Assessing the functional properties of tenogenic primed mesenchymal stem cells in ex vivo equine tendon and ligament explants: A preliminary study

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ABSTRACT

Injuries to equine tendons and ligaments are career-compromising, causing reduced performance and premature retirement. Promising treatment alternatives have been investigated in the field of mesenchymal stem cells (MSCs). In this study, the tissue adherence and protein expression of tenogenic primed mesenchymal stem cells (tpMSCs) after administration to ex vivo tendon and ligament explants is investigated. First, collagen type I (COL I) and smooth muscle actin (SMA) expression was assessed in explants treated with both cell types. Subsequently, cell adhesion to the explants and the amount of COL I and SMA positive cells was evaluated. The explants revealed a significantly higher COL I and lower SMA expression in tpMSCs compared to native MSCs. Second, equine superficial digital flexor tendon and suspensory ligament explants were cultivated, and a lesion was treated with both cell types. Subsequently, cell adhesion to the explants and the amount of COL I and SMA positive cells observed in the lesions treated with tpMSCs. The results of these explant co-cultures may demonstrate at least a part of the mechanism of action and functional properties of tpMSCs in restoring function to tendons and ligaments.

1. Introduction

Injuries to equine tendons and ligaments are career-compromising, causing reduced performance and premature retirement (Bonilla-Gutierrez et al., 2019; Clegg, 2012). An evaluation in UK racehorses revealed that 46% of the injuries were located in tendon and ligaments (Williams et al., 2001). In that study, 90% of lesions occurred within the superficial digital flexor tendon (SDFT) with the remainder being suspensory ligament (SL) injuries (Clegg, 2012; Williams et al., 2001). Current treatments offer symptom management, but do not restore normal anatomical or physiologic function. Promising treatment alternatives have been investigated in the field of regenerative medicine, more specifically mesenchymal stem cells (MSCs) targeted at tissue repair. Several ex vivo tendon models have been developed to study the healing mechanisms of MSCs for tendon injuries in horses. However, today, there is no generally accepted physiologically correlated ex vivo tendinopathy model which includes extracellular matrix and vascular supply to properly investigate regenerative treatments.

Extracellular matrix of tendons and ligaments contains collagen type I (COL I; 95%), collagen type III (COL III; 1–3%), elastin, glycoproteins (tenascin and tenomodulin), proteoglycans (decorin) and scleraxis (Meeremans et al., 2021; Spaas et al., 2012). As previously described, an
inflammatory phase occurs after an acute tendon injury (Depuydt et al., 2021; James et al., 2008; Meeremans et al., 2021; Spaas et al., 2012) followed by a proliferation phase and finally a remodeling or maturation phase where a higher amount of tissue-specific cells and matrix are produced. The natural healing process results in scar tissue containing smaller and less organized fibrils of COL III and leads to a loss of original tensile strength and elasticity (Shojaei and Parham, 2019). The healing process of tendons and ligaments is slow and the final disorganized scar tissue contributes to a high reinnjury rate (Beets et al., 2013; Clegg, 2012; Crevier-Denoix et al., 1997; Dyson, 2004; Smith, 2008). In order to obtain both structural and functional repair of tendon tissue the components of the extracellular matrix should be restored. This includes a high COL I content that is key for a functional, high-quality healing of the tendon tissue and is reported to be an important indicator of tendon matrix synthesis (James et al., 2008; Romero et al., 2017). Additionally, a lower distribution of COL III and smooth muscle actin (SMA) is seen with functional healing. The SMA protein is incorporated in myofibroblasts that play an important role in the post-inflammatory events after tendon injury (Nichols et al., 2019). An increased number of myofibroblasts results in the synthesis of abundant amounts of COL III causing the formation of persistent scar tissue (Cadby et al., 2014; Hinz, 2007; Williams et al., 1980), reduced blood flow and eventually chronic tendinopathies (Kvist et al., 1985).

MSCs are proving to be a promising primary treatment due to their ability to modulate mechanisms that result in a more functional repair with reduced inflammation and more normal tissue (Ahberg et al., 2018; Conze et al., 2014, Godwin et al., 2012; Rico et al., 2013; Romero et al., 2017; Smith et al., 2013; Spaas et al., 2012). Brandt et al. reported that these beneficial effects of native MSCs in acute tendon injuries can be compromised by an inflammatory environment (Brandt et al., 2018). Absence of beneficial treatment effect with native MSCs in surgically induced SDFT core lesions was also reported by Geburek et al. (Geburek et al., 2013). Unlike native MSCs, tenogenic priming of the MSCs is a key factor to their regenerative effects in tendon healing (Broeckx et al., 2012).

The use of peripheral blood-derived tenogenic primed MSCs (tpMSCs) has already been described in horses suffering from tendon injuries resulting in promising therapeutic effects (Beets et al., 2017; 2013; Broeckx et al., 2012; Depuydt et al., 2021). However, the mode of action of tpMSCs in tendon regeneration is still to be fully understood. There is an urgent need for biological methods to investigate their mode of action. The goal of this study was to demonstrate biologically active factors that correlate with the functional properties of tpMSCs in the tendon and ligament healing process. In the current study, COL I and SMA protein expression was evaluated for both MSC types in cytospins in a first experiment. Next, the adherence of native MSCs and tpMSCs is compared using ex vivo tendon and ligament explant cultures. Finally, the amount of COL I and SMA positive cells present in the ex vivo tendon lesion treated with both MSCs and tpMSCs was assessed.

2. Materials and methods

2.1. Isolation and tenogenic priming of mesenchymal stem cells

Tenogenic primed MSCs were prepared from the peripheral blood from three different adult donor horses and characterized as previously described (Broeckx, 2012; Spaas et al., 2013). Briefly, blood was collected aseptically from the vena jugularis in sterile EDTA tubes. The blood collection from the donor horses (EC 2018_002) was approved by an ethics committee with independent members evaluating the application as approved by the Flemish government (permit number: LA1700607). After gradient centrifugation, the interphase was isolated, seeded and cultivated in culture medium (Dulbecco’s modified Eagle medium low glucose (DMEM; Life Technologies) supplemented with fetal bovine serum (FBS; Sigma-Aldrich)). Subsequently, the MSCs were cultured until passage 5 and thoroughly characterized (i.e. viability, morphology, presence of cell surface markers, and trilineage differentiation) as previously described (Spaaes et al., 2013), prior of being frozen as intermediate cell stock. After characterization, cells were thawed, further cultivated and tenogenic primed by adding tenogenic growth factors to the culture medium. The specific growth factors and the markers used for characterization of the tenogenic priming cannot be disclosed due to company policies. At 80% confluence, the cells were trypsinized and resuspended at a concentration of 3 x 10^6 tpMSCs in 1 mL DMEM supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich). The samples were cryopreserved at −80 °C until further use. The used tenogenic primed MSCs are commercially available (RenutEnd®, Boehringer-Ingehelm). For each use, tpMSCs were thawed, drawn into a syringe and processed immediately after thawing. The donor horses were screened for equine pathogens as previously reported by our group (Broeckx, 2012) at Böse laboratory (Harsum, Germany).

2.2. Preparation of cytospins

For this experiment, native MSCs and tpMSCs were thawed and cytospins prepared by centrifugating 200,000 (tp)MSCs on an adhesion slide. All cytospins were fixed in acetone at −20 °C. After washing with phosphate buffered saline, the cytospins were stained for COL I and SMA as previously described by our group (n = 12 for each cell type/staining) (Depuydt et al., 2021).

2.3. Explants and creation of the lesion

For the current purpose, explants were taken from the superficial digital flexor tendon (n = 33) of two horses and the suspensory ligament (n = 21) of one horse, both without evidence of musculoskeletal disease that were euthanized for reasons unrelated to the study. There is no information available considering the anamnesis or age of the horses. After isolation of the SDFTs, the tendons were clinically normal without presence of swelling or injuries. Explants were isolated within 1 h of euthanasia at the Pathology Department of the Faculty of Veterinary Medicine, Ghent University. A sterile 8 mm punch was used together with a sterile forceps and bistoury knife (see Fig. 1 on the left). All biopsies were placed in Hank’s Balanced Salt Solution (HBSS, Life Technologies) supplemented with 2% AB/AM and shipped to the laboratory at room temperature.

Next, one central lesion was made parallel with the fiber bundles on the top surface of each explant using a scalpel blade (n = 15, Swann Morton) (see Fig. 1 in the middle). The incision was not made over the biopsy and there was no removal of tendon tissue. All explants were positioned in 24-well plates with the lesion side upwards before addition of the treatment.

2.4. (tp)MSC treatment for cell adhesion investigation

The vials containing the (tp)MSCs were thawed in the palm of a hand and were added at a concentration of 500,000 (tp)MSCs per 10 µL of culture medium to the explants (n = 9 for each MSC product) (see Fig. 1 on the right). Three negative controls without addition of stem cells were included. In this case, 10 µL of culture medium was added. The explants were sealed all from one horse. Afterwards, 1 mL of culture medium was added to each well. The explants were cultivated at 37 °C and 5% CO₂ in a humidified incubator.

After 24 h of culture, the cell adhesion efficiency was assessed. Therefore, the tendon explants were transferred as such to 4% formaldehyde at room temperature to allow fixation of the tissue. Following overnight fixation of the tendon explants at room temperature, paraffin sections were prepared (4 µm) and a Mason’s Trichrome staining was performed to evaluate the cell adhesion efficiency.

Per explant, one picture was taken over the entire length of the lesion at 25-fold magnification. Next, multiple pictures were taken over the entire length of the lesion at 200-fold magnification to evaluate for...
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38x404]deparaffinized and rehydrated in xylene and decreasing concentrations of alcohol in H2O. Sections were cut and mounted on adhesion slides. Next, slides were percentage of COL I and SMA. For each staining, 5 explants were prepared.

For this experiment, the same methods were used as described above for harvesting SDFT explants. Next, 15 explants were treated with tpMSCs, 9 explants with native MSCs and 9 explants without addition of stem cells. The same explants were used as for the cell adhesion investigation described above together with additional samples derived from the SDFT of a second horse. After 24 h of culture and overnight fixation of the tendon explants at room temperature, paraffin sections were prepared.

Immunohistochemistry was performed to evaluate the distribution percentage of COL I and SMA. For each staining, 5 µm thick tendon sections were cut and mounted on adhesion slides. Next, slides were deparaffinized and rehydrated in xylene and decreasing concentrations of alcohol in H2O (xylene, 96 % alcohol, 80 % alcohol, 70 % alcohol, 60 % alcohol and 100 % H2O).

2.6. Immunohistochemistry

For the sections from SDFT explants, the immunohistochemical staining for the detection of COL I and SMA were performed as previously described by our group (Depuydt et al., 2021). In brief antigen retrieval was performed for all explants. Next, sections were incubated with the primary polyclonal rabbit COL I antibody (1:500; ab138492; Abcam) or with the primary monoclonal mouse SMA antibody (1:200; M0851; Agilent). Next, the secondary antibody (respectively EnVision horseradish peroxidase (HRP) anti-rabbit; K4003; Agilent and EnVision HRP anti-mouse; K4001; Agilent) was incubated. Visualization was performed in a 3,3-diaminobenzidine solution (DAB; K3468; Agilent). The cell nuclei were counterstained with hematoxylin, after which the sections were dehydrated, and coverslips were applied. For each staining, a positive control sample was included. This was an equine skin section for the COL I staining and a section of the equine stomach for the SMA staining.

Pictures were taken from the lesion site. The individual cells were scored for positive staining and not the entire tissue slide. Protein expression was presented as % positive stained cells on the total cells counted.

### Table 1

<table>
<thead>
<tr>
<th>Score system</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>Score 0</td>
<td>No cellularity at the lesion site</td>
</tr>
<tr>
<td>Score 1</td>
<td>0 to 30 cells at the lesion site</td>
</tr>
<tr>
<td>Score 2</td>
<td>30 to 100 cells at the lesion site</td>
</tr>
<tr>
<td>Score 3</td>
<td>&gt; 100 cells at the lesion site</td>
</tr>
</tbody>
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2.7. Data analysis

For the cytospins and explants treated with (tp)MSCs for the investigation of protein expression, data were analyzed in IBM SPSS Statistics. The normal distribution assumption was checked using the Shapiro-Wilk test. Normally distributed data were analyzed using the independent samples T-Test and other data using the independent samples Mann-Whitney U Test at the 5 % significance level.

For the SDFT and SL explants treated with (tp)MSCs for evaluation of cell adhesion, data were analyzed in IBM SPSS Statistics. To compare the presence of score >1 for cellularity at lesions site between treatments, a Fisher’s Exact test was performed at the 5 % significance level.

3. Results

3.1. Cytospins

The expression of COL I and SMA were evaluated in equine native MSCs and tpMSCs using light microscopic immunocytochemistry. The brown color in the cytoplasm of the cells indicates cells positive for protein expression. The mean percentage of tpMSCs expressing COL I (66.2 ± 16.2 %) was significantly higher compared to native MSCs (14.2 ± 3.0 %) (P < 0.001; Fig. 2). On the other hand, a significantly lower mean percentage of tpMSCs expressing SMA (13.3 ± 6.3 %) was observed in comparison to native MSCs (80.2 ± 2.5 %) (P < 0.001; Fig. 2).

3.2. Explant study for investigation of cell adhesion

A score for cellularity at the lesion site was assigned to each tendon and ligament explant. The explants treated with tpMSCs showed a remarkably higher cellularity at the lesion site when compared to native MSCs, both for SDFTs and SLs (Fig. 3A-B). In conclusion, 9 tendon and 9 ligament explants treated with tpMSCs and 0 explants treated with native MSCs received a score >1, resulting in a statistically significant difference for both SDFT and SL explants (P < 0.001).

3.3. SDFT explants for protein expression investigation

The protein expression of COL I and SMA is depicted in Fig. 4. The brown color in the cytoplasm of the cells indicates cells positive for protein expression. In the tendon explants where no (tp)MSCs were added, no cells positive for both COL I and SMA could be detected. When counting the percentage of positive cells per explant in the lesion, significantly more cells in the SDFTs treated with tpMSCs were positive.
for COL I ($81.1 \pm 4.3\%$) when compared to the native MSC treated tissue ($9.5 \pm 0.9\%$) (Fig. 4; $P = 0.036$). On the contrary, significantly more cells were positive in the explants treated with native MSCs for SMA expression ($86.4 \pm 2.2\%$) when compared to the tpMSC group ($15.8 \pm 10.0\%$) (Fig. 4; $P < 0.001$).

### 4. Discussion

This study is the first to investigate the adherence and protein expression of equine native and tenogenic primed MSCs in an ex vivo tendon and ligament explant culture. In contrast to in vitro tenocyte cultures, models like tendon explants are very interesting due to the intact tissue architecture and cell-extracellular matrix interaction (Wunderli et al., 2020a). The current study shows that tpMSCs are capable of adhering to tendon and ligament biopsies in a greater extent than native MSCs. Additionally, it can be concluded from cytospin evaluations that tpMSCs show a significantly higher COL I and significantly lower SMA expression compared to native MSCs. Additionally, SDFT tissue treated with tpMSCs shows a significantly higher percentage of COL I and significantly lower percentage of SMA positive cells when compared to SDFT lesions treated with native MSCs. This indicates that the tenogenic priming of the MSCs elicits a significantly higher expression of proteins that play a crucial role in structural and functional tendon repair.

Native MSCs have beneficial effects which makes them a viable treatment option for tendon and ligament lesions, however, tenogenic priming allows a more targeted and effective repair of the tissues as demonstrated clinically and supported by the mechanisms demonstrated in the current research. Tenogenic priming also mitigates any concern that the cells may be compromised by their environment and not function as intended. Although not in horses, ectopic bone formation has been reported in rabbits and mice after administration of bone marrow-derived MSCs (BM-MSCs) into acute tendon lesions (Dressler et al., 2005; Fang et al., 2014; Harris et al., 2004; Shojaee and Parham, 2019). Tenogenic priming before clinical application ensures appropriate differentiation and mitigates improper differentiation associated with ectopic bone formation (Gomiero et al., 2016). Differentiation of MSCs has been performed previously by several research groups for the treatment of tendon injuries (Dale et al., 2018; Roth et al., 2018; Younesi Soltani et al., 2021). Younesi Soltani et al. (2021) collected human ASC samples and primed them using platelet-derived growth factor-BB and differentiation growth factor-6. After 14 days of culture, a higher expression of both COL I and COL III could be observed (Younesi Soltani et al., 2021). In the present in vitro study, one of the proteins involved in formation of scar tissue (i.e. SMA) was expressed in a significantly lower amount. Similar findings were also observed in an in vitro study performed by Costa-Almeida et al. (2018) in which native human adipose derived MSCs (ASCs) were co-cultivated with tendon explants in an indirect trans-well system (Costa-Almeida et al., 2018). After 7 cultivation days, a significantly lower deposition of COL III was observed compared to a single ASC culture. Additionally, Long et al. (2018) showed that co-cultivating of human tenocytes and ASCs using trans-wells resulted in a higher COL I production (Long et al., 2018).

The in vitro results of the present study are in line with an in vivo study performed by our group where a significantly higher COL I expression and a significantly lower COL III and SMA expression 16 weeks after treatment of a surgically induced SDFT core lesion with tpMSCs in comparison to the placebo treated limbs was demonstrated (Depuydt et al., 2021). In addition, ultrasound tissue characterization (UTC) demonstrated that tpMSC treated limbs had a significantly higher percentage of echo type I in comparison to the placebo group. This echo type corresponds to healthy tendon structure consisting of fully aligned intact tendon structures with a high amount of collagen type I fibers (Bosch et al., 2011). The percentages of echo type III and IV (generated by mainly fibrillar/amorphous matrix and fluid) were significantly lower in the tpMSC treated tendons in comparison to the placebo group. This data indicates that the tpMSCs are capable of expressing important proteins of the tendon extracellular matrix, resulting in a restoring a higher quality tendon structure and function.

Besides the many advantages when working with tendon explants, these models also come with some limitations. An important challenge is the loss of tissue homeostasis and understanding which factors are necessary to maintain a healthy tendon environment in culture conditions. Secondly, in vivo tendons are physiologically loaded causing...
moderate tension of the tissue which is not accounted for in an explant model. A future perspective is the use of mechanical loading to mimic the physiological tensile strength of a functional tendon. This was evaluated by Burk et al. assessing the effect of mechanical loading on decellularized SDFT scaffolds treated with equine ASCs for 24 h (Burk et al., 2016). The scaffold culture promoted parallel alignment of more elongated ASCs, which decreased in the mechanically stimulated group. Additionally, in this latter study, COL I expression was downregulated in mechanically stimulated scaffolds, while COL III levels were higher when compared to monolayer controls. These findings are also seen in the naturally tendon healing process (James et al., 2008). However, to obtain a structurally and functionally healed tendon that is not prone to re-injury, it is important that the COL I/COL III ratio remains high. Therefore, in contrast to the latter ASCs, tpMSCs in the current investigation elicit a high expression of COL I and a low expression of SMA. This is of major importance for a functional and structural high-quality tendon healing with lower chance of reinjury. Future studies can be performed where the tendon and ligament explants are cultivated for a longer period of time. This allows an evaluation of the adhesion capacities and possible differences in protein expression patterns in function of time. Moreover, more biopsies could be isolated from different horses to assess if these results are repeatable in tissue sections derived from other horses. In addition, the biomechanical properties of the treated tendon tissue could be assessed in a controlled loading device (Wunderli et al., 2020b). Biomechanical testing of tendon explants is performed by tensile loading to failure. This evaluation could include tendons with a created lesion that are treated with placebo, native MSCs and tpMSCs. After treatment, explants can be fixed in a loading device to

Fig. 3. Representative images of a Masson’s Trichrome staining of the suspensory ligament versus superficial digital flexor tendon explants at 25-fold magnification. Different groups are illustrated: addition of tpMSCs (A), addition of native MSCs (B) and addition of culture medium = negative control (C). Boxes represent 200-fold magnifications.
Fig. 4. Immunohistochemistry of SDFT explants treated with native MSCs or tpMSCs. The representative images of COL I and SMA positive cells in tissue derived from an equine SDFT seeded with native MSCs and tpMSCs are taken at a 1000-fold magnification. The brown color in the cytoplasm of the cells indicates cells positive for protein expression. The SDFT explants treated with tpMSCs show a significantly higher amount of COL I positive cells (upper panel) and a significantly lower amount of SMA positive cells (lower panel). MSCs = mesenchymal stem cells; tpMSCs = tenogenic primed mesenchymal stem cells; COL I = collagen type I; SMA = smooth muscle actin. An * indicates P < 0.05.

5. Conclusion

In conclusion, treatment of tendon and ligament explants with tpMSCs showed a significantly higher attachment to the tissue compared to native MSCs. Moreover, tpMSCs demonstrated protein expression consistent with tissue repair with significantly higher levels of COL I and lower amount of SMA expression. This ex vivo explant model is a valuable method for demonstrating the mechanism of action and functional potential of tpMSCs in tendon healing.

CRediT authorship contribution statement

Eva Depuydt: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Writing – original draft. Koen Chiers: Supervision, Writing – review & editing. Lore Van Hecke: Conceptualization, Data curation, Formal analysis, Methodology, Investigation, Supervision, Writing – review & editing. Jimmy Saunders: Ann Martens: Supervision, Writing – review & editing. Frederik Pille: Supervision, Writing – review & editing. Jan H. Spaas: Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

JHS, ED, and LVH were all employed by BIVMB at the time of the study. The content of this manuscript contains a commercially available stem cell product (RenuTend®) owned and patented by BIVMB.

The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


Cadby, J.A., Busch, E., Godbout, C., Van Weeren, P.R., Sneede, J.G., 2014. Differences between the cell populations from the peritenon and the tendon core


