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Tunica albuginea allograft: a new model of LaPeyronie's disease with penile curvature and subtunical ossification

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The pathophysiology of LaPeyronie's disease (PD) is considered to be multifactorial, involving genetic predisposition, trauma, inflammation and altered wound healing. However, these factors have not yet been validated using animal models. In this study, we have presented a new model obtained by tunica albuginea allograft. A total of 40, 16-week-old male rats were used. Of these, 8 rats served as controls and underwent a 10 × 2-mm-wide tunical excision with subsequent autografting, whereas the remaining 32 underwent the same excision with grafting of the defect with another rat's tunica. Morphological and functional testing was performed at 1, 3, 7 and 12 weeks after grafting. Intracavernous pressure, the degree of penile curvature and elastic fiber length were evaluated for comparison between the allograft and control groups. The tissues were obtained for histological examination. The penile curvature was significantly greater in the allografted rats as compared with the control rats. The erectile function was maintained in all rats, except in those assessed at 12 weeks. The elastin fiber length was decreased in the allografted tunica as compared to control. SMAD2 expression was detected in the inner part of the allograft, and both collagen-II- and osteocalcin-positive cells were also noted. Tunica albuginea (TA) allograft in rats is an excellent model of PD. The persistence of curvature beyond 12 weeks and the presence of ossification in the inner layer of the TA were similar to those observed in men with PD. Validation studies using this animal model would aid understanding of the PD pathophysiology for effective therapeutic interventions.
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Keywords: allograft; animal model; osteogenesis; penile curvature; penile surgery; peyronie's disease

INTRODUCTION

LaPeyronie's disease (PD) is an acquired disorder of the tunica albuginea (TA) of the corpus cavernosum resulting in penile deformity, pain, and erectile dysfunction (ED). The prevalence of this disease has been reported to be between 3.2% and 8.9%.[1,2] The psychological impact of PD can be devastating for men and their partners.[3–5] PD progresses in two phases: an acute phase that can last for up to one year, followed by a chronic phase. Formation of the plaque, the penile curvature, and the pain develop during the acute phase. The chronic phase is characterized by the stabilization of the curvature, decrease in penile pain, and development of ED. During the acute phase, medical treatment is focused at controlling the penile deformity and reducing the penile pain. During the chronic phase, verapamil injection, interferon or collagenase with or without application of physical force such as vacuum and stretching devices have been prescribed with varying degrees of success. In refractory cases, surgery is done to restore the normal penile shape.[4,6,7]

Although not fully understood, the pathogenesis of PD is believed to involve trauma to the penis as an inciting event.[8,9] Following such an event, extravasation of blood within the TA induces an abnormal inflammatory response, leading to fibrosis and formation of a calcified plaque that may cause penile curvature.[8–12] Whereas regression of the curvature has been reported in few men,[13] most patients continue to progress.[14,15] An autoimmune response may also play a role in the progression of the patient. However, investigation of the involvement of human leukocyte antigen haplotypes found in known autoimmune diseases (HLA-DQ5, HLA-DCw2, HLA-DR3, B7) has yielded conflicting results.[16–18] In another study, increased serum levels of anti-elastin antibodies have been noted.[19] Additionally, decrease in androgen synthesis in aging men could allow for an increase in auto-antibody production and contribute to the progression of the fibrotic plaque.[20]

Further, to elucidate these processes, robust animal models are necessary. Although several animal models of PD have been reported previously,[21–29] their efficiency is restricted by inconsistencies in the timing and duration of penile plaque formation, and by the lack of visible penile plaques, clear curvature, and/or calcification.[21–23,25–30] To address these deficiencies, we hypothesized that an allograft of the TA in the rat could provoke an immune reaction, mimicking the conditions of human PD.

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MATERIALS AND METHODS

Study design

Forty male Sprague-Dawley rats (16 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). Of these, thirty-two rats underwent a tunical excision of 10 × 2 mm width with closure of the defect by using an excised tunica from another rat as an allograft. The 32 rats in the allografted group were then randomized into 4 subgroups of 8 animals each for analysis of penile curvature, erectile function, and histology at 4 different time points (1, 3, 7, and 12 weeks after grafting). The remaining 8 rats served as controls and were assessed at 12 weeks. Following functional testing, the animals were euthanized and penile tissue was harvested for histological examination. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco, USA.

Surgical procedure

Under 3% isoflurane anesthesia, the ventral side of the penis was exposed via a midline peno-scrotal incision for two rats at the same time. The corpus spongiosum was dissected away from the left TA in each rat. A 10 mm long by 2 mm wide area of TA was excised from the left corpus cavernosum on both rats (**Figure 1a**). The resected area of TA from one rat served as an allograft for the other rat. 8–0 polypropylene continuous sutures were used to secure the allograft over the tunical defect (**Figure 1b**). The surgical wound was closed in two layers. Control rats underwent the same TA resection, and autograft was performed with the tunical resected specimen in the same manner.

Penile curvature assessment

Under inhalant anesthesia the penis was entirely dissected, the polypropylene suture removed, and a 25-G needle was inserted in the right proximal corpus cavernosum. Saline was injected into the corpus cavernosum to induce an artificial erection. Photographs were taken with a digital camera (Powershot A590, Canon, Japan) when the penis reached maximum erection. The penile angle was analyzed with Measurim software (TICESVT freeware, http://artic.ac-besancon.fr/svt/ressources_logiciels.htm) in a standardized fashion.

Erectile function assessment

Erectile function was assessed after measuring the penile curvature. Under the influence of ketamine (100 mg kg^{-1}) and midazolam (5 mg kg^{-1}) anesthesia, the major pelvic ganglion and cavernous nerve were exposed bilaterally via midline laparotomy. A 25-G butterfly needle was inserted into the proximal left corpus cavernosum, filled with a 250 U ml^{-1} heparin solution, and connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA) for intracavernous pressure (ICP) measurement. The ICP was recorded at a rate of 10 samples per second. A bipolar stainless-steel hook electrode was used to stimulate the cavernous nerve (CN) directly (each pole 0.2 mm in diameter, separated by 1 mm) with an aid of a signal generator (National Instruments, Austin, Texas, USA) and a custom built constant-current amplifier generating monophasic rectangular pulses with stimulus parameters of 5 mA, 20 Hz, pulse width of 0.2 ms, and duration of 50 s. The CN was stimulated at three sites of either side separately, and the maximum amplitude of ICP during nerve electrostimulation was calculated from the baseline value and was included for statistical analysis in each animal. Systemic blood pressure was recorded using a 25-G butterfly needle that was inserted into the aorta at the level of the iliac bifurcation for calculating the ratio of ICP increase to mean arterial pressure (MAP). After functional testing, animals were euthanized by intraperitoneal injection of pentobarbital (200 mg kg^{-1}) followed by bilateral thoracotomy. Penile tissue was then harvested for histological analysis.

Histology

Freshly dissected tissue was fixed for 4 h with cold 2% formaldehyde and 0.002% picric acid in 0.1 mol l^{-1} phosphate buffer, followed by overnight immersion in buffer solution containing 30% sucrose. Tissues were frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and stored at −80°C until use. Sections of 5 μm and 12 μm were cut for Hart's staining, which were adhered to charged slides, and air dried for 10 min, and rehydrated with phosphate buffered saline.

Histochemistry

Masson's trichome staining for collagen and smooth muscle content and Von Kossa's staining for calcium salt were performed according to previously described protocols.[27] Hart's staining was performed on 12 μm section to visualize the entire length of the elastic fibers and allow statistical analysis. For the analysis of the length of the elastic fiber, the mean length was compared between the groups on ×400 power field.

Immunohistochemistry and immunofluorescence

Immunohistochemisty and immunofluorescence were performed according to previously described protocols.[31] Slides were incubated with primary antibodies at 4°C overnight. For immunofluorescence, slides were incubated in 1:500 dilution of secondary antibody conjugated with Alexa fluor 488 (Invitrogen, Carlsbad, CA, USA). Nuclear staining was performed by incubating for 4 min in 4′,6-diamidino-2-phenylindole staining (DAPI; D-3571, Invitrogen).

For immunohistochemistry, an avidin–biotin–enzyme complex (ABC) staining kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) was used, with diaminobenzidine (DAB) as chromogen. Sections were counterstained with hematoxylin (bluish staining of all cell nuclei), whereas positive (antigen expressing) cells were stained with DAB (brown color).

The primary antibodies used for immunohistochemistry are: CD 3 (1/200), CD 45 (1/150), SMAD2 (1/500), Collagen 2 (1/500), Osteocalcin (1/200). All antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All data are expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using Prism 5.0 (Graphpad Sofware, La Jolla, CA, USA). Penile curvature angle and hemodynamic data were analyzed using a one-way ANOVA with repeated measures and Bonferroni *post hoc* test for multiple group comparisons. For the comparison of elastic fiber on Hart's staining, the length of each elastic fiber was assessed using Pro-Image 5.0 on ×400 power field in each group and was compared using ANOVA, followed by Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Tunica albuginea allograft causes penile curvature

The angle of penile curvature was assessed by saline injection into the corpora cavernosa. Control-treated rats displayed a straight penis with almost no curvature. The TA allograft transplantation consistently resulted in significant curvature of the penis (**Figure 1c**). No difference was observed in penile curvature between the different time-points in the allografted group; however, each time-point in the allografted group was significantly different compared with the control group ($P < 0.0001$) (**Figure 2a** and **Table 1**).

Tunica albuginea allograft does not affect erectile function before 12 weeks post-transplantation

Erectile function was tested by electrostimulation of the distal end of the CN at 1, 3, 7, and 12 weeks post-transplantation, in each respective group. Control rats displayed normal ICP/MAP ratios. Most of the post-transplantation time-points were not significantly different from control group, except for the 12-week subgroup, which reached the statistical significance ($P < 0.05$). (**Table 1**, **Figure 2b**).

Histology

Tunica albuginea allograft results in decreased elastic fiber length

Elastic fibers of the tunica albuginea were evaluated using Hart's staining. In control rats, the mean elastic fiber length was 275 ± 12 µm. There was no significant difference among the allografted groups (145 ± 28; 150 ± 27; 156 ± 11; 169 ± 17 µm for 1, 3, 7 and 12 weeks, respectively); however, each allografted subgroup was significantly different from control group ($P < 0.0001$). Overall, the tunica of the allografted rats showed a random rearrangement of elastic fibers, which were significantly shorter than those of the control rats. (**Table 1**, **Figure 3**).

Tunica albuginea allograft leads to intense scarring and cartilage formation

Masson's trichrome staining revealed intense inflammation and cellular infiltration at one week post-transplantation, and such reactions subsided over time. When the grafted tunica was rejected from the corpus cavernosum, a new tunica appeared underneath the allograft. This new tunica, however, was rebuilt in an irregular fashion with disappearance of the two original layers organization (external longitudinal and internal circular) (**Figure 4**). Cartilage-like areas were also observed in 30% of the cases with lacunae chambers and matrix material which fills space between lacunae. This finding is highly suggestive of hyaline cartilage organization.

Tunica albuginea allograft leads to chronic inflammation and localized osteogenesis

Immunohistochemistry revealed the presence of CD3 and CD45 (**Figure 5f** and **5g**) positive cells in every sample up to 12 weeks of post-transplantation, suggesting an ongoing chronic inflammatory process. No such cells were found in control animals. Transforming growth factor-beta (TGF-β) and SMAD2 were also consistently found at the inner part of TA post-transplantation (**Figure 5b** and **5a**). Von Kossa's staining revealed the presence of calcium salt in 30% of the samples (**Figure 5c**). Collagen-II, and osteocalcin were consistently found after 3 weeks of transplantation, and were still present at 12 weeks of post-transplantation. (**Figure 5d** and **5e**).

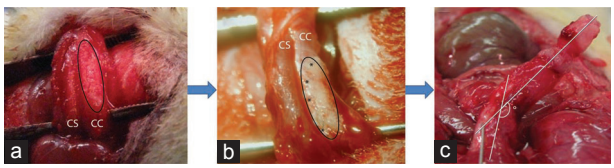

Figure 1: Surgical procedure. (**a**) tunica albuginea resection; (**b**) aspect after allografting; (**c**) aspect at 12 weeks during artificially induced erection. CC: corpus cavernosum; CS: corpus spongiosum. The grafted area is localized by an ellipsis. *: suture.

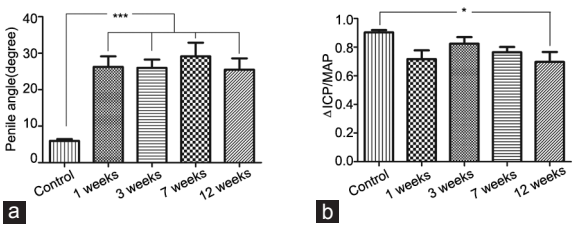

Figure 2: Statistical analysis. (**a**) penile curvature was significantly observed at every time point in allografted groups compared to control group (ANOVA). (**b**) erectile function was not significantly different compared to control group. ICP: intra cavernous pressure; MAP: mean arterial pressure.

DISCUSSION

PD pathogenesis is not fully understood; therefore, an animal model for PD can only approximate molecular findings and clinical observations. One of the most accepted theories is micro trauma as the initiating event, which induces fibrin deposition and stimulates the TGF-β1 pathway, leading to fibroblast-to-myofibroblast differentiation and collagen deposition. Pertaining to genetic factors as an etiology, the data have not been consistent. Nevertheless, the statistical association between PD and Dupuytren's contracture,[32,33] the significant presence of anti-elastin antibodies in PD patients' serum,[19] and the high prevalence of PD in Caucasians further suggest a genetic predisposition in PD.[9]

Various models of PD animal have been attempted using TGF-β1 or fibrin injection, surgical trauma, or genetic alteration. The first model of PD animal was reported by El-Sakka *et al.*[10,25] in the late 1990's by TGF-β1 injection in the rat TA. This model offers distinctive advantages in the study of PD due to its ability to induce chronic inflammation and fibrosis in the tunical tissue over time with just a single administration of the

Table 1: Penile angles, erectile function and elastic fiber length for 1, 3, 7, 12 weeks and for control groups

Group	*Front penile angles (degree)*	*ICP/MAP*	*Elastic fiber length (µm)*
Control	5.95±0.49	0.90±0.02	275±12
1 week	26.24±2.91***	0.72±0.06	145±28***
3 weeks	25.99±2.27***	0.82±0.05	150±27***
7 weeks	29.15±3.73***	0.76±0.04	156±11***
12 weeks	25.48±3.09***	0.70±0.07*	169±17***

ICP/MAP: intracavernous pressure/mean arterial pressure; ***: $P<0.0001$ versus control; *: $P<0.05$ versus control

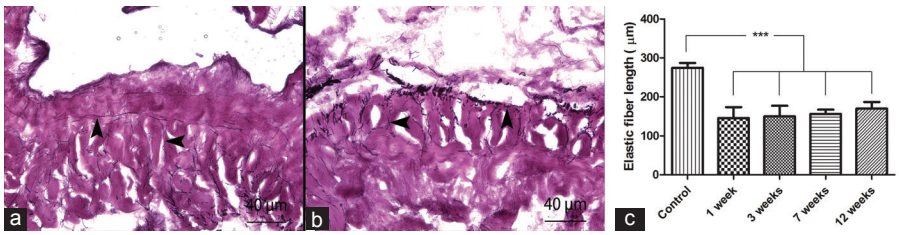

Figure 3: Elastic fiber analysis. (**a**) control (autografted tunica), (**b**) allografted tunica, (**c**) elastic fiber length was significantly decreased in allografted tunica compared to normal tunica ($P < 0.0001$; Mann-Whitney). Elastic fibers are designed by *arrows*. Hart staining. Scale bars = 40 µm.

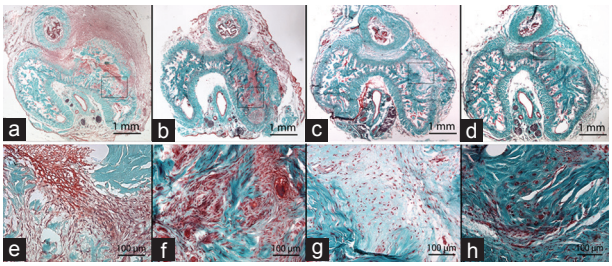

Figure 4: (a): Trichrome staining 1 weeks after allograft; (**b**) after 3 weeks; (**c**) after 7 weeks, (**d**) after 12 weeks (×20 magnification); (**e–h**): ×200 magnification of (**a–d**) area of interest.

TGF-β1 compound. This interesting phenomenon is likely attributed to the ability of TGF-β1 to promote its own synthesis through the feedback transcriptional stimulation. Additionally, there was extension of the fibrotic process into the corporal body in the TGF-β1 injected rats. However, no significant penile curvature or cartilage formation was found. Apparently, a single injection of TGF-β1 was insufficient to induce curvature, as Piao *et al.*[29] have reported that repeated injection of adenovirus expressing TGF-β1 could lead to significant curvature. A fibrin injection model has been performed by Davila *et al.*[23,24] with an outcome of stimulating results. This model achieves TGF-β1, iNOS, ROS and myofibroblast induction, but not curvature or cartilage formation. The Tsk genetic mouse[27] can achieve penile curvature and cartilage formation, but does not reflect the natural course of PD. Indeed, it is more of a systemic fibrosis model than PD, and the use of genetic mice is costly.

To our knowledge, this is the first report of using a TA allograft as a means to create a PD animal model. The strongest evidence for its PD phenotype is the presence of a curvature that lasted for up to 12 weeks. These results suggest that the penile curvature developed early following graft transplantation and subsequently remains stable over time.

The penile curvature seen in this model could be due to both the increase of collagen content under the TA and the disruption of elastic fibers in the TA. The presence of SMAD2 after 3 months indicates an ongoing process, which is also observed in human disease. The presence of cartilage was observed to be 30% in the allografted rats , which is comparable to human PD cases. In addition, collagen-II and osteocalcin were also observed in the allografted TA, suggesting the presence of chondrocytes and osteoblasts, respectively. However, we do not recognize the induction of TA allograft on chondrogenesis and osteogenesis except for the suggestion of the possible involvement of stem cells that have been observed in human TA (Vernet *et al.*)[34] under the influence of TGF-β. Cartilage is not often described in Peyronie's disease, but in acute phase in human, a penile plaque is palpable but not ossified. No histology has been performed at this stage in human, as the treatment is chiefly medical, and surgery reserved for the chronic phase. Penile ossification has been mainly described in chronic phase[35] on tunical resection. Cartilage is known to be the precursor of endochondral ossification,[36] thus could also be the precursor of the ossified plaque seen in chronic phase. Therapies targeting ossification pathways from cartilage formation could reduce penile ossification and curvature. This model could be helpful in testing new medical therapies that could prevent penile curvature in restricting the disease progression.

This study is limited by the inconsistency of cartilage and ossification formation and the lack of quantitative protein assessment (polymerase chain reaction (PCR) or western blot). Moreover, this model depends on the surgical technique and so morphological and functional outcomes could differ among the teams. The 12-week-allografted group has shown a decrease of the erectile function, as seen in chronic phase in men. Longer assessment could have refined the study of the variation of this parameter within time.

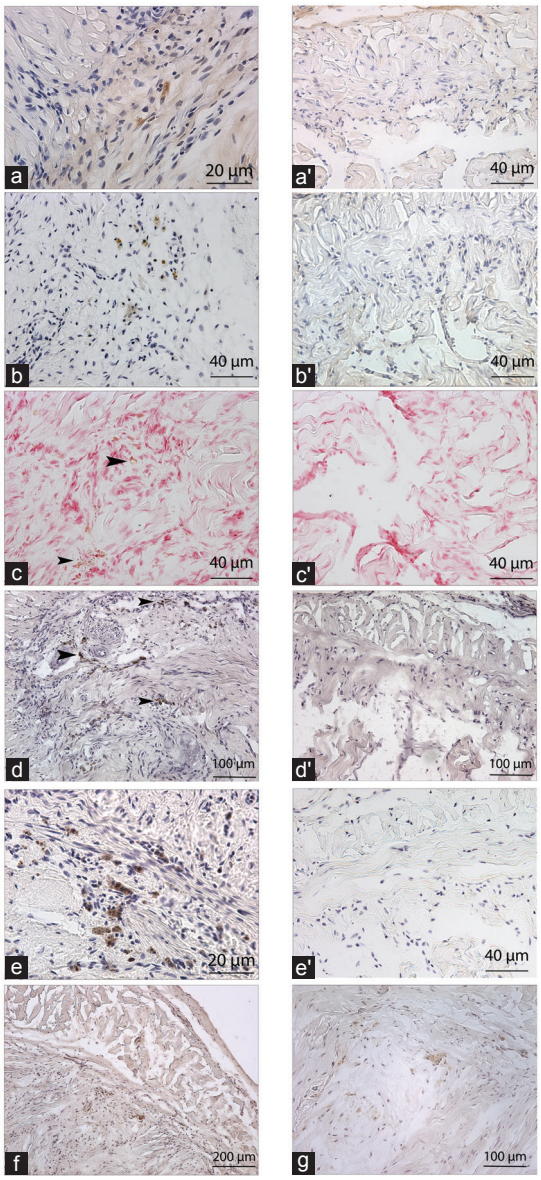

Figure 5: Immunohistochemistry and histochemistry. (**a**): TGF-β1 (×1000); (**b**): SMAD 2 (×400); (**c**): von Kossa staining showing calcium salts (×400); (**d**): Collagen II (×200); (**e**): osteocalcin (×400); (**f**): CD 3 (×100); (**g**): CD 45 (×200). Positive staining is designated by arrows. (**a'**), (**b'**), (**c'**), (**d'**), (**e'**): negative control for TGF-β1, SMAD 2, Von Kossa, Collagen II and Osteocalcin, respectively.

CONCLUSIONS

The TA-allografted rat is an excellent model of PD. Specifically, the persistence of curvature, and the presence of cartilage in the inner layer of TA are similar to human PD. This model could lead to a better understanding of the pathology of PD and more effective therapies.

AUTHOR CONTRIBUTIONS

LF and TFL designed the experiments; LF and XQ performed the animal experiments; HO, GW, LF and TMF performed the histology; LF, HZ performed the statistical analysis; LF, LB, AKW, TFL and CL wrote the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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