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Functional Tissue Engineering of Autologous Tunica Albuginea: A Possible Graft for Peyronie's Disease Surgery ☆

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Abstract

Objectives: The aim of the present study was to generate a tissue engineered type of mechanically stable graft suitable for surgical replacement of the tunica albuginea penis.

Methods: Porcine fibroblasts isolated from open fascia biopsies were seeded on decellularized collagen matrices and then cultivated in a bioreactor under continuous multiaxial stress for up to 21 days ($n = 12$). Static cultures without mechanical stress served as controls. Cell proliferation, cell alignment, and de novo synthesis of extracellular matrix proteins (proteoglycans, procollagen I, elastin) in these grafts was evaluated by hematoxylin-eosin, pentachrome, and immuno-staining. Additionally, the enzymatic isolation of porcine fibroblasts from Ø4 mm skin punch biopsies ($n = 8$) was evaluated.

Results: Mechanically strained cultures of fibroblasts showed a homogeneous multilayer matrix infiltration and a regular cell alignment in the direction of strain axis after 7 days, as well as a de novo production of extracellular matrix proteins compared to the static control. A large amount of viable fibroblasts was easily obtained from small skin punch biopsies.

Conclusion: This study shows that continuous multiaxial stimuli improve proliferation and extracellular matrix synthesis of mature fibroblasts reseeded on a biological matrix making this a feasible autologous tissue engineered graft for penile surgery. For the clinical setting fibroblasts harvested from small skin biopsies can be a comfortable cell source.

Keywords: Peyronie's disease; Penile deviation; Plaque surgery; Tunica albuginea penis; Tissue engineering; Cell culture; Small intestinal submucosa; Mechanical stress

1. Introduction

A variety of materials have been used as grafts for substitution of tunica albuginea penis mainly after plaque incision or resection in Peyronie's disease surgery. Whereas viable autologous grafts of vein or dermis are most frequently used in clinical routine nowadays [1–4], several other biological materials have been evaluated in animal experiments and human studies as possible off-the-shelf solutions. Among them are decellularized biological materials, such as small intestinal submucosa (SIS) [5–8], dermis [9], cadaveric pericardium (e.g. Tutoplast) [10–14], collagen fleece coated with tissue sealant (TachoComb) [15] or tunica

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albuginea matrix [16]. After first promising results with SIS one group has recently reported a significant rate of unfavourable outcome in pediatric patients treated with SIS for corporal grafting in proximal hypospadias with severe chordee [17]. The initial lack of viable cells inside the SIS bound graft may account for these results.

The aim of the present study was to generate a tissue engineered type of mechanically stable graft not only consisting of an adequate amount of extracellular matrix (ECM), mainly collagen and elastin network as in the above described decellularized materials, but also of functional cells, i.e. fibroblasts. In order to allow for physiological cell reseeding and generation of viable matrices the constructs were subjected to multiaxial stress during in vitro cultivation.

2. Methods

Primary porcine fibroblast cell cultures were obtained by enzymatic isolation from open fascia biopsies ($n = 12$). The fresh biopsy tissue was minced into small pieces of 1 mm^2 and 1 g of this material was incubated using a shaker in 12.5 ml Dulbecco's modified Eagle medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 0.06% collagenase A (Sigma, Munich, Germany) for 20 hours and afterwards in 12.5 ml of 0.05% trypsin and 0.02% ethylenediamine tetra-acetic acid (EDTA) (Biochrom, Berlin, Germany) for 1 hour at 37 °C with 5% CO_2 and 95% humidity. Further cell expansion over two passages was performed subsequently in T25, T75 and finally T175 culture flasks under standard cell culture conditions using DMEM supplemented with 10% fetal calf serum (FCS) (Invitrogen-Gibco, Karlsruhe, Germany) and 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany). Cell passage was performed at 80% cell confluency. Finally the cells were characterized by immunochemical staining of the respective cytospins from these cultures with monoclonal antibodies against vimentin (clone LN-6, Sigma, Munich, Germany; positive in fibroblasts), CD31 (clone LCI-4, Serotec, Düsseldorf, Germany; positive in endothelial cells and negative in fibroblasts) and desmin (clone DE-U-10, Sigma, Munich, Germany; positive in myocytes and negative in fibroblasts).

Additionally, two punch biopsies were taken with a Ø4 mm punch biopsy needle from porcine skin ($n = 8$) and the dermis was sharply dissected from the epidermis and cut into 1 mm^2 pieces. From this material fibroblasts were isolated according to the same technique as described above for the fascia biopsies omitting the one hour incubation in trypsin/EDTA. Cells were cultivated over two passages (passage performed at 100% confluency) and finally characterized immunochemically for vimentin, CD31 and desmin expression with the above mentioned antibodies.

Fascia derived fibroblasts were then seeded on acellular collagen matrices in a density of 1×10^5 cells/cm^2. These matrices (Ø50 mm, thickness ca. 200 µm) were harvested from porcine small intestinal submucosa. After removing the small bowel mucosa by mechanical means, the remaining graft was decellularized according to a modified protocol from Meezan et al. using 4% sodium-deoxycholate [18].

The reseeded cell-matrix composites were initially cultivated under static conditions in a newly designed bioreactor (Fig. 1) for 48 hours allowing the cells to attach to the matrix. After this initial period the matrix was exposed to continuous multiaxial stress (strain 5%, frequency 0.2 Hz) in this bioreactor for another 2, 7, 14 or 21 days, respectively ($n = 3$ for each group). The same number of permanent static cultures without mechanical stress served as controls. Throughout the whole experiment all cultures were exposed to a continuous flow of 5 ml/min culture medium, i.e. DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml amphotericin B (Biochrom, Berlin, Germany), in this bioreactor system at 37 °C with 5% CO_2 and 95% humidity.

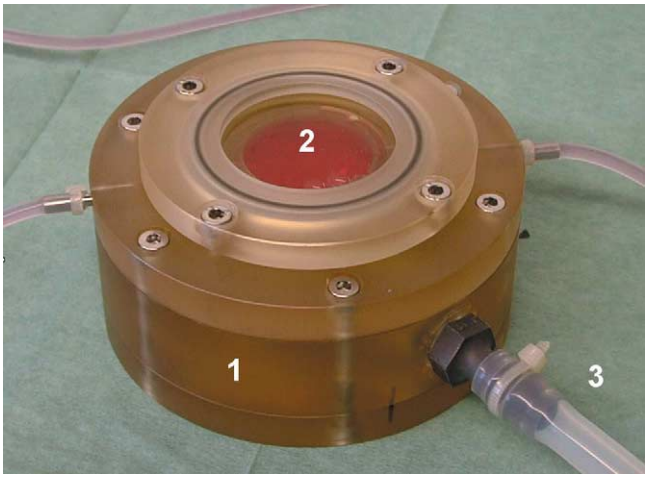

Fig. 1. Mechanical bioreactor with continuous perfusion of upper culture compartment (2) and air pressure (3) induced multiaxial stress performed on cell-matrix composite horizontally placed between the culture (2) and the pressure compartment (1).

Cellular metabolic activity of a defined segment of the reseeded matrix was demonstrated by sequential MTS-formazane essays (CellTiter96® Aqueous One Solution Reagent, Promega, Mannheim, Germany) after 2 days of mechanical stimulation and at the end of cultivation.

Cell density and cell alignment as well as matrix infiltration was evaluated by hematoxylin-eosin (H&E) staining; de novo synthesis of ECM proteins was proven by Movats pentachrome staining and by respective immunohistochemistry against procollagen I (M-38, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) of the harvested grafts.

3. Results

When fibroblast cultures harvested from porcine fascia were analysed for purity and viability, they showed 98% viable mature fibroblasts, 2% myocytes and no endothelial cells in the immunostainings of the cytospins against vimentin, CD31 and desmin. Following two passages over a total period of 4 weeks a final cell count of $2-2.5 \times 10^6$ cells was achieved from 1 g of solid fascia tissue.

Cells isolated from the dermis of two Ø4 mm skin biopsies were grown to confluency before passaging and cell count after 3 weeks and one passage was

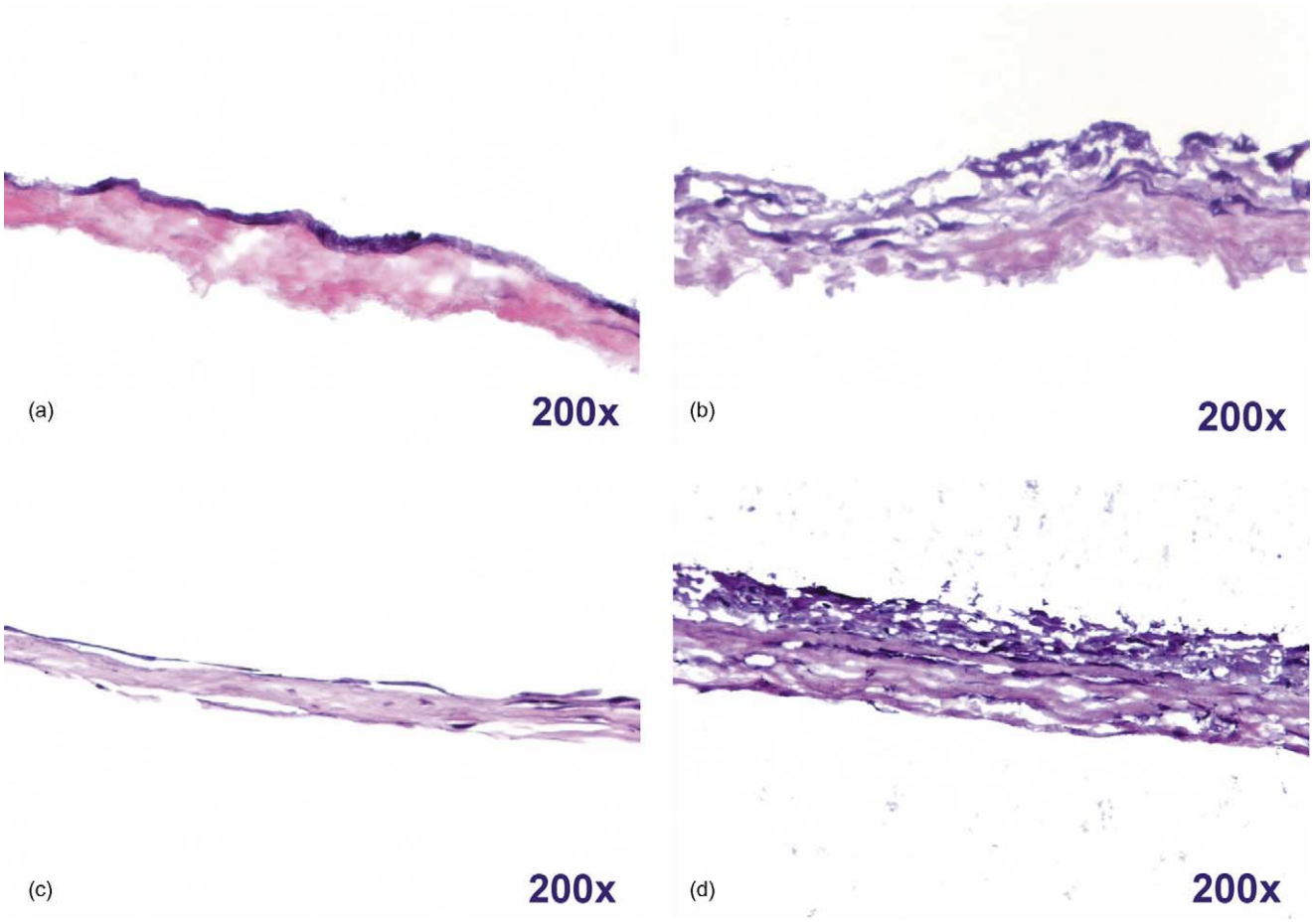

Fig. 2. Results of static and mechanical culture of reseeded porcine fibroblasts on acellular small intestinal submucosa matrix (H&E) (a) static and (b) mechanical culture after 7 days (c) static and (d) mechanical culture after 21 days.

$2.5-3 \times 10^6$ cells and increased to $4-8 \times 10^6$ cells after 6 weeks and two passages. Over 95% of these cells were identified as fibroblasts by the respective cytospin immunostainings.

Already 7 days after the initial cell reseeding procedure of the acellular starter matrix a homogeneous multilayer small intestinal submucosa matrix infiltration as well as a regular cell alignment in the direction of strain axis became evident in the mechanical cultures (Fig. 2b). In contrast to that only monolayers with very minor matrix infiltration were observed in the static cultures (Fig. 2a). This result was even more pronounced after 21 days under the respective culture conditions (Figs. 2c and d).

When matrix samples were analysed for metabolic activity, mechanical cultures clearly showed an all-time higher cell metabolism in the MTS-assay compared to the static system, which correlates with the amount of viable and living cells reseeded on these matrices (data not shown).

After day 14 the mechanical cultures showed a remodelling of collagen-matrix with beginning de novo synthesis of unformed matrix compounds like proteoglycans (stained blue by *Movats* pentachrome; Fig. 3b) as well as formed ECM-proteins like procollagen (stained brown by immunostaining against procollagen I; Fig. 4b) and elastic fibres (stained red by *Movats* pentachrome; Fig. 5). No comparable ECM-proteins typical of matrix remodelling were seen in the starter matrix or the static cultures at the respective time (Figs. 3a and 4a).

4. Discussion

Recent data have demonstrated a positive impact of in vitro applied mechanical pressure, strain and shear stress on proliferation, differentiation and de novo extracellular matrix synthesis of mature fibroblasts [19–21]. Therefore these techniques are used for tissue engineering of viable fibrous tissues, as e.g. ligaments and tendons in orthopedic and trauma surgery. The same principles seem to be feasible for the in vitro construction of fascia tissue for hernial repair in

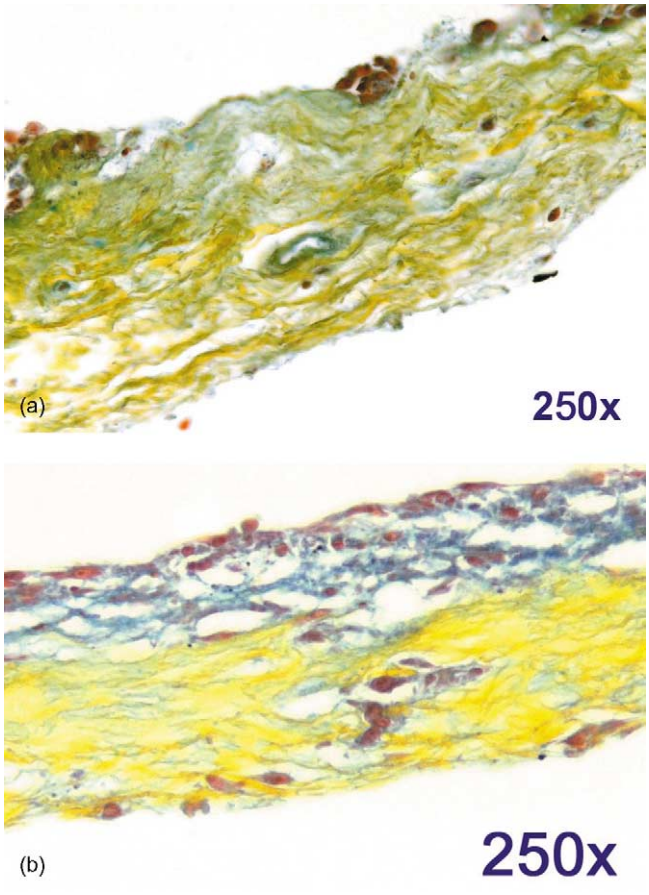

Fig. 3. Early matrix remodelling of proteoglycans (stained blue by *Movats* pentachrome) (a) static and (b) mechanical culture after 14 days.

abdominal surgery and of tunica albuginea for penile surgery in urology.

Sofar, fibroblast and myofibroblast cell cultures have only been used to investigate the pathophysiology of Peyronie's disease [22–26]. In these studies e.g. fibroblast cell proliferation, an increased production of collagen, mainly type I and III, and basic fibroblast growth factor (bFGF) have been taken as a cell culture model for plaque formation. Keeping this in mind, the use of fascia or skin fibroblasts in tissue engineered grafts for tunica albuginea replacement, as suggested in this article, might also bear some risks and unknown potentials in respect to fibrosis, resulting in graft shrinkage and recurrent penile deviation. It is not known how reseeded fibroblasts will behave after implantation and whether they will counteract or enhance postoperative fibrosis and shrinkage of the graft area in vivo by any of the above mentioned mechanisms. These issues have to be addressed and clarified by future in vitro and preferably in vivo experimental work.

On the other hand, an implanted acellular matrix will always be repopulated with blood cells first after the

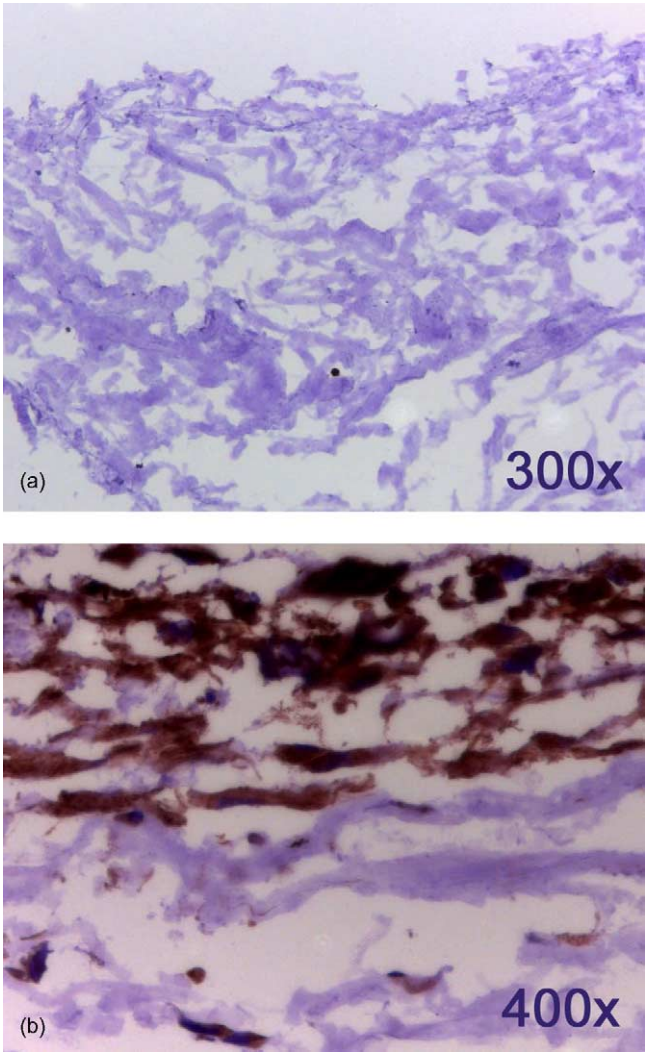

Fig. 4. Early matrix remodelling of procollagen (stained brown by immunostaining against procollagen I) (a) acellular starter matrix and (b) mechanical culture after 14 days.

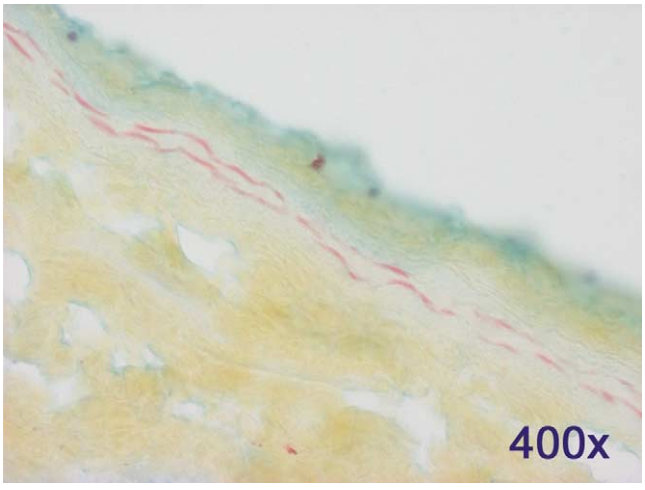

Fig. 5. Early matrix remodelling of elastin in mechanical culture after 14 days (stained red by *Movats* pentachrome).

operation procedure. This heamatoma-like infiltrated graft will then be reorganized by regular wound healing cascades involving a high risk of uncontrolled fibrosis in this granulation tissue [27,28]. This is considered to be different in tissue engineered grafts. Here viable and functional cells, in this case fibroblasts, are already in situ forming a tissue-like cell compound which can more easily be integrated into the surrounding local tissue after neovascularization [29–31]. The corpus cavernosum then provides an optimal underlying implantation site which facilitates a close contact of the graft to the circulating blood and by this will allow a fast and effective neovascularization of the cell seeded graft.

Our present in vitro data clearly support the findings that continuous multiaxial stimuli improve cell proliferation and ECM synthesis of reseeded mature fibroblasts, thus providing a useful bioreactor system to generate tissue engineered grafts. Only the mechanically strained grafts showed a multilayer cell composition and matrix infiltration as well as a beginning remodelling of extracellular proteoglycans, procollagen and elastin that are important for the functional anatomy of the reorganized tunica albuginea [32,33]. The biomechanical properties of these grafts are currently evaluated in our laboratory and other starter matrices are investigated for the reseeding experiments.

The alternative harvesting of autologous fibroblasts from single small skin punch biopsies as described in this paper and by other authors [34] provides a simple and useful way of cell retrieval for future animal models, as well as for later clinical application.

In analogy to previous studies performed by two of us (J. Wefer and N. Schlote) [16], using acellular tunica albuginea matrices without prior in vitro cell-reseeding, we are planning to initiate a study implanting respective tissue engineered patches in the rabbit model.

5. Conclusions

The future application of tissue engineered autologous substitutes for tunica albuginea in Peyronie's disease surgery should be considered for further research. Such cell-matrix-composites combine the advantages of implanting functional viable autologous cells, easily obtained from the patient, and using off-the-shelf matrix material for implantation.

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