

Seeding a human tendon matrix with bone marrow aspirates compared to previously isolated hBMSCs – An *in vitro* study

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Received 15 February 2011

Revised /accepted 14 May 2011

Abstract. Injuries of tendons and ligaments give rise to significant morbidity. Tissue engineering offers promising treatment concepts such as seeding a scaffold with human bone marrow stem cells (hBMSCs) to create high-quality tendon replacement tissue. hBMSCs are usually isolated and cultured prior to seeding. Studies evaluating if previous isolation is superior to seeding with bone marrow aspirates have not been published yet. The aim of this study was to compare these two seeding methods in terms of cell viability, proliferation and differentiation.

hBMSCs were harvested from the iliac crest during routine trauma surgery. In group A the scaffold (human achilles tendons) was seeded with bone marrow aspirates, while in group B hBMSCs were isolated, harvested and then seeded. Constructs were stimulated in perfusion bioreactors according to established protocols.

Mean cell proliferation was significantly increased ($p < 0.05$) on tendons seeded with bone marrow aspirates. Cell viability, the concentration of alkaline phosphatase in the perfused media and the synthesis of procollagen – III – polypeptide (PIIP) were not significantly different when comparing the two groups. The synthesis of procollagen-I-polypeptide (PIP) was significantly increased on tendons seeded with previously isolated hBMSCs ($p < 0.05$).

The results indicate that seeding a human tendon matrix scaffold with bone marrow aspirates may be equal to seeding with previously isolated hBMSCs. This new seeding method could facilitate and speed up the tissue engineering process.

Keywords: Tissue engineering, tendons, human bone marrow stem cells (hBMSCs), human tendon scaffold, cell proliferation, FGF-2 (fibroblast growth factor)

Abbreviations

hBMSCs: human bone marrow stem cells

RIA: Radio-Immuno-Assay

FGF-2: fibroblast-growth-factor 2

PIIP: procollagen – III – polypeptide

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PIP: procollagen – I – polypeptide
MSC: mesenchymal stem cells
ECM: extracellular matrix
IGF: insulin like growth factor
TGF: transforming growth factor
PDGF: platelet derived growth factor

1. Introduction

Tendon and ligament disorders are commonly seen in clinical practice, giving rise to significant morbidity. In the United States, approximately 32 million patients sustain degenerative or traumatic injuries of tendons and ligaments per year [1,2]. Reconstruction of the torn ligaments or tendons is frequently necessary to provide the joint with stability and prevent cartilage damage. Surgical repair of ligamentous injuries by use of autologous tendons or by re-adaptation is still state-of-the-art. However, the structural characteristics of the autologous tendon used as implant are altered and weakened. Furthermore the risk of donor site morbidity has to be kept in mind. Allografts from cadaveric donors are potential alternatives but carry the risk of disease transmission or immune reaction [2–4].

Therefore attention has been shifted towards tissue engineering [3]. Seeding a human tendon matrix scaffold with hBMSCs (human bone marrow stem cells) is a promising concept to create tendon replacement tissue which is comparable in structural characteristics and mechanical strength to the original tendon ligament – without the disadvantages mentioned above.

It is current standard to isolate and expand hBMSCs before seeding [5], potential advantages being an increased number of cells [6,7]. This includes centrifugation, resuspension, supplementation, incubation and passaging. This process is sophisticated, costly and time consuming. To our knowledge there is no evidence that isolation of hBMSCs before seeding is superior to simply seeding a scaffold with bone marrow aspirates. If it was possible to leave out the whole procedure of stem cell isolation, it would make the tissue engineering process cheaper, quicker and easier.

Therefore, the aim of this study was to compare seeding a human tendon matrix with bone marrow aspirates to previously isolated hBMSCs in terms of cell viability, proliferation and differentiation. We hypothesize that seeding the tendon matrix with bone marrow aspirates may be superior to seeding with isolated hBMSCs due to cytokines and growth factors contained in the aspirate.

2. Methods

Human BMSCs were harvested from the iliac crest during routine trauma surgery (6 healthy donors, age 35–48 years). 35 ml aspirates were harvested from every donor, and later split into group A (17.5 ml) and group B (17.5 ml). All procedures were approved of by the institutional ethical committee, and informed consent was obtained from all donors. The samples were aspirated in a syringe that was filled with 1000 IU of heparin and were processed within 24 hours. In group A the scaffold was directly seeded with bone marrow aspirates (17.5 ml aspirate). The cell aspirates used for direct seeding were a mixture of all cells retrieved from the bone marrow.

In group B, hBMSCs were isolated, harvested and then seeded. A density gradient (Biocoll, Biochrom AG, Berlin, Germany) was used in order to select cells of an equivalent molecular weight. For the 2-dimensional cell culture a fibroblast differentiation media (DMEM/HAM's F-12 (1:1) with L-Glutamine,

10% FCS (Biochrom AG, Berlin, Germany), 5 $\mu\text{g/ml}$ ascorbic acid, 3 ng/l FGF-22 (Biochrom AG, Berlin, Germany) was used. After the first passage, cells were pooled and 6 cell cultures from the same cell pool were used for the experiments ($n = 6$). Cells of the 2nd passage were counted and 106 cells were resuspended in the above mentioned media and then seeded on the collagen scaffold.

In order to see if we were using stem cells we performed a FACS analysis after the 2nd passage and before seeding the scaffolds. At this point cell phenotype was analyzed using the following protocol: cells were counted and 106 viable (trypan blue negative) bone marrow mononuclear cells were stained with the following nondiluted antibodies (all obtained from BD, Franklin Lakes, USA): CD45-APC, CD235-APC, CD271-PE, CD73-PE. The fraction of bone marrow mesenchymal stem cells (PE+, APC-) that were selected using a fluorometric cell sorter (BD FACSAriaTM, BD, Franklin Lakes, USA) was $89 \pm 6\%$.

Group C was the control group. Only 3.5 ml cell culture media were added to the scaffolds which were placed in the bioreactor and incubated just as in group A and B.

The scaffold consisted of human Achilles tendons dehydrated and sterilized with gamma irradiation (Tutoplast® ACT, TUTOGEN Medical GmbH, Neukirchen a. Br., Germany), that had been washed for two weeks (7 washing cycles, each with 120-150 ml PBS + 2% Nebacetin) to reduce potential toxic agents from the manufacturing process. The scaffold was cut into pieces and the groups were cultured separately.

The incubation took place in six perfusion bioreactors, 7cm³, 37°C, 5%CO₂, 95% rHu, (DMEM/HAM's F-12, Biochrom AG, Berlin), supplemented with 10% fetal calf serum (FCS, Gibco, Germany), 100 U/ml Penicillin/Streptomycin (Gibco, Karlsruhe, Germany), 0.5 $\mu\text{g/ml}$ Amphotericin B (Biochrom, Berlin, Germany), 5 $\mu\text{g/ml}$ ascorbic acid (Sigma, Dahlkirchen, Germany) supplemented with FGF-2 (3 ng/ml, Pepro Tech, Offenbach, Germany) buffered with Hepes buffer (Roth, Karlsruhe, Germany; pH 7.0). Continuous rotation was applied during the first 24 h of incubation (1 degree/s). After 24h, the reactor was connected to the perfusion system with culture media (2 ml/min). The media was changed every 3–4 days. Harvesting and subsequent examination of cell viability, proliferation and differentiation was performed after 24 hours, 1, 2 and 3 weeks.

The following tests were performed:

2.1. Cell proliferation

CellTiter 96® AQueous one solution cell proliferation colorimetric assay (MTS – Test, Promega). The samples were put immediately after harvesting into a 6-well plate (Nunc GmbH & Co KG, Wiesbaden) and incubated with 20 μl MTS (3 (4,5-Dimethyl-2-yl) – 5 (3-Carboxymehoxyphenyl) – 2 (5-sulfophenyl) – 2H-Tetrazolium) (Promega GmbH, Mannheim). After 60, 90 and 120 minutes incubation period at 37° in saturated water vapor (95%) and 5% CO₂ the reaction was terminated with a stop solution (SDS 10%) in a 96-well plate (Nunc GmbH & Co KG, Wiesbaden) and the absorption with 490 nm in the ELISA spectrometer was determined.

2.2. Cell viability

Samples were processed within 4 hours and acridine orange and fluorescein green solutions were alternately added for 10 minutes (Live/Dead Assay Kit, Invitrogen GmbH, Karlsruhe, Germany); 0.19 mg/ml in 0.1 M citrate acid and 0.3 M Na₂PO₄7H₂O pH 2.5). After rinsing with distilled water, a cover glass was put on top of the sample. The specimens were examined with a fluorescent light microscope in the dark chamber. Cells with green fluoroscopy were counted as viable cells. The number of viable cells was counted in 3 fields of view at 100x magnification. Mean values were used for further analysis.

2.3. Light microscopy

Freezing and paraffin cuts were conducted from all samples, then stained with monoclonal fibroblast antibodies (fibroblasts CD-90, Thy-1, Dianova GmbH, Hamburg) and DAPI. After air drying, H&E staining solution was added for 10 minutes (Sigma Chemical). Other samples were stained by von Kossa after published protocols [8]. After rinsing with distilled water, a cover glass was put on top of the sample. The specimens were investigated with the help of a light microscope (Olympus CX41, Olympus Deutschland GmbH, Hamburg, Germany). Samples of 2 patients were analyzed.

2.4. Synthesis of extracellular matrix

Procollagen – III – polypeptide (PIIP), procollagen – I – polypeptide (PIP) synthesis and total protein synthesis were analyzed. Samples were treated immediately with a solution of collagenase A 0,6% (Roche Diagnostics GmbH/Roche Applied Science, Penzberg, Germany) for 48 hours and aliquots were frozen and stored at -80°C until assayed. Synthesis of collagen III was investigated by quantification of the c-terminal propeptides of collagen III. The procollagen, intact PIIINP [125I] radioimmunoassay kit was purchased from Orion Diagnostica, Espoo, Finland, and performed in accordance with the manufacturer's recommendations. For the determination of the total amount of protein, a micro Lowry assay for microplates was used. The reagents were purchased in a kit (No. 690-A: Sigma Chemical, St. Louis MO). A standard curve was prepared (data not shown, linear fit, six points, $n = 3$, $R^2 = 0.98$). After standards and samples were diluted and transferred to the microplate, 200 μl of biuret reagent was added to each well and mixed thoroughly with repeated pipeting. Biuret reagent was prepared by mixing 0.5 ml of 1% cupric sulfate with 0.5 ml of 2% sodium potassium tartrate, followed by the addition of 50 ml of 2% sodium carbonate in 0.1 N NaOH. The mixture was then allowed to incubate at room temperature for 10–15 minutes prior to the addition of 20 μl per well of 1.0 N Folin & Ciocalteu's reagent. Samples were mixed immediately with repeated pipeting with each addition. Color was allowed to develop for 30 minutes at room temperature and the absorbance measured at 650 nm and blanked on the water only control. Although in these experiments the plates were read immediately, the reaction was found to be stable for up to an hour. All absorbance determinations were made using an ELISA microplate reader (BP 800, Biohit, Finland) with the reader controlled by an external PC running KC3 data reduction software (BioTek Instruments, Winooski, VT). Regression analysis and statistics of the curve were performed using KC3.

2.5. Osteogenic differentiation

A Von – Kossa staining and a colorimetric assay of Alkaline Phosphatase (ALP) in the media was performed with the help of the Department of Clinical Chemistry of Hannover Medical School. The same kind of serum (fetal calf serum) was used for all experiments.

2.5.1. Statistical analyses

Statistical analyses were performed using version 17.0 of the Statistical Package for the Social Sciences (SPSS). The location of the data collected was calculated by the analysis of descriptive parameters such as the mean and associated standard deviation. Then means of the samples were compared by an analysis of variance with post-hoc tests to locate the sources of significance. To avoid the risk of alpha-inflation generally linked to multiple testing, Scheffé correction was integrated. All figures were created using SPSS version 17.0. The significance level was set at 5% for all calculations.

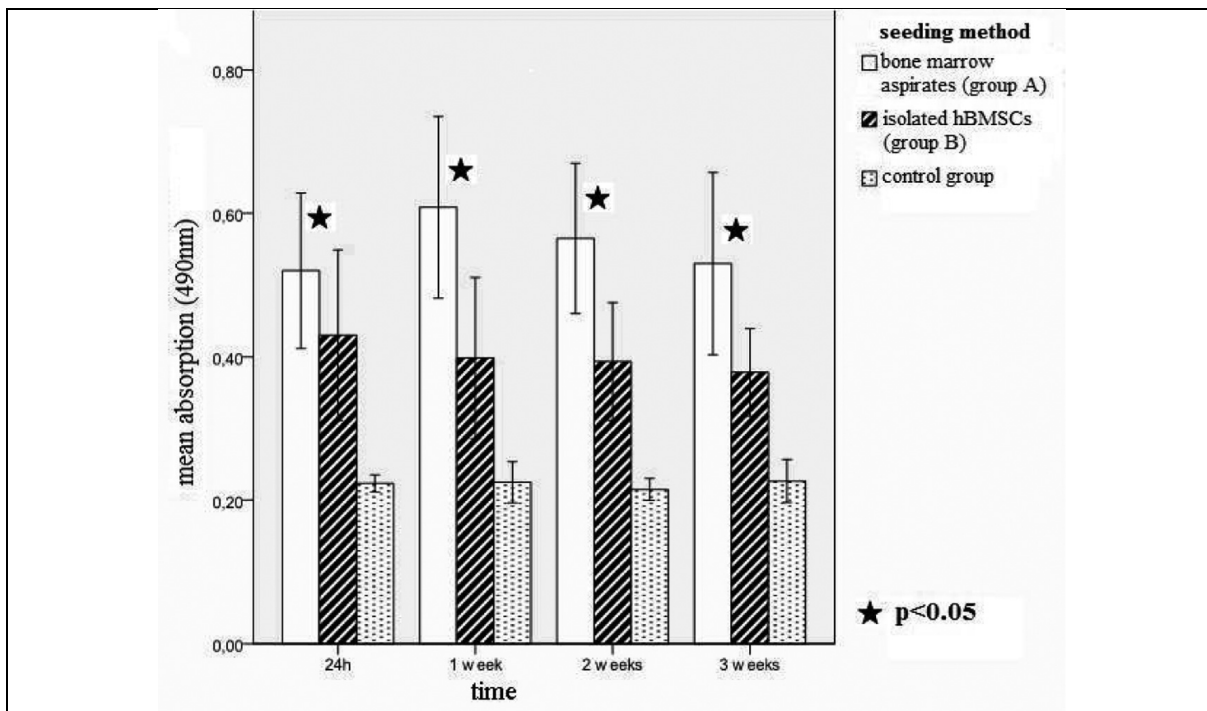


Fig. 1. Mean cell proliferation: Mean cell proliferation was measured with the MTS test, the reaction was terminated after 90 minutes incubation period. Absorption was then determined at 490 nm in the ELISA spectrometer. The asterisks show statistically significant differences between group A and B ($p < 0.05$).

3. Results

3.1. Cell proliferation

Mean cell Proliferation (mean \pm standard deviation) was significantly increased ($p < 0.05$) in group A (bone marrow aspirates) compared to group B (previously isolated hBMSCs) and to the control group (group A 24h 0.52 ± 0.11 , 1 week 0.61 ± 0.13 , 2 weeks 0.57 ± 0.1 , 3 weeks 0.53 ± 0.13 vs. group B 24h 0.43 ± 0.12 , 1 week 0.4 ± 0.12 , 2 weeks 0.4 ± 0.12 , 3 weeks 0.38 ± 0.12). The increase was statistically significant at all examined incubation periods. Proliferation remained fairly constant over all time intervals measured (Fig. 1).

3.2. Cell viability

Cell viability (%viable cells \pm standard deviation) was increased in group A (bone marrow aspirates) compared to group B (previously isolated hBMSCs) after 24h, 1 week and 2 weeks of incubation (group A: 24h 15.67 ± 4.73 , 1 week 19.0 ± 7.94 , 2 weeks 29.67 ± 17.96 vs. group B 24h 13.67 ± 12.66 , 1 week 17.67 ± 15.89 , 2 weeks 24.67 ± 2.52). After 3 weeks the tendons seeded with previously isolated hBMSCs showed a slightly higher percentage of viable cells in the Live/Dead Assay (group A: 21.0 ± 10.4 vs. group B 24.67 ± 15.31). No statistical significance was reached at any time point (Fig. 2). Assessment of cell viability showed that hBMSCs had successfully seeded onto the decellularized tendon slice in both groups.

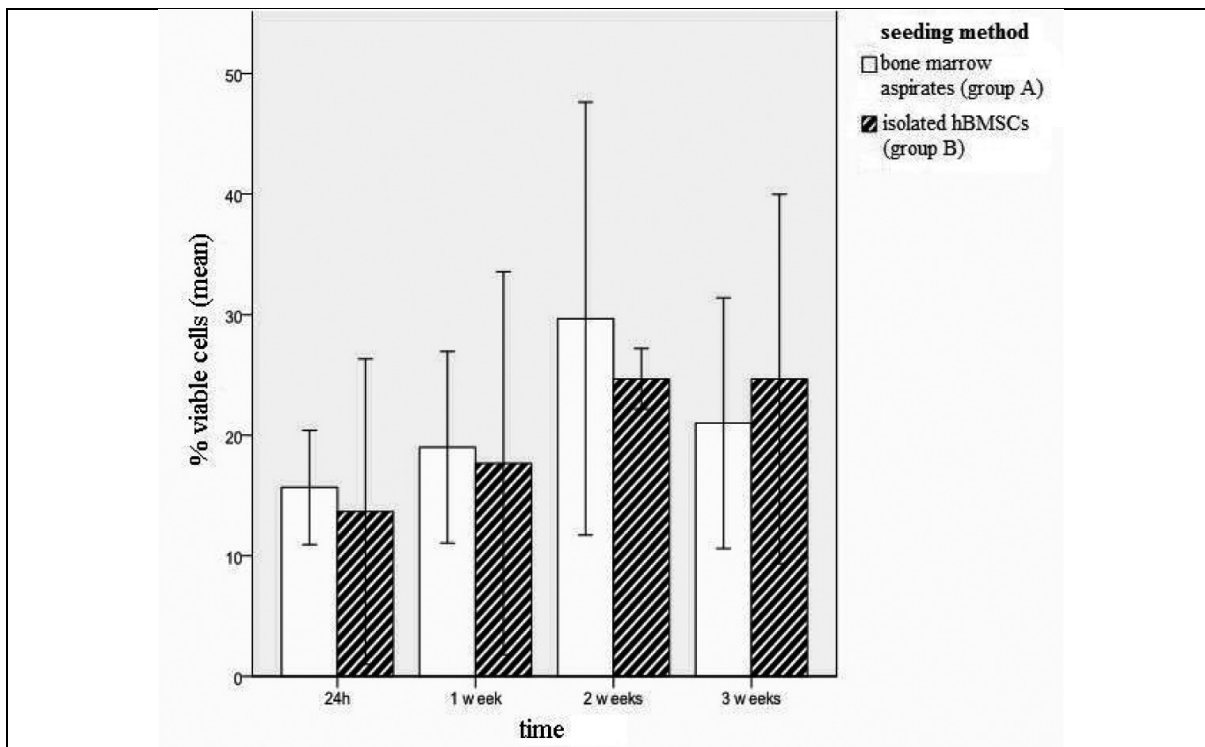


Fig. 2. Cell viability: The percentage of viable cells was counted with a Live/Dead Assay. Mean values were used for further analysis. No statistically significant difference between tendons seeded with bone marrow aspirates (group A) and tendons seeded with previously isolated hBMSCs (group B) was noted.

Synthesis of extracellular matrix was measured by the amount of synthesized procollagen III propeptide (PIIP) / total protein (g/g), values are presented as mean \pm standard deviation. The media contained fetal calf serum. There was no significant difference between group A and B (Fig. 3). The amount of synthesized PIIP was slightly higher in group A (bone marrow aspirates) after 1 and 2 weeks of incubation while there was a higher synthesis in group B (previously isolated hBMSCs) after 3 weeks of incubation (group A after 1 week 0.57 ± 0.19 , 2 weeks 0.54 ± 0.18 , 3 weeks 0.51 ± 0.15 vs. group B after 1 week 0.35 ± 0.17 , 2 weeks 0.44 ± 0.25 , 3 weeks 0.57 ± 0.32).

Additionally, the amount of synthesized procollagen I propeptide (PIP) was measured (PIP / total protein, results are presented as mean \pm standard deviation). The amount of synthesized PIP was statistically significantly higher ($p < 0.05$) in group B (Fig. 4). It was in group A after 1 week 0.23 ± 0.08 , 2 weeks 0.20 ± 0.31 , 3 weeks 0.19 ± 0.40 vs. group B after 1 week 0.26 ± 0.11 , 2 weeks 0.24 ± 0.77 , 3 weeks 0.25 ± 0.21 .

The concentration of alkaline phosphatase (measured in IE/L, values presented as mean \pm standard deviation) in the perfused media was not significantly different when comparing group A (bone marrow aspirates), group B (previously isolated hBMSCs) and the control media of group C (group A after 1 week 8.34 ± 0.96 , 2 weeks 8.22 ± 0.91 , 3 weeks 8.35 ± 0.80 vs. group B after 1 week 9.45 ± 1.28 , 2 weeks 8.59 ± 0.24 , 3 weeks 9.05 ± 0.40 , control media group C 9.04 ± 1.02 , see Fig. 5). The von Kossa stain did not show any signs of ossification in either group.

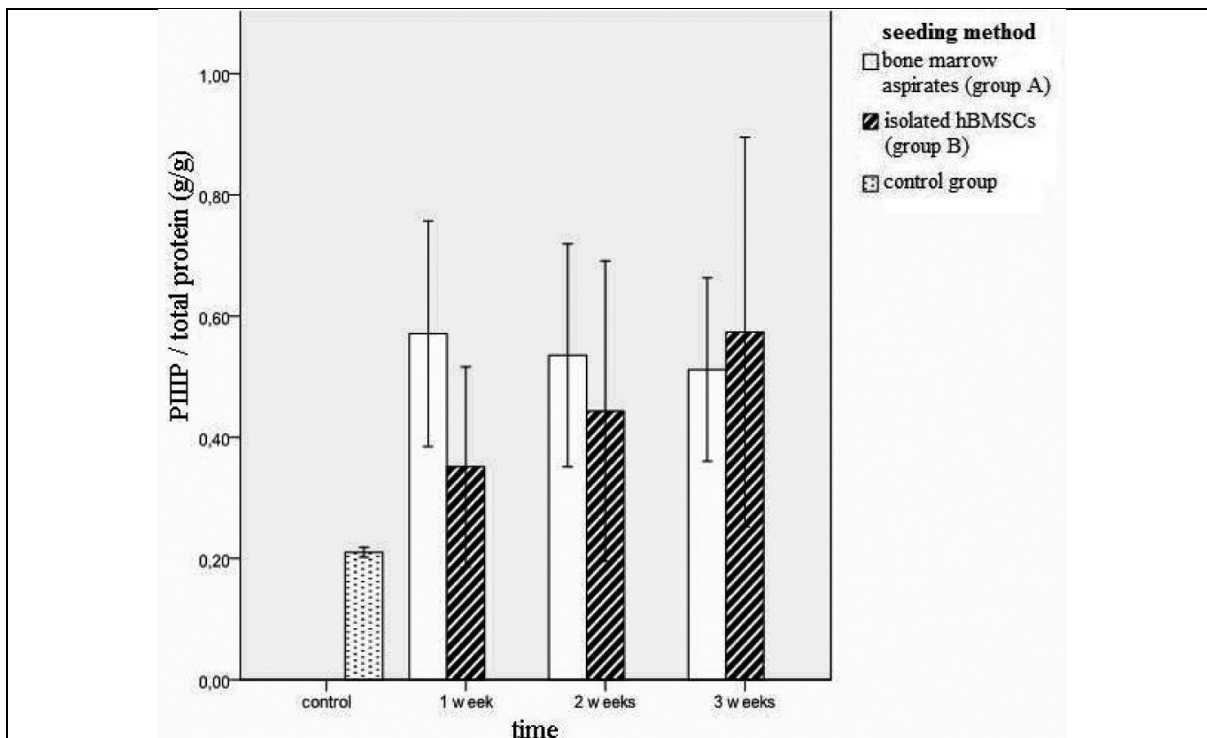


Fig. 3. Synthesis of extracellular matrix (synthesis of PIIIP): The synthesis of ECM was measured by the amount of synthesized procollagen III polypeptide (PIIIP) / total protein (g/g) in the media. Mean values were used for further analysis. There was no significant difference between the groups. Fetal calf serum was contained in the media.

4. Discussion

Several *in vivo* and *in vitro* experiments have shown the potential of mesenchymal stem cells (MSC) retrieved from the bone marrow or from adipose tissue for tendon engineering [9,10]. Ge et al. demonstrated hBMSCs' superior proliferation and collagen synthesis compared to differentiated fibroblasts [11]. It is current practice to isolate and expand hBMSCs before seeding – potential advantages being an increased number of cells [6,7]. However, there is no evidence that previous isolation of hBMSCs is superior to seeding a scaffold with bone marrow aspirates. Our results demonstrate a significantly increased cell proliferation following seeding with bone marrow aspirates. This could be due to cytokines and growth factors contained in the aspirate. Previous studies have shown that proliferation and differentiation of hBMSCs into a fibroblast-like phenotype depends on the culture media and especially on bFGF/FGF-2 [5]. Cytokines found in bone like IGF, FGF and PDGF are well known to promote various physiological parameters such as proliferation and they orchestrate with the TGFs [7]. The cell aspirates used for the direct seeding (group A) were a mixture of all cells retrieved from the bone marrow, which typically consists of red cells (mainly pro-erythrocytes) and white blood cells. Typically, during the washing period which was performed by constant continuous perfusion, pro-erythrocytes and lymphocytes are eliminated and hBMSCs and endothelial cells become adherent [12]. Both cell types have certainly contributed to the proliferation rates measured. However, both cells do make contributions to ligament regeneration. Endothelial cells have been shown to play an essential role in cardiomyocyte differentiation and are capable of inducing angiogenesis [13–15].

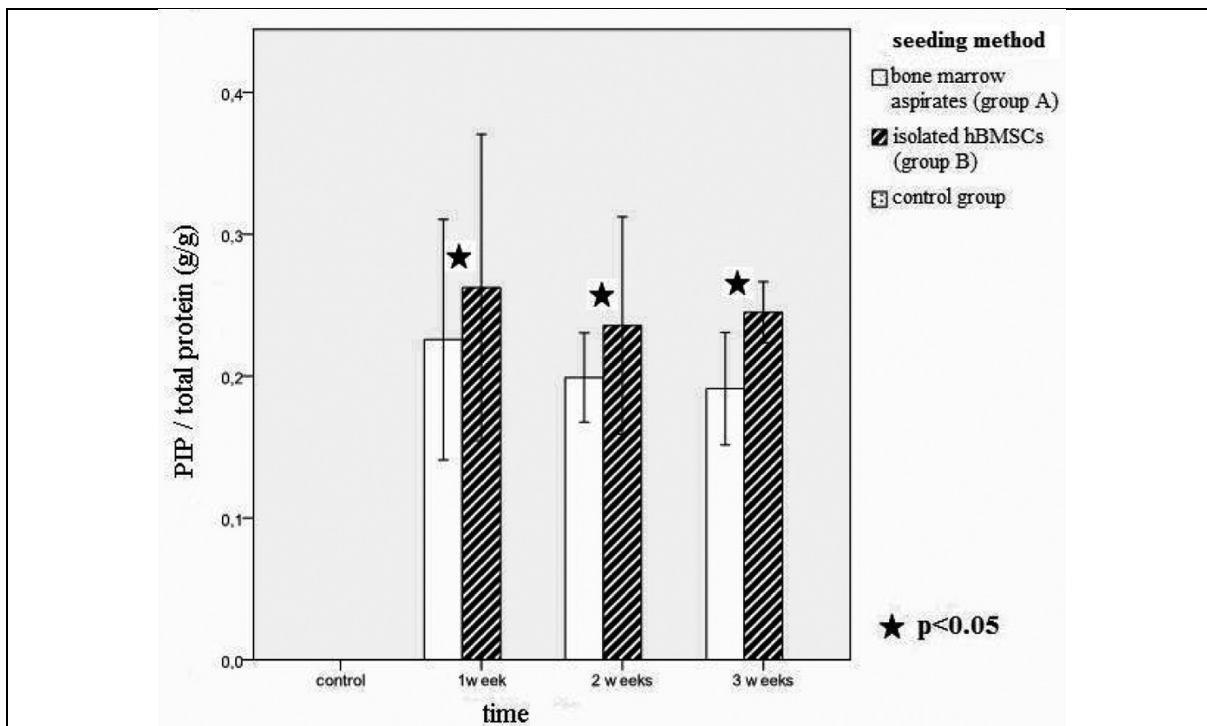


Fig. 4. Synthesis of extracellular matrix (synthesis of PIP): The synthesis of ECM was measured by the amount of synthesized procollagen I polypeptide (PIP) / total protein (g/g) in the media. Mean values were used for further analysis. There was a statistically significant difference ($p < 0.05$) between group A and B (marked by asterisks). Fetal calf serum was contained in the media.

Cell viability was rather low in both groups after seeding (about 20%). There are probably several reasons for this – a considerable number of cells are lost in the process of seeding and due to the rapid change of cell environment. Furthermore, we used a tendon that was conserved with a solvent. Its toxicity has to be taken into account. However, the rates of this study are similar to what is reported by other authors. Hankemeier et al. report apoptosis rates of 70–80% in a cell culture after one to three weeks [5].

Concentration of PIIP is a sign of collagen III synthesis and PIP of collagen I synthesis. Synthesis of PIP was significantly increased on tendons seeded with previously isolated hBMSC while there was no difference between the groups concerning PIIP synthesis. This finding hints that the selection process by density centrifugation does not seem to enhance the amounts of PIIP synthesized in a 3-dimensional cell culture later. Contrary to our expectation, however, the cytokines contained in bone marrow did not result in an increased PIP and PIIP production on tendons seeded with bone marrow aspirates [7].

With regard to stimulation protocols we used a protocol similar to the one outlined by Hankemeier et al. [5] including rotational mechanical stimulation and continuous perfusion to induce a fibroblastic differentiation of the hBMSCs. hBMSCs and fibroblasts depend on mechanical stimulation for cell proliferation, migration and collagen synthesis [16,17]. Magnitude, frequency and duration of mechanical stimulation as well as growth factors influence the cellular response [18–21]. Rotational stress is an important factor fostering differentiation of hBMSCs into a fibroblastic phenotype [22,23]. In a culture of hBMSCs the main stimulus for cell proliferation is continuous perfusion which is crucial for the development of the tissue-engineered construct [19,24,25].

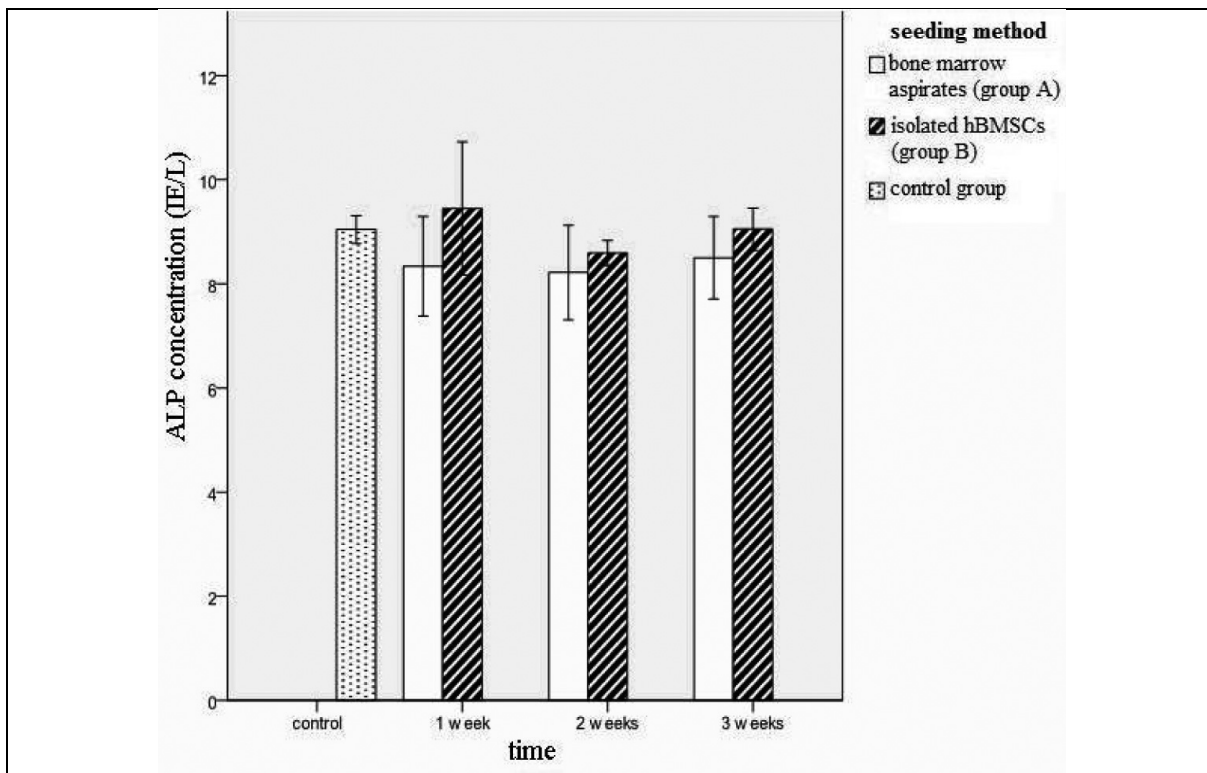


Fig. 5. Osteogenic differentiation: With regard to osteogenic differentiation the concentration of alkaline phosphatase (ALP) in the perfused media was measured with a colorimetric assay (measured in IE/L). No statistically significant difference between group A and B was found.

As for the scaffold we used native decellularized tendon tissue because it is in many ways an ideal scaffold environment for cell seeding, adhesion and survival, containing native collagen, with a normal tendon structure and organization. However, we performed HE-stains to evaluate the cellular ingrowth into the tendon-scaffold and found only few cells in deeper layers (results not shown). This observation is similar to the results reported by Omae et al. who seeded a decellularized multilayer tendon scaffold with BMSC [1]. To overcome this problem Ingram et al. made use of ultrasonication to produce a microscopically more open porous matrix. They denied any adverse effects on the scaffold's architecture and reported that human tenocytes penetrated into the center of the scaffold [26]. These results are very promising but we believe that further studies are necessary regarding this issue. We worry that the creation of a more open porous matrix might result in a worsening of biomechanical characteristics and degradation of the scaffold in the long run.

Acellular human dermal scaffolds like GraftJacket® show that a relatively dense matrix has its place in clinical use if it provides sufficient primary stability and it has been demonstrated that a remodeling can take place over time [27,28]. All in all there seems to be no ideal scaffold so far – no cell-collagen construct has been able to achieve sufficient mechanical properties [1,29,30] and synthetic scaffolds are associated with different problems regarding tissue regeneration and healing [31,32]. Silk matrices are a potentially attractive scaffold material [22] and attempts have been made to have fibroblasts generate their own extracellular matrix [31].

Finally, two limitations to our study have to be mentioned. Firstly, our experimental setting is rather

complex and thus we could perform only a limited number of experiments. Secondly, we did not evaluate the mechanical property of this newly engineered tendon. It may be significantly weaker than that of natural tendon. Therefore further *in vitro* and *in vivo* studies are necessary to elucidate the benefits of such cell seeded constructs which should include evaluations of the mechanical stability of such scaffolds.

5. Conclusions

Despite major advances in recent years the process of tendon tissue engineering continues to be a highly sophisticated enterprise. Our group conducted this trial evaluating a new – and much simpler – seeding technique. It could facilitate, cheapen and speed up the tissue engineering process because the results suggest that it may not be necessary to isolate the stem cells before seeding the scaffold but rather seed the scaffold with bone marrow aspirates.

Acknowledgements

We are grateful for the financial support of the “Deutsche Arthroshilfe” and the German Society of Orthopaedic Traumatologic Sports Medicine (GOTS). The authors would like to thank R. Meister for supervising the cell culture experiments and histologic analysis of the samples.

Competing interests

The authors declare that they have no competing interests.

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