

Tissue-engineered sling with adipose-derived stem cells under static mechanical strain

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Abstract. The implantation of a suburethral sling is an important treatment for stress urinary incontinence (SUI). However, the slings used current have a number of inherent limitations, such as tissue rejection and infection. The present study investigated the potential of engineering sling tissue *in vitro* using adipose-derived stem cells (ADSCs). The ADSCs were obtained from Sprague-Dawley rats and were characterized *in vitro*. The ADSCs were seeded on polyglycolic acid (PGA) fibers that formed a scaffold with a shape mimicking a sling complex. The results demonstrated that following *in vitro* culture for 12 weeks under static strain, neo-sling tissue could be generated using ADSCs. With increasing culture time, the engineered neo-sling tissue exhibited a significant improvement in biomechanical properties, including maximal load and Young's modulus ($P < 0.05$), and the tissue and collagen structures matured. Furthermore, differentiated ADSCs cultured under static strain were maintained their myoblast phenotype within the PGA scaffolds. These results indicate that ADSCs may serve as a novel cell source for tissue sling engineering and could improve treatment for patients with SUI.

Introduction

Stress urinary incontinence (SUI) is a common and embarrassing urologic problem (1,2). Among the various treatment techniques available, the sling procedure has become the mainstay of surgical treatment for the management of SUI.

However, these slings are associated with complications, including infection and urethral erosion (3-6). Following the advancements in tissue engineering research, a biodegradable tissue engineered sling may offer a promising alternative for the treatment of SUI (7,8).

Over the past 10 years, numerous studies have reported injectable stem cell therapies that achieved a high success rate in repairing SUI models. Muscle-derived stem cells (9), adipose-derived stem cells (ADSCs) (10,11), bone-marrow-derived mesenchymal stem cells (BMSCs) (12), fibroblasts and myoblasts (13) have been used for the repair of damaged sphincter muscle, which reveals a promising approach for sling engineering *in vitro*.

In the present study, a polyglycolic acid (PGA) scaffold was selected as the suburethral sling material because of its good biocompatibility and suitable degradation rate (14). Considering the fact that the majority of sling engineering studies have reported the short-term results of *in vivo* sling engineering, ADSCs and PGA were employed to explore the possibility of generating a sling complex *in vitro* for a relatively longer observation period. In future, it would be optimal to provide off-the-shelf engineered sling products so that patients can benefit from sling grafts that are immediately available.

Materials and methods

Isolation and culture of ADSCs. The Sprague-Dawley rats were obtained from the Animal Research Center of Fudan University (Shanghai, China). A total of 20 4-month-old female Sprague-Dawley rats (body weight, 240 ± 20 g) were housed in pairs at 23°C and 50-70% humidity, with a 12 h light/dark cycle and free access to water and food pellets. Adipose tissues were obtained from the inguinal regions of Sprague-Dawley rats. The experimental protocol was approved by the Research Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai). Isolation and culture of ADSCs was performed as described previously (15,16). Briefly, the samples were digested with 0.10% collagenase I (Sigma-Aldrich, Inc.; Merck KGaA, Darmstadt, Germany) through shaking at 37°C for 1 h. Following digestion, collagenase I was neutralized with an equal volume of basic growth medium containing Low Glucose Dulbecco's Modified Eagle's Medium (LG-DMEM; HyClone; GE Healthcare

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Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (both Gibco; Thermo Fisher Scientific, Inc.). After centrifugation at 37°C for 5 min at 1,500 x g, cells were resuspended in the basic growth medium and cultured at 37°C with 5% CO₂. The culture medium was changed every 3 days. When the culture dishes reached 80-85% confluence after ~4 days, the cells were passaged with trypsin-EDTA. ADSCs at passage 2 were used for the following experiments.

Characterization of ADSCs *in vitro*. The specific cell surface antigens, cluster of differentiation (CD) 90, 44 and 34, of ADSCs were characterized by flow cytometry analysis. Briefly, cells were incubated with fluorescein isothiocyanate-tagged antibodies, including anti-rat CD90 (1:100; cat. no. 11-0900; eBioscience; Thermo Fisher Scientific, Inc.), anti-rat CD44 (1:100; cat. no. MCA643FA; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and anti-rat CD34 (1:100; cat. no. 11-0341, eBioscience; Thermo Fisher Scientific, Inc.) for 30 min at 4°C, then washed three times using PBS containing 4% FBS. Flow cytometry was performed using fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Data were analyzed by FlowJo software version 7.6 (Tree Star, Inc., Ashland, OR, USA). The ADSCs were cultured and induced using an appropriate medium. For osteogenic differentiation, ADSCs were induced in DMEM supplemented with 10% FBS, β -glycerol phosphate, dexamethasone and ascorbic acid for 3 weeks, and analyzed with Alizarin Red S staining as previously described (17,18). For adipogenic differentiation, ADSCs were incubated in DMEM supplemented with 10% FBS, 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin for 2 weeks, and analyzed by Oil Red O staining as previously described (19).

Preparation of cell-PGA constructs. A custom-made spring formed with a stainless steel frame was used to provide constant strain as described previously (20). Briefly, 50 mg PGA fibers (~20-30 μ m in diameter) were arranged into a cord shape with a length of 4.5 cm and a diameter of 0.4 cm, and then secured onto a custom-made spring. The scaffolds were sterilized with 75% ethanol, washed with PBS and then pre-incubated at 37°C in DMEM supplemented with 10% FBS to enhance cell attachment. Subsequently, the ADSCs were collected and resuspended in culture medium at a density of 4x10⁷ cells/ml followed by seeding onto the PGA fibers. After 7 days of culture, the cell-scaffold construct was examined using a scanning electron microscope (SEM) as reported previously (21). After the SEM examination, the subsequent culture period included a 4-week-long myoblast differentiation phase and an 8-week-long proliferation phase. Induction of myoblast differentiation was performed by adding 10 μ mol/l 5-azacytidine (5-Aza, Sigma-Aldrich, Inc.; Merck KGaA), 5% FBS and 5% horse serum (Gibco; Thermo Fisher Scientific, Inc.) to LG-DMEM. After the 4-week-long differentiation phase, the medium was replaced with basal culture medium containing LG-DMEM supplemented with 10% FBS.

Analysis of the cell scaffolds. At 4 weeks (following the 4-week-long myoblast differentiation phase), 8 weeks (4-weeks

into the proliferation phase) and 12 weeks (following the 8-week-long proliferation phase), specimens of *in vitro* engineered slings were collected, and their length and diameter was measured. Randomly selected samples were taken and fixed with 4% paraformaldehyde at 4°C for 24 h followed by three washes in PBS, prior to being paraffin embedded and sectioned to a 5-8 mm thickness for hematoxylin and eosin (HE) staining as reported previously (21) in order to examine the tissue structure, particularly the cellular density and rate of PGA degradation. In addition, the sections were subjected to Masson's trichrome staining as described previously (21) in order to examine collagen production. Myoblast formation was evaluated by immunohistochemical staining. The tissue sections were stained with primary antibodies directed against desmin (1:100; Abcam, Cambridge, UK) and α -smooth muscle actin (α -SMA; 1:100), followed by the addition of a goat anti-rabbit secondary IgG conjugated to horseradish peroxidase (EnVision+ system; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). The antibody staining was visualized with the Liquid DAB⁺ Substrate Chromogen system (cat. no. K3467; Dako; Agilent Technologies, Inc.) prior to counterstaining with hematoxylin at 37°C for 15 min.

Biomechanical analysis. The *in vitro* engineered slings were collected at 4, 8 and 12 weeks as described above. The constructs were subjected to mechanical tests using a biomechanical analyzing instrument (Instron Model 4411; Instron, Norwood, MA, USA). The length of the tested slings was set to 2 cm between two grippers. The grippers were then gradually moved apart at a speed of 25 mm/min until complete rupture of the tissue in order to calculate the maximal (max) load. Additionally, the Young's modulus (MPa) was calculated from the linear slope of a stress-strain curve as described previously (22).

Statistical analysis. All quantitative data are presented as the mean \pm standard deviation. A one-way analysis of variance was performed to analyze the differences in mechanical properties between different time points. P<0.05 was considered to indicate a statistically significant difference. SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

Morphological and differentiation characteristics of ADSCs. Primary cultured ADSCs generated from fresh rat adipose tissue proliferated rapidly, reaching 80% confluence within 5 days. These cells were assessed for the expression of cell surface markers that are considered to define adult stem cells, and identified to express CD90 (Fig. 1A) and CD44 (Fig. 1B). However, the cells were negative for the hematopoietic stem cell marker CD34 (Fig. 1C). Additionally, ADSCs were tested for their ability to differentiate into other cell types. ADSCs cultured in adipogenic medium accumulated lipid vacuoles, which were confirmed by Oil Red O staining (Fig. 1D). Furthermore, ADSCs cultured in osteogenic medium deposited calcium as detected by Alizarin Red S staining (Fig. 1E). These data indicate that ADSCs exhibits multipotentiality.

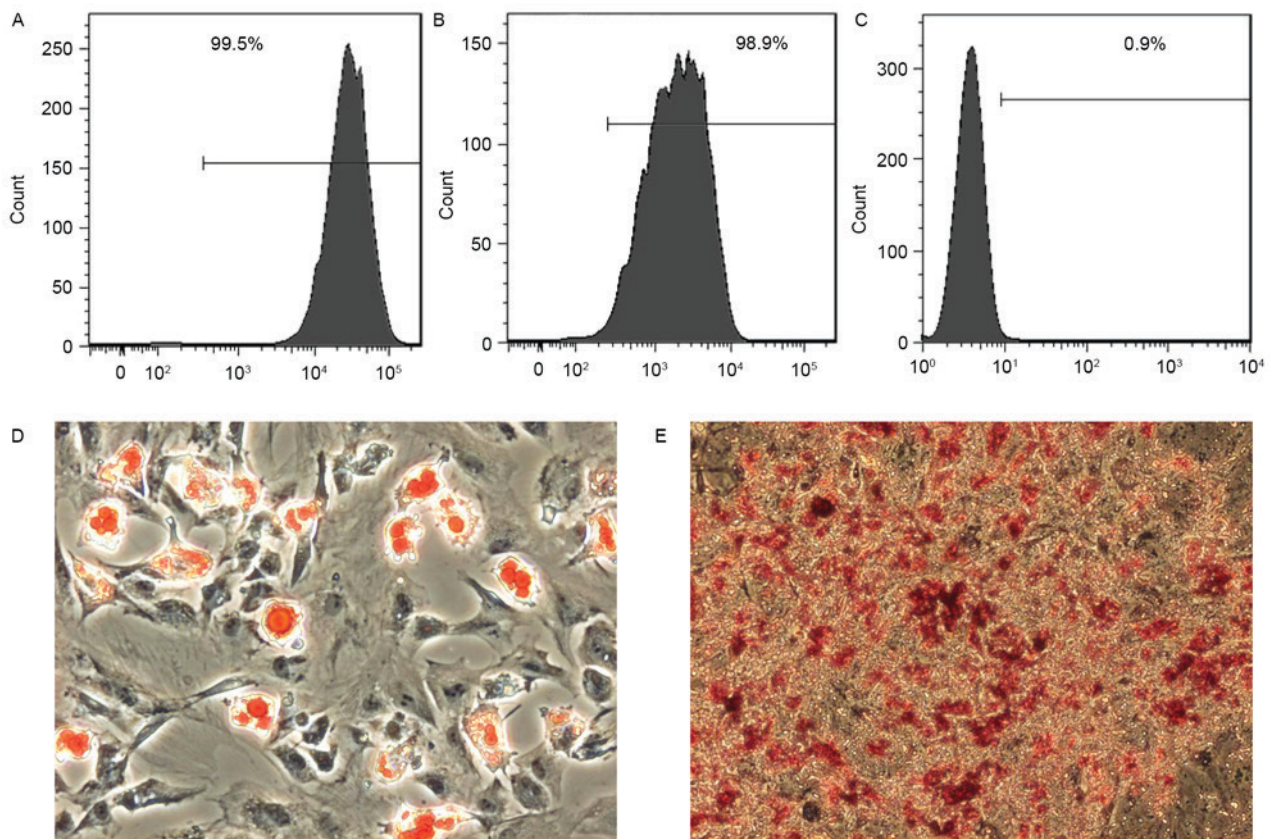


Figure 1. Characterization of the ADSC scaffolds. ADSC expression of (A) CD90, (B) CD44 and (C) CD34 was analyzed using flow cytometry. (D) Oil Red O staining revealed lipid vacuoles in the cytoplasm of the ADSCs. (E) Alizarin Red S staining revealed calcium deposits as red granules in the ADSC. Magnification, x100. ADSCs, adipose-derived stem cells; CD, cluster of differentiation.

Culture and characterization of the ADSC-PGA complex. At passage 2, the ADSCs were observed to be able to maintain good proliferation (Fig. 2A), and were thus used for sling engineering by seeding them onto a PGA scaffold under a constant strain (Fig. 2B). A total of 7 days after seeding, phase-contrast microscopy and SEM revealed good cell attachment on the scaffold and secreted extracellular matrices filling the space between the fibers (Fig. 2C and D), indicating good biocompatibility between the ADSCs and PGA fibers.

Gross observation and mechanical properties of the engineered slings. As illustrated in Fig. 3A, the ADSC-PGA construct appeared as a sling-like structure with a cord-like shape during the first 4 weeks of *in vitro* culture. The engineered slings exhibited a smoother surface at 8 (Fig. 3B) and 12 (Fig. 3C) weeks of culture. However, at 12 weeks the sling-like structure became much thinner with a more mature tissue appearance when compared with the constructs at 4 weeks. Furthermore, a biomechanical analyzing instrument was used to examine the mechanical properties of the *in vitro* engineered slings. As presented in Fig. 3D-F, the engineered slings exhibited different patterns of stress/strain curves when they were subjected to mechanical testing at 4, 8 and 12 weeks, respectively. In order to further associate tissue structure features and their mechanical properties in the slings, the max load and Young's modulus were investigated. After 4, 8, and 12 weeks of culture, the max load reached 0.26 ± 0.02 N, 0.84 ± 0.06 N and 1.05 ± 0.06 N, respectively (Fig. 3G). Furthermore, Young's modulus at 4

(0.26 ± 0.03 MPa), 8 (0.64 ± 0.16 MPa) and 12 weeks (0.94 ± 0.11 MPa) increased with time (Fig. 3H). These results demonstrate that the engineered slings had gradually increased stress/strain curves, max load and Young's modulus values over time, with significant differences between slings cultured for 4, 8 and 12 weeks of culture ($P<0.05$).

Histological assessment of the *in vitro* engineered slings. The *in vitro* engineered slings were assessed by HE and Masson's trichrome staining (Fig. 4). This revealed that the ADSC-PGA constructs were primarily composed of PGA fibers, with less matrix deposition observed at 4 weeks of culture (Fig. 4A and D). When cultured for 8 weeks, the slings exhibited a structure of longitudinally aligned collagen fibers and cells with increased matrix deposition (Fig. 4B and E). At 12 weeks, the parallel alignment of the cells and collagen fibers was enhanced further (Fig. 4C and F), suggesting that a longer cultivation enhances tissue remodeling and maturation. To further investigate whether ADSCs could be induced to differentiate into myoblasts and whether the differentiated ADSCs could maintain their contractile phenotype when seeded onto the PGA scaffold, immunohistochemical staining for desmin and α -SMA was performed. As illustrated in Fig. 4G-L, the tissue of the engineered slings was identified to contain cells expressing desmin and α -SMA, which increased over time. Complete degradation of the scaffold material was identified after 12 weeks of culture (Fig. 4C and F).

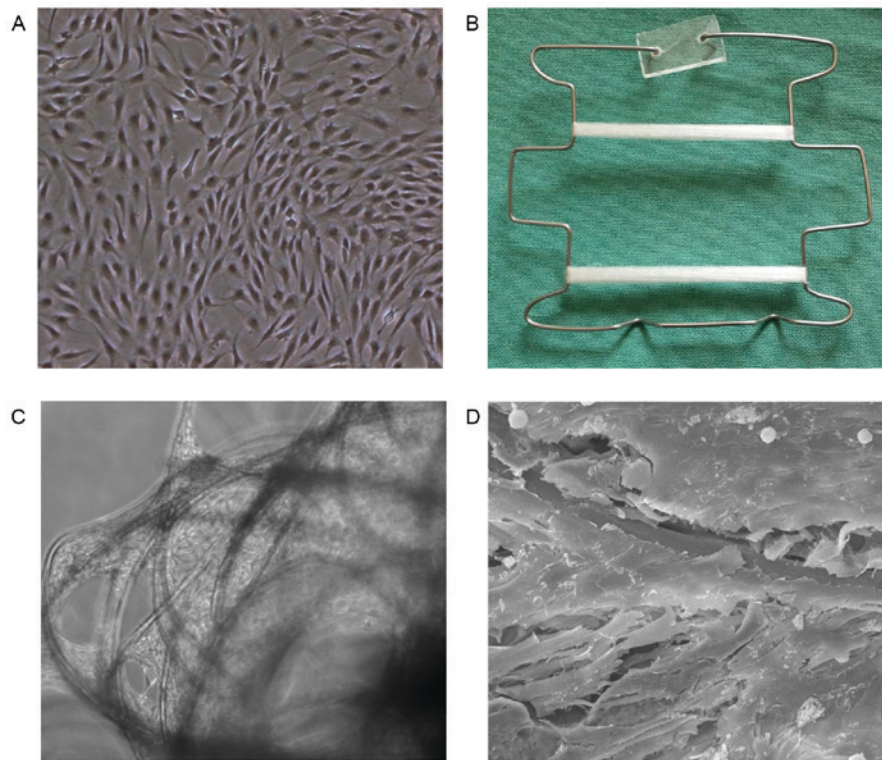


Figure 2. *In vitro* culture of the ADSC-PGA constructs. (A) ADSCs cultured for 3 days. Magnification, x40. (B) PGA scaffold secured onto a custom-made stainless steel frame. The ADSC-PGA construct was cultured for 7 days, and matrix production was examined with (C) phase-contrast microscopy (magnification, x40) and (D) scanning electron microscopy (magnification, x500). ADSCs, adipose-derived stem cells; PGA, polyglycolic acid.

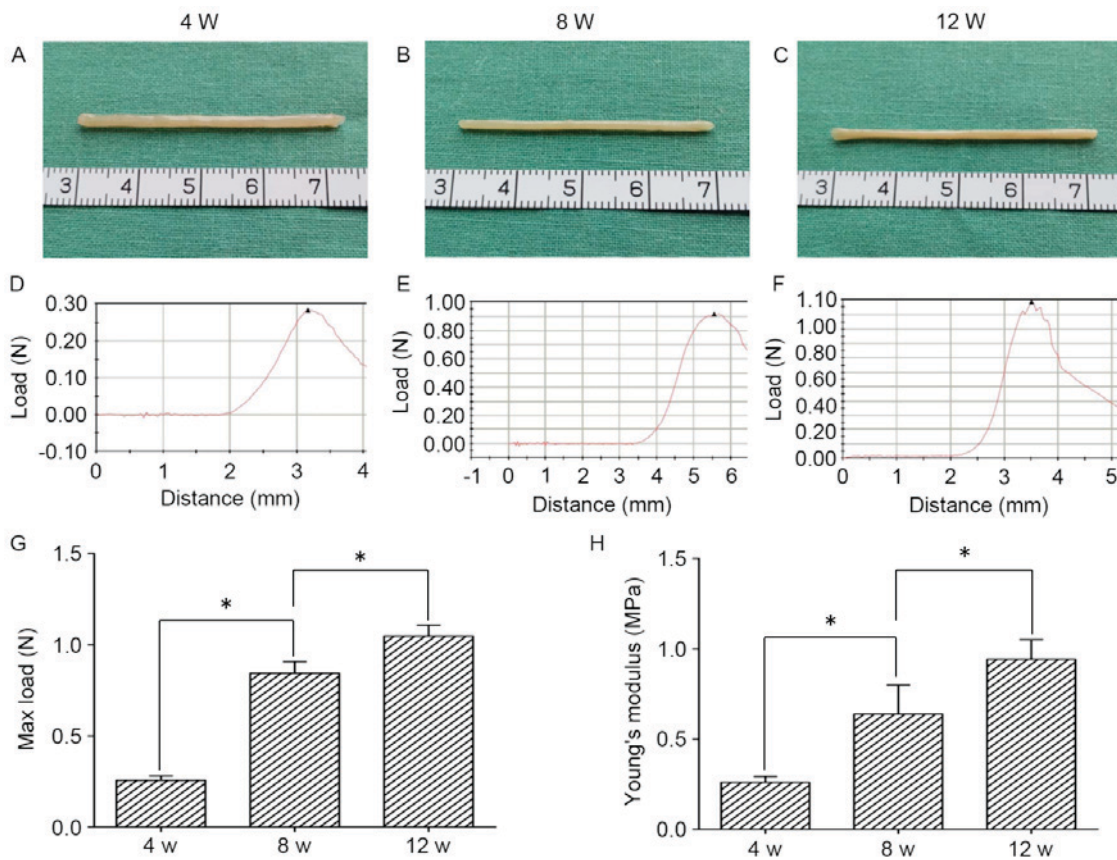


Figure 3. Gross view and mechanical property analyses of *in vitro* engineered slings. Gross view of the ADSC-based engineered slings cultured for (A) 4, (B) 8 and (C) 12 weeks *in vitro*. Representative stress/strain curves for ADSCs engineered slings at (D) 4, (E) 8 and (F) 12 weeks of culture. Comparison of the (G) max load and (H) Young's modulus of the engineered slings at various culture time points. * $P < 0.05$. ADSCs, adipose-derived stem cells; max load, maximal load.

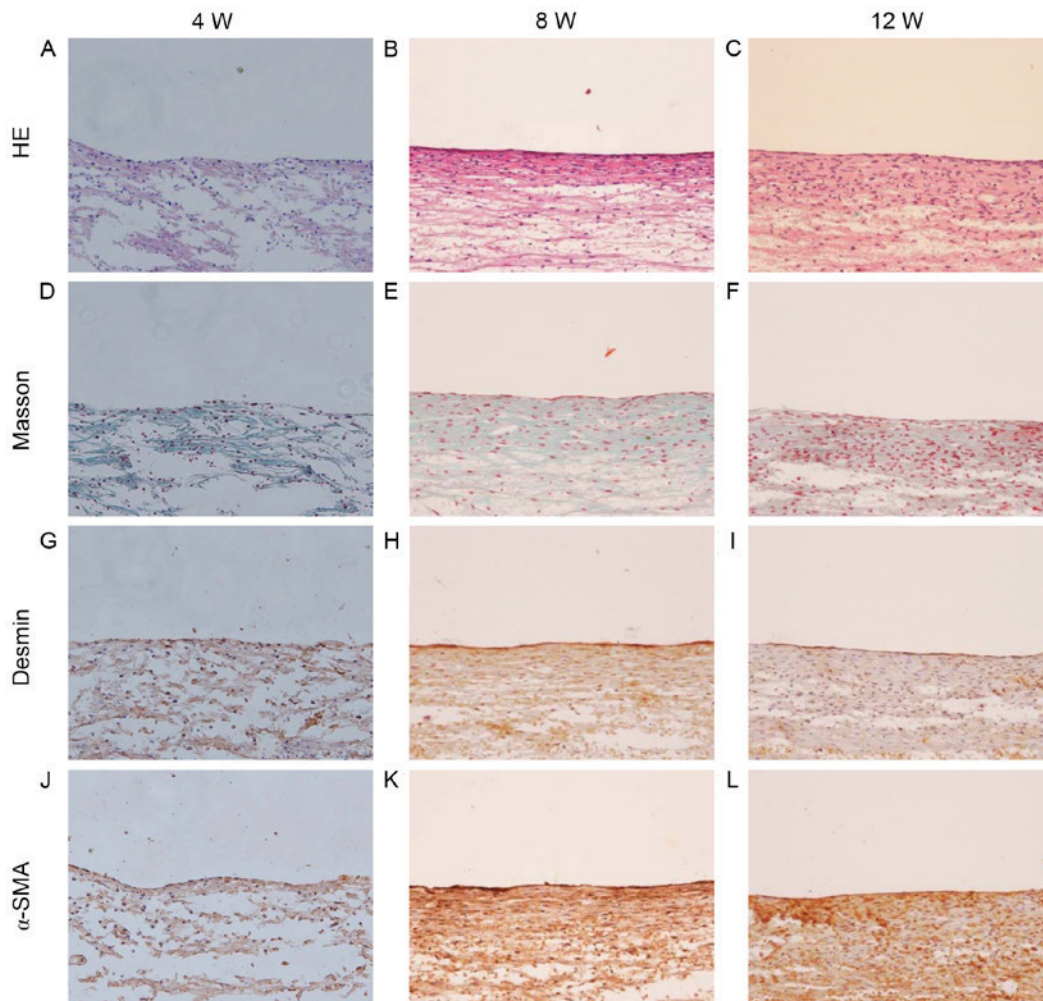


Figure 4. Histological evaluation of the tissue engineered slings. Hematoxylin and eosin staining at (A) 4, (B) 8 and (C) 12 weeks of culture. Masson staining of the *in vitro* engineered slings at (D) 4, (E) 8 and (F) 12 weeks of culture. Immunohistochemistry staining for desmin at (G) 4, (H) 8 and (I) 12 weeks of culture. Immunohistochemistry staining for α -SMA at (J) 4, (K) 8 and (L) 12 weeks of culture. Magnification, x200. α -SMA, α -smooth muscle actin.

Discussion

Previous studies have reported the success of sling engineering for treating stress urinary incontinence (7,8). An important issue in sling engineering is finding an appropriate cell source for engineering a transplantable sling graft. ADSCs have proven to be an excellent cell source, which have the advantage of being able to be harvested in abundance and causing fewer traumas to the donor site. Furthermore, ADSCs possess a similar pluripotency and self-renewal potential to BMSCs (23). Previously, the periurethral injection of ADSCs has been demonstrated to allow the active functional recovery of deficient sphincters (24,25). Therefore, ADSCs are likely to become the primary cell source for sling engineering. PGA has been widely used in tissue engineering due to its biocompatibility and degradability. Previous studies have revealed that by forming a preliminary tissue structure PGA could substantially degrade *in vitro*, avoiding the accumulation of degradation products at the implantation site, which may adversely affect tissue regeneration by causing fibrosis (26,27).

The present study investigated the feasibility of engineering a relatively thick sling by promoting the myoblast differentiation of ADSCs seeded onto PGA scaffolds under mechanical

loading stress. This revealed that a relatively thick sling could be engineered after 12 weeks of *in vitro* culture. Furthermore, the engineering of a sling requires a relatively long time to allow for tissue maturation, which leads to improved mechanical properties. In the present study, the engineered sling could reach a gross diameter of >2 mm and exhibited enhanced mechanical properties in a time-dependent manner. Furthermore, histological examination revealed that the collagen fibers and myoblasts were highly compacted, which increased with increased culture times, and complete degradation of the scaffold material was identified after 12 weeks of culture.

The present preliminary study demonstrated the feasibility of engineering a sling *in vitro* using a tissue engineering approach. The method used in the present study may aid in the future clinical treatment of SUI. In addition, following *in vitro* mechanical loading, the engineered sling tissue matured and demonstrated enhanced mechanical properties, which demonstrated that mechanical loading serves an important role in the maturation of tissue-engineered slings. However, there were several limitations in the present study. The tension-loaded slings were observed to be thinner with increased engineering times. Therefore, a constant strain without relief, which is non-physiological, may not be the best way to exert mechanical

loading stress. Previous studies have demonstrated that tissue quality and mechanical strength could be markedly improved when periodic mechanical loading was applied in a bioreactor system (28,29). Thus, a dynamic strain may be a more appropriate approach to pursue in future.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that a sling-like tissue structure could be generated *in vitro* by culturing the cell scaffold for a relatively long time. The results of the present study identified that differentiated ADSCs could maintain their myoblast phenotype when cultured on the PGA scaffold under a static strain. In addition, as engineering times increased, the engineered sling tissues exhibited a notable improvement in histological structure and mechanical properties. However, the slings under constant strain were observed to be thinner over time, and so the application of a bioreactor system to exert dynamic strain in future is warranted.

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