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Evaluation of autologous skeletal muscle-derived factors for regenerative medicine applications

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Objectives

Regenerative medicine is an emerging field aimed at the repair and regeneration of various tissues. To this end, cytokines (CKs), growth factors (GFs), and stem/progenitor cells have been applied in this field. However, obtaining and preparing these candidates requires invasive, costly, and time-consuming procedures. We hypothesised that skeletal muscle could be a favorable candidate tissue for the concept of a point-of-care approach. The purpose of this study was to characterize and confirm the biological potential of skeletal muscle supernatant for use in regenerative medicine.

Methods

Semitendinosus muscle was used after harvesting tendon from patients who underwent anterior cruciate ligament reconstructions. A total of 500 milligrams of stripped muscle was minced and mixed with 1 mL of saline. The collected supernatant was analysed by enzyme-linked immunosorbent assay (ELISA) and flow cytometry. The biological effects of the supernatant on cell proliferation, osteogenesis, and angiogenesis in vitro were evaluated using human mesenchymal stem cells (hMSCs) and human umbilical cord vein endothelial cells (HUVECs).

Results

The supernatant contained several GFs/CKs, with especially high levels of basic fibroblast growth factor, and CD34+ cells as the stem/progenitor cell fraction. With regard to biological potential, we confirmed that cell proliferation, osteoinduction, and angiogenesis in hMSCs and HUVECs were enhanced by the supernatant.

Conclusions

The current study demonstrates the potential of a new point-of-care strategy for regenerative medicine using skeletal muscle supernatant. This attractive approach and readily-available material could be a promising option for tissue repair/regeneration in the clinical setting.

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Keywords: Muscle, Cytokine, Growth factor

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Article focus

- The potential of muscle as a new approach of the regenerative medicine was evaluated.
- Supernatant from minced muscle in saline was analysed as the candidate for tissue repair / regeneration in point-of-care approach.
- Cell characterisation, the ability of osteogenesis and angiogenesis in supernatant of muscle were analysed.

Key messages

- Supernatant of muscle included CD34+ and CD44+ cell fractions and several growth factors, especially bFGF.
- Supernatant of muscle accelerates cell proliferation, osteogenic differentiation and angiogenesis.
- These results suggest that supernatant of muscle can be applied regenerative medicine in easy way.

Strengths and limitations

- Strength: This study shows the effect of supernatant muscle obtained in easy way on tissue repair / regeneration.
- Limitations: The therapeutic trials in vivo animal studies were not performed.

Introduction

Regenerative medicine is an emerging field aimed at the repair and regeneration of various tissues. A range of materials, such as stem/progenitor cells, cytokines (CKs) and growth factors (GFs), have been utilised as feasible and accessible candidates for tissue repair and regeneration, and their efficacy has been confirmed[1]. However, the procurement and preparation of these factors involves invasive, costly, and time-consuming procedures[2]. Therefore, we adopted the concept of a point-of-care approach as a simpler and more favourable option for use in the daily clinical setting.

Previous studies have demonstrated that skeletal muscle contains various factors such as stem/progenitor cells and CKs/GFs.[3-5] Moreover, several reports have shown comparable healing of cartilage and bone treated with muscle-derived cells (MDCs). These facts suggest that skeletal muscle contains cells that are favourable for musculoskeletal tissue repair.[6-8] It has been reported that skeletal muscle mass makes up approximately 35% of body weight, therefore we hypothesised that skeletal muscle could be a feasible target to obtain a patient's autologous tissue as a biological source for tissue repair with a point-of-care approach.[9] In this study, we tested the hypothesis that various factors, including stem/progenitor cells and CKs/GFs, would be easily obtained from skeletal muscle supernatant and that this supernatant would have therapeutic potential.

The purpose of this study was to characterise the cell population and the amounts of CKs and GFs in muscle-derived supernatant obtained via a point-of-care approach and to investigate its angiogenic and osteogenic potentials for future clinical applications in the field of orthopaedic surgery.

Materials and Methods

This study was approved by the Ethics Committee of the Graduate School of Biomedical Sciences, Hiroshima University.

Human skeletal muscle and preparation of supernatant from muscle tissue. Skeletal muscle from the semitendinosus tendon was harvested from a total of ten patients who underwent anterior cruciate ligament (ACL) reconstruction. Stripped muscle was minced in the operating room, and then transferred to the bench for further processing. Minced tissue, 500 mg of each sample, was mixed well with 1 mL of saline and centrifuged at 900 rpm for two minutes. The supernatant was collected, transferred to a new tube and analysed as described below. The number of samples available for each experiment varied on the size of the available muscle and therefore the amount of supernatant available from the individual specimen processing.

Experimental design. Two experiments were performed in this study. First, to characterise the supernatant from skeletal muscle that presumably contains growth factors, cytokines and cells, enzyme-linked immunosorbent assay (ELISA) and flow cytometric analyses were used. For the ELISA, vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), and stromal cell-derived factor-1 (SDF-1) were evaluated. For detection and characterisation of the cell fraction in skeletal muscle supernatant, the sample was stained with the stem/progenitor cell markers CD34, CD44, CD45 and CD105, and analysed by flow cytometry.

Second, the biological potential of muscle supernatant was evaluated *in vitro*. To confirm the effect on cell proliferation, we added supernatant from muscle to human mesenchymal stem cells (hMSCs) and evaluated proliferation using a cell-growth assay at days one and three. Osteoinductive and angiogenic potentials of muscle supernatant were investigated using hMSCs and the tube formation assay with human umbilical vein endothelial cells (HUVECs).

ELISA analysis. VEGF, IGF-1, bFGF and SDF-1 were quantitatively evaluated by ELISA using the Quantikine system (R&D Systems Inc., Minneapolis, Minnesota). According to the manufacturer's protocol, standards and pretreated samples were pipetted into the 96 wells. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for each growth factor was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of growth factor bound in the initial step. The colour development was stopped and the intensity of the colour was measured using a microplate reader set to 450 nm. The obtained values were used to calculate the target protein concentration from a standard curve.

Flow cytometric analysis. Flow cytometry was conducted using the skeletal muscle supernatant. Specific antibodies were used for cell labelling: CD34 antibody for stem/progenitor cells, CD44 and CD105 antibodies for MSCs, and CD45 antibody for haematopoietic lineage cells (all from BD Biosciences, Franklin Lakes, New Jersey). Samples underwent regular flow cytometric profiling with a FACSCalibur Analyser and CellQuest Pro Software (BD Biosciences Immunocytometry Systems, San Jose, California). Dead cells were excluded from the plots on the basis of propidium iodide (PI) staining (Sigma-Aldrich Corp., St Louis, Missouri). Cells were washed twice with Hanks' balanced salt solution (HBSS) containing 3.0% heat-inactivated foetal bovine serum (FBS) and incubated with monoclonal antibodies for 30 minutes at 4°C after Fc recepter blocking reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The stained cells were washed three times with HBSS/3.0% FBS, resuspended in 0.5 mL of HBSS/3.0% FBS/PI, and analysed. The following monoclonal antibodies were used to identify the

CD34+/CD45dim subpopulation (the stem/progenitor cell fraction), and the CD44+/CD105+ subpopulation (the MSC fraction): CD34-FITC (clone RAM34), CD44-APC (clone G44–26), CD105-PE (clone 266), CD45-FITC (clone HI30), IgG1-PE isotype control, and IgG1-FITC (all from BD Biosciences Pharmingen San Diego, California).

Cell culture. Primary human bone marrow mesenchymal stem cells (hBMMSCs; Life Technologies Corp., Carlsbad, California) were cultured in StemPro MSC SFM (Life Technologies) with antibiotics (at a final concentration of 100 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B; Nacalai Tesque, Kyoto, Japan) in humidified air containing 5% CO_2 at 37°C. Cultured and passaged MSCs were utilised for experiments as described below.

Cell-growth assay. Cell-growth assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Cultured MSCs, passage 3, were inoculated into a 96-well plate (2000 cells/well). We then added 5 µL phosphate buffered saline (PBS) to wells of the control group (n = 10) and 5 µL muscle supernatant to the muscle supernatant group (n = 10) on day 0 with pre-incubation performed for 24 or 72 hours in a humidified incubator. Substrate (highly water-soluble tetrazolium salt, WST-8) was then delivered into each well, and after two hours of incubation the absorbance was measured at 450 nm using a microplate reader.

Osteoinductive culture of MSCs and evaluation. For osteogenic induction, cells were cultured to 80% confluence in a 24-well plate, and the medium was replaced with osteogenic induction medium using the StemPro osteogenesis differentiation kit (Life Technologies, Grand Island, New York). At three, six, nine, and 12 days after induction of osteogenesis, the medium was changed and 10 µL of PBS or 10 µL muscle supernatant was added to the cultured dishes in each group. All experiments were carried out in triplicate. After 14 days of osteoinduction, gene expression was analysed by real-time polymerase chain reaction (RT-PCR) and cultures were stained with alizarin red. Runt-related transcription factor 2 (RUNX2), collagen type I alpha 1 (COL1A1) and osteocalcin were used as bone-related genes. Total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, California) and total RNA yields were calculated and quality was determined using absorption spectrochemical analysis. cDNA was synthesised using 2 µg of total RNA with a SuperScript VILO kit (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed using the following TaqMan gene expression assay probes (Life Technologies): RUNX2 (Hs00231692_m1), COL1A1 (Hs01076777_m1), osteocalcin (Hs01587814_g1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_m1). The GAPDH gene was used as a control to normalise differences in total RNA levels in each sample. A threshold cycle (CT) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the delta-delta CT (ΔΔCT) values and results are expressed as 2-ΔΔCT. The value of each control sample was set at 1 and the mean biological duplicate of each sample was used to calculate the fold-change of target genes.

For qualitative analysis of bone formation, alizarin red staining was performed.[10] Aliquots of 1.0 mL of 10% formalin were added to each well and cells were fixed for 15 minutes at room temperature. The formalin solution was then removed, and 500 µL of 40 mM alizarin red stain (pH 4.1) was added to each well. The plates were incubated at room temperature for 20 minutes with gentle shaking, after which the dye solution was pipetted out and the stained monolayers were visualised by microscopy.

Tube formation assay. Angiogenesis is the process of generating new capillary blood vessels. We examined the effects of muscle supernatant on angiogenic activity and tube formation of human umbilical vein endothelial cells (HUVECs). HUVECs were purchased from Lonza Group AG (Basel, Switzerland) and pre-cultured with endothelial basal medium-2 (EBM-2) (Lonza, Basel, Switzerland) and then reseeded at a density of 1.5×10^4 cells/well in 96-well plates pre-coated with Matrigel Matrix (Merck Millipore, Billerica, Massachusetts). We then added 5 µL of muscle supernatant to the supernatant group and incubated at 37°C overnight in a tissue-culture incubator. All experiments were carried out in triplicate. We investigated total tube length at 12 and 24 hours by means of Image J analysis of digital images according to the method described in previous reports.[11]

Statistical analysis. All data are expressed as mean and standard deviation (SD). The Mann-Whitney U test was used to detect differences between the two groups. A p-value less than 0.05 was considered statistically significant.

Results

ELISA analysis revealed that skeletal muscle supernatant contained detectable amounts of CKs and GFs (VEGF 3.80 pg/mL, SD 8.16, SDF-1: 3.58 pg/mL, SD 8.50, bFGF: 325.6 pg/mL, SD 210.2, IGF-1: 82.0 pg/mL, SD 58.2). Flow cytometric analysis demonstrated the presence of CD34+ and CD44+/CD105+ cells as the stem/progenitor cell fraction, although the actual number of cells in the muscle supernatant was very small (Fig. 1).

Cell-growth assay at 24 and 72 hours after addition of muscle supernatant revealed that cell growth was significantly increased compared with the PBS group at both time points (Fig. 2).

In the muscle supernatant group, alizarin red staining at 14 days showed larger stained areas compared with

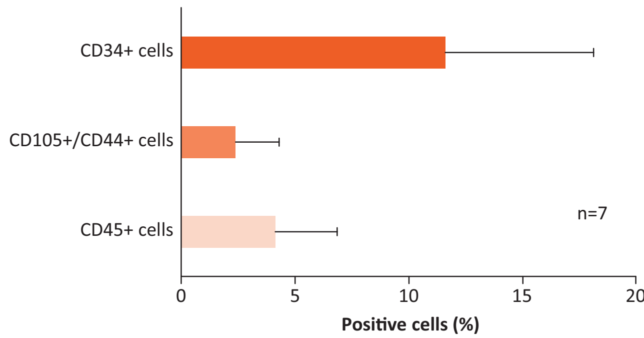

Fig.1

Flow cytometric analysis of supernatant from skeletal muscle (n = 7). The stem/progenitor cell fraction, CD34+ and CD105+/CD44+ cells, was confirmed. The presence of cells of the haematopoietic lineage, CD45+ cells, was also demonstrated.

the PBS group (Fig. 3a). Moreover, the expression levels of RUNX2, COL1a1, and osteocalcin were significantly upregulated in the muscle supernatant group compared with the PBS group (all $p < 0.05$, Fig. 3b).

The tube formation assay showed that the total tube length was significantly greater in the muscle supernatant group compared with the PBS group at both the 12 and 24 hour time-points ($p < 0.05$ for both) (Fig. 4).

Discussion

In this study, we showed that the supernatant from skeletal muscle contains various proteins and cell types and has favourable biological effects on cell proliferation, osteoinduction and angiogenesis. To our knowledge, this is the first report to demonstrate the therapeutic potential of skeletal muscle supernatant for tissue repair, aiming for future clinical application following the point-of-care concept.

Many factors such as surrounding tissues, blood supply, nutrient delivery, biomechanical forces, and the supply of several growth factors can affect tissue healing. In an attempt to provide these conditions, many approaches have been applied,[12-14] however, a time-saving, low-cost and less invasive approach is desirable in practical clinical settings. Hence, the concept of point-of-care has been widely accepted and applied in the field of orthopaedic surgery such as platelet-rich plasma (PRP) administration and fibrin clot delivery for tissue repair and regeneration.[15,16]

It is reported that skeletal muscle releases abundant CKs and GFs following traumatic injury[11] and also that muscle-derived stem cells have great potential to maintain and contribute to the repair and regeneration of muscle tissue. In addition to this biological potential, the anatomical distribution of skeletal muscle in the human body makes it easy to obtain skeletal muscle tissue from a

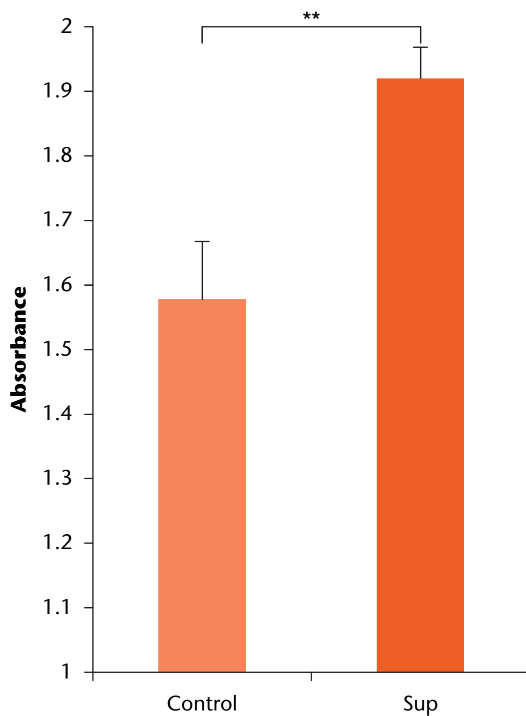

Fig.2a

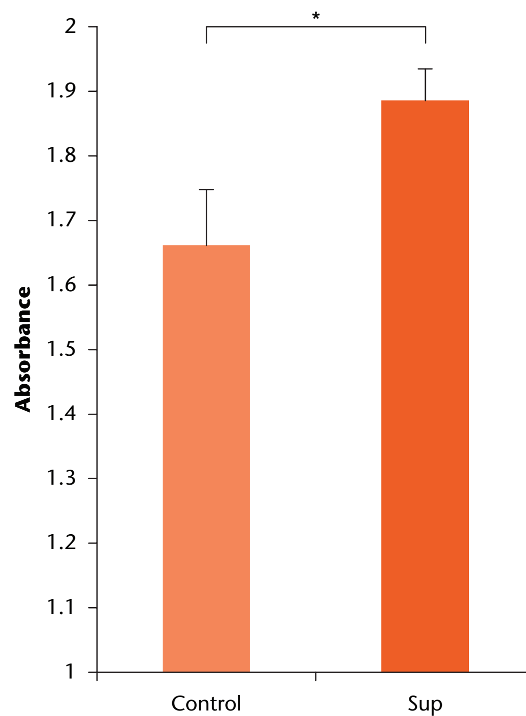

Fig.2b

Cell-growth assay (WST-8) at 12 hours (a) and at 24 hours (b) after the addition of skeletal muscle supernatant (n = 10) (*; p < 0.01, **; p < 0.05). The PBS group was treated with 5 µL PBS and the Supernatant group was treated with 5 µL of muscle supernatant (n = 10). Data are shown as means and standard deviation (*; p < 0.01,**; p < 0.05). The asterisks indicate that cell growth in the group treated with skeletal muscle supernatant was significantly higher than that of the PBS-treated group both at 24 and 72 hours.

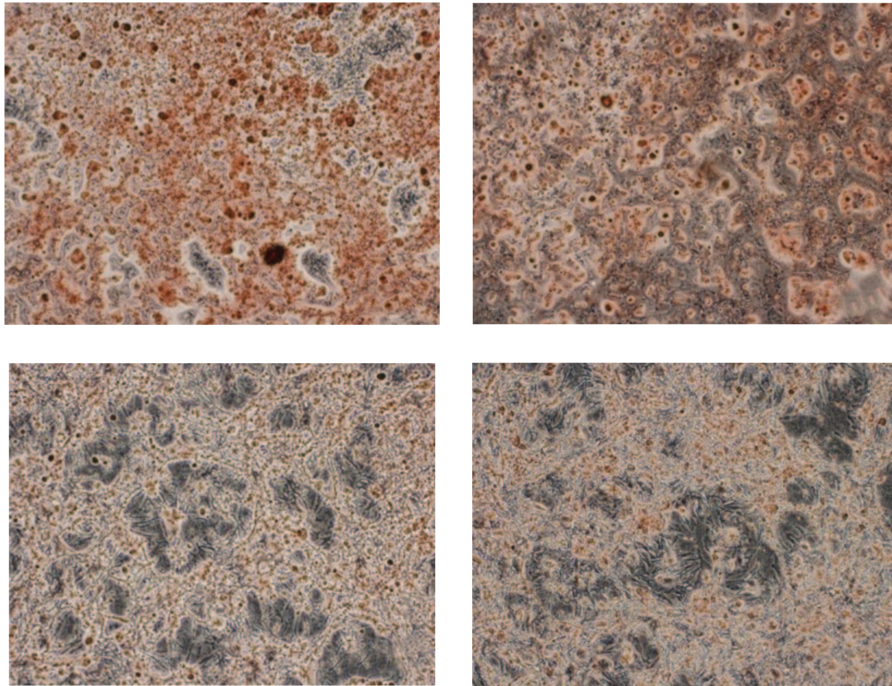

Fig.3a

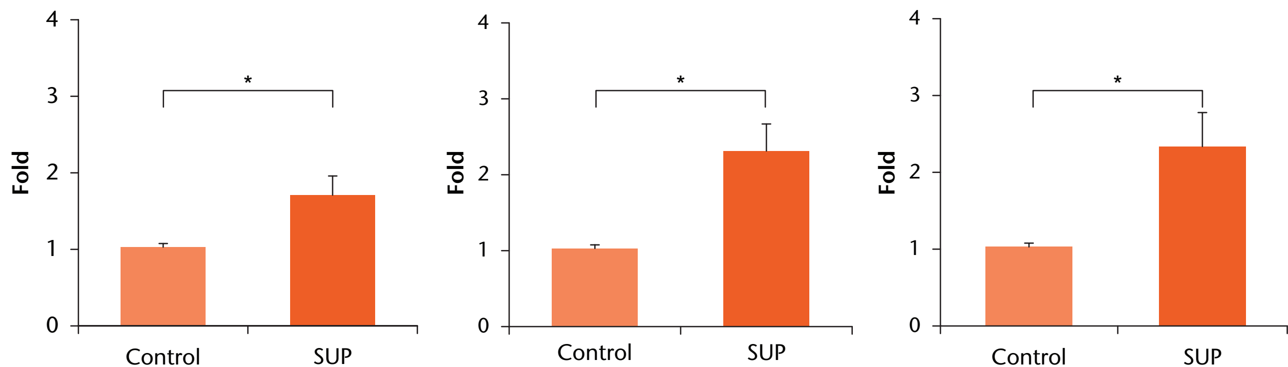

Fig.3b

Osteogenic differentiation capacity: a) Photomicrographs showing alizarin red staining at 14 days after addition of skeletal muscle supernatant. Control cultures were treated with PBS: top row: supernatant group; bottom row: control group; b) expression levels of RUNX2 (left), COL1a1 (middle) and osteocalcin (right) at 14 days after addition of skeletal muscle supernatant, analysed by real-time PCR (n = 6) (*; $p < 0.05$).

surgical site. Therefore, we considered that skeletal muscle would be a feasible autologous candidate tissue for the point-of-care approach.

In our study, muscle supernatant could not only promote cell proliferation, but also accelerate osteogenic differentiation of MSCs and angiogenesis of HUVECs, which suggests that muscle supernatant produced by a simple, easy and rapid method has effects on tissue repair and regeneration. In particular, bFGF is recognised as playing an important role in osteogenesis and angiogenesis. Many studies suggest that bFGF contributes to the repair and regeneration of a variety of injured tissues including bone,[17-19] cartilage,[18] nerve tissue,[19] and wound healing.[20,21] In the current study, we confirmed the abundant content of bFGF in muscle supernatant (325.6 pg/mL, SD 210.2). According to previous reports, PRP contains smaller amounts of bFGF (37.9 pg/mL, SD 7.8),[22] a fact which supports the suggestion that skeletal muscle supernatant may have superior biological properties compared with PRP.

Regarding another aspect of skeletal muscle supernatant, it was confirmed that a number of cells, such as CD34+ and CD44+/CD105+, exist in the supernatant. CD34+ cells are commonly known not only as haematopoietic stem/progenitor cells, but also as endothelial or osteogenic progenitor cells, and this fraction has

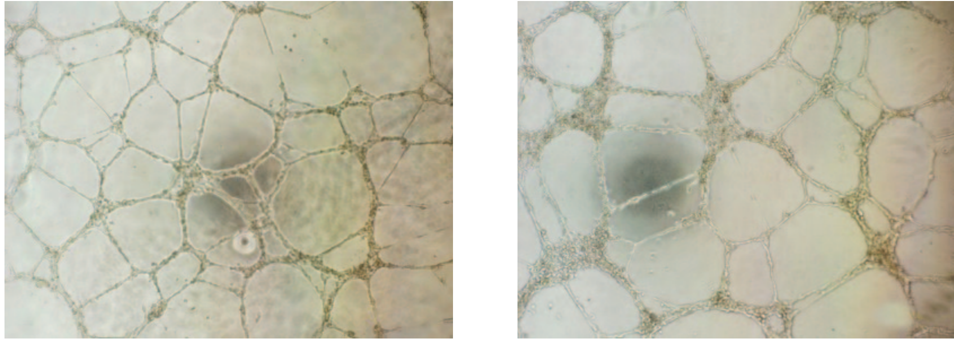

Fig.4a

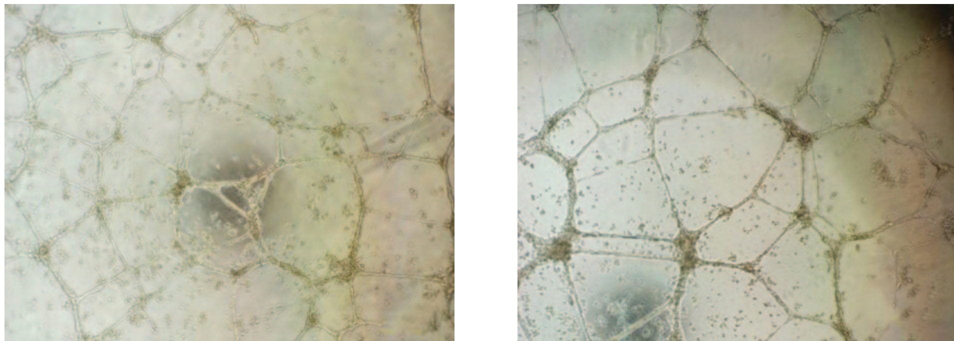

Fig.4b

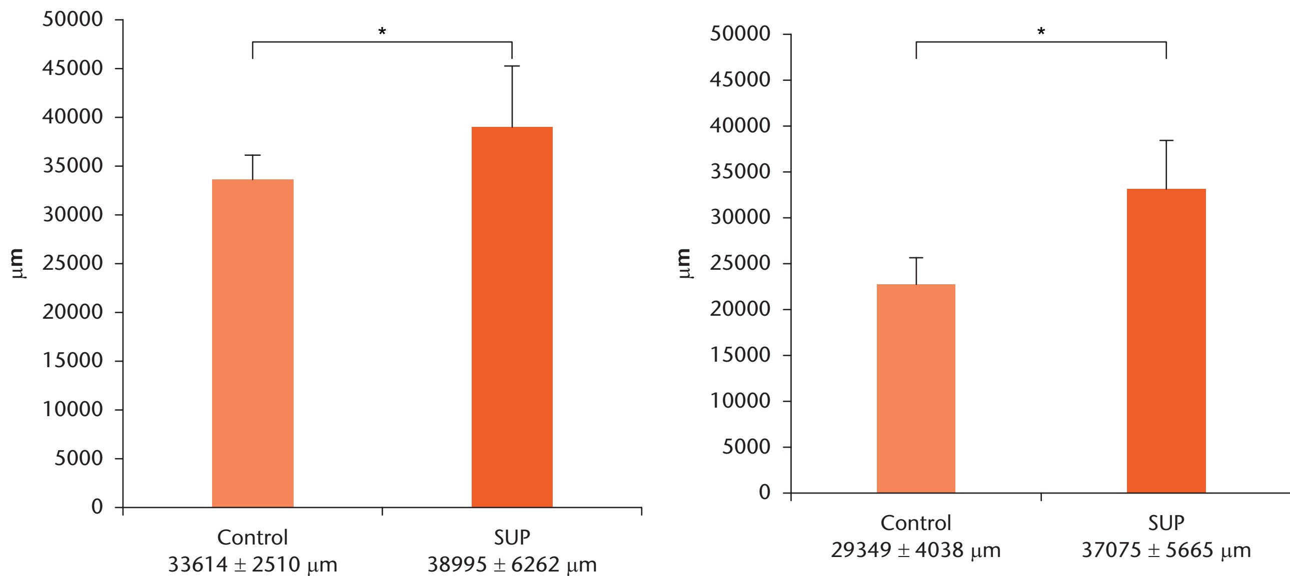

Fig.4c

Photomicrographs of tube formation in cultures of HUVECs at 12 (a) and 24 hours (b) after addition of skeletal muscle supernatant. Control cultures were treated with PBS: (a) supernatant group at 12 hours (left) and control group at 12 hours (right); (b) supernatant group at 24 hours (left) and control group at 24 hours (right). Total tube length measured at 12 (left) and 24 hours (right) after addition of skeletal muscle supernatant (c) (n = 6) (*; $p < 0.05$). The control group was treated with 5 µL PBS and the supernatant group was treated with 5 µL of muscle supernatant (n = 6). Data are expressed as mean and standard deviation (*; $p < 0.05$).

great therapeutic potential for tissue repair via angiogenesis *in vivo*.[23] Although the populations of these cell fractions are very limited in the supernatant, their beneficial effect at the administered site would still be evident due to their naturally limited frequency in the human body.

As demonstrated in this study, skeletal muscle tissue can be obtained from the semitendinosus tendon.

Therefore, the surgical reconstruction of the ACL, especially using autologous hamstring tendon, would be a favourable clinical target of this point-of-care approach. Repair of ACL injury still involves several problems such as combined injuries of cartilage and meniscus,[24,25] healing of the tendon–bone interface,[26,27] and maturation of grafted tendon.[28] To accelerate tissue repair and maturation, muscle tissue supernatant could be a promising option. Based on the biological properties of muscle supernatant, this could be applicable to various surgeries such as repair of fractures, tendon injuries, and wounds.

There are several limitations to this study. Firstly, the effective amounts of CKs and GFs or stem/progenitor cells necessary for tissue repair is still unclear. The obtainable volume of muscle is limited. In our study, the average amount of semitendinosus muscle harvested was approximately 1.5 g, and this yielded approximately 3.0 mL of muscle supernatant, which contained approximately 0.9 ng bFGF. The application of muscle supernatant would be mainly for local use such as in meniscus tear and cartilage defects. Hence, this approach might lead to successful results when used for tissue repair and regeneration depending on the administration site, although the volume of factors would be very limited. Second, muscle supernatant may contain factors with the adverse effects of exposure to proinflammatory cytokines. Our data demonstrated that muscle supernatant in large quantities could accelerate cell proliferation, osteogenesis and angiogenesis. The effect on chondrogenesis, neurogenesis, and tendon healing should also be examined to confirm its universal effect. Moreover, the orchestration of CKs and GFs that we presented in this study has to be investigated. Finally, the therapeutic potential *in vivo* was not analysed in animal studies. Further investigation will be needed before future clinical application can be undertaken.

In conclusion, the current study presents the potential of a new point-of-care strategy for regenerative medicine using skeletal muscle supernatant. This attractive approach and material could be a promising option not only for use in knee surgery but also in other tissue repair and regeneration procedures in the clinical setting.

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Author Contribution

- M. Yoshikawa: Acquisition of data, Analysis and interpretation of data, Drafting of manuscript.
- T. Nakasa: Study conception and design, Critical revision of the article for important intellectual content.
- M. Ishikawa: Acquisition of data.
- N. Adachi: Final approval of the article.
- M. Ochi: Final approval of the article.

ICMJE COI Statement

- None declared.

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