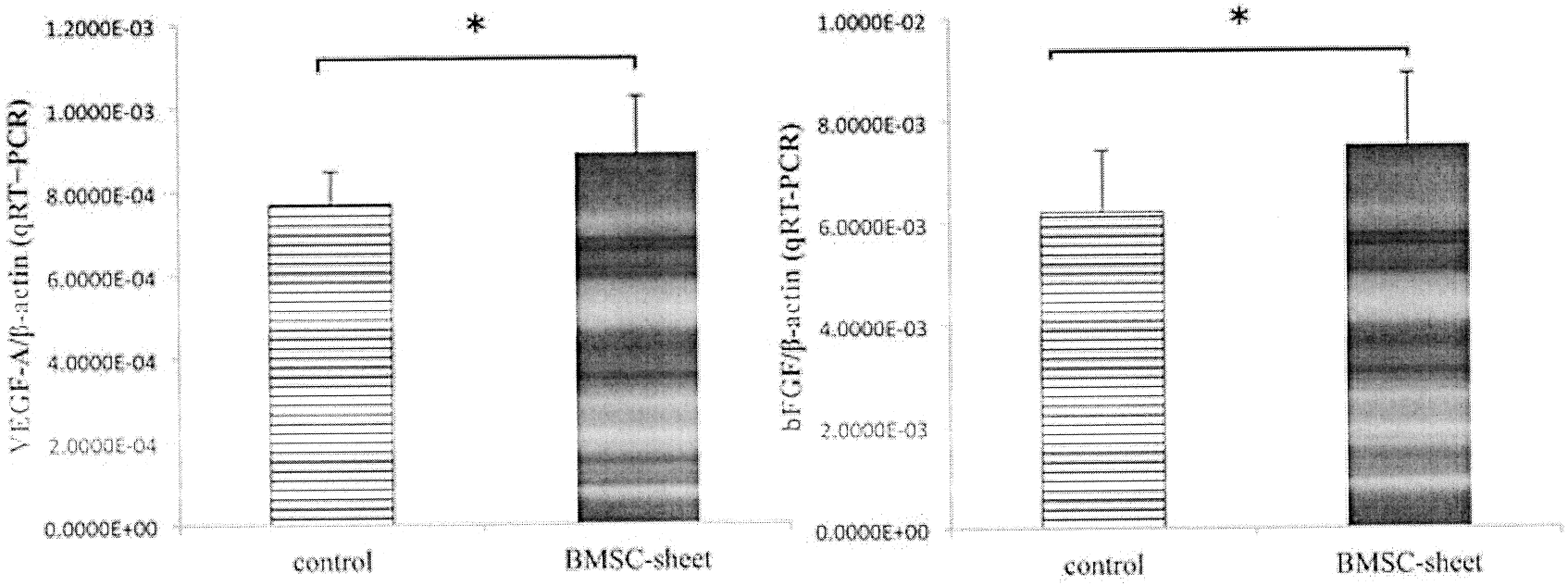

*: $p < 0.05$, Student's *t*-test

*: $p < 0.05$, Student's *t*-test

Figure 7
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