Annals of Plastic Surgery

Preliminary report of in vitro reconstruction of a vascularised tendon-like structure: a novel application for adipose derived stem cells (ADSCs)

--Manuscript Draft--

Manuscript Number:	SAP-D-11-00476R1
Full Title:	Preliminary report of in vitro reconstruction of a vascularised tendon-like structure: a novel application for adipose derived stem cells (ADSCs)
Article Type:	Research
Keywords:	tendon regeneration; tendon tissue engineering; tenocytes; adipose stem cell
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Abstract:	Introduction. A greater supply of tendinous tissue can be obtained through tissue engineering technology with increasing application of adult stem cells. It is well known that adipose derived stem cells (ADSCs), found in abundance in adipose tissue, have the same differentiating capacity as mesenchymal stem cells (MSCs) yet have the advantage of being easily isolated. In the present study, we combined the great facility of ADSCs to differentiate with the application of an external mechanical stimulus to successfully create an in vitro reconstructed tendon like structure with a microcapillary network. Materials and methods. Hyalonect® meshes were used as scaffold. Human ADSCs were seeded onto the biomaterials, and the cell/scaffold constructs were cultured under mechanical stress for up to 15 days. Human tenocytes were used in the same conditions as control. Performance were assessed by histology, immunochemistry, ultrastructure, and biomolecular analysis. Results. ADSCs seeded onto Hyalonect® adhered and differentiated along the entire surface of the biomaterial and began to infiltrate within its structure. Subsequently, endothelial cells migrated, forming capillary in the new extracellular matrix. Conclusions. This technique allowed for the creation of a vascularized tendon equivalent that could easily be detached from the bioreactor, thus facilitating its implant at the lesion site. These results highlight biological performance of biodegradable hyaluronic acid-based (HYAFF-11) scaffolds, which were shown to be suitable for deposition of the autologous extracellular matrix critical for ADSCs differentiation.

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Padova, 07 February 2012

Dear William C. Lineaweaver, MD, FACS
Editor in Chief Annals of Plastic Surgery

Thank you very much for giving me the opportunity to revise the manuscript according to the Reviewers suggestions. We revised the manuscript as suggested by the reviewers, maintaining the research message that the aim of the work has not been the isolation and differentiation of tenocytes from tendon biopsy, but the quality comparison of in vitro reconstructed tissue-engineered tendon-like tissue starting from tendon derived adult stem cells vs. adipose derived stem cells.

With the hope that this revised manuscript fulfils the scientific requirements of *Annals of Plastic Surgery*, I look forward to hearing from you.

Best Regards,

Vincenzo Vindigni, MD, PhD
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Reviewer Comments:

Reviewer #1: SAP-D-11-00476: Preliminary report of in vitro reconstruction of a vascularised tendon-like structure: a novel application for adipose derived stem cells (ADSC)

Structured abstract:

The most important numbers should be stated in the abstract to provide some quantitative data. The abstract should be divided into sections (introduction, materials and methods, results, conclusion). Why is the performance of the scaffold material called "exceptional"? - No other material was used for comparison.

The abstract was rewritten according to reviewer's suggestions.

Introduction:

A short overview over the state of the art in tendon tissue engineering should be given. The authors should explain in what way they expect to clarify yet unclear aspects of tendon tissue engineering with their approach.

We changed the introduction following reviewer's indications.

Materials and methods

Biomaterials

A tendon graft needs long-term mechanical stability. AS HYAFF-11 is resorbable, does it fulfill this requirement sufficiently? What was the size of the construct? What was the cell concentration for seeding of the scaffold?

The aim of the research was to clarify the role of ADSCs in tendon tissue engineering. We completely agree with the reviewer that hyaluronic acid biomaterial have poor mechanical properties at this moment. Therefore we clarified in the discussion that our results stimulate future research to improve their mechanical properties, as we have previously done with resorbable micro-vascular prosthesis (Zavan B, Vindigni V, Lepidi S, Iacopetti I, Avruscio G, Abatangelo G, Cortivo R. Neoarteries grown in vivo using a tissue-engineered hyaluronan-based scaffold. FASEB J. 2008 Aug;22(8):2853-61).

HYAFF-11 was used to create Hyalonect® meshes of 50-µm-thick fibers with a specific weight of 100 g/m2 (2 x 1 cm rectangles).

We clarified that the concentration of tenocytes and ADSCs seeded onto biomaterials was 2.5×10^5 tenocytes/cm^2.

Immunohistochemical analysis

The authors say that they made stainings for von Willebrand factor, but they do not show these images- why?

We added Figure 4b.

Bioreactor

Why did the authors use exactly these parameters for stretch, load and cycle? Were the optimal parameters determined in a pilot study? A picture of the bioreactor would be helpful for the reader to get a notion of this device.

We added a picture of bioreactor (Figure 1).

Yes it is. We performed preliminary tests and chose the parameters that ensured the optimal results. The mechanical loading was applied for 120 min a day (40 min every 8 hours) with an amplitude of 1/10 length of the constructs, and with a frequency of 6 times per minute, each lasting for 5 s with an interval of 5 s.

Results

Monolayer culture

Tenocyte morphology- why was the staining with the fibroblast antibody done? Fibroblast staining is often very unspecific, a negative control is missing, and morphology could also be evaluated under phase-contrast microscopy without staining.

The aim of the work has not been the isolation and differentiation of tenocytes from tendon biopsy, but the quality comparison of in vitro reconstructed tissue-engineered tendon-like tissue starting from tendon derived adult stem cells vs. adipose derived stem cells. We used a fibroblastic marker to identify cell derived from tendon biopsy only for this purpose.

Culture with mechanical stress

When the authors want to prove that tenocytes align in the direction of traction, image 2a and 2c are not very convincing, especially image 2a. Also on 2c, the cells form a layer on the artificial fiber, but I cannot see any alignment. What was the direction of the traction vector?

The direction of the traction was indicated by blue arrows in figures.

The arrangement of the images is confusing. Images with and without mechanical stimulation should be placed adjacent to each other.

We arranged the images according to the reviewer's suggestions.

Images 3a-3c are not suitable to prove an alignment of matrix fibers- how was the fiber alignment without mechanical stimulation?

We added figures 2e and 2f showing the absence of alignment without mechanical stimulation.

TEM- what is the additional information of the TEM images? Alignment of collagen fibers cannot be proven convincingly with that. Is TEM really necessary to show fat droplets in a cell? Why not just do a fat staining?

We deleted TEM images according to the reviewer's suggestions.

MTT assay: Image 5a, first two columns- these look almost equal, are they really significantly different from each other with a p-value <0.01? The numbers shown by the columns should be given in the text.

We added all the information requested.

Real-Time PCR: The sequences of the primers used should be indicated. Standard deviations should be shown in the figures and the numbers should be written in the text. Significant differences should be marked in the figures.

We added all the information requested.

Microcapillary network: where ADSCs negative for CD31 initially? The authors should show these initial images if they want to prove transformation.

We addeded the requested image (Figure 4c).

Discussion

The potential benefits and drawbacks of the observed neovascularization should be discussed. It is known that there is enhanced vacularization at the repair site in case of intrinsic tendon healing, but does an overall increase of vascularization in a tendon graft improve its biomechanical properties?

We did not discuss that the vascularisation improve biomechanical properties, we discuss that strongly improve the engraftment and then the survival of the cells.

Figure 1 and 2:

The nomenclature (3D culture, 3D in bioreactor, 3D statical culture) is confusing. Why not just call it monolayer, stimulated, non-stimulated?

The referee is right, we changed the nomenclature.

Preliminary report of in vitro reconstruction of a vascularised tendon-like structure: a novel application for adipose derived stem cells (ADSCs)

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CONFLICT OF INTEREST

None of the authors has any commercial associations that might pose or create a conflict of interest with information presented in this article. Such associations include consultancies, stock ownership or other equity interests, patent licensing arrangements, and payments for conducting or publicizing a study described in this article. No intramural or extramural funding supported any aspect of this work.

ADSCs for tendon tissue engineering

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ADSCs for tendon tissue engineering

ABSTRACT

Introduction. A greater supply of tendinous tissue can be obtained through tissue engineering technology with increasing application of adult stem cells. It is well known that adipose derived stem cells (ADSCs), found in abundance in adipose tissue, have the same differentiating capacity as mesenchymal stem cells (MSCs) yet have the advantage of being easily isolated. In the present study, we combined the great facility of ADSCs to differentiate with the application of an external mechanical stimulus to successfully create an in vitro reconstructed tendon like structure with a microcapillary network.

Materials and methods. Hyalonect® meshes were used as scaffold. Human ADSCs were seeded onto the biomaterials, and the cell/scaffold constructs were cultured under mechanical stress for up to 15 days. Human tenocytes were used in the same conditions as control. Performance were assessed by histology, immunochemistry, ultrastructure, and biomolecular analysis.

Results. ADSCs seeded onto Hyalonect® adhered and differentiated along the entire surface of the biomaterial and began to infiltrate within its structure. Subsequently, endothelial cells migrated, forming capillary in the new extracellular matrix.

Conclusions. This technique allowed for the creation of a vascularized tendon equivalent that could easily be detached from the bioreactor, thus facilitating its implant at the lesion site. These results highlight biological performance of biodegradable hyaluronic acid-based (HYAFF-11) scaffolds, which were shown to be suitable for deposition of the autologous extracellular matrix critical for ADSCs differentiation.

Keywords: Adipose Derived Stem cells; tendon; adipose tissue; hyaluronic acid; endothelial cells; tissue engineering; extracellular matrix; type I collagen

INTRODUCTION

Tendons are soft connective tissue that connect muscle to bone, forming a musculo-tendinous unit whose primary function is to transmit tensile loads generated in muscle to move and stabilize joints.[1] Tendon injuries are a common but challenging clinical problem. The most widespread clinical therapy is direct end-to-end suturing, which joins the ends of tendons while providing sufficient mechanical strength to prevent gapping. Some injuries heal poorly using this type of surgery and thus require subsequent treatment with grafts.[2] Artificial grafts for this kind of operation are not yet available and the use of natural grafts is difficult. Autografts from patients can result in donor site morbidity, and allografts from cadavers, apart from being limited in supply, can incite harmful immune responses. Frequently, in both cases, the graft does not match the strength of undamaged tissue.

Since being defined in 1988, tissue engineering has offered great potential in the treatment of these difficult injuries. In theory, tissue engineering has sought to enhance biologic activity by delivering cells and/or a biologic scaffold to a repair site in an attempt to augment the healing response. To enhance their properties in culture, researchers have even stimulated these constructs with various chemical (growth factors and cytokines) and mechanical signals.[3-7] Our research activities have focused for many years on tissue engineering using the benzyl ester of hyaluronic acid in "smart scaffolds" that can be processed into several types of devices such as tubes, membranes, non-woven fabrics, gauzes and sponges. All these scaffolds are highly biocompatible within the human body, do not elicit any adverse reaction and are resorbed by the host tissues. We have cultured human dermal fibroblasts, hepatocytes, chondrocytes, endothelial cells, bone marrow mesenchymal stem cells and adipose derived stem cells (ADSCs) inside hyaluronic acid based scaffolds. Under these conditions and in the presence of specific cell medium-promoting drugs, cells attach to the biomaterial fibers, proliferate, differentiate and colonize the entire device.[8-11]

Three fundamental issues in tendon engineering are: (1) to find an ideal cell source for engineering a suitable tendon construct; (2) to regenerate a capillary network inside the structure to facilitate in vivo graft of the tendon substitute, and (3) to reproduce mechanical stimuli in vitro, required for human cells to form a new tendon tissue structure with parallel collagen fibers and elongated cells. Tenocytes were used to reconstruct tendon like tissue, but, this approach would be difficult to put in clinical practice, because autologous tendon harvest is necessary to extract cells.[12,13] Therefore, to satisfy the first two issues, we decided to use human ADSCs, that which are found in abundance in adipose tissue, have the same differentiating capacity as mesenchimal stem cells yet have the advantage of being easily isolated, and can be induced to differentiate in endothelial cells.[14-16] Recent studies demonstrated that collagen fibers and tendon cells can be oriented along the direction of the mechanical stress and can upregulate synthesis of the main components of tendinous esxtracellular matrix (ECM) in the presence of a mechanical force.[17-19] In vitro cyclic strain allows an increased production of TGF-β, FGF and PDGF by human tendon fibroblast.[17-19] Expression of collagen types I and III, fibrinonectin and elastin genes was found to have increased when compared with non-stretched controls in which no ligament matrix was found.[17-19] Therefore, we used a bioreactor system consisting of five pneumatic actuators, able to apply tensile strains to the constructs in culture to direct cellular differentiation and promote extracellular matrix (ECM) development, to satisfy the third issue (Fig. 1).

MATERIAL AND METHODS

Biomaterials

Biomaterials used in the present study were derived from the total esterification of hyaluronan (synthesized from 80-200 kDa sodium hyaluronate) with benzyl alcohol, and are referred to as HYAFF-11. The final product is an uncross-linked linear polymer with an undetermined molecular weight; it is insoluble in aqueous solution yet spontaneously hydrolyzes over time, releasing benzyl alcohol and hyaluronan. HYAFF-11 was used to create Hyalonect® meshes of 50-µm-thick fibers with a specific weight of 100 g/m^2 (2 x 1 cm rectangles). These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano Terme, Padova, Italy).

Cell culture

Tenocytes

Tenocytes were isolated from hand tendon (5 cm) obtained from five healthy female patients with ages ranging from 35 to 45 years. Briefly, tendons were cut into small pieces (2–3 mm^2) and tenocytes were isolated by sequential trypsin and collagenase digestion. These cells were then cultured with DMEM medium supplemented with 10% fetal bovine serum (FBS) (Bidachem) plus 2 mM l-glutamine and penicillin/streptomycin (cDMEM). Medium was changed twice a week and cells harvested by trypsin treatment.

Adipose-derived stem cells (ADSCs)

ADSCs were extracted from adipose tissue of five healthy female patients undergoing cosmetic surgery procedures, following guidelines from the Clinic of Plastic Surgery, University of Padua. Adipose tissues were digested with 0.075% collagenase (Type 1A; Sigma–Aldrich) in Krebs–Ringer buffer (modified KRB: 125mM NaCl, 5mM KCl, 1mM Na3PO4, 1m MMgSO4, 5.5mM

glucose, and 20mM Hepes [pH 7.4]) for 60 min at 37°C followed by 10 min with 0.25% trypsin. Floating adipocytes were discarded, and cells from the stromal-vascular fraction were pelleted, rinsed with media, and centrifuged, and a red cell lysis step in NH4Cl was completed for 10 min at room temperature. The viable cells obtained were counted using the tryptan blue exclusion assay and seeded at a density of 10 x 10^5 cells per square centimeter for in vitro expansion.[17]

Bioreactor

A custom-made bioreactor provided a dynamic stretch to the constructs (Fig. 1). The mechanical loading was applied for 120 min a day (40 min every 8 hours) with an amplitude of 1/10 length of the constructs, and with a frequency of 6 times per minute, each lasting for 5 s with an interval of 5s.

Immunohistochemical analysis

Cryostatic sections (7 μm) were used for morphological and immunohistochemical analyses. Briefly, sections were layered over gelatin-coated glass slides, fixed with absolute acetone for 10 min at room temperature, and cryopreserved at –20°C until use. Primary antibodies were used to determine collagen I (SIGMA), CD31 (DAKO), fibroblasts (DAKO) and vWF (DAKO). Negative controls were performed by replacing primary antibody with normal rabbit serum or phosphate-buffer salt solution. Immune reactions were revealed by the Fast Red Substrate (Sigma), and counterstained with hematoxylin (Sigma). Immunofluorescent studies were performed using rabbit monoclonal anti-human von Willebrand factor and CD-31 antibody.

After a one-hour incubation, samples were rinsed with buffer solution, and the second antibody was added for one hour. The second antibody was TRITC-conjugated pure goat anti-mouse IgG (1:200 dilution) and CyTM2-conjugated pure goat anti-rabbit IgG (1:200 dilution) (Jackson Immunoresearch, West Grove, PA). Samples were washed in PBS solution for 10 min, and then

treated with an 80% glycerol/20% PBS solution. Sections were evaluated under a Zeiss fluorescence microscope. Negligible background was found in controls where primary antibody was omitted.

Real time RT-PCR

For each target gene, primers and probes were selected using Primer3 software. Gene expression was measured using real-time quantitative PCR on a Rotor- GeneTM 3500 (Corbett Research).

Collagen 1: TGAGCCAGCAGATCGAGA/ACCAGTCTCCATGTTGCAGA
Collagen 2: CAGCAAGAGCAAGGAGAAGAAAC/GTGGTAGGTGATGTTCTGGGA
Adiponectin: GATGAGAGTCCTGGGTGTGAG/CTGGGTAGATATGGGATTCAAGAGA
Tenascin: CCCTGCTCACTTGGACTGA/GCATTGTAGGAGGTGGAGGGAA
Scleraxis (SCXA): AGCTACATCTCGCACCTGG/TGTTTGGGCTGGGTGTTCTC
PCR reactions were carried out using primers at 300 nM and the SYBR Green I (Invitrogen) (using 2 mM MgCl2) with 40 cycles of 15 seconds each at 95°C and 1 minute at 60°C. All cDNA samples were analysed in duplicate. Fluorescence thresholds (Ct) were determined automatically by the software with efficiencies of amplification for the studied genes ranging between 92% and 110%. For each cDNA sample, the Ct value of the reference gene R16 was subtracted from the Ct value of the target sequence to obtain the ÄCt. The level of expression was then calculated as 2-ΔCt and expressed as the mean±SD of quadruplicate samples of two separate runs. Relative quantification of marker gene expression is given as a percentage of the beta actin product and the t-test was applied.

Statistical Analysis

The one-way analysis of variance (Anova test) using the software package Excel (Microsoft office 2000) was used for data analyses. Repeat measurement analysis of variance (Re-ANOVA) and paired t tests were used to determine if there were significant ($p<0.05$) changes. Repeatability was calculated as the standard deviation of the difference between measurements of the test performed.

RESULTS

Monolayer culture, not-stimulated

Tenocytes obtained from digestion of human tendon reached confluence after 15 days of monolayer culture. These cells showed an extended fibroblast-like morphology, visible using anti-fibroblast immunohistochemistry (Fig. 2a). ADSCs, obtained by digestion of lipoaspirates and expanded in DMEM complete medium in monolayer conditions, showed an extended, fibroblast-like morphology with a well defined cytoplasm enriched in lipid droplets (in red) (Fig. 2b).

Tenocytes and ADSCs culture in scaffolds, not-stimulated

Tenocytes (visible in Fig. 2c inside the black circle) seeded onto biomaterial (cell density: 2.5×10^5 tenocytes/cm^2) adhered to the scaffold fibers (Fig. 2c; black arrows) and maintained their extended fibroblast-like phenotype. Also ADSCs (visible in Fig. 2d inside the black circle) (cell density: 2.5×10^5 ADSCs/cm^2) adhered along the entire surface of the biomaterial fibers (black arrows, Fig. 2d), and lipid droplets were visible within the cytoplasm. Both cell types were randomly dispersed around the fibers of the non-woven mesh (Fig. 2e and 2f), filling the inner spaces of the scaffold.

Tenocytes and ADSCs culture in scaffolds plus mechanical stress

Tenocytes and ADSCs seeded onto Hyalonect were placed inside the special chambers of the bioreactor and cultured under mechanical stress (traction) for up to 15 days. Histologically, tenocytes demonstrated a morphology and growth extended in the same direction of the traction, adhering to the hyaluronic acid fibers (Fig. 3a and 3c). ADSCs showed the same behavior: they were lined up parallel to the traction vector and adhered to and colonized the biomaterial fibers (Fig. 3b and 3d). Immunohistochemistry using anti-collagen type I confirmed the initial lining up of

matrix fibers, which were also parallel to the traction vector, whether secreted by tenocytes (Fig. 3e) or ADSCs (Fig. 3f).

Microcapillary network

A well-defined microcapillary network formed by ADSCs was reconstructed inside the tendon-like structure (Fig. 4a, 4b and 4d). Positive staining of CD31 and vWF, markers of mature endothelial cells, demonstrated the endothelialization of tendon constructs (Fig. 4b and 4d, white arrows). Thus, ADSCs seeded onto the hyaluronan derived scaffold, after 15 days of tensile culture, adhered and differentiated along the entire surface of the tendinous layer and began to infiltrate within the scaffold structure. Subsequently, endothelial cells migrated, forming capillary-like ring structures in the newly synthesized extracellular matrix. Moreover, in the interstitial spaces of the scaffold, endothelial cells were organized into ring aggregates, similar to capillary structures, in which a lumen was clearly evident (Fig. 4a, 4b and 4d).

MTT and Real time PCR

The biocompatibility of Hyalonect® as well as the tensile force of tenocytes and ADSC were evaluated using the MTT test of stretched and not stretched cultures from days 4 to 15 (Fig. 5a, 5b). Both cell types showed a progressive increase in proliferation during the 15 days of culture, confirming the compatibility of the biomaterial with these cells. MTT values were higher in scaffolds in traction than in mechanical stress-free cultures (Fig. 5a, 5b).

Expression of collagen type I and type II transcriptase, the tendon markers, scleraxis and tenascin, and the adipocyte marker, adiponectin, was studied (Fig. 6 for tenocytes [a, 4 days; b, 14 days]; Fig. 6 for ADSc [c, 4 days; d, 14 days]).

Tenocytes and ADSC (in all conditions) had the highest expression of matrix proteins such as collagen I, while collagen II, which is more typical of cartilage tissue, was expressed in ADSC traction free samples and no expressed in tenocytes and in ADSc cultures on stretched conditions.

The adipose tissue marker, adiponectin, was expressed exclusively in ADSC in the first days of culture, when cells showed no signs of differentiation (Fig. 6c). Its expression decreased in the following weeks of traction-challenged ADSC culture (Fig. 6d). Adiponectin was not expressed in tenocyte cultures (Fig. 6d).

Scleraxis expression, a marker highly specific to tenocytes, was higher in traction- challenged tenocyte cultures (Fig. 6a, 6b). Its expression in ADSC cultures was initially low (Fig. 6, 6c, 6d), but then increased in the second week of culture under mechanical stress (Fig. 6d). This finding indicated that a partial differentiation of cultured ADSC into tenocytes occurred when cells were challenged with traction.

DISCUSSION

Tendons are able to heal naturally, but their pre-injury conditions are not restored, owing to the development of scar tissues at the wound site, the biomechanical properties of which are inferior to uninjured tendon. The loss of mechanical competence is mainly due to a distorted ECM composition and a misalignment of collagen fibrils in the scar tissue.

To improve the quality of repaired tendons, various surgical repair techniques using sutures and soft tissue anchors have been developed. However, surgically repaired tendons are still functionally inferior. With the advent of tissue engineering (TE) technology, new hope has been turned to the repair of tendon lesions *in situ* by integrating engineered, living substitutes of their native counterparts *in vivo*. Historically, tendon-like mechanical properties were considered the primary requirement for a tissue engineered scaffolding material. However, because TE is aimed at the regeneration of a functional neo-tissue rather than at replacing damaged tissue with an artificial

prosthesis, the scaffold must have similar mechanical properties to that of tendons.[19] Ideally, the scaffold should not only promote cell proliferation and differentiation but also restore the natural ECM composition and histological structure of tendon. Prior to implantation, TE constructs often undergo a certain period of *in vitro* culture, which is traditionally carried out under mechanically static culture conditions. Externally applied cyclic strain under *in vitro* conditions has enormous effects on various functions of tenocytes, such as their metabolism, proliferation, orientation and matrix deposition. In the past decade, significant progress has been made in tendon tissue engineering including cell sourcing, and understanding the biological effects of mechanical stretch on cells.[20-24] The discovery of adult stem cells that possess regenerative capabilities has opened new possibilities for treating damaged tendon tissue that is slow to repair after injury. Unlike autologous bone grafts that can be harvested in large quantities from large bones like the pelvis, autologous tendon tissue is not readily available for use as grafting material. The ability to use small portions of tendons to isolate and expand cells that could form tendon tissue in vivo offers a new strategy for improving the current means of tendon repair. In light of these considerations, the aim of the present study was the in vitro reconstruction of a tendon-like tissue employing ADSCs.[25,26]

In a previous report,[8] we demonstrated the ability to isolate, amplify and differentiate ADSCs in vitro into several tissues such as cartilage, bone and adipose tissue. The latter, once implanted in vivo, not only became well integrated within the host tissue, but provoked a surprising revascularization process.

This interesting plasticity of ADSCs was exploited for the in vitro reconstruction of a vascularised tendon-like structure by exposing a cellularized hyaluronic acid-based scaffold to cyclic loading in a bioreactor. Mechanical stress is known to play a significant role in modulating cell behaviour within tendon tissue, the morphology of which is naturally driven by endogenous tension.

When ADSCs were isolated from adipose tissue and amplified in monolayer conditions, they showed a typical adipose phenotype with a lipid droplet cytoplasm (Fig. 1b). When seeded into

hyaluronan-based scaffolds in static conditions, ADSCs maintained their differentiated aspect (Fig. 1b). Even tenocytes/fibroblasts isolated from tendons maintained their elongated shape both in monolayer (Fig. 1a) and in three-dimensional (3D) culture (Fig. 1e). These cells as well as ADSc adhered more deeply into the scaffold fibers when cultured in 3D conditions (Fig. 1f).

When the cellularized biomaterial was placed inside a mechanical force-inducing bioreactor, ADSCs began to acquire a new phenotype more similar to tendon than adipose tissue (Fig. 2b). Cells were more elongated and lined up parallel to the traction vector (Fig. 2b, 5d, red arrows), and continued to adhere to and colonize within the biomaterial fibers, evolving the same features of adult tenocytes previously isolated from tendon (Fig. 2a). Its ability to secrete extracellular matrix was well evident (Fig. 3b, 3c; Fig. 3a, 3b): collagen type I fibers secreted by ADSCs were typically arranged in longitudinal rows in close proximity to the cell and the lipid droplets (Fig. 4c) present in the cytoplasm of non-mechanically stressed ADSC were replaced with collagen fibers (Fig. 4a, 4b).

Real time PCR was performed to monitor the most representative markers of phenotype for adult tenocytes (type I collagen, tenascin, scleraxis), adipocytes (adiponectin) and chondrocytes (type II collagen) in tenocytes and ADSCs. As Figure 6 (a, b) illustrates, at 3 and 14 days of culture, tenocytes expressed only tenogenic markers which then increased when cells were placed under mechanical stress, indicating that tendon cells can respond to altered mechanical load with a net gain in collagen synthesis. Proliferation of both cell types (Fig. 5) was also stimulated by periods of repetitive tension.

The intriguing behavior of ADSCs under tension was well documented by gene expression. In the first few days of culture, no evident difference in terms of marker expression was evident in stretched and not stretched conditions (Fig. 6a). In both cases, ADSCs showed adipogenic (i.e., adiponectin) tenogenic (type I collagen, tenascin, scleraxis), and chondrogenic (type II collagen) markers, indicating that cells were probably in an indifferentiated state. However, after 14 days of

mechanical stress, chondrogenic markers disappeared (Fig. 6b) adipogenic gene expression decreased, and tenogenic gene expression notably increased.

Lastly, a critical feature of ADSCs plasticity observed under these conditions was their ability to acquire endothelial characteristics (Fig. 7). When forming a new blood vessel, i.e. the process of angiogenesis, endothelial cells must proliferate, migrate and invade surrounding tissues. Induction of angiogenesis is mediated by a number of growth factors and cytokines produced by cells and the surrounding host tissue. Endothelial cells also require collagens and adhesion molecules for their growth. Under our experimental conditions, ADSCs were not only able to create an endothelium promoting milieu, but were able to drive themselves into vascular-like cells, creating a capillary-like structure inside the cultured tendon-like tissue.

In conclusion, with the present work we demonstrate that in the presence of external mechanical stimuli, adipose derived stem cells created a tendon-like construct with a microcapillary network. A well-orchestrated basic tendon structure was generated with a good extracellular matrix enriched with an endothelialized structure. This technique allows for the creation of a vascularized tendon structure that can be easily detached from the bioreactor, thus facilitating its implant at the lesion site. Formation of these microvessel-like structures within the tendon-like structure was probably promoted by the well documented angiogenic properties of ADSCs;[27-29] by the precursors of endothelial cell that could be derived from the original adipose tissue, and by the commitment of ADSCs promoted by the cell–cell interactions in the presence of extracellular matrix previously deposited by the stem cell population inside the scaffold interstice.[27-29] Both of these requirements, the presence of stem cells with angiogenical properties (inside adipose tissue cell population) and the secretion of their ECM molecules, were present in this reconstructed tendon substitute. A pre-formed capillary-like structure, i.e., the observed diffusion of autologous endothelial cells within the substitute, should significantly accelerate neovascularization of the tendon-like structured once grafted. Lastly, these results highlight the successful biological performance of degradable

hyaluronic acid based scaffolds, which were highly suitable for deposition of the autologous extracellular matrix critical for ADSCs differentiation, and stimulate future research to improve mechanical properties of this biomaterial.

We could conclude that: (1) physical factors influence ADSCs activity. Mechanical stimuli, generated in vitro by a bioreactors, could improve tendon like structure before surgery; and (2) strategies for orthopaedic tissue repair could benefit from the properties of ADSCs.

References

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FIGURE LEGEND

FIGURE 1. The bioreactor.

FIGURE 2. Morphologic analyses performed after 15 days of tenocytes and ADSCs in monolayer (a; b) and onto scaffold (c; d; e; f), not-stimulated:

a) Immunostaining reaction of anti-fibroblast on tenocyte monolayer cultures (10x).
b) Oil red staining for lipid droplets on ADSCs monolayer cultures (5x).
c) Contrast phase image of tenocytes culture onto scaffold (20x): tenocytes are inside black circle; black arrows indicate Hyalonect fibers. (scale bar 50 µm)
d) Contrast phase image of ADSCs culture onto scaffold (20x): ADSCs are inside black circle; black arrows indicate Hyalonect fibers. (scale bar 50 µm)
e) Haematoxylin/eosin staining of tenocytes culture (5x): red arrow indicates tenocytes; black arrows indicate Hyalonect fibers. (scale bar 50 µm)
f) Haematoxylin/eosin staining of ADSCs culture onto scaffold (10x): red arrow indicates ADSCs; black arrows indicate Hyalonect fibers. (scale bar 50 µm)

FIGURE 3. Morphologic analyses of extracellular matrix production performed after 15 days of tenocytes (a - e) and ADSCs (b - f) cultured onto biomaterial and stimulated (blue arrows show the direction of the traction):

a) Haematoxylin/eosin staining tenocytes (10x): red arrows indicate tenocytes; black arrows indicate Hyalonect fibers. (scale bar 50 µm)
b) Haematoxylin/eosin staining of ADSCs (10x): red arrows indicate ADSCs; black arrows indicate Hyalonect fibers. (scale bar 50 µm)

c) Haematoxylin/eosin staining of tenocytes (20x): red arrows indicate tenocytes; black arrows indicate Hyalonect fibers. (scale bar 50 µm)

d) Haematoxylin/eosin staining of ADSCs in bioreactor (20x): red arrows indicate ADSCs; black arrows indicate Hyalonect fibers. (scale bar 50 µm)

e) Immunostaining reaction of anti type I collagen on tenocyte cultures (40x). (scale bar 50 µm)

f) Immunostaining reaction of anti type I collagen on ADSCs cultures (40x). (scale bar 50 µm)

FIGURE 4. Morphologic analyses performed after 15 days of microcapillary network inside ADSCs cultures onto biomaterials and in stretched conditions.

a) Haematoxiline/eosine (5x): white arrows indicate the microcapillary network; black arrows indicate Hyalonect fibers. (scale bar 50 µm).

b) Immunostaining reaction of vWF cell marker: white arrows point microvascular structures (40x). (scale bar 50 µm).

c) Negative control for CD 31

d) Immunostaining reaction of CD31 cell marker: white arrows point microvascular structures; black arrows indicate Hyalonect® fibers (40x). (scale bar 50 µm).

FIGURE 5. Vitality rate of tenocytes (a) and ADSCs (b) cultured into Hyalonect® scaffolds. The graph represents the mean +SD of three different experiments at various time points. The one-way analysis of variance (Anova test) of the software package Excel (Microsoft office 2000) was used for data analyses. Repeat measurement analysis of variance (Re-ANOVA) and paired t tests were used to determine if there were significant ($p<0,5$) changes. Repeatability was calculated as the

standard deviation of the difference between measurements of the MTT test performed. Anova test: * p < 0,05; * * p<0,01; * * * p<=,001

FIGURE 6. Real time PCR performed to monitored the most representing markers of adult tenocytes (type I collagen, tenascin, SCXA), adipocytes (adiponectin) and chondrocytes (type II collagen) were test in tenocytes and in ADSc cultures with the aim to test the phenotype acquired by the cells. a) and b) gene expression of tenocytes (a at 4 days; b at 15 days); c) and d) gene expression of ADSCs (c at 4 days; d at 15 days). Red bars in not-stretched conditions. Pink bars in stretched conditions.

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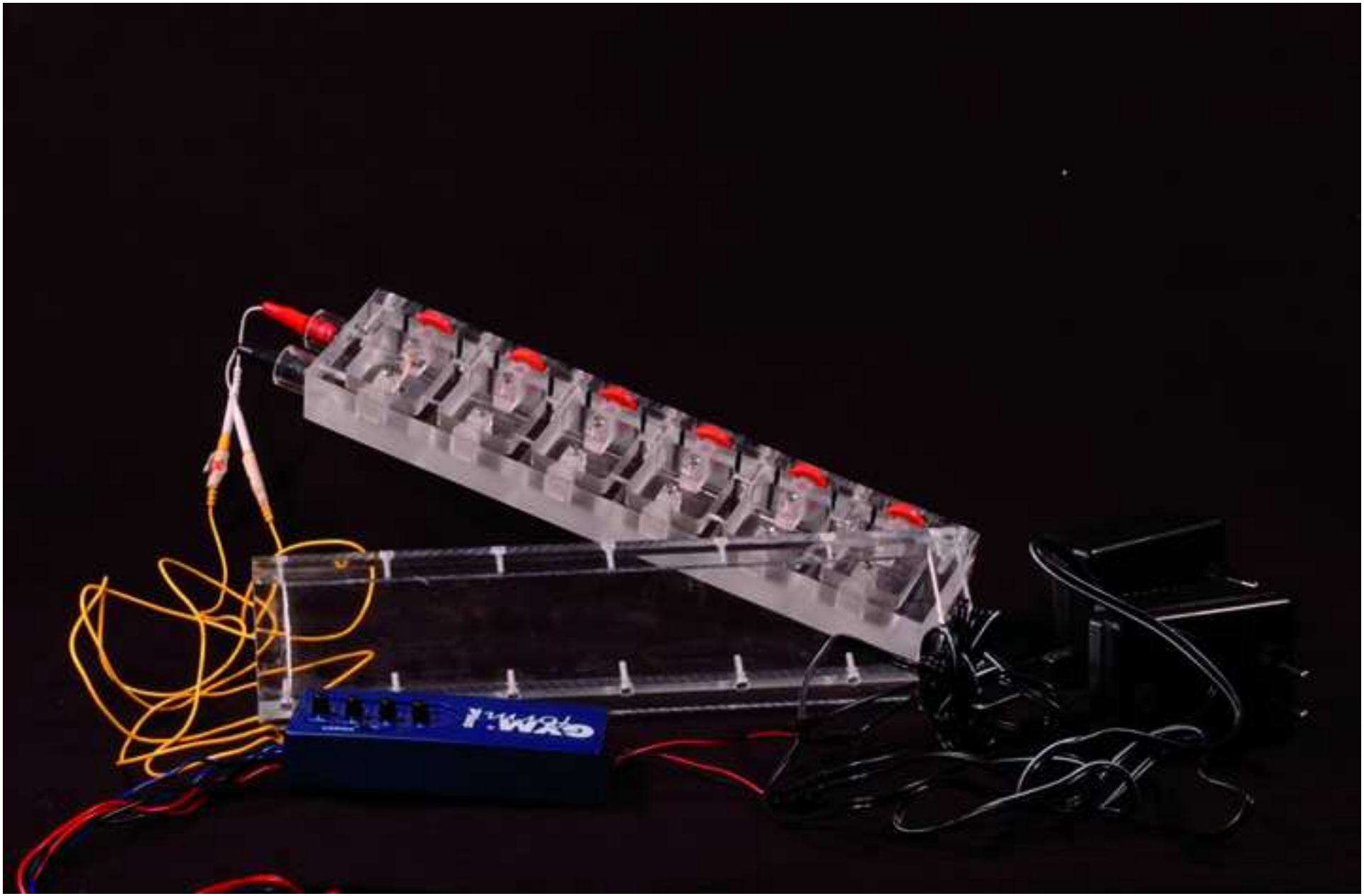

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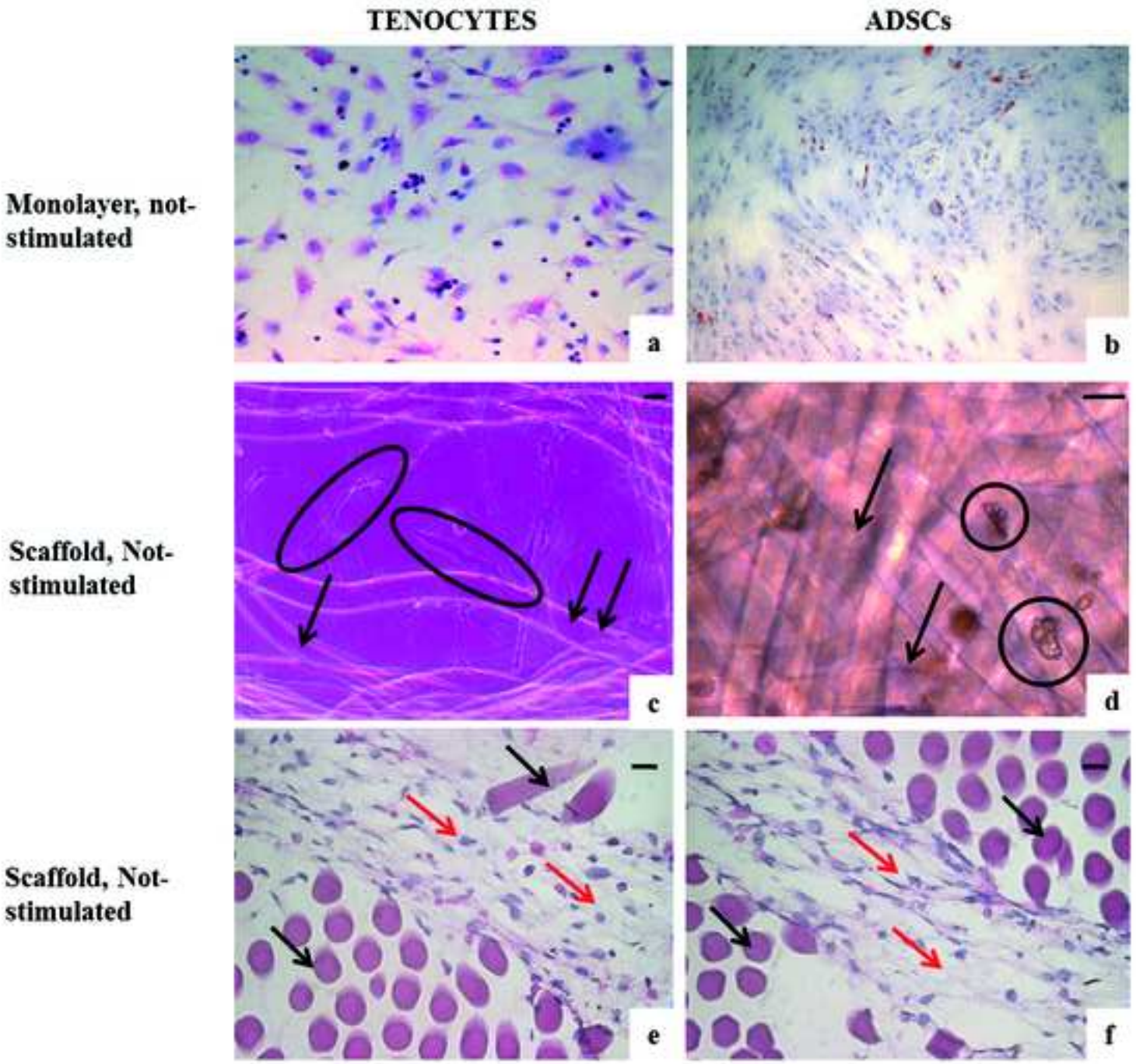

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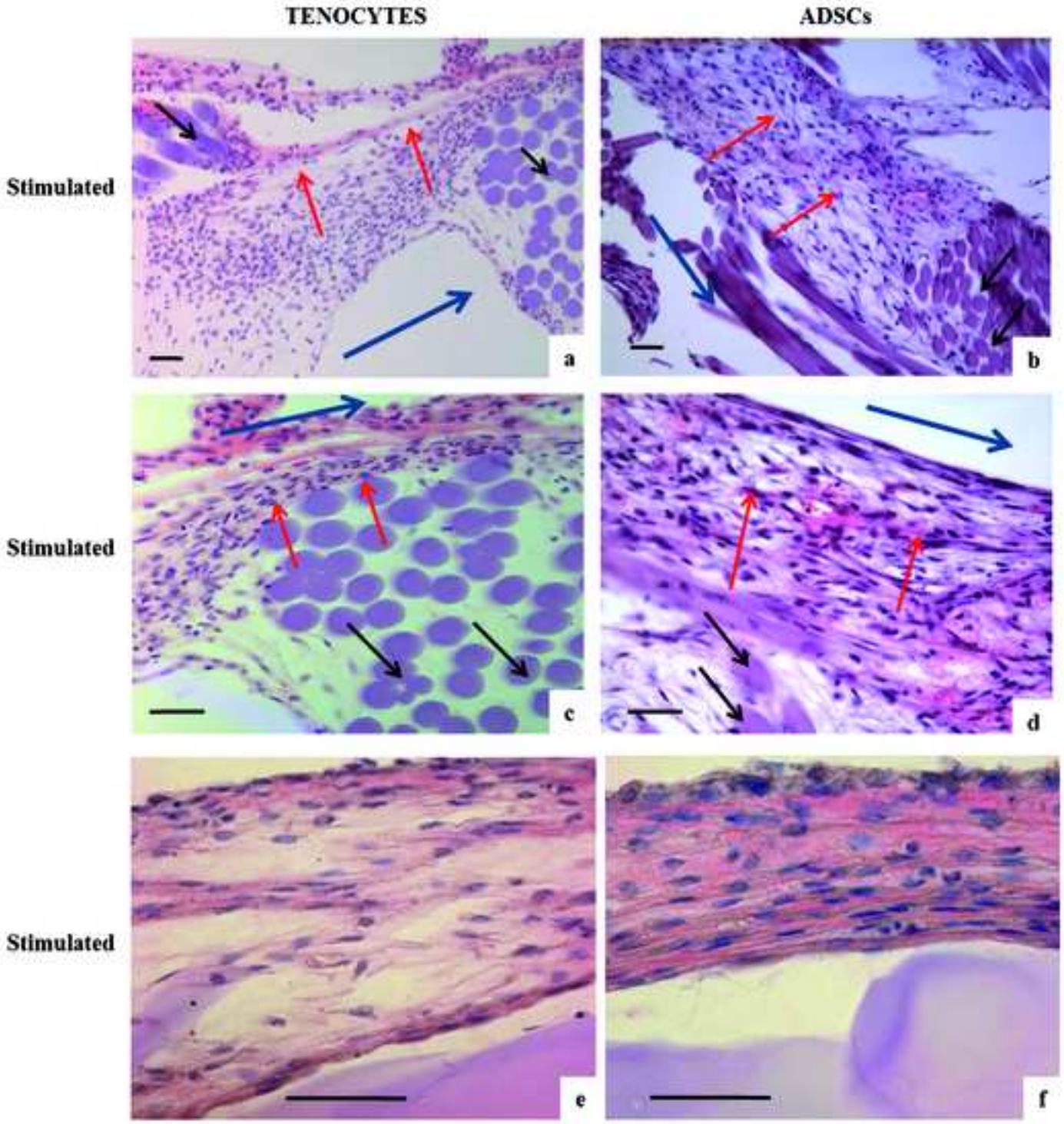

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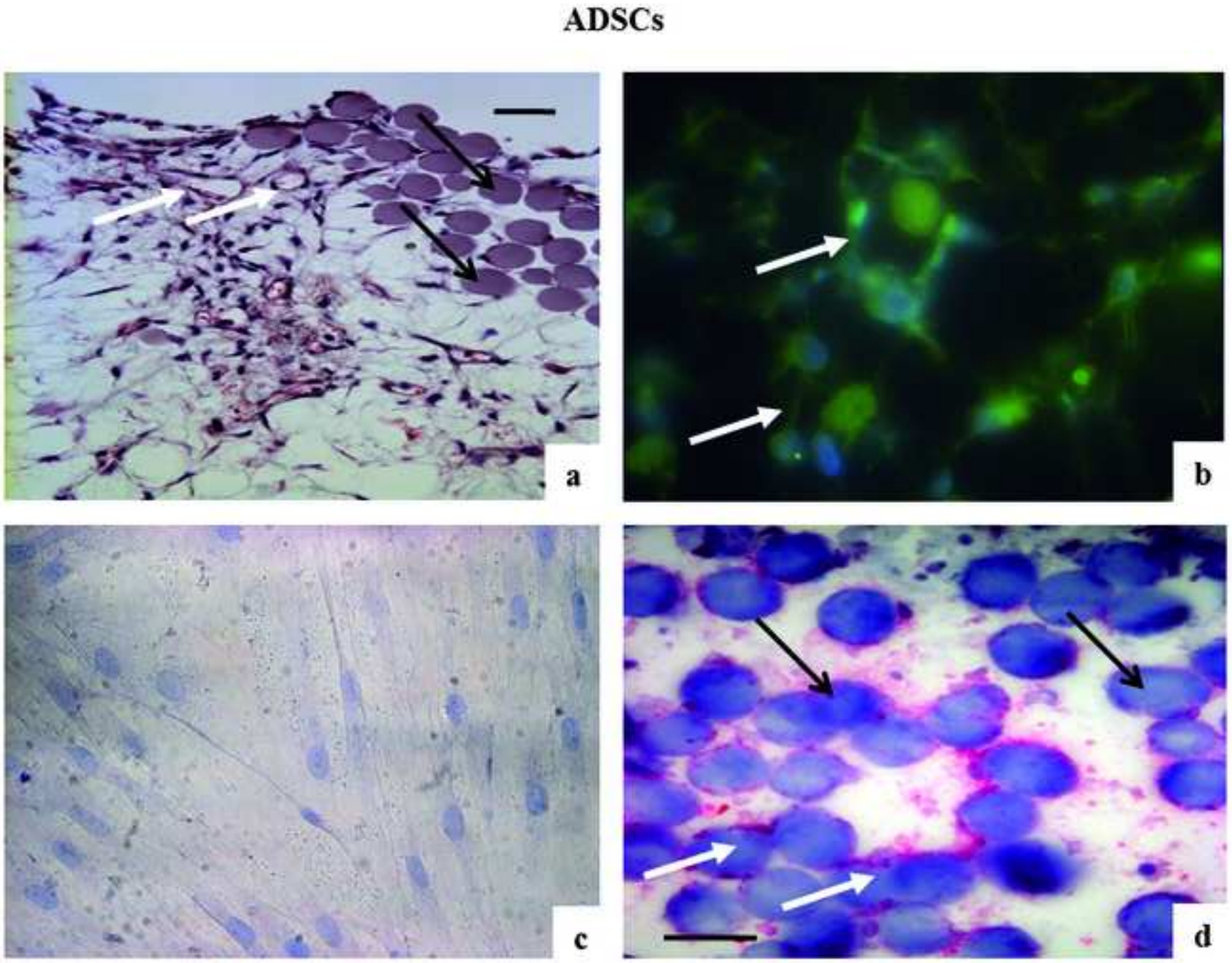

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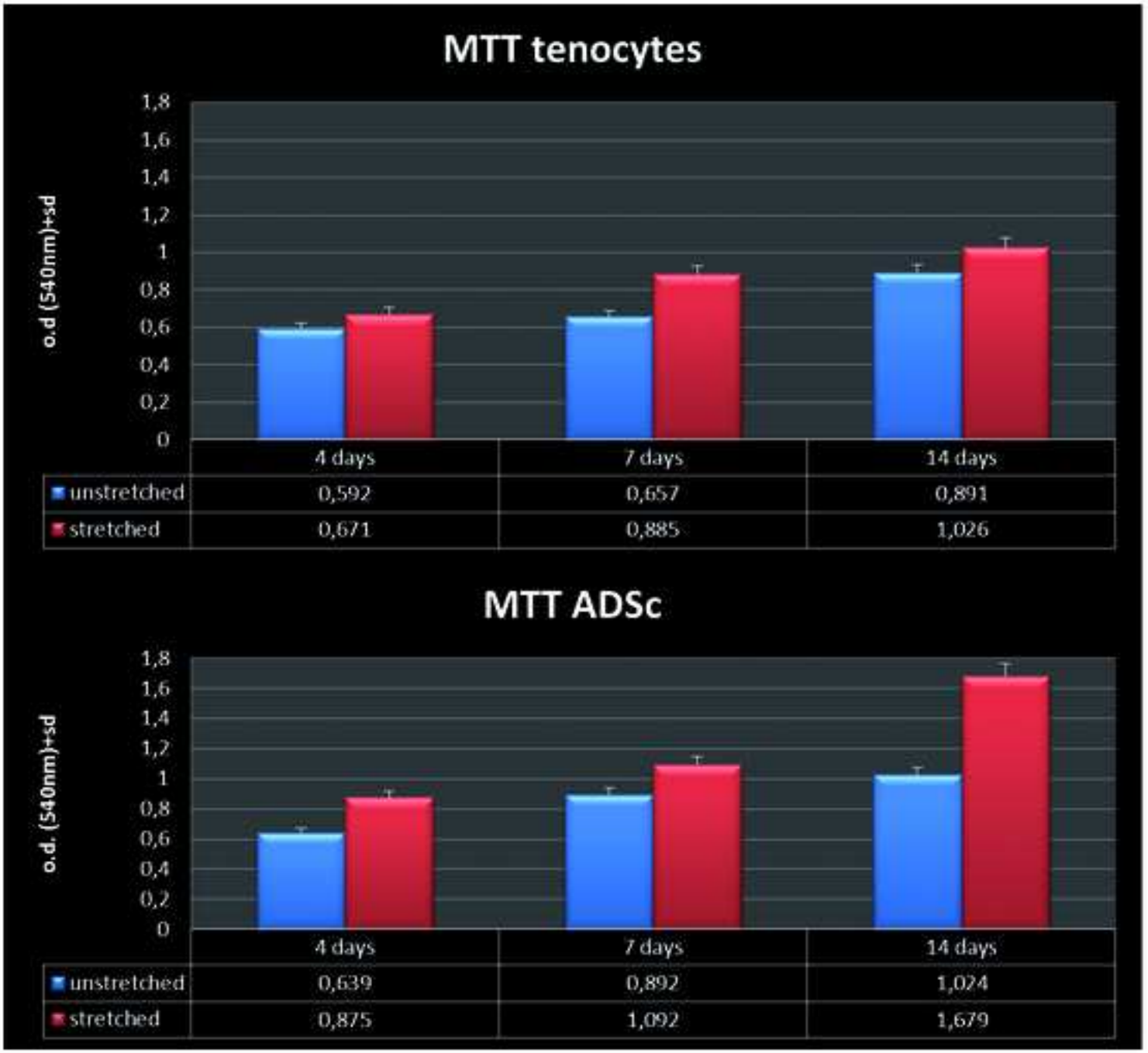

Figure 6
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