Penile Enhancement Using Autologous Tissue Engineering with Biodegradable Scaffold: A Clinical and Histomorphometric Study

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ABSTRACT

Introduction. Autologous tissue engineering with biodegradable scaffolds is a new treatment option for real penile girth enhancement.

Aim. The aim of this article is to evaluate tissue remodeling after penile girth enhancement using this technique.

Methods. Between June 2005 and May 2007, a group of 12 patients underwent repeated penile widening using biodegradable scaffolds enriched with expanded autologous scrotal dartos cells. Clinical monitoring was parallel to histological investigation of tissue remodeling. During second surgical procedure, biopsies were obtained 10–14 months after first surgery (mean 12 months, N = 6) and compared with those obtained after 22–24 months (mean 23 months, N = 6), and control biopsies from patients who underwent circumcision (N = 5). Blind evaluation of histomorphometrical and immunohistochemical finding was performed in paraffin sections.

Main Outcome Measurements. Penile girth gain in a flaccid state ranged between 1.5 and 3.8 cm (mean 2.1 ± 0.28 cm) and in full erection between 1.2 and 4 cm (mean 1.9 ± 0.28 cm). Patients’ satisfaction, defined by a questionnaire, was good (25%) and very good (75%).

Results. In biopsies obtained 10–14 months after first surgery, highly vascularized loose tissue with collagen deposition associated with small foci of mild chronic and granulomatous inflammation surrounding residual amorphous material was observed. Fibroblast-like hyperplasia and small vessel neoangiogenesis occurred intimately associated with the progressive growth of vascular-like structures from accumulation of CD34 and alpha-smooth muscle actin-positive cells surrounding residual scaffold-like amorphous material. Capillary neoangiogenesis occurred inside residual amorphous material. In biopsies obtained after 22–24 months, inflammation almost disappeared and tissue closely resembled that of the dartos fascia of control group.


Key Words. Penile Enhancement; Penile Widening; Dartos Cell Culture; PLGA Scaffolds

Introduction

Autologous tissue engineering with biodegradable poly-lactic-co-glycolic acid (PLGA) scaffolds is a new and safe therapeutic approach for penile girth enhancement. Preliminary experience[1] showed significantly lower complication rate than previously described procedures [2–7]. Other advantages are simplicity, low morbidity, reduced operative time, and good results [1]. Despite promising clinical results, data on tissue remodeling following scaffold-driven autologous tissue engineering have yet to be investigated. In order to observe tissue changes and long-term results, we used histomorphometric and immunohistochemical methods to analyze bioptic samples.
obtained at different times after surgery from patients who underwent repeated penile widening using autologous cultured scrotal dartos cells and biodegradable scaffold. We documented tissue thickening with morphological and vascular characteristics resembling those of normal dartos fascia what encourage further research of scaffold-driven autologous tissue engineering for plastic surgery procedures of the penis.

**Methods**

**Patient Selection**

From June 2005 to May 2007, 12 patients underwent repeated penile widening using tissue engineering with biodegradable PLGA scaffolds (Regen Biotech, Seoul, South Korea). The age ranged from 21 to 36 years (mean 28 years), and the time between the first and second surgery, i.e., biopsy ranged from 7 to 24 months. Two operations are not planned initially. Although all patients were satisfied with results of the first surgery, they wanted repeated procedure in order to gain additional penile girth. Thus, we used this fortunate occasion to perform histomorphometrical investigation of results of previous surgery. The study was approved by the local Institution Bioethics Committee and all patients were thoroughly informed about surgery and biopic sampling and written informed consent obtained. In order to evaluate tissue remodeling at different times, biopsies were obtained during repeated surgical procedure and patients divided in two groups according to the lower or higher than the mean (17 months) interval of time. Consequently, biopsies obtained after 10–14 months (Group A, mean 12 months, \( N = 6 \)) were compared with those obtained after 22–24 months (Group B, mean 23 months, \( N = 6 \)) using as control biopsy specimens from patients aged from 19 to 31 years (mean 25 years, \( N = 5 \)) who underwent circumcision.

**Cell Isolation and Scaffold Preparation**

Two pieces of scrotal dartos fascia are harvested in local anesthesia and transferred to the laboratory, as well as 100 mL of blood for isolation of serum. Specimen was washed three times in phosphate-buffered saline (PBS), and then put in sterile test tube containing 5× concentrated antibiotics–antimycotic cocktail for cell cultures (Sigma–Aldrich, St. Louis, MO, USA), Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen, San Diego, CA, USA) as medium and 5% of autologous serum. The specimen was then incubated 2 hours at 37°C, placed in sterile Petri dish, cut into small pieces and collected by Pasteur pipette into a solution of Collagenase IV (Sigma–Aldrich; final concentration 2 mg/mL) and incubated overnight at 37°C. Successively, cell suspension was centrifuged for 10 minutes at 1,800 rpm. Cell pellet was washed in PBS twice, and cells seeded into culture flask containing DMEM supplemented with 15% of autologous serum. Cells are grown and maintained at an appropriate temperature (37°C) and gas mixture (5% CO₂). After 5 days, cells were washed with PBS and medium replaced to reach confluence. Cells were subsequently split until they reached \( 10 \times 10^7 \) per flask, which usually takes 3 weeks. Scaffolds (Regen Biotech) were soaked into 75% ethanol for 2 hours at 4°C, dried under laminar hood, washed with sterile H₂O three times and placed into glass, covered with aluminum foil and placed into vacuum desiccator under 760 mm Hg pressure for 2 hours, washed with PBS, and put into incubator to reach temperature 37°C.

**Cell Seeding**

Harvested primary cells were detached with 0.5% trypsin–ethylenediaminetetraacetic acid (EDTA), washed in PBS and re-suspended in DMEM supplemented with 20% of autologous serum. Cell suspension was seeded into scaffolds by dropping on surface. Scaffolds were then rolled for 2 hours to allow cell attachment. Finally, scaffolds were filled with medium and put into incubator overnight; 24 hours later, they were ready for use.

**Surgical Procedure**

Penile degloving for implantation of PLGA scaffolds between dartos and Buck’s fascia was performed up to penile base. Two scaffolds seeded with cells were placed onto penile shaft next to each other and fixed to the Buck’s fascia with 3-0 Vicryl suture to prevent their movement (Figure 1) [1]. Penile skin with dartos was pulled back using 4–5 spatulas placed over scaffolds in order to prevent their disruption, movement, and sticking to the tissue (Figure 2). Subcoronal incision was closed in two layers [1]. In all patients with high insertion of the scrotum ventrally (penoscrotal web) its release was performed [8]. Elastic compressive dressing was applied for 2 weeks after surgery to prevent movement and rubbing between scaffolds and surrounding tissue. Oral antibiotics were administrated for 5 days postoperatively and urethral catheter not used. Patients were discharged next day and sexual inter-
course not allowed for 6 weeks after surgery. During the second penile widening operation, small biopsy specimens that included skin and the full thickness of fascia tissue were taken. Clinical follow-up was up to 24 months. A structured patient-rated questionnaire was used for satisfaction evaluation of the final surgical result and for

the appraisal of genital appearance and function [1]. Briefly, patients ratings for flaccid and erect penile girth were “satisfied” or “dissatisfied,” and satisfaction for appearance ranged from 1–5, with 1 being very dissatisfactory, 2 dissatisfactory, 3 good, 4 very good, and 5 excellent.

Histomorphometry and Immunohistochemistry
Biopsy specimens were fixed in 4% buffered formaldehyde and embedded in paraffin. Serial sections were stained with Hematoxylin–Eosin, Masson’s trichrome or employed for immunohistochemistry. For the latter, after antigen retrieval, immunoperoxidase staining with positive and negative control was performed [9], using 3′-diaminobenzidine as chromogen and Hematoxylin counterstaining.

Sections were incubated with antibodies anti-α-smooth muscle actin (α-actin, Dako, Glostrup, Dakopatts, Denmark; 1:50 dilution in Tris-buffered saline) anti-CD34 (Ventana Benchmark, Ventana Medical System, Tucson, AZ, USA; 1:50), anti-CD3 (Dako, 1:100), anti-CD20 (Dako, 1:100), antivimentin (Dako, 1:40) and anti-Ki-67 (Ventana, 1:100). Sections were observed using a Nikon Eclipse E800 photomicroscope (Nikon Instruments, Florence, Italy) and images acquired with a DS-Fuji digital (Nikon) and a high-sensibility AxioCam color camera (Carl Zeiss AG, Aalen, Germany). For each case, the ratio of the score with the number of analyzed fields was calculated. Blinded evaluation of semiquantitative immunoreactions was estimated in at least 10 randomly selected fields by two of the authors, with an interobserver reproducibility of >95%.

Phenotypic characterization of expanded dartos cells before implantation was performed in sparse seeded cells in the presence of serum and quantified by immunofluorescence, as previously reported [10], using Rhodamine-conjugated antimouse Ig (Jackson immunoResearch Labs, West Grove, PA, USA) as secondary antibody.

Scanning Electron Microscope
For the scanning electron microscope, fragments of PLGA scaffolds were dried in a E3100 critical point drier (Polaron Equipment Limited, Wafford, UK) with CO₂ transition fluid, mounted on aluminum stubs with silver print and coated with a 20-nm gold layer in an E500-PS3 sputter coater (Polaron). Photographs were performed in a scanning electron microscope (LEO 400, LEO Electron Microscopy, Oberkochen, Germany) at 10 kV.
Statistical Analysis
Results were analyzed by means of Student’s t-test. The differences were considered statistically significant for value of \( P < 0.05 \). Results are shown as mean ± standard error of the mean.

Results
Clinical Outcome and Patient Satisfaction
An example of clinical result is shown in Figure 1D. As reported in Table 1, we performed the objective measurement of penile girth before and after first surgery in patients undergoing repeated penile widening using tissue engineering with scaffolds [11]. We did not perform measurements after the second surgery because our aim was the investigation of clinical and histomorphometrical results after the first surgery. Penile body circumference in a flaccid state after first surgery ranged between 10.5 and 13.5 cm (mean 11.7 cm), and with a gain of 1.5–3.8 cm (mean 2.1 ± 0.28 cm). In full erection, the range was from 11 to 14.5 cm (mean 12.9 cm) with a gain of 1.2 to 4 cm. (mean 1.9 ± 0.28 cm). There were no complications after surgery in this series of patients. Sensitivity was preserved in all patients. All patients had temporary penile shortening due to lack of scaffolds elasticity and all of them regained preoperative penile length in erection after biodegradation of scaffolds in average 2 months after both first and second the surgery. After 7–24 months of follow-up, all patients were satisfied with flaccid and erect penile girth and the quality of their sex life, and expressed good (25%) and very good (75%) satisfaction of penile appearance, with no macroscopic scar and capsule formation or relevant postsurgical complications.

Morphological, Phenotypic and Morphometric Findings
Immunofluorescence revealed that expanded dartos cells resulted vimentin positive, and 25.8 ± 3% and 25.5 ± 2% resulted CD34+ and \( \alpha \)-actin+, respectively (Figure 3E,F).

In control biopsies, the deep dartos appeared as loose connective tissue characterized from small collagen bundles accumulation and fascicles of \( \alpha \)-actin-positive smooth muscle fibers, with abundant vessels and nerves (Figure 4A,I) [12]. The majority of vessels appeared of medium or large size, from CD34-positive endothelial cells surrounded from multiple layers of \( \alpha \)-actin-positive cells, featuring a myocytic or pericytic phenotype. In Group A biopsies, microscopic examination revealed small inflammatory foci with macrophages, lymphocytes, and rare multinucleated giant cells, surrounding collagen deposition with fibroblast-like cell hyperplasia and neoangiogenesis. Inflammatory foci surrounded amorphous material with a vague trabecular pattern compatible with residual collapsed scaffold matrix (Figure 4E–H). Inside the amorphous material, thin capillaries containing blood cells were observed. The progressive enlargement of these small vessels coincided with growth and/or fusion of adjacent large vascular-like structures and thickening of their \( \alpha \)-actin-positive wall (Figure 4M–O). At the deep edge, focal accumulation of interspersed elastic fibers, resembling those typical of Buck’s fascia [9], was detected (not shown). Morphometric examination (Figure 5) documented increased fibroblasts number in areas adjacent to granulomatous reaction compared to control (\( P < 0.05 \)). These interstitial elongated cells were vimentin-positive and \( \alpha \)-actin-negative,
confirming their fibroblastic phenotype. Interestingly, CD34-positive capillaries and cells among layers of α-actin-positive cells were detected around luminal amorphous material, still surrounded by occasional giant cells and scattered inflammatory cells. Different phases of organization of these vascular-like structures could be observed even in the same area. CD34-positive capillaries present inside the wall of these vascular-like structures progressively elongated and intimately inserted in their wall (Figure 6C,D). Although the number of vessels per mm² in Group A was higher than control ($P < 0.05$), the percentage of vascular area did not differ, was compatible with a progressive vascular remodeling from the outset of small neovessels. As reported in Figure 5, immunostaining also revealed more numerous Ki-67-positive cells in Group A compared to control biopsies ($P < 0.05$; Figure 6), in the interstitium as well as in the wall of vascular-like structures.

Figure 3 (A) Detail of the porous aspect of biodegradable poly-lactic-co-glycolic acid scaffold, taken with scanning electron microscopy. (B) Scrotal dartos-derived cells after expansion in vitro, at confluence. Photographic documentation (C) of the surgical technique of scaffold implantation and (D) final result showing 24-month girth gain. (E–F) Immunofluorescence of scrotal dartos-derived cells showing α-actin (E) and (F) CD34-positive cells. E,F: Original magnification, 400×.

Figure 4 (A–C) Vascular and smooth muscle network in deep dartos of (A) control, (B) 12 months, and (C) 23 months after autologous tissue engineering and poly-lactic-co-glycolic acid scaffold implantation, as documented by anti-α-actin immunostaining. (D–H) Microscopic appearance at different magnification of progressive tissue and vascular remodeling 12 months after autologous tissue engineering and scaffold implantation, with progressive appearance and dilatation of thin capillaries containing blood cells inside residual scaffold-derived amorphous material, thickening and enlargement of new vascular structures. (I) Microscopic appearance of deep dartos in control biopsy. (J,M) Masson’s trichrome stainings, (K,N) anti-CD34 and (L,O) anti-α-actin immunostainings of serial sections of deep dartos in biopsies obtained, and (J–L) biopsy obtained 12 months and (M–O) 23 months after autologous tissue engineering and scaffold implantation, showing the progressive collagen deposition as blue staining after tissue-engineered scaffold implantation and the presence of adjacent new vascular-like structures in different phases of maturation. Original magnification, A–C and J–O: 100×; D–I: 200×.
structures. No dense collagen deposition with α-actin-positive cells resembling myofibroblast-driven fibrous scaffold encapsulation was detected. Although not significant due to the high variability, interstitial CD3+ and CD20+ lymphocytes were only tendentially increased in Group A.

In Group B biopsies, inflammatory foci were greatly reduced in number and size; the micro-

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scopic appearance, including medium- and large-size vessel density (Figure 4M,O), closely resembled that of the control. Morphometric analysis documented the reduction of inflammatory cells (Figure 5). Also the number of vessels, fibroblasts/mm², and Ki-67⁺ cells were reduced compared to Group A and similar to control values.

Discussion

The present study documented that penile girth enhancement using autologous tissue engineering combined with biodegradable scaffold was due to the de novo synthesis and accumulation of mechanically stable dartos tissue. Autologous tissue engineering with expanded scrotal dartos cells and PLGA biodegradable scaffold generated fibroblast hyperplasia, highly vascularized connective tissue and vasculogenesis typical of adult deep penile dartos, with only a mild chronic inflammation. Previously reported methods for penile girth enhancement, including autologous lipofilling and autologous dermal-fat grafting [13–16], yielded limited positive results that, in some cases, were not permanent. Various investigators explored the use of different tissue cell grafts combined with different types of natural or synthetic scaffolds to successfully generate vascularized tissue constructs capable of supporting chemical and/or mechanical stimulations [17–19]. Tissue-engineered scaffold have been previously generated for other urological necessities with the aim of obtaining constructs that can accommodate adequate amounts of cells and tissue and structural properties needed for long-term preservation of tissue integrity [20]. Our present and previous reports [1,21] documented the excellent biocompatibility and degradation properties of PLGA scaffold. Although there were no complications in this series of patients, postoperative subcutaneous seroma, lymphedema of preserved prepuce, epidermal inclusion cyst, partial skin necrosis, and wound infection are possible [4,5,22–25]. All these complications could be treated conservatively except excessive lymphedema, where surgical excision is needed.

Earlier studies with nonwoven mesh scaffolds found that pore size, pore orientation, fiber structure, and fiber diameter can influence cell behavior and tissue development [26,27]. The porosity of present scaffold appeared adequate for migration and growth of dartos-derived expanded cells, communication for growth factors, nutrients and oxygen diffusion, and a good support for extracellular matrix deposition. Autologous dartos cells engineered in biodegradable scaffolds is a new treatment option for achieving real penile girth enhancement. Histomorphometric analysis allows analyzing and quantifying normal tissue components. Masson’s trichrome staining reveals collagen accumulation, and CD34 and α-actin immunostaining the presence of endothelial and myocytic cells, respectively; the latter are abundant in normal adult dartos fascia [12]. Histomorphometric analysis after immunoreaction for inflammatory cells also documented the presence of residual chronic inflammatory infiltrates surrounding scaffold material in 1-year biopsies. After 2 years, scaffold matrix was reabsorbed and PLGA scaffold-driven tissue engineering induced accumulation of highly vascularized connective tissue, closely resembling the normal deep human dartos fascia, with no residual inflammatory signs. Interestingly, in 12 months biopsies, small capillaries were detected adjacent to developing complex vascular structures around residual amorphous
scaffold-like material. The presence, inside the amorphous material, of developing thin wall capillaries with blood cells strongly suggests a progressive re-endothelialisation, as also supported by the presence of elongated CD34+ cells among parietal α-actin+ cells. The increased number of vessels per mm2 with no difference in the overall vascularized area at that time was in line with the progressive vascular remodeling. The clinical advantage suggests that transplantation of dartos cells favors the development of vascular structures similar to those of adult penile body, with a tissue-specific vascular plasticity. As matter of fact, the adult dartos appears highly vascular, with many arteriolar and venular structures with abundant layers of α-actin-positive myocytic/pericytic cells [9]. One of the major challenges in tissue engineering is the generation of large three-dimensional vascular structures capable of furnishing an adequate blood supply [28]. Vasculogenesis we described likely develops through two main phases, i.e., the stimulation of small neoangiogenesis and of the growth of vascular structures mimicking those typical of the tissue to be replaced [29]. Which type of cells contributes to the scaffold-driven vasculogenesis in the deep dartos? Our in vitro data indicated that expanded cells, although morphological similar, are heterogeneous in their phenotype and express in small percentages α-actin and CD34 antigens. Other experiments are needed to quantify the contribution of scaffold-supported proliferation of a resident subpopulation of adult dartos precursors or if in vitro expansion of dartos cells provides substantial enrichment of these resident precursors. Although the inflammatory reaction was generally mild, the presence of developing vascular structures with CD34+ capillaries and elongated cells proceeding α-actin+ cell accumulation was in keeping with vasculogenesis from recruitment of resident or circulating vascular precursors. These data support the hypothesis that dartos tissue contains precursors capable of contributing to a scaffold-driven vasculogenesis with characteristics similar to the tissue of origin [30]. The possibility that circulating cells of hematopoietic origin can

Figure 6 In the deep dartos biopsy obtained 12 months after autologous tissue engineering and scaffold implantation, (A–D) anti-CD34 immunostainings show the progressive elongation of CD34-positive capillaries inside the wall of new developed vascular structure after tissue-engineered scaffold implantation; arrowhead, an isolated elongated CD34-positive cell in the wall of developing vascular structure; (E) The progressive thickening by α-actin-positive cell accumulation is also shown. (F) Ki-67 immunostaining shows an isolated positive nucleus inside vascular wall of developing vascular-like structure. Original magnification, A–D and F: 200×; E:100×.
differentiate into vascular cells has been previously hypothesized [31]. Nevertheless, using hematopoietic chimeras, less than 1.4% of endothelial cells derive from circulatory hematopoietic progenitors during the 4-month period, and only around 10% of cells in sponge-induced granulation tissue vessels [32]; moreover, transdifferentiation into smooth muscle cells is not supported from experimental models of vascular remodeling after injury [33]. It appears highly suggestive to hypothesize that resident vascular precursors are not homogeneous but promote de novo vasculogenesis with differential characteristics according to the tissue of origin.

Conclusions

Autologous dartos cells engineered in biodegradable scaffolds is a new and promising treatment option for achieving real penile girth enhancement. Clinical advantage derives from stable remodeling from neovasculogenesis, fibroblast-like hyperplasia and collagen accumulation typical of the highly vascularized normal connective tissue of human adult dartos.

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References