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Research article

Tissue engineering approaches to develop decellularized tendon matrices functionalized with progenitor cells cultured under undifferentiated and tenogenic conditions

Daniele D'Arrigo [1,§], Marta Bottagisio [1,2,§], Silvia Lopa [1], Matteo Moretti [1,3,4], and Arianna B. Lovati [1,*]

[1] Cell and Tissue Engineering Laboratory, IRCCS Galeazzi Orthopaedic Institute, Milan, Italy
[2] Department of Veterinary Medicine (DiMeVet), University of Milan, Milan, Italy
[3] Regenerative Medicine Technologies Lab, Ente Ospedaliero Cantonale (EOC), Lugano, Switzerland
[4] Swiss Institute of Regenerative Medicine (SIRM), Lugano, Switzerland

[§] Daniele D'Arrigo and Marta Bottagisio contributed equally to this work.

* **Correspondence:** Email: arianna.lovati@grupposandonato.it; Tel: +39-026-621-4069.

Abstract: Tendon ruptures and retractions with an extensive tissue loss represent a major clinical problem and a great challenge in surgical reconstruction. Traditional approaches consist in autologous or allogeneic grafts, which still have some drawbacks. Hence, tissue engineering strategies aimed at developing functionalized tendon grafts. In this context, the use of xenogeneic tissues represents a promising perspective to obtain decellularized tendon grafts. This study is focused on the identification of suitable culture conditions for the generation of reseeded and functional decellularized constructs to be used as tendon grafts. Equine superficial digital flexor tendons were decellularized, reseeded with mesenchymal stem cells (MSCs) from bone marrow and statically cultured in two different culture media to maintain undifferentiated cells (U-MSCs) or to induce a terminal tenogenic differentiation (T-MSCs) for 24 hours, 7 and 14 days. Cell viability, proliferation, morphology as well as matrix deposition and type I and III collagen production were assessed by means of histological, immunohistochemical and semi-quantitative analyses. Results showed that cell viability was not affected by any culture conditions and active proliferation was

maintained 14 days after reseeding. However, seeded MSCs were not able to penetrate within the dense matrix of the decellularized tendons. Nevertheless, U-MSCs synthesized a greater amount of extracellular matrix rich in type I collagen compared to T-MSCs. In spite of the inability to deeply colonize the decellularized matrix in vitro, reseeding tendon matrices with U-MSCs could represent a suitable method for the functionalization of biological constructs, considering also any potential chemoattractant capability of the newly deposed extracellular matrix to recruit resident cells. This bioengineering approach can be exploited to produce functionalized tendon constructs for the substitution of large tendon defects.

Keywords: decellularized tendon; undifferentiated mesenchymal stem cells (U-MSCs); extracellular matrix; type I collagen; tenogenic differentiation (T-MSCs); functionalized tendon constructs; cell repopulation and static culture

Abbreviations

bFGF	basic Fibroblast Growth Factor
CM	Complete Medium
DAB	3’-Diaminobenzidine
ddH$_2$O	Double-distilled water
H&E	Haematoxylin and Eosin
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
ROI	Region of Interest
RT	Room Temperature
SE	Standard Error
T-MSCs	Tenogenic Mesenchymal Stem Cells
TE	Tissue Engineering
TGF-β	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
U-MSCs	Undifferentiated Mesenchymal Stem Cells

1. Introduction

Tendon disorders are very common musculoskeletal injuries and represent a major clinical problem with a continuously increasing prevalence [1]. In particular, tendon ruptures and retractions with an extensive tissue loss represent a great challenge in surgical reconstruction, counting for 21.5 and 221 per 100,000 person-years for Achilles and finger tendons, respectively [2]. Along with long time recovering, these injuries are directly associated with high health and socioeconomic costs [3]. Nowadays, to restore tendon gaps, surgical approaches mainly consist in the use of autologous or allogeneic grafts. Nevertheless, these grafts often do not match the physiological properties of the

native tendon [4]. This limit, together with the lack of donor tissue and the morbidity of the sampling site, as well as an augmented operating time and risks of disease transmission represent the major drawbacks of in the use of human tendon grafts [5,6].

For these reasons, in the last few years, a growing number of tissue engineering (TE) strategies have been developed. Due to the combination of cells, growth factors and scaffolds, TE is a valid approach to substitute or regenerate damaged tissues, thus recovering their physiologic functionality [4,7]. In this context, finding an effective material to be used as a scaffold for tendon reconstruction is mandatory and the use of xenogeneic tissues represents a promising perspective to overcome the challenge of generating large sized grafts with structural, mechanical and biochemical composition that resembles the native tissue [4,8].

Equine species was identified as the best candidate for tendon xenografts in terms of limited risks of disease transmission, appropriate dimensions, and large availability independent from any ethical concern [9]. To be suitable for clinical uses, a method to decellularize equine tendons has been developed to keep unaltered the biochemical features, as well as the tissue architecture [10,11]. In fact, the decellularization makes biological matrices safe from an immunogenic point of view and suitable to support both the adhesion and migration of resident cells from the tendon tissue stumps [12]. In view of moving towards the clinical practice, these matrices could serve as carriers for transplanting viable cells in damaged tendons, as a result of reseeding steps [12], and optimally integrate with the host tissue, thus facilitating and accelerating tendon regeneration.

With this purpose, a broad variety of cell types has been used to reseed decellularized tendons, such as tenocytes and dermal fibroblasts, or mesenchymal-derived lineages like adipose-derived MSCs, tendon stem progenitor cells, and bone marrow-derived MSCs [9]. Using tenocytes for tendon TE strategies has considerable drawbacks. In fact, tenocytes are terminally differentiated cells with a limited proliferative capacity. Furthermore, the tendon cellularity is low and in vitro expansion of tenocytes is necessary to obtain a sufficient number of cells to reseed the matrix, thus requiring large sample retrievals associated with donor site morbidity and potential cell de-differentiation [13,14]. On the contrary, MSCs, used in several clinical trials as the cell therapeutic gold standard [15,16,17], are characterized by a high proliferation rate and collagen synthesis. Furthermore, the use of MSCs is encouraged because of their immunomodulatory ability, which is a favorable feature for cell transplantation [18]. However, the success of TE strategies to functionalize decellularized tendon matrices is not only related to cell repopulation, but also to cell proliferative and differentiating properties. With this aim, a culture medium suitable for the terminal differentiation of bone marrow MSCs toward the tenogenic lineage in monolayer was previously developed [19] using an innovative cocktail of growth factors making a step forward with respect to the existing literature [20,21,22]. The main assumption underlying the terminal differentiation of cells is the capability to resemble the native environment, thus better restoring tissue damages. Nevertheless, while inducing the cell terminal differentiation, the cell cycle is affected with an influence on cell proliferation and presumably on their viability [23] as well as on the potential release of adverse signals. Thus, the "terminal differentiation" of MSCs towards a specific lineage might not be the optimal approach in the case of transplantation in vivo.

The aim of this work is the evaluation of the behavior of undifferentiated or tenogenic-differentiated MSCs after their seeding onto decellularized equine tendons as a potential biological

scaffold for tendon repair. Cell survival rate and replicative capacity along with the deposition of collagen matrix have been assessed in vitro over an incubation period of 24 hours, 7 and 14 days.

2. Materials and Methods

2.1. Study design

In this study, two experimental groups were established: MSCs cultured under tenogenic (T-MSCs) and undifferentiating (U-MSCs) conditions seeded onto decellularized tendon matrices. For each condition, equine tendon matrices previously decellularized and sterilized were used as described elsewhere [10,11]. These matrices were seeded with rabbit bone marrow MSCs and cultured for 24 hours, 7 and 14 days in two different culture media. The experiments were performed in triplicate. At each time point, cell viability, proliferation and collagen matrix deposition were assessed by means of viability assay, histology and immunohistochemistry.

2.2. Preparation of the scaffolds

Equine superficial digital flexor tendons were collected from three different adult horses (n = 3) at the slaughterhouse, then decellularized and sterilized as described elsewhere [10,11]. Briefly, tendons were cut in 0.3 cm-thickness slices and treated with 1% tri-n-butyl phosphate buffered in 1M Tris-HCl pH 7.8 for 24 hours at room temperature (RT) under agitation, rinsed in ddH_2O and stored in PBS at 4 ℃ for 24 hours. After the immersion in 0.0025% DNAse-I in PBS at RT under agitation for 4 hours, the specimens were incubated in 3% aqueous solution of peracetic acid (stock solution 32%) under agitation at RT for 4 hours. Finally, slices were rinsed in ddH_2O for 15 minutes, then in PBS for 15 minutes, and decontaminated by means of β-irradiation at 15 kGy using a 10 MeV source (Bioster S.P.A., Seriate, Bergamo, Italy). Then, the decellularized tendon matrices were dry-stored at –80 ℃ until use. To be used as biological scaffolds for cell culture, decellularized tendons were thawed and cut into small pieces of 0.5 cm × 0.5 cm × 0.3 cm.

2.3. Culture and expansion of rabbit bone marrow MSCs

Rabbit bone marrow MSCs (Oricell™, Cyagen Biosciences, Inc.; Cat. No. RBXMX-01001, Santa Clara, CA) were used for this study, as already described [19]. Cells were seeded at a density of 6000 cells/cm^2 and cultured in complete medium (CM) composed of Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (Hyclone, GE Healthcare Life Sciences, UT), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES (all from Thermo Fisher Scientific). Medium was changed twice a week and cells were sub-cultured after reaching 90% of confluence.

2.4. Recellularization of decellularized tendons with undifferentiated and tenogenic-differentiated MSCs

The decellularized tendons were placed individually in 12-wells culture plates. MSCs at passage 3 were seeded onto the scaffolds at a density of 150,000 cells/cm^2 and cell attachment onto the scaffolds was induced by incubating the constructs for 2 hours. Afterwards, the constructs were statically cultured in CM added with 10 ng/ml basic fibroblast growth factor (bFGF; Peprotech EC Ltd., London, UK) to maintain cells undifferentiated (U-MSCs) or cultured in tenogenic medium (T-MSCs) composed of CM supplemented with 100 ng/ml BMP-14, 50 ng/ml VEGF, and 1 ng/ml TGF-β3 (all from Peprotech) [19]. The cell response to different media onto the biological scaffolds were assessed after 24 hours, 7 and 14 days. Each experiment was performed in triplicate. In particular, analyses were carried out to test cell viability, proliferation, cell morphology, matrix deposition and distribution, and type I and III collagen production.

2.5. Cell viability

To evaluate cell viability of U-MSCs and T-MSCs onto the decellularized matrices, Live & Dead viability test (Thermo Fisher Scientific) was performed according to the manufacturer's instructions. Briefly, after 24 hours, 7 and 14 days of culture, the reseeded constructs were labeled with Live & Dead stain consisting in 2.5 μl calcein AM and 10 μl ethidium homodimer-1 dissolved in 5 ml of PBS. Then, the samples were incubated at 37 ℃ in the dark for 15 minutes before microscopic analysis. Live cells appeared green, while dead cells fluoresced red.

2.6. Histological process

Samples were fixed in 10% buffered formaldehyde for 24 hours at RT, dehydrated, paraffin embedded and longitudinally sectioned at 3.5 μm. Sections were stained with Haematoxylin-Eosin staining (H&E) to assess the cellular components, their morphology and newly formed matrix. Photomicrographs were captured using Olympus IX71 light microscope and Olympus XC10 camera (Olympus Corporation, Shinjuku, Tokyo, Japan). The sections were qualitatively analyzed for cell morphology and the presence or absence of newly formed matrix.

2.7. Immunohistochemistry

To evaluate proliferating cells in the U-MSCs and T-MSCs groups, immunostaining was performed for proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase. The U-MSCs group was also analyzed to assess the distribution of type I and III collagen within the newly formed matrix. After removal of the paraffin and dehydration, immunostaining was carried out with a reaction time of 60 minutes for primary anti-PCNA antibody (PC-10, Thermo Fisher Scientific) at a dilution of 1:1000, anti-collagen type I (1:200 dilution) and type III (1:2000 dilution) (Sigma-Aldrich Corporation, Saint Louis, MO). Then, sections were exposed to a biotinylated anti-mouse secondary antibody (1:200 dilution; Vinci Biochem Vinci, Florence, Italy) for 30 minutes. The signal was detected by means of the streptavidin-biotin method coupled with the

3'-Diaminobenzidine (DAB) chromogen system (Vinci Biochem). Counterstaining was performed by haematoxylin, and sections were microscopically analyzed. Negative control was carried out by omitting the primary antibodies. The positive control consisted in skin and derma of rabbit ears.

2.8. Histomorphometry and semi-quantitative evaluations

Semi-quantitative analyses were performed to assess the cell proliferation, the amount of newly formed matrix and the percentage of type I or III collagen deposed within the matrix. To do this, three photomicrographs at 200× for each sample were evaluated using Image J 1.45s® software (open source: http://rsbweb.nih.gov/ij) through the identification of three independent regions of interest (ROI, 144,947 μm^2).

The amount of newly formed matrix was assessed on H&E-stained sections. The matrix area was calculated by removing the white area and the area occupied by cells from the total ROI. Data are reported as μm^2.

The cell proliferation was evaluated on immunostained slices for PCNA. The area occupied by cells within the ROI was analyzed after removing both the white and the newly formed matrix areas by means of a color threshold. Similarly, the whole area occupied by PCNA positive and negative stained cells was measured. Data are reported as the percentage occupied by proliferating cells on the total ROI.

Type I or III collagen within the newly synthesized matrix was evaluated on immunostained slices. As described above, after removing the white and cellular area, the amount of the matrix positive for type I or III collagen was measured and reported as the percentage on the total ROI.

2.9. Data and statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software. Data were verified for normal distribution using Kruskal-Wallis test. Comparisons between U-MSCs and T-MSCs were analyzed using an unpaired t-test in the case of normal distribution and Mann Whitney test for nonparametric data. Comparisons among time points were analyzed using one-way analysis of variance (ANOVA) coupled with Bonferroni's post hoc test in the case of normal distribution and Dunnett's test whether nonparametric. Data are expressed as the mean ± standard error (SE). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Cell viability

The evaluation of the viability of U-MSCs and T-MSCs onto the decellularized tendons was performed by Live & Dead Viability test, as shown in Figure 1. After 24 hours, both T-MSCs and U-MSCs were viable. Cells were homogeneously distributed onto the entire tendon surface. T-MSCs were uniformly elongated and covered completely the construct surface. Differently, U-MSCs showed a mixture of rounded and elongated shapes. Specifically, rounded cells were randomly

distributed onto the surface, while elongated cells were strictly connected to each other forming a compact layer onto the construct. After 7 days of culture, both T-MSCs and U-MSCs proliferated, and a slight increase of dead cells was observed, mainly in the T-MSC group. The same morphological differences described above appeared more evident with greater number of rounded cells in the U-MSC group.

After 14 days, some dead cells were present in both groups, with an appreciable increase in their total amount. Morphologically, T-MSCs appeared elongated and formed a very compact layer. Otherwise, most of U-MSCs maintained their rounded shape and others formed a thin layer.

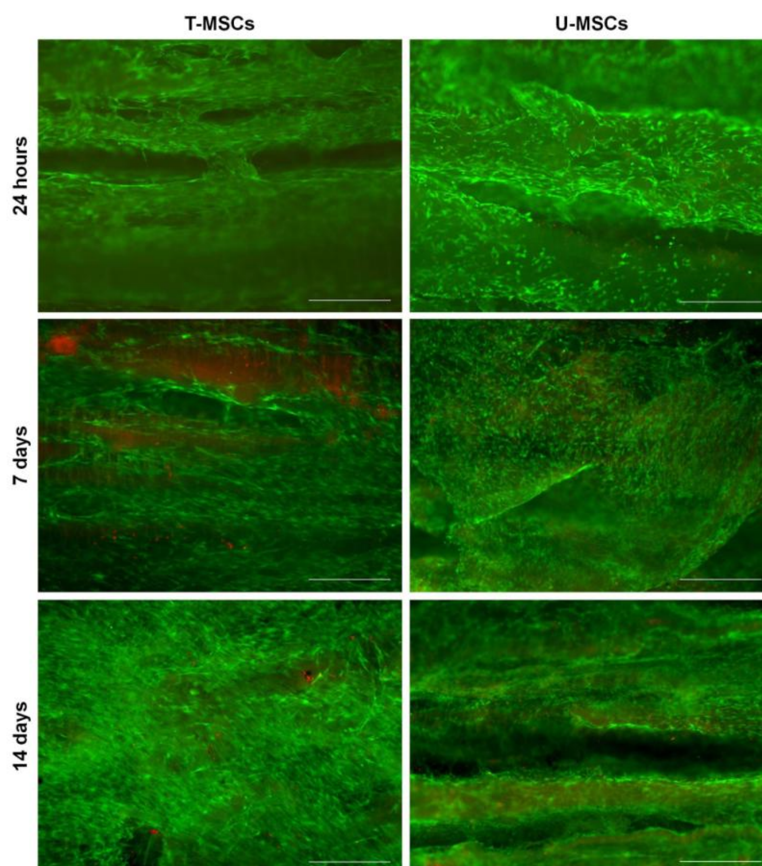

Figure 1. Live & Dead assay for the evaluation of cell viability in the T-MSC and U-MSC groups after 24 hours, 7 and 14 days. Viable cells are green stained, while dead cells are red stained. Magnification: 4×, scale bar: 500 μm.

3.2. Architecture of the constructs: cell morphology and matrix deposition

H&E-stained sections, reported in Figure 2, showed both parallel collagen fiber bundles organized in a very orderly way within the decellularized tendon matrix, and a complete absence of resident cell nuclei and debris, confirming the effectiveness of the decellularization protocol. In general, the seeded MSCs did not penetrate or colonize the tendon matrix, as expected in a static culture.

At 24 hours after reseeding, T-MSCs appeared elongated and tightly connected onto the external construct surface. Confirming data observed in Live & Dead assay, U-MSCs were rounded shaped and distributed onto a looser, less organized newly deposed extracellular matrix onto the decellularized tendon surface. After 7 days, T-MSCs assumed a tenogenic-like morphology with scarce cytoplasm and small nuclei and formed a continuous monolayer onto the newly formed matrix deposed onto the tendon surface. In the U-MSC group, cells seemed less connected and migrated within the new extracellular matrix, which began to acquire a more dense structure. At the last time-point (14 days), T-MSCs lost their strict connections, showing bigger and rounded nuclei, as well as an augmented cytoplasm volume, and the newly deposed matrix was no more detected. Moreover, these cells tended to detach from the tendon surface. On the contrary, U-MSCs maintained the morphology seen on day 7. Moreover, a greater number of cells penetrated within the newly synthesized matrix, which appeared qualitatively thicker compared to that observed at previous time points.

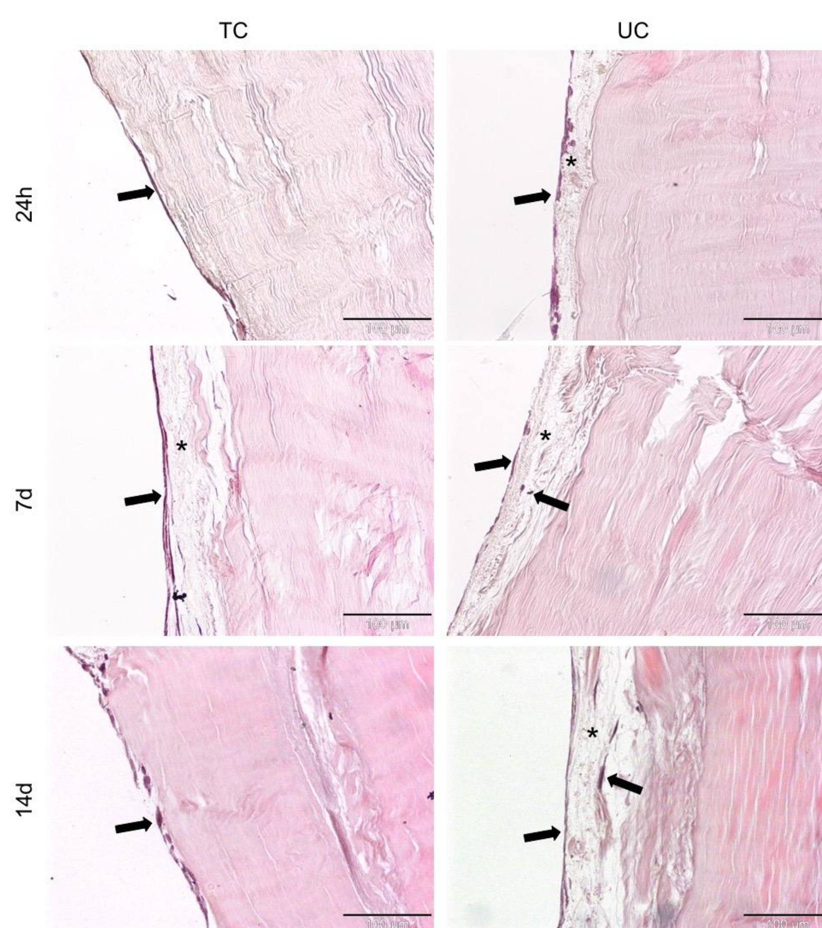

Figure 2. Photomicrographs of H&E-stained sections of the T-MSC and U-MSC groups cultured onto the decellularized tendon matrix. Arrows indicate the reseeded cells both on the surface and within the newly synthesized matrix. Asterisks (*) point out the new synthesized matrix. Magnification: 20×, scale bar: 100 μm.

3.3. Immunohistochemistry

3.3.1. PCNA

Cell proliferation was investigated by immunohistochemistry using a primary antibody directed against the Proliferating Cell Nuclear Antigen (PCNA). As shown in Figure 3, most of T-MSCs and U-MSCs were PCNA-positive at 24 hours after seeding. The signal intensity decreased at succeeding time points. This phenomenon was evident in the T-MSC group already after 7 days, while in the U-MSC group it was observed on day 14. On day 14, the majority of the cells resulted no longer PCNA-positive in both the experimental groups.

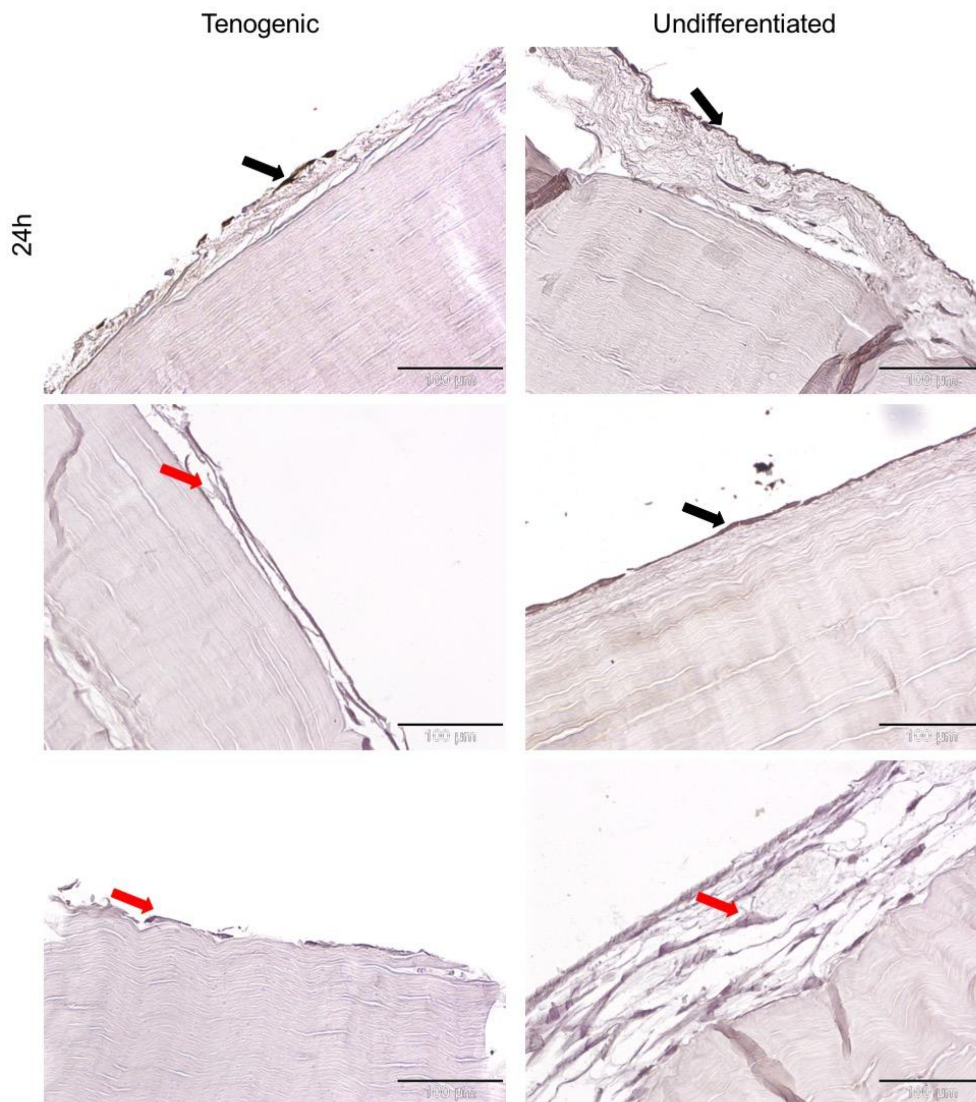

Figure 3. Photomicrographs of immunostained sections of the T-MSC and U-MSC groups cultured onto the decellularized tendon matrix. Black arrows indicate PCNA-positive cells; red arrows indicate cells negative for PCNA. Magnification: 20×, scale bar: 100 μm.

3.3.2. Type I and III collagen

Immunohistochemical analyses of type I and III collagen were performed to characterize the newly synthesized matrix deposed only in the U-MSC group. Indeed, a poor evidence of newly deposed matrix was detected in the T-MSC group only on day 7. In all the three experimental time points, type I collagen qualitatively appeared strongly present and homogeneously distributed within the newly deposed matrix. Differently, a spotted positivity for type III collagen was visible associated with a more intense signal within the decellularized tendon matrix (Figure 4).

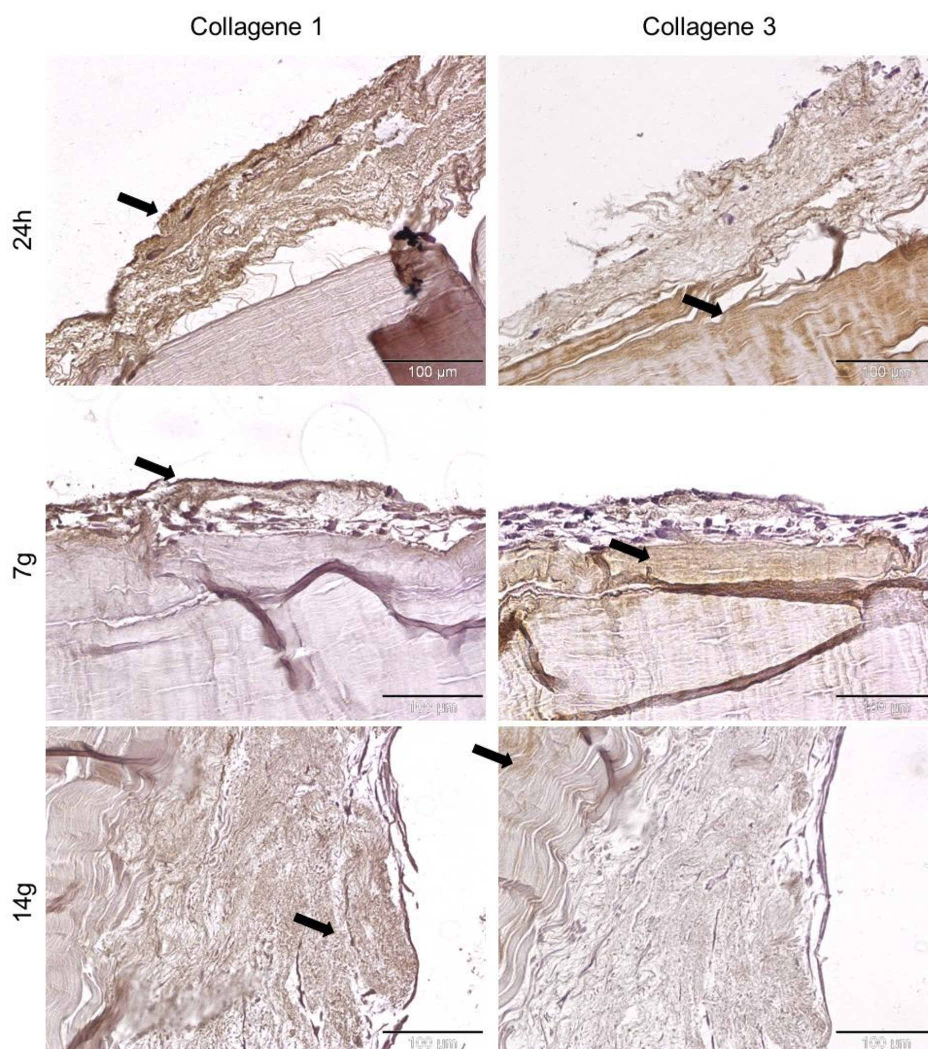

Figure 4. Qualitative comparison of the immunostaining analysis for type I and III collagen in the U-MSC group. Arrows point out the positivity for each collagen antigen. Magnification: 20×, scale bar: 100 μm.

3.4. Semi-quantitative histomorphometric analysis

The semi-quantitative histomorphometric analysis of the cell proliferation rate showed a decreasing trend over time in both the experimental groups (Figure 5a). There was not a significant

difference in cell proliferation rate between the T-MSC and U-MSC groups at 24 hours. The T-MSC group showed a drastic reduction of cell proliferation on day 7 and 14 ($p < 0.01$ and $p < 0.001$, respectively). No differences were found in this group between 7 and 14 days. Similarly, the U-MSC group depicted a consistent decrease in proliferating cells over time ($p < 0.001$). More importantly, the T-MSC group showed a significant decrease of cell proliferation compared to the U-MSC group on day 7 ($p < 0.001$).

The newly formed matrix showed a significant difference between T-MSCs and U-MSCs, with greater matrix deposition in the U-MSC constructs compared to the T-MSC ones, at 24 hours and on day 14 ($p < 0.001$ and $p < 0.01$, respectively). No significant differences were observed in the T-MSC group at any time point. Otherwise, a significant increase of the matrix deposition was found in U-MSCs on day 14 with respect to both 24 hours and day 7 ($p < 0.05$) (Figure 5b).

Within the newly formed matrix in the U-MSC group, the total collagen remained almost constant over time. Nevertheless, the percentage of type I collagen increased simultaneously with the decrease of type III collagen. In particular, a significant amount of type I collagen was detected with respect to type III collagen at any time point ($p < 0.001$). In fact, type I collagen deposition increased significantly over time with $p < 0.05$ between 24 hours and 7 days, and $p < 0.001$ between 24 hours and 14 days. On the contrary, the production of type III collagen was very limited and significantly diminished on day 14 compared to the initial time point (24 hours) ($p < 0.001$) (Figure 5c).

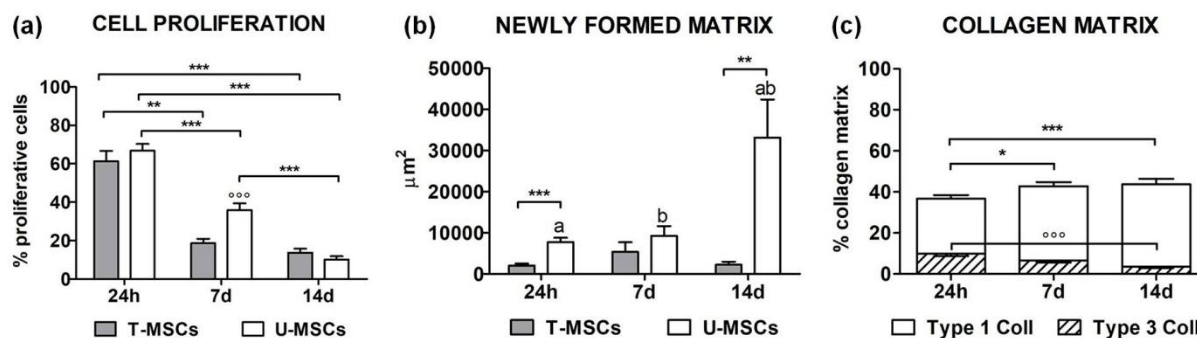

Figure 5. Semi-quantitative histomorphometric analyses. a: Cell proliferation rate in T-MSC and U-MSC constructs, data are reported as the percentage of area occupied by proliferating cells (%); b: Newly formed matrix in T-MSC and U-MSC constructs (μm^2); c: Collagen matrix deposition in U-MSC constructs, data are reported as the percentage of area occupied by type I or type III collagen (%). Highly significant difference was found between type I and III collagen at any time point ($p < 0.001$). a, b, * $p < 0.05$; ** $p < 0.01$; ***, °°°$p < 0.001$.

4. Conclusions

The aim of this study was the identification of suitable culture conditions for the generation of a functionalized biological construct to be used as a tendon graft, following the TE triad (growth factors, scaffold and cells). To do this, the functionalization of a decellularized tendon matrix

reseeded with bone marrow MSCs directly cultured in undifferentiated or tenogenic medium onto a biological scaffold was evaluated.

Live & Dead viability assay demonstrated that cell viability was not affected either by the culture conditions or by the culturing time in both the T-MSC and U-MSC groups. This result is supported by the immunohistochemical analysis for Caspase-3 (data not shown) and is in accordance with data reported by Ozasa and colleagues who demonstrated that bone marrow MSCs seeded onto decellularized tendons were viable up to 14 days after seeding [24]. In addition, as shown by anti-PCNA immunohistochemistry, while the proliferation rate decreased in both the T-MSC and U-MSC groups over time, about 20% of cells were still proliferating 14 days after reseeding. In support of this result, Kryger and colleagues demonstrated that both undifferentiated bone marrow MSCs and tenocytes derived from the epitenon continued to proliferate without signs of cellular senescence up to 8 weeks in an in vitro culture on decellularized tendons [25]. Overall, our results indicate that this approach is suitable to furnish an adequate and initial proliferative cue in order to start the regenerative process.

The H&E staining verified the complete absence of nuclei or cellular debris within the decellularized tendon matrix, which displayed a physiologic structure consisting in organized and parallel collagen bundles. This finding represents a further evidence of the effectiveness to obtain a xenogeneic decellularized tendon matrix, as previously described [9,10,11]. Notwithstanding, cells were not able to efficiently penetrate within the tendon matrix when cultured in static conditions, as also supported by other studies using similar approaches [24,25,26]. This phenomenon could be related to the highly compact structure of the tendon, which makes the cell infiltration difficult even after the decellularization process. It could be also hypothesized that the interference with the cell colonization could be due to the presence of a basement membrane containing epithelial cells that covers tendons, with the function to keep tenocytes inside tendon fibers and to prevent the spontaneous development of adhesions [27]. To overcome these limitations, a dynamic in vitro cell culture in sophisticated bioreactors can be used, as already proven by others [28,29,30]. Anyway, Thorfinn and colleagues reported that tenocyte-reseeded decellularized tendons were deeply repopulated by resident cells derived from the surrounding tissues after in vivo transplantation in rabbit tendon defects [26]. Moreover, an in vitro study demonstrated that tenocytes have high migratory capability and reseeded decellularized tendons are characterized by greater colonization rate compared to the unseeded matrix [31]. Bone marrow MSCs are well known to synthesize extracellular matrix, and they have been often employed for the production of cell-derived matrices used as scaffolds in TE [32]. Indeed, under specific culture conditions, bone marrow MSCs, have proven to be able to deposit various types of extracellular matrix with different compositions resembling different tissues such as bone, fat and cartilage [32]. However, so far, no attempts have been made to develop a tendon-specific matrix using this approach.

Based on these findings, this study investigated if bone marrow MSCs seeded onto decellularized tendon matrices and cultured in tenogenic conditions could better functionalize the engineered constructs compared to undifferentiated progenitor cells. Unexpectedly, in the present study, U-MSCs produced a greater amount of extracellular matrix than T-MSCs. Furthermore, the new matrix synthesized by U-MSCs was richer in type I collagen rather than type III at all the experimental time points, contrary to what expected by an immature tendon matrix [33]. This means

that U-MSCs had the ability to synthesize type I collagen immediately after seeding and to protract this capacity over time better than the T-MSCs. This ability in synthesizing type I collagen might be an essential signal for the chemoattraction of resident tenocytes after implantation, indirectly accelerating both the cell repopulation and the healing process [31]. Due to this feature, despite the inability to deeply colonize the decellularized matrix in vitro, the use of U-MSCs seems to represent a suitable approach for the TE functionalization of tendon constructs for three main reasons. First of all, no growth factors need to be used within the culture medium, thus avoiding all the concerns related to their use in cell-based therapies [34]. Secondly, in the case of *in vivo* implantation, seeded cells will find a tenogenic environment able to induce a proper differentiation process with no need for an extensive in vitro cell manipulation. Finally, the extracellular matrix rich in type I collagen synthesized by the U-MSCs onto the decellularized scaffold can act as a chemoattractant surface for the resident cells.

In conclusion, the good survival and proliferation of U-MSCs was demonstrated to be associated with great deposition of new type I collagen extracellular matrix onto decellularized tendon scaffolds. With the prospect of clinical applications, this approach could offer a valid substrate in the case of substitution of large tendon defects. The advantages of this technique are mainly related to both large number of reseeded decellularized tendon xenografts that can be obtained avoiding the use of autologous or allogeneic grafts, and the lack in using exogenous growth factors. Further *in vivo* studies should be required to investigate the effectiveness of this approach as a substitute for the tendon reconstruction.

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Conflict of Interest

All authors declare no conflict of interest in this paper.

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