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ORIGINAL ARTICLE

Operational Andrology

Microsurgical tunica albuginea transplantation in an animal model

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Several andrological diseases require surgical repair or reconstruction of tunica albuginea, which envelops the corpora cavernosa penis. Despite intense research efforts involving a variety of biological materials, such as skin, muscle aponeurosis, human dura mater, tunica vaginalis, and pericardium, engineered tunica albuginea suitable for graft use is yet to be obtained. The study investigates microsurgical tunica albuginea allotransplantation in an animal model with the purpose of creation of an organ-specific tissue bank to store penile tissue, from cadaveric donors and male-to-female trans-sexual surgery, for allogeneic transplantation. Materials were tunica albuginea tissue explanted from 15 donor rats, cryopreserved at -80°C , gamma-irradiated, and implanted in 15 recipient rats, of which three rats were used as controls. Penile grafts were explanted at different time intervals; after macroscopic evaluation of the organ, the grafts were processed to morphological, histochemical, and immunohistochemical examinations by light microscopy. Detection of pro-inflammatory cytokines was also performed. Examination of the tunica albuginea allografts collected 1, 3, or 6 months after surgery and of control tunica albuginea fragments showed that tunica albuginea implants achieved biointegration with adjacent tissue at all-time points. The integration of cryopreserved rat tunica albuginea allografts, documented by our study, encourages the exploration of tunica albuginea allotransplantation in humans. In conclusion, the effectiveness and reliability of the tunica albuginea conditioning protocol described here suggest the feasibility of setting up a tunica albuginea bank as a further tissue bank.

Asian Journal of Andrology (2017) 19, 694–699; doi: 10.4103/1008-682X.192034; published online: 24 January 2017

Keywords: penile tunica albuginea; rat model; transplant

INTRODUCTION

Advances made in the past decade have considerably improved andrological surgery, enabling more effective Peyronie's disease (PD) treatment, tumor resection, organ reconstruction, and penile augmentation. All surgical approaches to such conditions require repair or reconstruction of tunica albuginea (TA), which envelops the corpora cavernosa penis.^{1,2} Despite intense research efforts involving a variety of biological materials, such as skin, muscle aponeurosis, human dura mater, tunica vaginalis, and pericardium, engineered TA suitable for graft use is yet to be obtained.^{3–5} Recently, acellular matrices prepared from heterologous tissues have not achieved the expected results in PD penile reconstruction.^{6–8}

The human penis is structurally and functionally unique; this characteristic, and the distinctive biochemical, structural, and ultrastructural features of its extracellular matrix (ECM), set it apart from the organ of all other mammals and from all other human organs, hampering the search for biocompatible materials.^{6,9} Advances in microsurgery and transplantation immunology have made allogeneic tissue transfer the method of choice to manage patients with severe penile conditions.¹⁰ Clinical studies have documented composite tissue allograft transplantation.^{11,12} The survival of animal allografts,

supported by new immunosuppressant combinations, has encouraged further attempts at human allotransplantation. Experimental rat studies have been conducted by Akyurek *et al.*¹⁰ and Seyam *et al.*¹³ to test the feasibility of allogeneic transplantation. Notably, Hu *et al.*¹⁴ have described the first penile transplantation in a 44-year-old male using a partial, not whole TA graft; immunosuppression was not required because the tissue had been cryopreserved and sterilized by gamma irradiation according to the transplanted ligament tissue protocol, resulting in an inert graft.¹⁵

In a previous study,¹⁶ our group investigated human TA cryopreservation methods using an *ad hoc* panel of histological and ultrastructural tests, preparatory to setting up an organ-specific tissue bank to store penile tissue from cadaveric donors or male-to-female trans-sexual surgery for allogeneic transplantation. The present study, a continuation of the same project, investigates microsurgical TA allotransplantation in an animal model.

MATERIALS AND METHODS

Animals

The animal handling protocol for the study was approved by the Institutional Animal Care and Use Committee in accordance with

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Received: 24 November 2015; Revised: 09 March 2016; Accepted: 29 September 2016

institutional guidelines (project IACUC No. 125). The research project was approved by the Italian Health Ministry (project No. 32161). A total of 33 six-week-old male Wistar rats weighing 200–250 g, purchased from Harlan Laboratories (Milano, Italy), were housed in plastic cages at the animal facility of the University of Catania. They were bred and raised in a pathogen-free room with controlled temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($45\% \pm 5\%$) in a 12/12 h light/dark cycle. Food and water were available *ad libitum* and cages were cleaned once a week. Three rats were used as controls, 15 as donors, and 15 as recipients.

Surgical procedure

Surgical procedures were performed under sterile conditions. After overnight fasting, rats were weighed and anesthetized with an intramuscular injection of tiletamine/zolazepam (100 mg kg^{-1} body weight, bw) and dexmedetomidine ($25 \mu\text{g kg}^{-1}$ bw).

Samples of normal TA were collected from donor rats after penile degloving, using a TA incision with an eye scalpel, according to Nesbit's technique.¹⁷ In particular, the ventral side of the penis was exposed via a midline penoscrotal incision. The corpus spongiosum was dissected away from the TA. A 2–4 mm² wide area of TA was excised by an eye scalpel (**Figure 1a**). Donor rats then received an overdose of anesthetic. TA samples were cryopreserved at -80°C for 7 days, and then thawed at 37°C and sterilized by gamma radiation (dose 25 kGy ; dose rate 112 Gy s^{-1}). Recipient rats were anesthetized as described above; a TA strip the same size as the graft was removed and the TA grafts were implanted. After accurate hemostasis, the graft was sutured with 5-0 Vicryl and the cutaneous wound closed in planes. The rats received antibiotic therapy (cefalexin 20 mg kg^{-1} bw) and an analgesic (tramadol 5 mg kg^{-1} bw). The 15 recipient rats were sacrificed with an overdose of anesthetic at 1 month (five rats), 3 months (five rats), or 6 months (five rats). Control rats were operated on to collect TA tissue but did not undergo graft implantation. The blood of each experimental animal was collected and centrifuged for 20 min at 1000 g . Serum samples were separated for detection of pro-inflammatory cytokines and frozen at -80°C in individual aliquots for each assay.

Macroscopic evaluation

Before sacrifice, a tourniquet was applied at the base of the penis under anesthesia, and a full rigid erection was induced by injecting 2 ml of normal sterile saline, to assess any deformity of the penis. Macroscopic evaluation of the organ did not evidence any penile deformity. It was followed by graft collection and morphological examination by light microscopy.

Histological and histochemical analysis

Sections from implanted and control rats were processed in the same manner. After fixation in 10% buffered formalin for 24 h and overnight washing, grafts were dehydrated in graded ethanol and paraffin-embedded with preservation of their anatomical orientation. Sections 4–5 μm in thickness were obtained according to routine procedures, mounted on silane-coated slides, and air-dried. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained with hematoxylin-eosin (H&E) for routine histological observation and to assess tissue integrity. Sections for histochemistry were treated with Mallory's trichrome, which stains nuclei red, collagen blue, and smooth muscle orange; Weigert's elastic stain, which stains elastic fibers purple; and Picrosirius red stain for collagen. All these chemicals and reagents were purchased from Bio-Optica (Milano, Italy).

Immunohistochemistry

For the immunohistochemical studies, the TA grafts were fixed overnight in 10% neutral buffered formalin. After overnight washing in

water, they were dehydrated in graded ethanol and paraffin embedded. They were then cut into 5- μm -thick sections using a microtome (Hn40, Reichert-Jung, Saarbruecken-Gersweiler, Germany) and placed on silanized glass slides. After rehydration, endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 for 10 min. Nonspecific antibody binding was blocked by treatment with normal horse/goat serum diluted 1:20 in phosphate-buffered saline (PBS), 0.1% bovine serum albumin (Roche Applied Science, Germany). Sections were treated ($3 \times 5 \text{ min}$) in capped polypropylene slide holders with citrate buffer (pH 6) using a microwave oven (750 W) to unmask antigen sites.

The following primary antibodies were used: rabbit anti-collagen I, anti-collagen III, and anti-collagen IV polyclonal antibodies (Novus Biologicals, Littleton, CO, USA). The primary antibodies were applied directly onto sections at 1:50 dilution. Slides were incubated overnight in a humid chamber at 4°C . Sections were then washed in PBS, treated with a biotinylated antibody, and detected using peroxidase-labeled streptavidin, both incubated for 10 min at room temperature (LSAB+System-HRP, Dako Milano, Italy).

Positive controls were Keloid for collagen I, extracellular matrix (ECM) components in pituitary gland for collagen III, and basal lamina of spleen for collagen IV. Negative control sections were processed like the experimental slides, except that they were incubated with PBS instead of the primary antibody.

The immunoreaction was assessed using a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) after incubating sections in 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA) for 4 min. Sections were lightly counterstained with Mayer's hematoxylin and then mounted on GVA mount (Zymed Laboratories, San Francisco, CA, USA).

Computerized image analysis

To quantify immunohistochemical staining, 10 sections/sample were analyzed in a stepwise fashion as a series of consecutive fields at $\times 40$ magnification; the stained area was expressed as pixels/field. Randomly selected fields from each section were analyzed, and the percentage area staining for collagen I, collagen III, and collagen IV was calculated using AxioVision Released 4.8.2 image analysis software and an AxioVision 4 Module AutoMeasure (Zeiss, Göttingen, Germany), to quantify immunolabeling in each field. Values from all images were averaged. Computerized image analyses were performed separately by three investigators. Digital pictures were taken using a Zeiss Axiocam camera (Göttingen, Germany).

Detection of pro-inflammatory cytokines

Some pro-inflammatory cytokine concentrations, such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), were measured by a specific commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, USA). All assays were performed as specified by the manufactures of the respective kits. The minimum detectable dose of IL-6 was typically $<0.70 \text{ pg ml}^{-1}$, of IL-1 β $<1.0 \text{ pg ml}^{-1}$, and of TNF- α ranged from 0.5 to 5.5 pg ml^{-1} . According to the manufacturer's protocol, absorbance was measured at 450 nm in an automatic microplate photometer. The sample values were then read as a function of the standard curve. The concentrations were expressed as pg ml^{-1} .

Statistical analysis

Statistical analysis was performed using SPSS software (Released 16.0, Chicago, IL, USA). Comparisons between means were tested

with nonparametric Kruskal–Wallis test. $P < 0.05$ was considered statistically significant.

Functional assessment of penile erection following graft placement

We performed a functional assessment of the penile erection with administration of acid N-methyl-D-aspartic acid (NMDA) directly into the paraventricular nucleus (PVN) of five recipient rats sacrificed at 1 month. After TA grafts transplantation, recipient rats were implanted with a stainless steel cannula aimed at the PVN. They were anesthetized with an intramuscular injection of tiletamine/zolazepam ($100 \text{ mg kg}^{-1} \text{ bw}$) and dexmedetomidine ($25 \text{ } \mu\text{g kg}^{-1} \text{ bw}$), and placed in a stereotaxic apparatus. A small hole was made in the skull, and a stainless-steel guide cannula ($500 \text{ } \mu\text{m}$ outer diameter) was implanted stereotaxically at the following coordinates: 1.5 posterior to the bregma, 0.4 mm lateral to midline, and 7.8 mm ventral to the dura. Two stainless-steel anchoring screws were fixed to the skull, and the cannula was secured in place by acrylic dental cement. Three days after stereotaxic surgery, penile erection was induced by NMDA microinjections in the PVN of the conscious and freely moving rats. They were placed individually into a Plexiglas cage and injected with NMDA (50 ng) into the PVN unilaterally in a volume of 100 nl using microsyringe. After NMDA injection, the rats were monitored to quantify the number of episodes of penile erection by a blind observer with video camera registration.

RESULTS

Hematoxylin-eosin stain

Examination of TA grafts recovered 1, 3, or 6 months after surgery and of control TA fragments showed a similar structure with a small number of circumflex vessels branching through TA tissue and a thick sheath of eosinophilic collagen fibers, and these data are shown in **Figure 1b–1f**. No significant differences in tissue integrity and no necrotic or hemorrhagic areas were detected in grafts explanted 1, 3, or 6 months from surgery. At higher magnification, the collagen fibers showed slit-like spaces that were probably related to cryopreservation at -80°C for 7 days, as shown in **Figure 1g**. No signs of myxoid degeneration have been observed between slit-like spaces. No significant chronic inflammation was noted in grafts collected 1, 3, or 6 months after surgery.

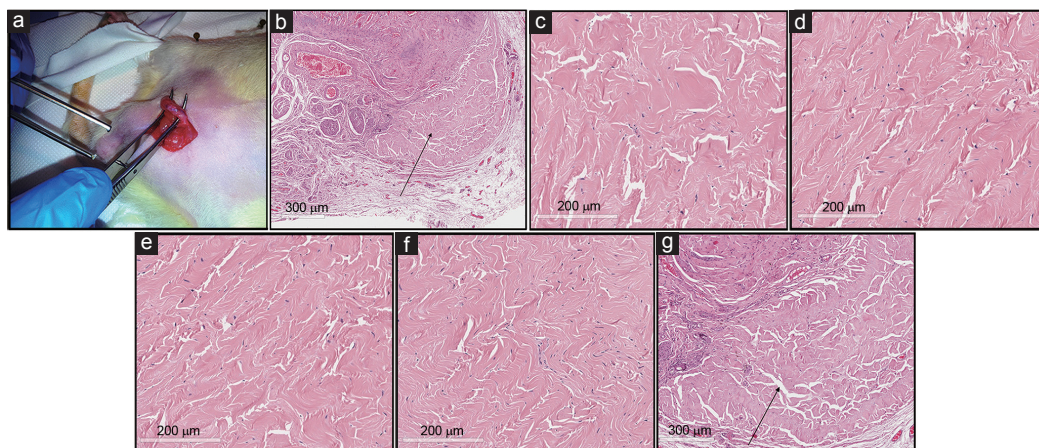


Figure 1: (a) Surgical identification of the corpora cavernosa. (b) Low-magnification photograph of a tunica albuginea graft after implantation showing a small number of circumflex vessels and a thick sheath of eosinophilic collagen fibers (black arrow) (H&E, $\times 25$). High-magnification photograph of (c) a control tunica albuginea fragment and of grafts collected (d) 1 month, (e) 3 months, and (f) 6 months from implantation (H&E, $\times 400$). (g) Slit-like spaces between collagen fibers after graft implantation (black arrow) (H&E, $\times 100$). Scale bar = $300 \text{ } \mu\text{m}$ in b and g, $200 \text{ } \mu\text{m}$ in c–f.

Mallory trichrome stain

Histological examination of grafts stained with Mallory's trichrome showed collagen fibers normally arranged in an outer longitudinal and an inner circular layer, as in control TA tissue, the responses are shown in **Figure 2a–2d**. A preserved, compact sheath of collagen fibers with normal orientation was seen in grafts collected 1, 3, or 6 months after surgery demonstrating absence of fibrosis development.

The quantitative data of staining intensity are reported in **Figure 2e**.

Weigert's elastic stain

Rare, intact elastic fibers of normal size were detected in grafts and control TA treated with Weigert's elastic stain (**Figure 3a–3d**). No significant differences in elastic fiber size and orientation were detected in grafts recovered at 1, 3, or 6 months.

The quantitative staining intensity data are reported in **Figure 3e**.

Immunohistochemical analysis

All TA samples expressed type I, III, and IV collagen (**Figure 4a–4d**, **5a–5d**, and **6a–6d**, respectively). Collagen I was more abundantly expressed than collagen III or IV both in grafts and in control TA tissue. There were no differences in the immunolocalization and/or immunoexpression of collagen types in the grafts collected at 1, 3, or 6 months. Both immunostained fibers and cells were visualized as brown reaction product.

The quantitative data on collagen I, III, and IV immunoexpression are reported in **Figure 4e**, **5e**, and **6e**, respectively.

Detection of pro-inflammatory cytokines

The release of IL- 1β , IL-6, and TNF- α , pro-inflammatory cytokines, was determined by ELISA kits in control and recipient rats. The results showed that, compared to the control group, the recipient rats displayed no alteration of IL- 1β , IL-6, and TNF- α values. Only at 1 month, the levels of serum IL- 1β , IL-6, and TNF- α were slightly but not considerably higher in the recipient group than that in the control group ($P > 0.05$) (**Table 1**).

Functional assessment of penile erection after NMDA administration

The erectile responses to NMDA administration was physiological (2.0 ± 0.3 penile erection per rat in the first 20 min) and not decreased after graft placement.

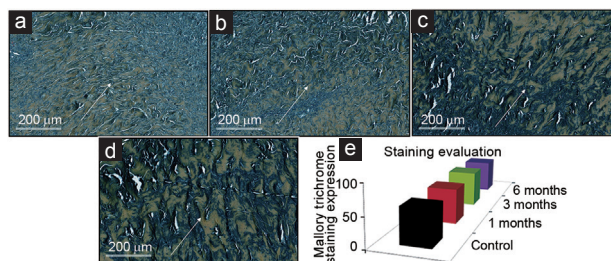


Figure 2: High-magnification photographs of Mallory trichrome stain (a) of a control tunica albuginea fragment and of grafts collected (b) 1 month, (c) 3 months, and (d) 6 months from implantation ($\times 400$). A preserved, compact sheath of collagen fibers with normal orientation was observed (white arrows). (e) Quantification of graft staining with Mallory trichrome after implantation. Values are expressed as mean \pm standard deviation. Scale bar = 200 μm .

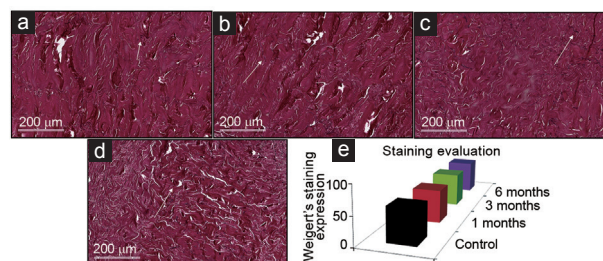


Figure 3: High-magnification photographs of Weigert's elastic stain (a) of a control tunica albuginea fragment and of grafts collected (b) 1 month, (c) 3 months, and (d) 6 months from implantation ($\times 400$). Rare, intact elastic fibers of normal size were detected (white arrows). (e) Quantification of graft staining with Weigert's elastic stain after implantation. Values are expressed as mean \pm standard deviation. Scale bar = 200 μm .

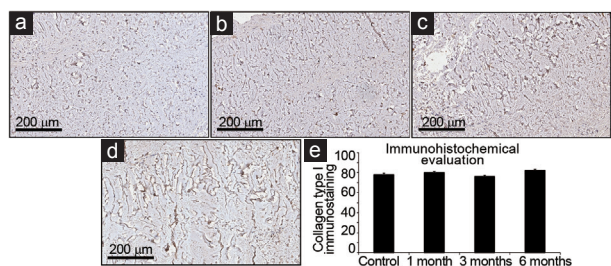


Figure 4: Collagen I immunopositivity (a) in a control tunica albuginea fragment and in grafts explanted (b) 1 month, (c) 3 months, and (d) 6 months from surgery ($\times 400$). (e) Quantification of collagen I immunostaining in tissue from control rats and in grafts collected 1, 3, and 6 months after surgery. Values are expressed as mean \pm standard deviation. Scale bar = 200 μm .

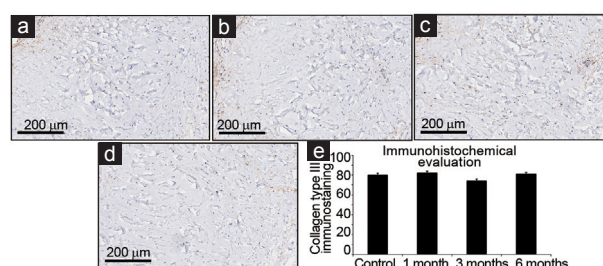


Figure 5: Collagen III immunopositivity (a) in a control tunica albuginea fragment and in grafts explanted (b) 1 month, (c) 3 months, and (d) 6 months from surgery ($\times 400$). (e) Quantification of collagen type III immunostaining in tissue from control rats and in grafts collected 1, 3, and 6 months after surgery. Values are expressed as mean \pm standard deviation. Scale bar = 200 μm .

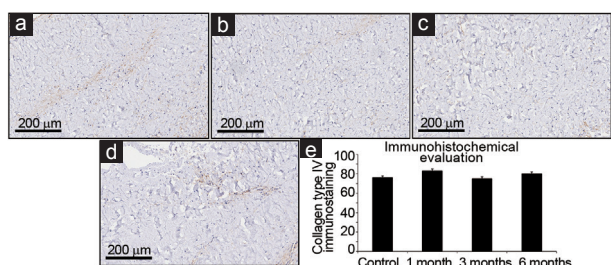


Figure 6: Collagen IV immunopositivity (a) in a control tunica albuginea fragment and in grafts explanted (b) 1 month, (c) 3 months, and (d) 6 months from surgery ($\times 400$). (e) Quantification of collagen type IV immunostaining in tissue from control rats and in grafts collected 1, 3, and 6 months after surgery. Values are expressed as mean \pm standard deviation. Scale bar = 200 μm .

DISCUSSION

PD is one of the most common male conditions, affecting nearly 10% of men.^{18,19} It is characterized by TA fibrosis and formation of plaques or even ectopic calcification or ossification causing painful erection and distortion, bending, or constriction of the erect penis. Penile curvature can impair the biomechanical properties of sexual intercourse, and reconstructive procedures are often required. Surgical treatment is usually performed in the late stage of PD, which is characterized by stable chronic disease. Three types of surgical approach can be adopted: (i) shortening of the convex TA portion, (ii) lengthening of the concave side with grafts, or (iii) penile implant surgery.²⁰

Human penis is structurally and functionally unique, and its ECM has such distinctive biochemical, structural, and ultrastructural features^{21,22} as to hamper heterologous transplantation and/or grafting of

synthetic matrices. Acellular matrix grafts obtained from heterologous tissue (bovine pericardium, porcine small intestinal mucosa) have not achieved the expected results.⁶⁻⁸ Synthetic grafts (Gore-Tex graft) induce a marked inflammatory reaction, which leads to fibrosis around the graft, and involve a higher risk of infection.²³ Acellular matrices are the main candidate materials for genitourinary tract reconstruction.^{16,24} Autologous TA grafts obtained by culturing autologous fibroblasts on a polyglycolic acid (PGA) scaffold currently seem to be the best option for tissue engineered penile surgery grafts.²⁵ Their chief disadvantage is cost while there is little evidence that they provide better results in terms of reduced scarring and restoration of erectile function.²³ Development of the ideal TA material is still an elusive goal.

The search for a suitable TA substitute for penile surgery has led to testing materials such as human amniotic membrane, lingual mucosa, and buccal mucosa.^{20,26,27} Salehipour *et al.*²⁶ have documented good integration of grafted amniotic membrane with surrounding tissue, successful reepithelization, increased collagen fiber deposition, reduction of elastic fibers in the upper portion of the repaired area, and no dysplasia in canine TA defects; however, the authors described no data on possible immunological reactions and their study is limited by a relatively short follow-up (12 weeks). Salem *et al.*²⁰ were the first to implant a lingual mucosal graft; they reported that it is feasible and reliable, and it provides satisfactory short-term results; the main limitations of their study were the short follow-up and the difficult approach to the donor site because of its vascularization and innervation. Most recently, Zucchi *et al.*²⁷ retrospectively assessed the surgical and functional efficacy of buccal mucosal graft corporoplasty in 32 patients and concluded that it is effective and easy to perform; buccal mucosa has exceptional inosculation and revascularization

Table 1: Serum levels of IL-1 β , IL-6, and TNF- α obtained through ELISA in different rats groups (n=30)

Rats	IL-1 β (pg ml ⁻¹)	IL-6 (pg ml ⁻¹)	TNF- α (pg ml ⁻¹)
Control 1 month	1.4 \pm 0.7	0.9 \pm 0.1	1.2 \pm 0.3
Recipient 1 month	2.1 \pm 1.1	1.3 \pm 0.3	1.9 \pm 0.6
Control 3 months	1.2 \pm 0.6	0.9 \pm 0.3	1.0 \pm 0.4
Recipient 3 months	1.6 \pm 0.3	0.8 \pm 0.1	1.1 \pm 0.3
Control 6 months	1.9 \pm 0.7	1.0 \pm 0.2	1.3 \pm 0.6
Recipient 6 months	1.9 \pm 0.3	1.0 \pm 0.3	1.8 \pm 0.5

IL-1 β : interleukin-1 β ; IL-6: interleukin-6; TNF- α : tumor necrosis factor- α ; ELISA: enzyme-linked immunosorbent assay

capacities, it heals quickly with little scar formation, and it has good elasticity and stretch properties, but graft collection is quite invasive.

In our study, the TA grafts achieved biointegration with adjacent tissue. There were no significant differences in graft take. This was demonstrated by integration of neighboring vascular structures and development of a thick sheath of partially hyalinized collagen fibers. Histological examination also showed preservation of both inner longitudinal and outer circular layer of collagen fibers. Weigert's stain demonstrated a normal distribution pattern of elastic fibers comparable to the one seen in control TA. By immunohistochemistry, the explanted TA grafts exhibited type I, III, and IV collagen immunoreaction, like normal TA. Finally, serum pro-inflammatory cytokines determination showed no significant change at 1, 3, or 6 months after surgical procedures.

The results of the present study, obtained in a rat model, may provide the scientific rationale for using cryopreserved TA tissue from cadaveric donors or male-to-female trans-sexual surgery to treat penile disease. However, Ferretti *et al.*²⁸ reported an immune reaction mimicking human PD induced by a TA allograft in rat, as reflected by chronic inflammation, decreased elastic fiber length, localized osteogenesis, and intense scarring with cartilage formation. The discrepancy with our results may be accounted for by our tissue processing protocol, which involved TA tissue cryopreservation at -80°C and sterilization by gamma irradiation before implantation. As reported by other researchers,²⁹⁻³¹ this treatment induces immunomodulation of the antigen response of biological structures without inducing morphological changes,³² thus reducing tissue immunogenic power.^{29,30} Finally, our grafts were sutured with 5-0 Vicryl degradable polymer suture, to reduce the risk of inflammation,³³ whereas Ferretti *et al.* used an 8-0 polypropylene continuous suture, which induces a higher inflammatory response.

The present findings suggest that TA has the potential to provide ideal graft material to address a variety of urogenital dysfunctions requiring allotransplantation, offering a significant breakthrough for reconstructive microsurgery.

CONCLUSIONS

The successful outcome of cryopreserved rat TA allotransplantation, reported in this study, supports and encourages the application of TA allotransplantation in humans. The feasibility and reliability of the approach, documented by our findings, suggest that a TA bank could be set up like many other tissue banks. We are aware of the differences between TA transplantation in rats and humans, and many additional complications may be observed in case of human transplantation. Nevertheless, this is a preliminary study and our findings need to be confirmed on larger series before performing microsurgical TA allotransplantation in humans. In conclusion, even though this is an experimental study involving small number of animals and a short

follow-up, further investigation is required before it can be extended to humans. Thus, our group will perform a third step of the project that will also include a clinical andrological evaluation. The present data suggest that TA allografting may be a novel treatment for PD.

AUTHOR CONTRIBUTIONS

C Loreto, RC, VC, and SS have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data. SS, RL, and GV performed the microsurgical technique. VC performed the animal handling protocol for the study. C Loreto, RC, GM, C Lombardo, and VC performed laboratory techniques. GS, C Loreto, SC, and VC helped evaluate the laboratory results. C Loreto, RC, SS, GV, VC, and RL revised the manuscript. All authors approved the final version and agreed to publish the manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

ACKNOWLEDGMENTS

We would like to thank the physicist Dr. M. Trimarchi, University of Messina and the veterinary Dr. M. Abbate, University of Catania, for their kind cooperation in the experimental protocol.

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