Master of Science

Continuous pumping for a gradient tendon-muscle engineering

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of the University of Ulsan

Department of Electrical, Electronic and
Computer Engineering

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Continuous pumping for a gradient tendon-muscle engineering

Supervisor: Professor Kyo-in Koo

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ABSTRACT

The interface between two tissues is considered the weakest point in our body, where injuries usually happen due to high-intensity exercise or doing sports. One of these examples is the myotendinous junction (MTJ), an interfacial zone between tendon and muscle. Tissue engineering aims to use biocompatible materials with tissue-specific cells to fabricate the target tissue for regeneration. However, making advanced single tissues does not solve the problem of interface injuries, as they can happen again after the surgery, due to the complex structure of the interface given not only by the existing proteins but also the mechanical properties. The gradient characteristic of MTJ in our body helps to reduce the force pressure from muscles to bones which most of the recent research about MTJ fails to replicate or rather uses complex systems. Therefore, making this gradient interfacial tissue is necessary for regenerating functions of the tendon-muscle unit. In this research, we propose a continuous extrusion technique by using a simple and accessible device to fabricate gradient tendon-muscle scaffolds which can be changed the parameters suitable for different targets. This device contains three capillaries: two inlets are perpendicular, and one outlet was connected with a plastic tube. The bio-inks were made by mixing collagen and decellularized extracellular matrix (dECM) derived from tendon and muscle tissue. By this way, the tissue-specific proteins were provided to help cells proliferation and differentiation. Then, the human cells: tenocytes and muscle cells, were mixed carefully in the tissue-specific bio-inks before printing. The pumping speeds were set for 10 uL/min for both syringe pumps, which were programmed sequentially. The printed time per construct were 4 minutes. And then, the scaffolds were kept in the incubator (5% CO_2 and 37°C) for 10 to 15 minutes to allow the gelation after printing. Scaffolds were cultured in the growth medium for 1 day and moved to the differentiation afterwards, and then collected at day 1 and day 7 for further examination. By pre-staining two type of cells with Cell Tracker solutions, we could monitor the cell location post printing and successfully fabricate a gradient tendon-muscle scaffold with three zones: tendon, muscle and the myotendinous junction, a gradient transition between two types of cells. The cells viability of the scaffolds, for both tendon and muscle, were observed

through images with more than 95% after 1 day and 7 days culturing. Electrical stimulation experiment was set up with the aim to increase the differentiation of muscle tissue. At the results, skeletal muscle cells in the stimulated group formed more aligned and densely packed myotubes compared to non-stimulated group.

Key words: tendon engineering, muscle engineering, interface engineering, gradient scaffold, decellularized extracellular matrix.

CHAPTER I: INTRODUCTION

Interfacial zones between tissues in our body can be divided into four categories: cartilage-bone, ligament-bone, tendon-bone, and muscle-tendon interfaces [1]. The transition zone between muscle and tendon (the myotendinous junction) is known as the weakest link in the tendon-muscle unit, leading it to be the most interface tissue involved in strain injury. The injuries that happen in this interface are prevalent due to the biomechanical inconsistency between these tissues which present high strain fields [1], especially in popular sports such as football [2].

Even though researchers have made great improvements in single tissue engineering, such as muscle tissue with good functions as in the natural [3], interfacial tissue engineering remains challenging in mimicking the myotendinous junction (MTJ) given that the complexity and gradient in their structure as well as mechanical demands [1]. In nature, the MTJ has a unique composition to transmit force from soft tissue (muscles) to hard tissue (bones) through tendon tissue. It is not a clear transition between muscle and tendon but is where muscle fibers interact with elements of the extracellular matrix (ECM) from tendon tissue [6, 7], helps to transmit the contractile force to the tendon, and finally to the bone. Therefore, providing ECM proteins for tendon-muscle tissue scaffolds is necessary in order to be able to mimic the native MTJ tissue.

In tissue engineering, cell encapsulated bioprinting is a technique in which cells are mixed with a hydrogel solution to form a bio-ink before printing [8]. In this technique, hydrogel plays an important role as its function is to mimic the characteristic of native ECM. A huge number of synthetic and naturally derived hydrogels have been used in bioprinting, namely gelatin, collagen, polyethylene glycol, and pluronic [9, 10]. However, there are some limitations regarding natural materials, for instance, they show poor physical properties during the culture period [9]. On the other hand, synthetic hydrogels lack the requirement for biological activities, such as poor absorbable or not support for cell adhesion [10]. For the past few years, decellularized extracellular matrix (dECM) from various tissue have been used as bio-ink to solve those problems including skeletal muscle, kidney, tendon,

bone, dermis, and lung [6, 11]. As derived from target tissue, dECM contain tissue-specific biochemical and physiological characteristics, such as growth factors and cytokines [12]. Furthermore, dECM can form stable hydrogels under physiological as well as be mixed with other hydrogels to enhance its mechanical properties, dECM becomes a promising material for tissue engineering [9, 14]. In these techniques, hydrogel plays an important role as its function to mimic the characteristic of native ECM. A huge number of synthetic and naturally derived hydrogels have been used in bioprinting, namely gelatin, collagen, polyethylene glycol, and pluronic [15]. However, there are some limitations regarding natural materials, for instance, they show poor physical properties during culture period [9]. On the other hand, synthetic hydrogels lack the requirement for biological activities, such as poor absorbable or not support for cell adhesion [9]. Finding a good material for printing process is necessary in tissue engineering.

Extra cellular matrix (ECM) is a three-dimensional network surround cells, contains collagens, fibronectin, laminins, proteoglycans/glycosaminoglycans, and other glycoproteins. Signals from the surface receptors were transmitted to cells through ECM. Therefore, ECM is responsible for many cellular functions, included survival, growth, migration, and differentiation [14]. For the past few years, decellularized extracellular matrix (dECM) from various tissue have been used as bio-ink to solve those problems including skeletal muscle, kidney, tendon, bone, dermis, and lung. Extra cellular matrix (ECM) is a three-dimensional network surround cells, contains collagens, fibronectin, laminins, proteoglycans/glycosaminoglycans, and other glycoproteins. Signals from the surface receptors were transmitted to cells through ECM. Therefore, ECM is responsible for many cellular functions, included survival, growth, migration, and differentiation [14]. For the past few years, decellularized extracellular matrix (dECM) from various tissue have been used as bio-ink to solve those problems including skeletal muscle, kidney, tendon, bone, dermis, and lung [8, 14]. As derived from target tissue, dECM contain tissue-specific biochemical and physiological characteristics, such as growth factors and cytokines [14]. Furthermore, dECM can form stable hydrogels under physiological as well as be mixed with other hydrogels to enhance its mechanical properties, dECM becomes a promising material for tissue engineering. As derived from target tissue, dECM contain

tissue-specific biochemical and physiological characteristics, such as growth factors and cytokines [15]. Furthermore, dECM can form stable hydrogels under physiological as well as be mixed with other hydrogels to enhance its mechanical properties, dECM becomes a promising material for tissue engineering [8].

In this study, we propose a continuous pumping technique with two bio-inks making by dECM from tendon tissue and muscle tissue to fabricate the tendon-muscle gradient structure with three parts: tendon, muscle and the myotendinous junction (MTJ). After seven-day of culturing, the cultured scaffolds were analyzed in view of cell viability and cell differentiation.

CHAPTER II: MATERIALS AND METHODS

2.1. Decellularized extracellular matrix (dECM) preparation

The details of the protocol to produce the tendon decellularized extracellular matrix (tdECM) and the muscle decellularized extracellular matrix (mdECM) are based on the protocol designed by P. J. Schaner [15]. Fresh porcine tendon and muscle were collected from a local slaughterhouse and minced into cubic pieces of approximately 2mm in length. Then, the sliced pieces were washed with deionized water for 6 hours to remove any remaining blood. Afterwards, to remove residual cells, the tissues were rinsed carefully in 0.3 wt% sodium dodecyl (151-21-3, Thermo Fisher Scientific, Massachusetts, United States) solution for 24h, and 3 wt% Triton X-100 (9002-93-1, Sigma-Aldrich, United States) solution for another 24 h while stirring gently. To wash out the remaining chemical detergents, tissues were treated with PBS for 24h. And then, they underwent the deep-frozen at −80 °C and lyophilized to powders. The tendon dECM and muscle dECM were stored at -20 °C until further use. All steps were conducted under aseptic conditions.

2.2. Tendon and muscle dECM hydrogel formation

2.2.1. Solubilization

The decellularized tendon ECM and muscle ECM powders were used to form tissue-specific hydrogels by enzymatic digestion in an acidic environment. The solubilization processes were done by strongly stirring 100 mg of each type of dECM in 0.5 M acetic acid solution (64-19-7, Sigma-Aldrich, United States) and 10 mg of pepsin (P6887-1G, Sigma-Aldrich, United States) for 48 h at room temperature with constant speed. The tendon dECM (tdECM) and muscle dECM (mdECM) solutions were separately stored at 4^{o}C.

2.2.2. Neutralization

Before each experiment, the tdECM hydrogel and mdECM hydrogel were prepared by using a one-ninth volume of 10X PBS to provide salinity and NaOH 10M to neutralize the dECM solutions. The whole process was conducted over ice to prevent the dECM gelation.

To test the gelation time of tdECM hydrogel and mdECM hydrogel were kept in the incubator (37ºC).

2.3. Collagen hydrogel preparation

A solution of 3 mg/mL type I collagen solution was prepared following the protocol published by AIM Biotech (Singapore) [16]. Briefly, 10 mg/mL of type I collagen (5133-20ML, Advanced Biomatrix, California, United States) was diluted in 10X PBS, neutralized with 0.5 N sodium hydroxide (NaOH), and then added deionized (DI) water to obtain the target concentration. All the procedure was performed over ice to prevent collagen gelation.

2.4. Bio-inks formulation

The bio-ink 1 was composed of 40mg/ml tdECM hydrogel and 3 mg/ml collagen with a ratio of 7:3. And, the bio-ink 2 was a mix of 20 mg/ml mdECM hydrogel and 3 mg/ml collagen with a ratio of 7:3. To avoid the gelation, the bio-inks were kept in ice before and during the experiment.

2.5. Cells culture

Human tenocytes were purchased (#TEN-F; ZenBio Inc.; Durham, NC, United States) and cultured in Dulbecco's Modified Eagle Medium-high glucose (LM 001-05, Welgene; Republic of Korea) supplemented with 20% fetal bovine serum (WG-S001-01, Welgene; Republic of Korea) and 1% Penicillin-Streptomycin (15140122, Thermo Fisher Scientific, Massachusetts, United States). Culture plates were coated with collagen type I (5133-

20ML, Thermo Fisher Scientific, Massachusetts, United States) with the concentration of 200 ug/mL for 24 h in the incubator and rinsed carefully with 1X Dulbecco′s Phosphate Buffered Saline (D5652-10X1L, Sigma Aldrich) before seeding cells. Cells were seeded at the density of 370,000-500,000 cells per collagen I coated T-75 culture flask.

Primary human skeletal muscle-derived cells were purchased (SK-1111, Cook Myosite Inc, United States) and cultured in Skeletal muscle myoblasts basal medium (SKBMTM-2, Lonza, Switzerland) supplemented with Growth Factors kit (SKGMTM-2, Lonza, Switzerland). The cell density was adjusted to 3 500 cells per cm^2 before seeding.

Both cells were cultured at 37°C in a controlled humidified atmosphere containing 5% CO_2. The mediums were replaced every two days. Upon 80-90%, cells were washed carefully two times with phosphate-buffered saline (PBS) and dissociated by 0.05% trypsin-EDTA (25300-062, Thermo Fisher Scientific, Massachusetts, United States). The cells were resuspended in the growth medium and seeded in new dishes to get the needed amount for the experiment.

2.6. PDMS device

The device was fabricated with PDMS (Dow-sylgard 550, Dow Corning) as shown in Figures 1(a)-(b). Three glass capillaries (580 μm inner diameter (ID), 1B100- 6, World Precision Instruments, United States) were used to fabricate the device. It includes a horizontal inlet (inlet 1), a vertical inlet (inlet 2), and an outlet connected with a Tygon E3603 tubing (721014, Sigma-Aldrich, United States). Capillaries were assembled in a cuboid shaped PDMS body by pouring a mixture of the PDMS solution over them. After curing at 70^0C for 5-6 hours, the capillaries were removed and autoclaved at 121°C for 15 minutes before conducting experiments. Figure 1c also includes a schematic of the resulting scaffolds.

Table 1. The pumping procedure.

Procedure	Pump 1 (Bio-ink 1)	Pump 2 (Bio-ink 2)
Step 1	ON (55 seconds)	**OFF**
Step 2	**OFF**	ON (55 seconds)
Step 3	ON (55 seconds)	**OFF**

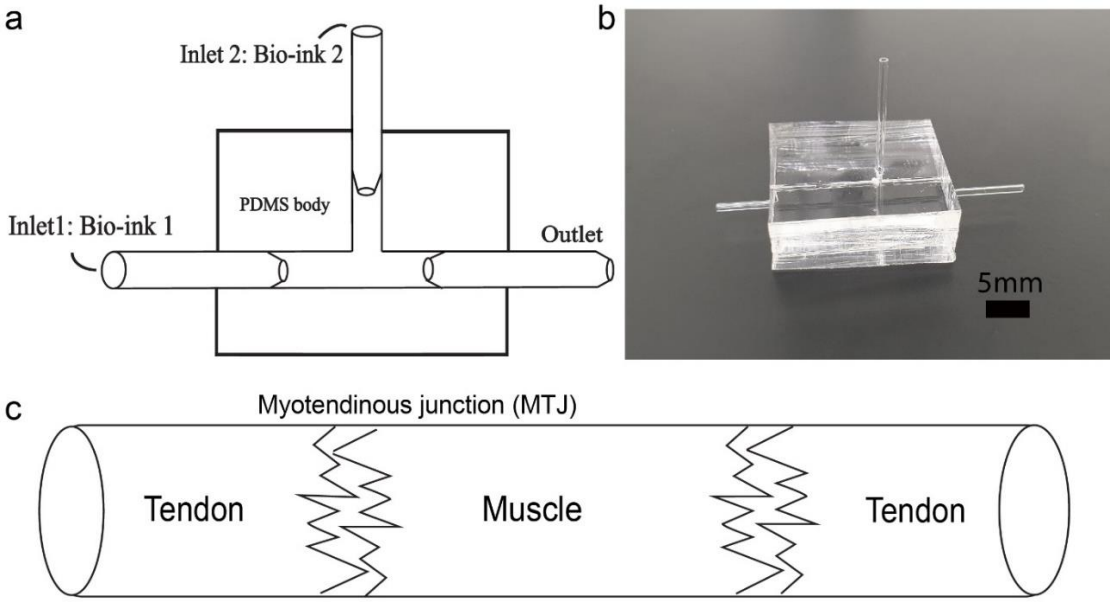

Figure 1. Experiment Concept (a) Schematic of the proposed generator. (b) The fabricated generator. (c) Schematic of the gradient tendon-muscle scaffolds with three zones: tendon, muscle, and myotendinous junction (MTJ).

2.7. Extrusion printing with micro-particles

To test our hypothesis of making a gradient scaffold by doing continuous extrusion of two different bio-inks, experiments with micro-particles were carried out. Two different colors of micro-particle based on polystyrene were used: dark blue (55463-10ML-F, Sigma-Aldrich, United States) represented for tendon cells mixed with bio-ink 1, and dark red (61946-5ML-F, Sigma-Aldrich, United States) represented for muscle cells mixed with bio-ink 2. By using two syringe pumps (11 Elite C300918, Harvard Apparatus, United States), continuous scaffolds were fabricated with the pumping speed 10 µL/min. After

printing, the microparticle-based scaffolds were kept in an incubator set with 37°C for 10 to 15 minutes for the gelation process. The micro-particles scaffolds were observed under the IX53 inverted fluorescent microscope (Olympus, Japan) and the images were captured using the cellSens software (Olympus, Japan).

2.8. Fabrication of gradient tendon-muscle scaffolds

The tendon-muscle scaffolds were fabricated using a simple device (Figure. 1). Expanded tenocytes and skeletal muscle cells at passage 6 were treated with 0.05% trypsin-EDTA to get single-cell suspensions. A mixture of bio-ink 1 and tenocytes was injected into inlet 1, and a mixture of bio-ink 2 and skeletal muscle cells was loaded into inlet 2. Cell density in two mixtures was adjusted to about 5×10^6 cells/mL. The pumping rate was set at 10 μL/min for both syringe pumps (11 Elite C300918, Harvard Apparatus, United States). After printing, scaffolds were polymerized into a plastic tube for 10 to 15 mins in the incubator (5% CO_2 and 37°C). Then, scaffolds were gently pushed out of the plastic tube by using a pit moved to the culture dishes containing growth media and cultured in the incubator (5% CO_2 and 37°C).

At 1 d after printing, the media were switched to the differentiation media: 97% Dulbecco's Modified Eagle Medium-low glucose (DMEM-11885084, Thermo Fisher Scientific, Massachusetts, United States), 2% horse serum (Thermo Fisher Scientific, Massachusetts, United States), 0.2 mg/ml 6-aminocaproic acid (60-32-2, Sigma-Aldrich, Unites States), 10 μg/ml insulin (wp-004, Thermo Fisher Scientific, Massachusetts, United States), 30 units/mL penicillin + 30 μg/mL streptomycin (15140122, Thermo Fisher Scientific, Massachusetts, United States), and 50 μg/mL gentamicin (GIB-15710-072, Thermo Fisher Scientific, Massachusetts, United States). The medium was changed every two days. Scaffolds were collected on day 1 and day 7 in culture for other analysis.

2.9. Fluorescent cell labelling and tracking

To monitor cell location, cells were fluorescently labeled before printing using a standard protocol. Tenocytes cells were labeled with the green fluorophore (CellTracker™ Green BODITY™ Dye, Thermo Fisher Scientific, Massachusetts, United States) and Skeletal muscle cells were labeled with the red fluorophore (CellTracker™ Red CMTPX dyes, Thermo Fisher Scientific, Massachusetts, United States).

Allowed the dye vial to warm to room temperature before dissolving the lyophilized product in Dulbecco's Modified Eagle Medium-low glucose (DMEM-11885084, Thermo Fisher Scientific, Massachusetts, United States) to a final concentration of 10 mM to get CellTracker™ Working Solution. Added 1 to 2 ml of the Working Solution to each cell plate. Cells were exposed to the dye agent for 30 - 35 minutes under growth conditions (37°C and 5% CO_2). Removed the solution and washed the cells with 5 mL PBS solution 2 times to remove all traces of serum that contains trypsin inhibitor. Added a corresponding volume of 0.05% Trypsin-EDTA solution to the plate and incubated for 3 minutes. Harvested the cells by centrifugation, and then mixed with the bio-inks in preparation for printing.

Constructs were visualized and imaged under a fluorescence microscope to track cell's location. Under two different filters, green fluorescence was visualized in tenocyte cells and red fluorescence was visualized in skeletal muscle cells.

2.10. Cell viability

After printing, the cell viability of the tendon-muscle constructs was measured using a LIVE/DEAD™ Viability/Cytotoxicity Kit (L3224, Thermo Fisher Scientific, Massachusetts, United States) at three different time points (1, 7, and 14 days in culture). The staining solution was made by adding 5 µl Calcein-AM and 20 µl ethidium homodimer-1 to 10 mL of DPBS. After removing the medium, added the staining solution to each construct and incubated in the dark for 30 min at 20-25°C. Rinsed the constructs carefully in PBS solution for 3 times, 5 minutes for each time. The nuclei of cells were

shown by staining DAPI (NucBlue® Live ReadyProbes™ Reagent, Thermo Fisher Scientific, Massachusetts, United States) for 15 minutes in incubating condition at 37°C, 5% CO2. Then, the printed constructs were observed under a fluorescence microscope.

To calculate cell viability, twelve images (3 images per experiment) were analyzed using ImageJ software (Fiji, NIH Image, United States, version 1.51 h). The fluorescent signal of the images was split into a green channel (live cell signal), a red channel (dead cell signal), and then measured the intensity of the two channels. The percent of cell viability was calculated from the measured signal intensity using the ratio of green intensity over the total intensity by the following formulation:

$$viability = \frac{green\ intensity}{green\ intensity + red\ intensity} \times 100 [17]$$

2.11. Electrical pulse stimulation

Electrical stimulation set-up for the scaffolds based on the protocol from Park *et al*. with some modifications [18].

After 4 days of pre-culture, the printed constructs were moved to stimulation dishes containing 10 mL of the culture media. Two platinum wires were immersed in the culture media and connected to the electrical stimulator at one end. The scaffolds were continuously stimulated for 2 days and 3 hours each day. The stimulation parameters were followed by 2 Hz for the frequency and 5 V for the voltage. For control, printed constructs were cultured in the stimulation dish without stimulation for 6 days. During the culture time, the medium was refilled after 2 days of stimulation.

Samples were collected and evaluated the differentiation of muscle tissue.

After 3 days of pre-culture, the printed constructs were moved to stimulation dishes containing 10 mL of the culture media.

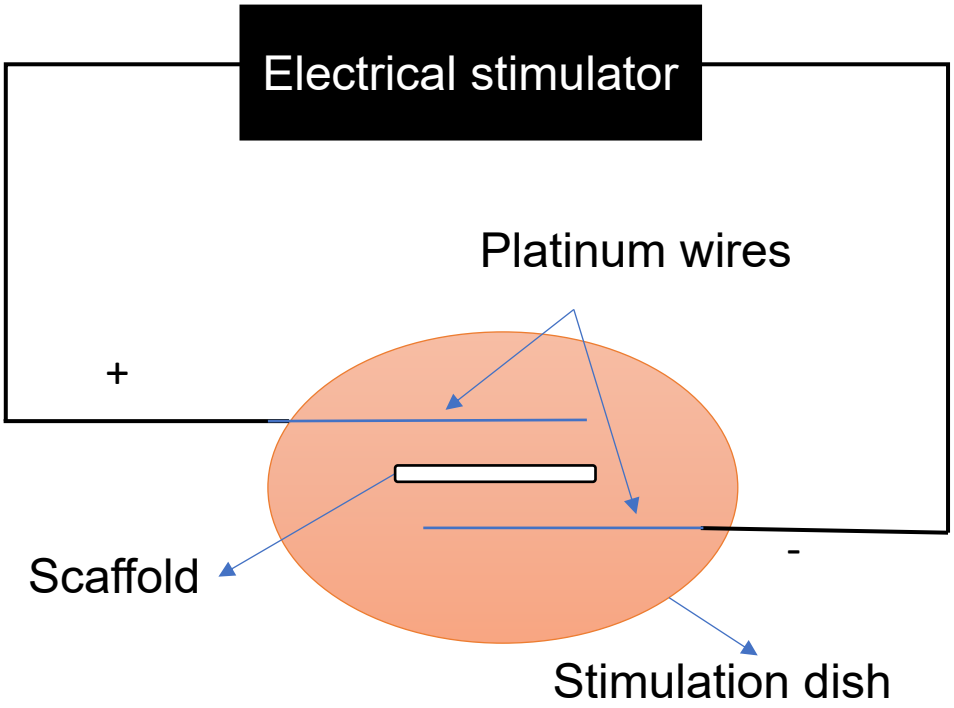

Figure 2. Schematic of electrical stimulation set-up. Scaffolds were stimulated by platinum wires connected with an electrical stimulator.

2.12. Immunohistochemistry

Tissue models were fixed in 4% paraformaldehyde (P6148 Sigma-Aldrich, United States) in PBS for 20 minutes at room temperature. Following fixation, tissues were washed carefully with PBS for 3 times, 5 min each time. After aspirate PBS, 0.1% Triton X-100 (T8787, Sigma-Aldrich, United States) in PBS was added for 4 minutes, washed in PBS, and then blocked in 1% bovine serum albumin (BSA, 37525, Thermo Fisher Scientific, Massachusetts, United States) for 30 minutes at room temperature.

For muscle tissue characterization, antibodies against myosin heavy chain (2265353, Thermo Fisher Scientific, Massachusetts, United States) were used with a dilution of 1:100 in PBS and incubated overnight at 4°C. After washing 3 times with PBS, samples were incubated with secondary antibody Alexa Fluor 488 (diluted in blocking buffer 1:500) for

1 hour at room temperature. After the PBS wash steps, samples were incubated with 4′,6-diamidino- 2-phenylindole solution (DAPI, NucBlue® Live ReadyProbesTM Reagent, Thermo Fisher Scientific, Massachusetts, United States) for 5 minutes.

2.13. Microscopic imaging and analysis

The cell-laden scaffolds were observed under the IX53 inverted fluorescent microscope (Olympus, Japan) and the images were captured using the CellSens software (Olympus, Japan). For Live/Dead staining and fluorescently prelabelled cell-laden scaffolds, the samples were then observed under a fluorescence microscope (Olympus, Japan). Then, the images were analyzed using the ImageJ 1.51h software (National Institutes of Health, United States).

CHAPTER III: RESULTS

3.1. Development of tissue-specific hydrogels

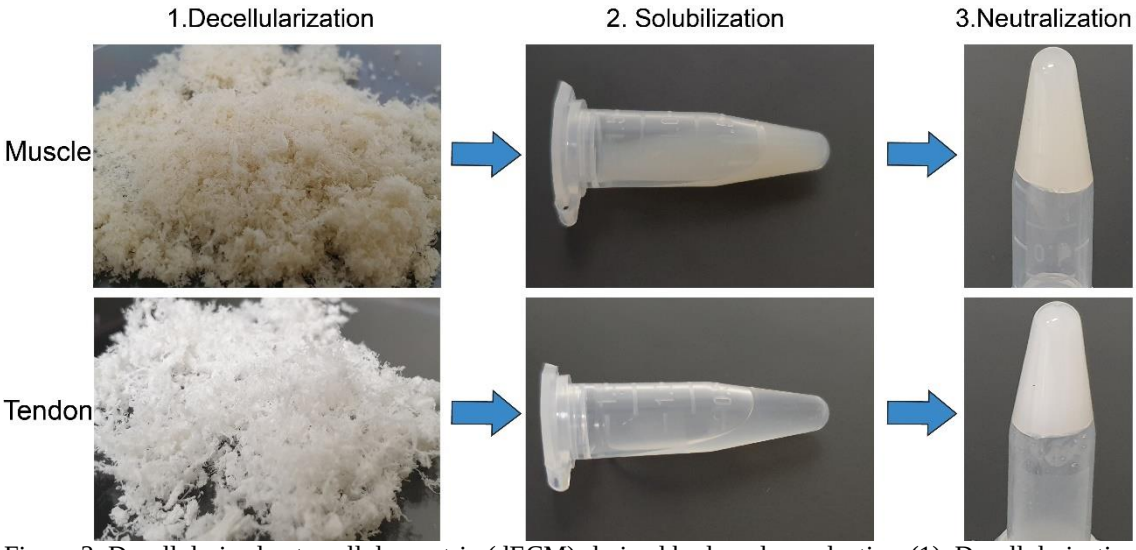

Figure 3. Decellularized extracellular matrix (dECM)-derived hydrogels production. (1). Decellularization (2). Solubilization (3). Neutralization.

Fresh muscle and tendon underwent three steps to become tendon and muscle dECM hydrogels: (1) Decellularization: remove the cellular components to get dECM powders (tendon and muscle dECM), (2) Solubilization: solubilize the dECM powders by using acid acetic and pepsin to form mdECM and tdECM solutions, and (3) Neutralization: neutralize the dECM solutions to a physiological pH and salinity to form mdECM and tdECM hydrogels (Figure 3).

The polymerization mechanism of dECM hydrogel is thermal crosslinking. In the condition of incubation at 37^{o}C, dECM hydrogels formed gels after 10 to 15 minutes (Figure 3.3).

3.2. Microparticle-based scaffolds

To test our hypothesis of making scaffold using our device and pumping method, experiments using micro-particles as a replacement for two types of cells were conducted

with inlet 1: blended bio-ink 1 with microparticles blue, and inlet 2: blended bio-ink 2 with microparticles red (Figure 4A).

The microparticle-based scaffolds were fabricated with three parts represented for the gradient tendon-muscle unit (Figure 4B). Under the microscope, we can observe one end of the fabricated scaffolds contains > 95% blended blue micro-particles in bio-ink 1 to act as tendons (Figure 4C.i), while, on the other end, >95% red micro-particles are represented as muscle (Figure 4C.iii). Transitions in color from blue to red along the scaffold's length give a visual indication of the resulting gradient (Figure 4C. ii). Thus, showing that a smooth transition between the two bio-inks was fabricated. From the results, it is possible to fabricate the gradient tendon-muscle constructs with our method.

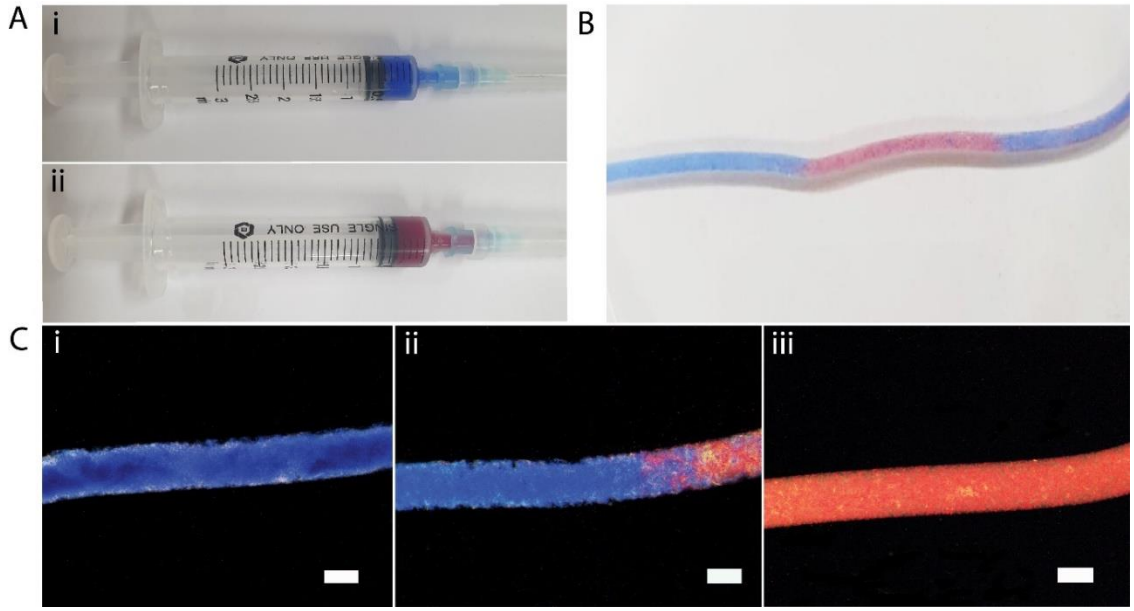

Figure 4. The fabricated micro-particle scaffold. A. bio-inks: (i) bio-ink 1 mixed micro-particles blue, (ii) bio-ink 2 mixed with micro-particles red; B. Microparticles-based Scaffolds. C. Three regions of the scaffolds under the microscope. Scale bar: 500 μm

3.3. Structure and composition of gradient tendon-muscle scaffolds

The gradient tendon-muscle scaffolds were fabricated using syringe pumps and our device. human tenocytes were fluorescently labelled with CellTracker Green and human skeletal

muscle cells were labelled with CellTracker Red to keep track of the location of cells and observe the cell-to-cell reaction after printing.

To prepare for printing process, bio-ink 1 was mixed with fluorescently labelled human tenocytes cells and bio-ink 2 was mixed with fluorescently labelled human skeletal muscle cells. And then, the mixtures were loaded into separate syringes for the printing process. The printing time per construct was approximately 4 minutes and the post-printing time was 15 minutes in the incubator (37^{o}C) for the gelation of the hydrogels. In the results, the scaffolds were 3 cm long with 0.8 mm diameter.

Cell location in the printed constructs was visualized under the microscope. Three different zones of scaffolds were distinguishable: the tenocytes cells were green, the skeletal muscle cells were red, and the myotendinous junction was the yellow part (Figure 5). The interface between two types of cells was smooth and showed the gradient transition of cell concentration between two tissues.

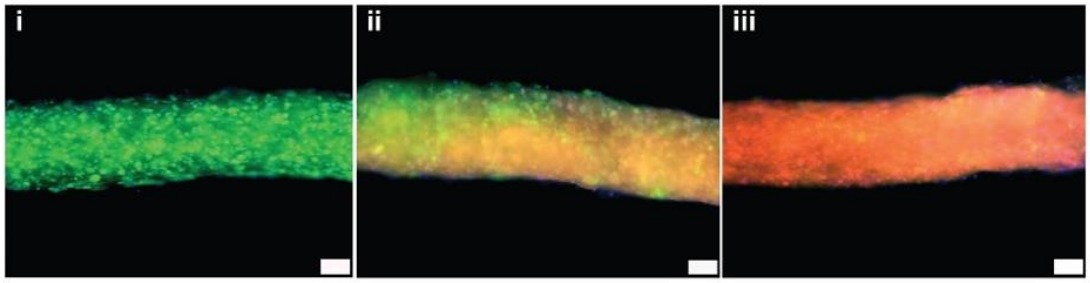

Figure 5. Fluorescent-labelled cell printed gradient tendon-muscle constructs. (i) tendon side, (ii) the MTJ, and (iii) muscle side. (Green: tenocyte cells; red: skeletal muscle cells; yellow: the interface region between two cells). Scale bar: 100 µm.

3.4. Cell viability in scaffolds

To check the cell viability throughout the printing process, the gradient tendon-muscle scaffolds were fabricated without fluorescently prelabeled steps for cells before printing. Samples were collected on day 1 and day 7 culturing in the proliferation mediums and evaluated the cell viability by using the Live/Dead kit. Only after one day, tenocytes and skeletal muscle cells started to change their morphology and increased the number. In the

case of skeletal muscle cells, they formed bundle-like structures after 7 days proving good proliferation and cells differentiation (Figure 6).

The quantitative results showed that two types of cells remained in good viability during the culturing time. On the muscle side, skeletal muscle cells with 97.60 ± 0.45 viability on day 1 retained good viability at 95.42 ± 1.54 on day 7 in culture. On another hand, tenocytes were 96.73 ± 2.15 viable on day 1 and recovered slightly with 97.12 ± 2.58 viability after culturing for 7 days (Figure 7). Therefore, our printing method and printing materials did not affect the cell viability and cell proliferation of the gradient tendon-muscle scaffolds.

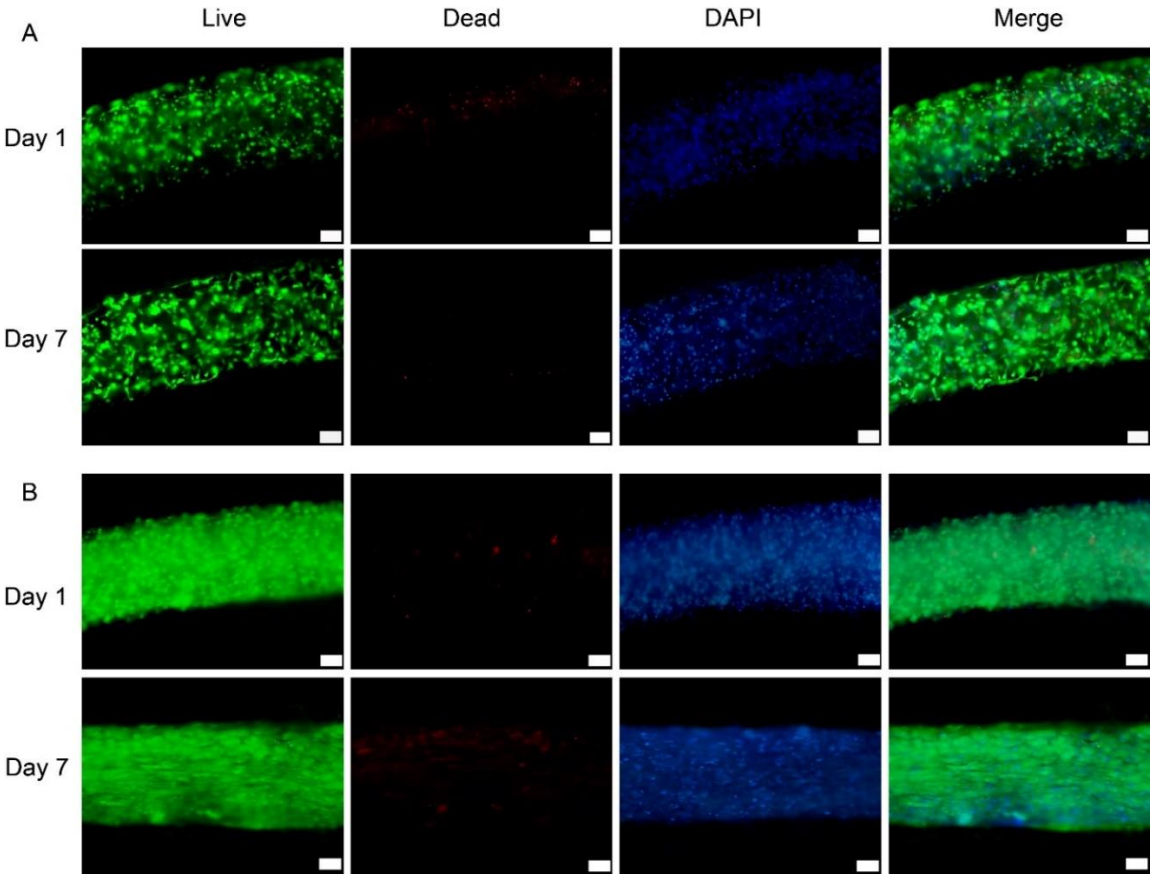

Figure 6. Cell viability after printing: Live/Dead images staining of (A) Tendon cells and (B) skeletal muscle cells were assessed for viability at day 1 and day 7 using a Live/Dead cytotoxicity assay (green: live, red: dead, blue: cell nuclei). Scale: 100 μm.

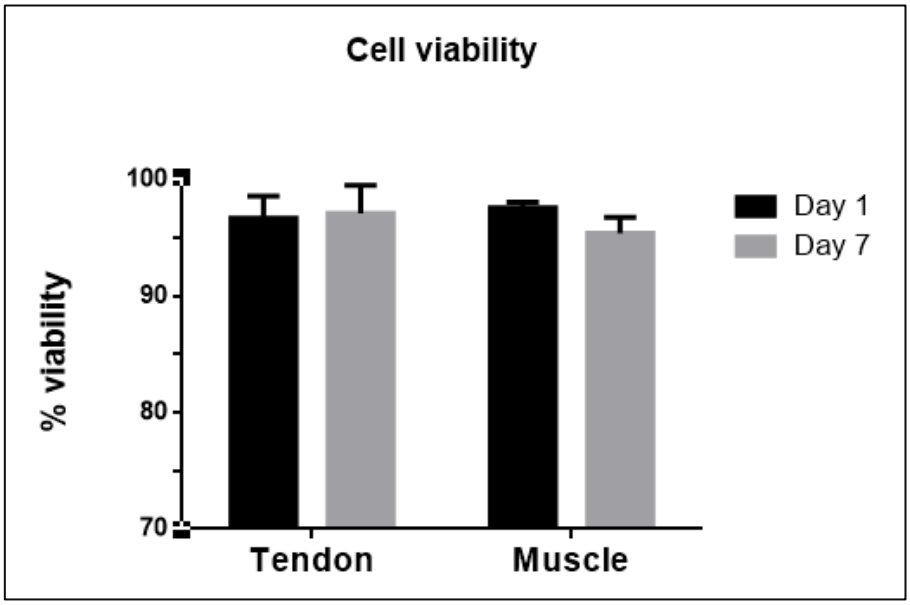

Figure 7. Quantitative data of cell viability

3.5. Skeletal muscle cells differentiation and electrical stimulation

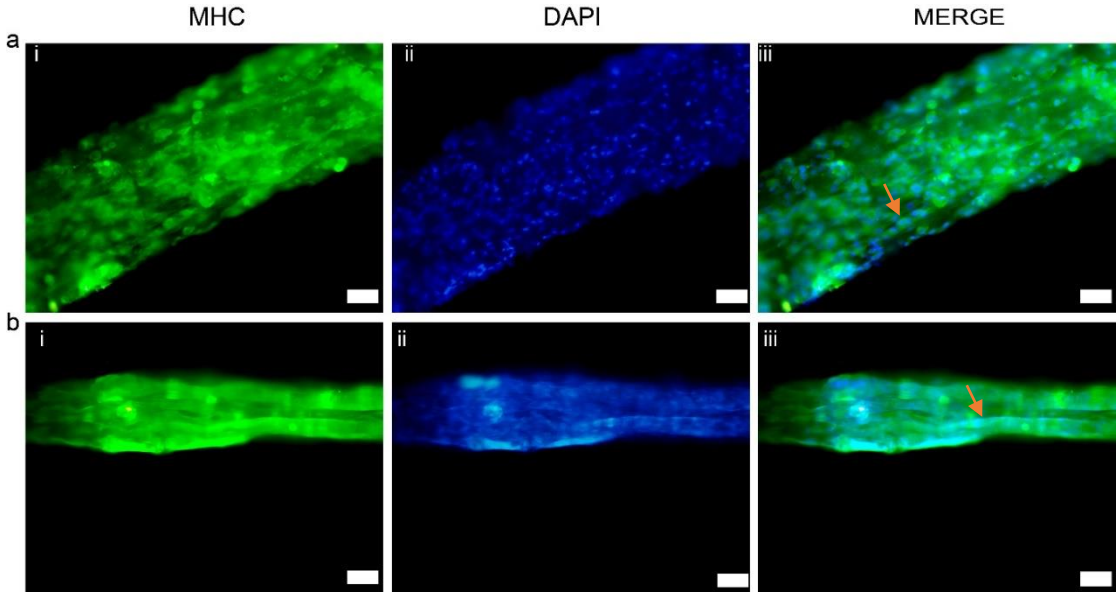

Figure 8. Effect of electrical stimulation on skeletal cells differentiation. Myosin heavy chain (MHC) staining at day 7 differentiation of (a) non-stimulated scaffolds and (b) electrically stimulated scaffolds (green: MHC, blue: cell nuclei, arrow: myotube). Scale bar: 50 μm

After printing, the gradient tendon-muscle scaffolds were kept in a proliferation medium for a day to give time for cell growth and environmental adaption before beginning tissue differentiation. After 7 days of culturing, we examined tissue composition and architecture by immunohistology to confirm tissue maturation and differentiation. Herein, we demonstrated on the maturation of muscle tissue by myosin heavy chain (MHC) immunostaining. During muscle differentiation, skeletal muscle cells fuse to become multi-nucleated and functional myotubes. As the result, multinucleated myotube development was seen throughout the entire scaffold (Figure 8.a).

To increase the differentiation of muscle tissue, printed scaffolds were electrically stimulated for 2 days for 3 hours per day. After the stimulation time, the myosin heavy chain (MHC) immunostaining result showed that the scaffolds were more compacted with a higher degree of myofiber alignment compared to un-stimulated scaffolds. Our data demonstrated that electrical stimulation promoted the differentiation of skeletal muscle cells (Figure 8.b)

CHAPTER IV: DISCUSSION

The extracellular matrix (ECM) is an extremely hydrated and viscoelastic network, that contains soluble macromolecules including cytokines, chemokines, and growth factors. These components' molecular concentration gradients are crucial for biological processes like morphogenesis, wound healing [21, 22], and chemotaxis [21]. Additionally, the characteristics of the gradients in the ECM and the local cell density can unite mechanically dissimilar tissues, such as tendon-muscle junction and bone-cartilage interfaces [22]. The ECM in the skeletal muscle of an adult includes three layers with different ECM compositions: the endomysium, the perimysium, and the epimysium [23]. To help transmit contractile forces from muscles to bones for motion, the epimysium layer is believed to be continuous with the tendon [26, 27]. This continuous section between the tendon and the muscle is known as the myotendinous junction (MTJ), a specific interface formed through the interaction of integrin with ECM proteins released by tendon and myotube [26]. On the other hand, the main component of tendons is the type I collagen fiber that usually is arranged in the same direction of the tensile force [27, 28]. Sutures have typically been used to regenerate injured MTJ, however, this treatment has a high risk of recurrence and causes fibrous tissue in the deficient area [31, 32]. Tissue engineering techniques have been investigated as a solution to this issue by using the functionally and biochemically properties of different biomaterials to mimic the target tissue [33, 34]. Even though the use of biomaterials and novel printing techniques have contributed tremendously to the advancement of MTJ regeneration, an ideal MTJ junction with a functional gradient is yet to be considered.

Herein, we propose a simple and valid method for the fabrication of artificial tendon-muscle scaffolds with a gradient interface to scale up tendon-muscle tissue engineering for clinical applications. To provide specific biochemicals for target tissues, decellularized extracellular matrix (dECM) from muscle and tendon were used as the main component of bio-inks for cell-laden scaffolds. However, the most frequent drawback of these hydrogels made from solubilized ECM is poor mechanical properties [19]. Different mechanical supporting materials have been used to address this problem, including mixing

with biological polymers [36, 37], or crosslinking using chemical [38] and UV photochemical processes [39]. Previous research revealed that crosslinkers do not produce sufficient mechanical characteristics or can cause inflammation[24], therefore adding biocompatible polymers would be a good option to achieve good mechanical stability. Herein, we combined the dECM hydrogel with collagen type I hydrogel, a key component of tendon ECM, with a ratio of 30% collagen type I and 70% dECM. For the printing process, two types of primary cells were used: tenocytes to create the tendon and skeletal muscle cells to mimic the muscle.

The used device is a simple cuboid shaped PDMS, which parameters can be changed according to the target diameter of scaffolds, compared to other works using 3D bioprinting systems [25, 26]. This is of great advantage for integrated gradient tissues that need to be modified depending on the application, e.g., scaffolds of small diameters for drug screening or scaffolds of big diameters for tissue regeneration. The pumping velocity and pumping time can also be changed depending on the scaffold size.

By changing the pump continuously every 55 seconds, we printed the tendon-muscle scaffold with a gradient interface of cells and materials between two tissues. By doing it this way, tendon cells interacted with muscle ECM to form a MTJ that resembles what happens in the human body. We have shown the precise position of tenocytes on the sides and skeletal muscle cells in the center of the scaffolds by using two different fluorescents, non-toxic, and living cell markers. Herein, a transition between two colors was observed directly after the printing process demonstrating the gradient necessary to mimic the MTJ junction. With a short printing time (almost 4 minutes per construct), the human tenocytes cells and skeletal muscle cells in our scaffolds survived with >95% viability after the printing process and the same happened after culturing for seven days. In the proliferation medium, tenocytes and skeletal cells expanded, and immunological marker gene expression analysis demonstrated that the skeletal muscle cells differentiated into myotubes.

In our body, the bioelectrical system with endogenous electrical fields in skeletal muscle cells mediates crucial physiological processes, such as muscle migration and development [42, 43]. The movement of charged ions within the extracellular matrix determines the

electrical characteristics of muscle tissues [29]. For the last decade, electrical pulse stimulation (EPS) has been used for the induction of animal and human muscle cells. In one study, by using EPS as an in vitro exercise model in C2C12 skeletal muscle cell line culturing, Nedachi *et al*. [45] reported the contraction of myotubes, accelerated glucose absorption, and increased insulin sensitivity by EPS for 24 hours at 1 Hz. In another work, Park *et al*. [18] stimulated C2C12 muscle cells seeded in collagen scaffolds with a rectangular signal of voltage and frequency. The outcome demonstrated that the stimulated group developed highly organized sarcomeres and that a certain electrical frequency may influence the production of type I collagen. With the target to promote the differentiation of skeletal muscle cells, we stimulated our printed scaffolds by using the protocol from Park but with some modifications. In the results, stimulated scaffolds showed highly aligned and matured myotubes compared to non-stimulated scaffolds revealed by the immunostaining technique.

In conclusion, we printed human primary skeletal muscle and tendon cells laden in tissue-specific bio-inks to create tendon-muscle gradient scaffolds. The tendon-muscle-tendon model could be fabricated with different sizes by changing the parameters of the device. After printing, skeletal muscle and tendon cells showed good viability (>95%), and the muscle cells differentiated into myotubes. Then, electrical pulse stimulation increased the maturation and alignment of myotubes.

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국문요약

주로 고강도 운동이나 운동으로 인해 부상이 발생하는 우리 몸의 가장 약한 지점으로 두 조직의 접점이 꼽힌다. 이러한 예들 중 하나는 힘줄과 근육 사이의 계면 영역인 근간 접합부(MTJ)이다. 조직 공학은 재생을 위한 표적 조직을 만들기 위해 조직 특정 세포와 생체 적합 물질을 사용하는 것을 목표로 한다. 그러나 고급 단일 조직을 만든다고 해서 기존 단백질뿐만 아니라 기계적 특성으로 주어진 계면의 복잡한 구조로 인해 수술 후 다시 발생할 수 있기 때문에 계면 손상 문제가 해결되지는 않는다. 우리 몸에서 MTJ 의 그레이디언트(gradient) 특성은 MTJ 에 대한 대부분의 최근 연구가 복제에 실패하거나 오히려 복잡한 시스템을 사용하는 근육에서 뼈로 가는 힘 압력을 줄이는 데 도움이 된다. 따라서 이 경사 계면 조직을 만드는 것은 힘줄-근육 단위의 기능을 재생하기 위해 필요하다. 본 연구에서는 간단하고 접근 가능한 장치를 사용하여 다양한 대상에 적합한 매개 변수를 변경할 수 있는 경사 힘줄-근육 스캐폴드(Scaffold)를 제작하여 연속 압출 기술을 제안한다. 이 장치에는 세 개의 모세혈관이 들어 있는데, 두 개의 입구는 수직이고, 한 개의 입구는 플라스틱 튜브로 연결되었다. 바이오 잉크는 힘줄과 근육 조직에서 유래한 콜라겐과 탈세 포화된 세포외 매트릭스(dECM)를 혼합하여 만들어졌다. 이 방법으로, 세포 증식과 분화를 돕기 위해 조직 특이적 단백질이 제공되었다. 그리고 나서, 인간 세포, 즉 장세포와 근육 세포를 인쇄하기 전에 조직 고유의 바이오 잉크에 조심스럽게 섞었습니다. 펌프 속도는 순차적으로 프로그래밍된 두 주사기 펌프 모두에 대해 10 uL/min 으로 설정되었습니다. 구성당 인쇄 시간은 4 분입니다. 그 후, 스캐폴드(Scaffold)를 인큐베이터(5% CO_2 및 37°C)에 10~15 분 동안 보관하여 인쇄 후 겔화가 가능하도록 하였다. 스캐폴드(Scaffold)는 배지에서 1 일 동안 배양한 후 분화로 이동한 후, 추가 검사를 위해 1 일째와 7 일째에 채취하였다. Cell Tracker 용액으로 두 가지 유형의 세포를 사전 염색함으로써, 우리는 인쇄 후 세포 위치를 모니터링하고 힘줄, 근육 및

근긴장 접합이라는 세 개의 구역으로 이루어진 그레이디언트(gradient) 힘줄-근육 비계를 성공적으로 제작할 수 있었다. 이것은 두 가지 유형의 세포 사이의 그레이디언트 (gradient) 전환이다. 힘줄과 근육 모두에 대한 비계의 세포 생존력은 1 일 및 7 일 배양 후 95% 이상의 영상을 통해 관찰되었다. 전기자극 실험은 근육 조직의 분화를 증가시키기 위한 목적으로 시작되었다. 그 결과, 자극 그룹의 골격근 세포는 자극되지 않은 그룹에 비해 더 정렬되고 빽빽하게 채워진 근관을 형성했다.

주요어: 힘줄 공학, 근육 공학, 인터페이스 공학, 그레이디언트 (gradient) 비계, 탈세 포화된 세포 외 매트릭스.