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CLINICAL & BASIC RESEARCH

Penile Girth Enhancement Using Amniotic Membrane in a Rabbit Model

A stereological study

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abstract: ***Objectives:*** This study aimed to evaluate the efficacy of penile girth enhancement (PGE) using amniotic membrane (AM) as a graft in a rabbit model. Additionally, quantitative histological data of the structure of the penis were obtained by stereological studies. ***Methods:*** This study was conducted at the Histomorphometry and Stereology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. In this study, 20 adult male rabbits of similar age and weight were allocated to two groups: sham surgery and surgery+AM. Both groups underwent surgery in which a longitudinal I-shaped midline incision was made in the tunica albuginea on the dorsal surface of the penis. The surgery+AM group underwent PGE using AM as a graft. The penile length and mid circumference were measured using a vernier caliper before and two months after the surgery. ***Results:*** The mean total volume and diameter of the penis significantly increased in the surgery+AM group (P <0.03 and P <0.04, respectively). On stereological evaluation, a significant increase in the mean volumes of the tunica albuginea and corpora cavernosa was observed in the surgery+AM group compared to the sham group (P <0.01 and P <0.03, respectively). Additionally, the mean volume densities of the collagen bundles, muscle fibres, cavernous sinuses, and the total number of fibroblasts and smooth muscle cells increased in the surgery+AM group compared to the sham group (P <0.05 each). No infections, bleeding or other complications were observed. ***Conclusion:*** The use of AM as a graft is a method that shows promising results for material use in penile enhancement. Thus, it may be considered for PGE in the future.

Keywords: Amniotic Membrane; Animals; Histopathology; Penis; Augmentation.

Advances in Knowledge

- *Penile girth enhancement (PGE) with amniotic membrane (AM) resulted in an increase in mean penile volume and diameter two months' post-procedure. Thus, AM may be considered for PGE in the future.*
- *The stereological evaluation showed a significant increase in the mean volumes of the tunica albuginea, corpora cavernosa, collagen bundles, muscle fibres, cavernous sinuses and the number of fibroblasts and smooth muscle cells in the penile tissue of AM group.*

Applications to Patient Care

- *The use of AM as a graft is a new method that could be helpful for material use in PGE.*
- *AM may be considered for human PGE in the future.*

The penis has historically been considered a sign of masculinity. Hence, its size has become a source of worry for many men. Today, some men seek ways of enlarging their penis in order to increase their self-confidence and to sexually satisfy their partners more.[1]

Penile girth enhancement (PGE) procedure is undergone by some males for cosmetic and psychological reasons, similar to breast enlargement undergone by females.[2] Patients with a small penis and those with special urological conditions such as micropenis, Peyronie's disease and trauma to the penis may benefit from this procedure.[3] The aim of PGE is to improve penile function and appearance. However, there are no suggested guidelines and specific techniques for PGE.[4]

Generally, PGE can be carried out via methods such as grafts, flaps, fillers and injections.[5] Graft procedure is one of the techniques for PGE, in which fat tissue is commonly used.[6] Other tissues such as small intestinal submucosa and temporalis fascia are also used in graft procedures.[3,7]

Many researchers have studied the impact of graft fat procedures on the penis and their studies have indicated the effectiveness of using graft fat in enhancing penile girth. For example, Zhang *et al*. evaluated the effectiveness and safety of using human acellular dermal matrix graft in the augmentation phalloplasty method.[8] Xu *et al*. also illustrated the effectiveness and safety of using dermal fat graft in the augmentation phalloplasty method among men with a small penis.[9] Similarly, Leungwattanakij *et al*.

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showed promising results using small intestinal submucosa for penis enlargement in a rat model.[3] In addition, Küçükçelebi *et al.* reported that the use of microvascular temporalis fascia strengthened the penis in humans.[7]

Considering the progress in society and increase in people's awareness of their sexual needs and demand for surgical treatment to enlarge the penis, researchers have made genuine attempts to develop new and effective methods for this purpose.

In the last decade, amniotic membrane (AM) has been shown to possess many properties that are indicative of its value in several medical applications. AM has also been used in many genitourinary surgeries.[10–13] AM is the deepest semitransparent layer of the embryonic membrane, which contains an avascular stromal matrix, a thick collagen layer, an overlying basement membrane and a single layer of cuboidal epithelium.[14] In this study, AM was used for PGE for the first time. AM transplantation has been used in surgical procedures in the fields of medicine, ophthalmology, dermatology, plastic surgery, urogenital system and ENT. Many researchers have described these applications separately, with each application using different techniques and giving different results.[14]

Rabbits have a vascular penis that contains two corpora cavernosa and a corpus spongiosum that encloses the urethra. In addition to the lack of a penile bone, this vascular penis has certain characteristics that make it more similar to a human penis. Therefore, it is a good animal model for studying the structure of the penis.[15] Stereology techniques have been increasingly applied for determining a variety of morphometric variables of three-dimensional structures.[16] To the best of the authors' knowledge, no study has evaluated the efficiency of the application of AM in PGE in a rabbit model using stereological methods for obtaining quantitative histological data. The chief advantage of stereological methods is the provision of unbiased and precise assessments. Thus, this study aimed to investigate whether the use of AM for PGE accelerates the regeneration of various parts of the penile tissue, leading to an increase in its size.

Methods

A total of 20 adult male New Zealand White (Oryctolagus cuniculus) rabbits (weight: 1,600–2,500 g; age: 18 weeks) were obtained from the University's Center of Comparative and Experimental Medicine. The rabbits were kept individually in cages with a 12-hour light–12-hour dark cycle at a room temperature of 22–24°C and humidity of 50%. The rabbits had access to water and food *ad libitum*. All animals were kept according to the guidelines of the Animal Care and Ethics Committee of the University. The rabbits were divided into two groups: sham and surgery+AM using simple random sampling (n = 10). In both groups, surgery was done by making a longitudinal I-shaped midline incision in the tunica albuginea on the dorsal surface of the penis. The second group (surgery+AM) also underwent PGE using AM.

All animals underwent the surgical procedure, but stereological studies were conducted on only six rabbits in the sham group and seven rabbits in the surgery+AM group.

Human AMs, provided by Burn and Wound Healing Research Center, were kept in alcohol (95%) till the time of application. At this centre, AMs are sourced from delivery rooms and are employed as biological dressings in burn patients. The AMs were received from women who had undergone deliveries and had no history of premature rupture of membrane, endometritis or meconium ileus. All women underwent seronegative tests for human immunodeficiency virus, hepatitis types B and C and syphilis.[17]

All rabbits were anesthetised using an intramuscular injection of ketamine (10–15 mg/kg) and xylazine (6–10 mg/kg). Supplement doses of ketamine were administered as needed to maintain a uniform level of anaesthesia. All animals were well shaved and prepared with a povidone-iodine topical antiseptic

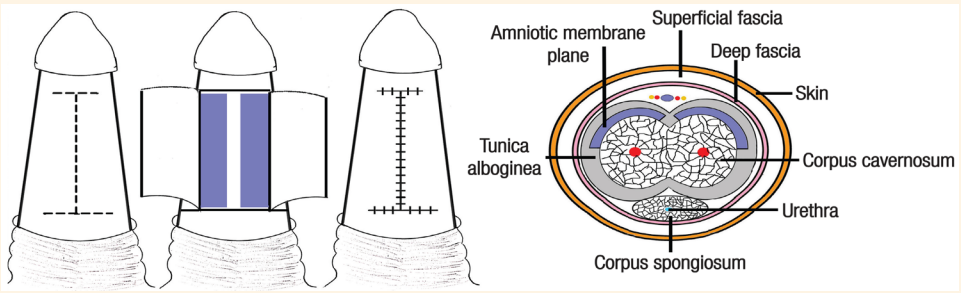

Figure 1: Surgical procedure was done by making a longitudinal I-shaped midline incision in the tunica albuginea on the dorsal surface of the penis and the AM graft was placed between the tunica albuginea and the corpus cavernosum on both left and right sides of the penis.

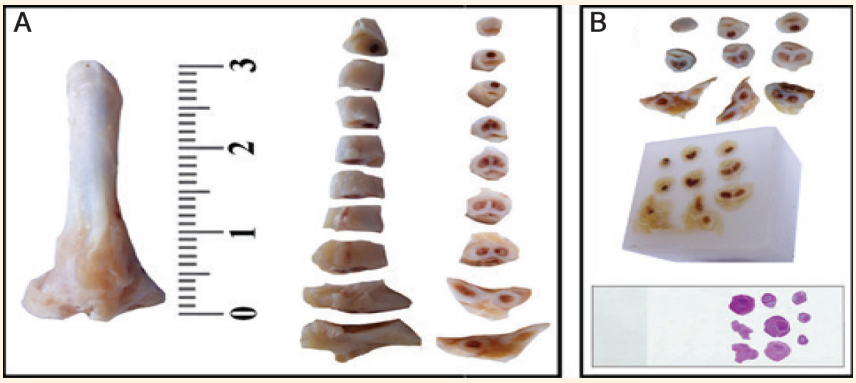

Figure 2: Processing technique. **A:** The penis was cut into 8–12 sections according to its length. **B:** The sections were embedded in paraffin blocks, sectioned, mounted on a slide and stained.

solution and then draped with sterile sheets. The penis was then exposed under aseptic conditions, and the glans was sutured with 4/0 nylon held with a mosquito clamp under gravity to stretch the penis downward.

In both groups, surgery was done by making a longitudinal I-shaped midline incision in the tunica albuginea on the dorsal surface of the penis. In the surgery+AM group, the AM graft (3 × 15 mm^2 piece) was placed on the dorsal surface of the penis between the edges of tunica albuginea and over the cavernosal tissue on both sides of the penis and was sutured with a 6-0 PDS (polydioxanone) [Figure 1].

All rabbits were housed individually and were given standard feed throughout the experiment. Antibiotics were also administered intramuscularly to all groups for three days. After the operation, the rabbits were observed for bleeding, haematoma, swelling, penile deviation and other complications.

The penile length and mid-circumference were measured using a digital vernier caliper (accuracy: 0.5 mm). The girth of the penis was measured at the mid-penile body in the flaccid state. The penile length during the flaccid state was measured from the palpable lower border of the pubic symphysis to the tip of the glans. The mean length and girth of each rabbit category were determined and compared with those of other rabbit categories.[18]

After two months, all the rabbits were sacrificed with deep anaesthesia. The penis and skin sutures were removed in its entirety by dissecting along the shaft to the crura and separating each crus from its point of attachment at the ischial tuberosity. The penis was divided into 8–12 sections according to its length at equal distance [Figure 2A]. The sections of each penis were processed, embedded, sectioned (4 µm thickness and 25 µm length) and stained (haematoxylin-eosin) [Figure 2B].[19]

Sections of 4-µm thickness were used to estimate the volume of the penis and the volume density of the penile components. The penis is composed of skin, penile fascias (superficial fascia or dartos fascia and deep fascia or buck's fascia), tunica albuginea, paired corpora cavernosa and a single corpus spongiosum that contains a spongy tissue and the urethra. In each penile section, the borders between the regions were identified and characterised [Figure 3A]. The corpora cavernosa contains fibrous tissues (collagen bundles), smooth muscle cells, cavernous sinuses and vessels.[19] The volumes of the fascia (superficial and deep fascia), tunica albuginea and corpora cavernosa were estimated

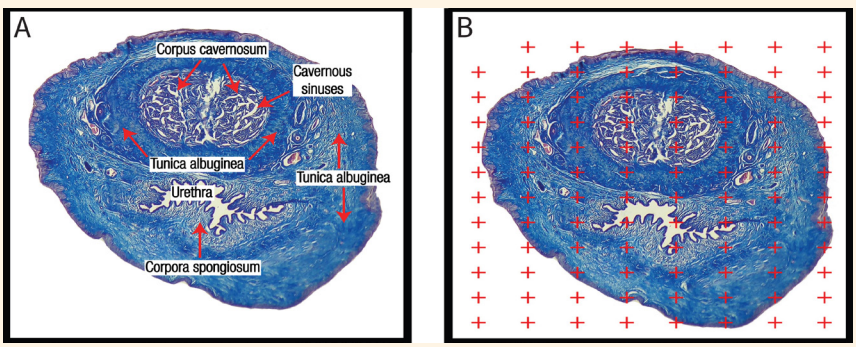

Figure 3: Assessment of the rabbit penile tissue. **A:** The penile components are indicated in the histological section by arrows. **B:** The volumes of the penis and penile components were assessed by Cavalieri's technique and the point-counting method.

Figure 4: Point-counting method was employed to estimate the volume density of the collagen bundles, smooth muscle cells, cavernous sinuses and vessels of the corpora cavernosa (**A**). Optical disector technique was used to estimate the numerical density of the fibroblasts and smooth muscle cells. The fibroblasts' or smooth muscle cells' nuclei coming into focus through scanning of the height of the disector were recorded (the arrow) (**B**).

using a video microscopy system and the software designed at the University's Histomorphometry and Stereology Research Center. The volumes of the penis and its components were estimated by using the 'Cavalieri method' at ×12 magnification [Figure 3B]:

$$V(\text{penile component}) = \Sigma p \times A(p) \times T$$

Where 'Σp' is the total number of points hitting the structure of interest, 'A (p)' is the area related to every grid point and 'T' is the distance between the sections.[19]

Estimation of the volume density of the collagen bundles, smooth muscle cells, cavernous sinuses and vessels of the corpora cavernosa

The volume density 'Vv' of collagen bundles, smooth muscle cells, cavernous sinuses and vessels was calculated by the 'point-counting method' and the following formula [Figure 4A]:

$$Vv(\text{structure/corpora cavernosa}) = P(\text{structure})/P(\text{corpora cavernosa})$$

Where 'P(structure)' shows the number of points placed on the mentioned structures and 'P(corpora cavernosa)' indicates the number of points superimposed on the corpora cavernosa. The total volume of each structure was calculated by the following formula:

$$V(\text{structure}) = Vv(\text{structure / corpora cavernosa}) \times V(\text{corpora cavernosa})$$

The numerical density 'Nv(fibroblasts or myocyte/cavernous bodies)' and the total number of fibroblasts and smooth muscle cells were estimated using the 'optical disector' technique utilised on 25-µm sections. The optical disector consists of an Eclipse microscope with a high numerical aperture (NA = 1.30) × 40 oil-immersion objective lens connected to a video camera that transmits microscopic live images to a computer monitor and an electronic microcator with digital readout for estimating the number of fibroblasts moving in the Z-direction. The numerical

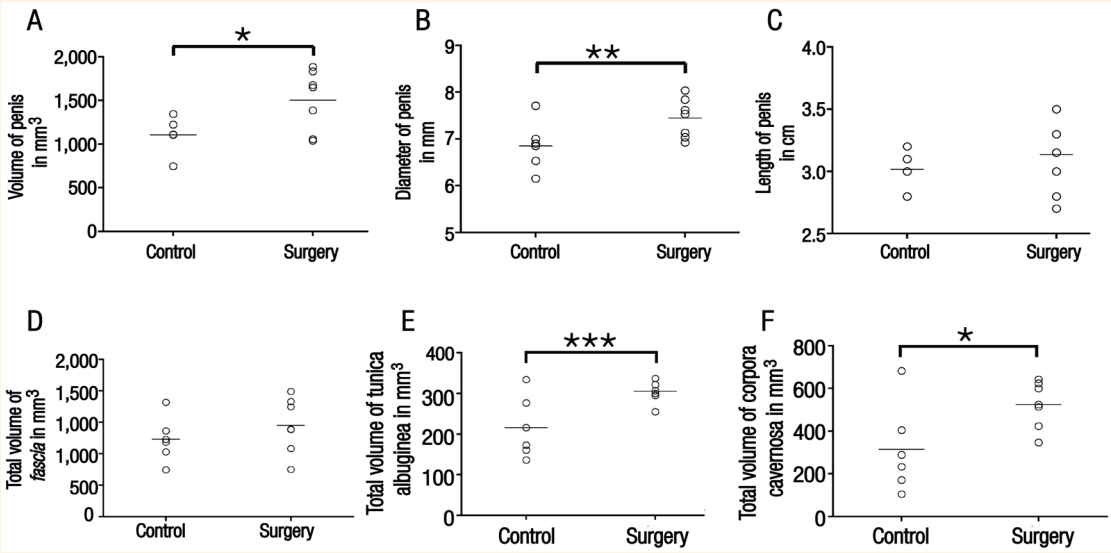

Figure 5: The aligned dot plots of the (**A**) total volume, (**B**) diameter and (**C**) length of (**D**) the fascia, (**E**) *tunica albuginea* and (**F**) *corpora cavernosa* of the penis in the sham and surgery + amniotic membrane groups. Each dot shows an animal and the horizontal bars represent the means of the parameters. Statistical significance was determined by the Mann-Whitney U test.

**P = 0.03, **P = 0.04, ***P = 0.01.*

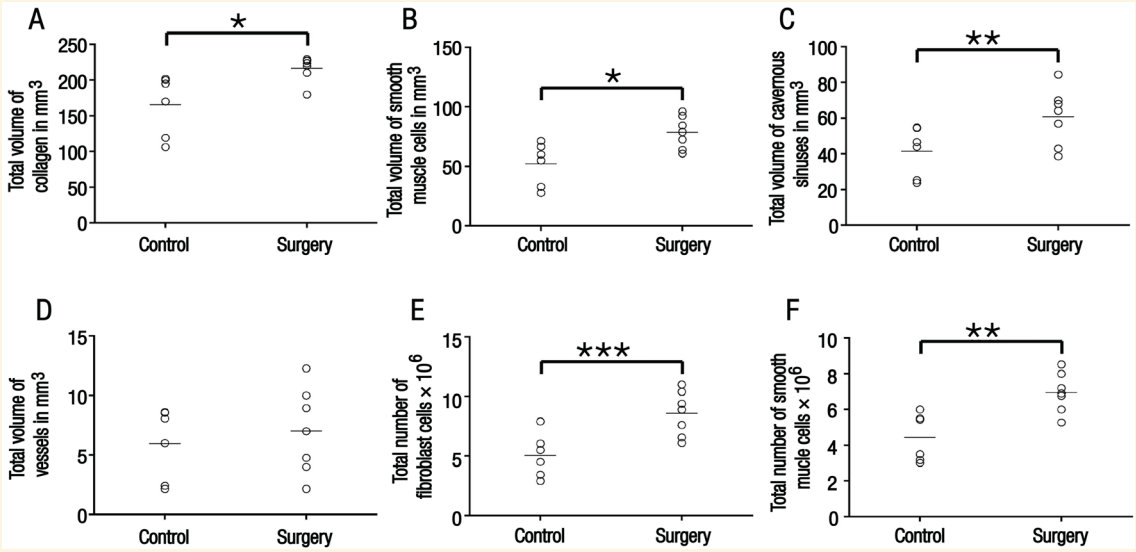

Figure 6: The aligned dot plot of the volume density of the (**A**) collagen bundles, (**B**) smooth muscle cells, (**C**) cavernous sinuses and (**D**) vessels and (**E**) the number of fibroblasts and (**F**) smooth muscle cells of the *corpora cavernosa* in the sham and surgery + amniotic membrane groups. Each dot represents an animal and the horizontal bars show the means of the mentioned parameters. Statistical significance was determined by Mann-Whitney U test.
**P = 0.01, **P = 0.03, ***P = 0.05.*

density (NV) of the fibroblasts and smooth muscle cells was estimated using the following formula:

$$NV(\text{fibroblasts or myocytes / cavernous bodies}) = \Sigma Q - /(\Sigma p \times (a/f) \times h) \times (t/BA)$$

Where '∑Q' is the number of sampled fibroblasts or myocytes, '∑P' is the number of disectors, 'a(f)' is the area of the frame, 'h' is the height of the disector and 't' is the mean section thickness and 'BA' is the block advance of the microtome. The upper and lower borders of each section were considered guard zones. The total number of fibroblasts or myocytes was estimated by multiplying the numerical density by V (cavernous bodies) [Figure 4B].[19]

Fibroblasts were identified according to their specific criteria (including plentiful and irregularly branched cytoplasms, a large ovoid euchromatic nucleus and a prominent nucleolus). Smooth muscle cells were also identified by their spindle shape and single central nucleus.[20]

GraphPad Prism software, Version 8.0 for Windows (GraphPad Software, San Diego, California, USA), was used to analyse the data. The data were compared through the Mann-Whitney U test and were presented as dot plots. Statistical significance was considered at $P < 0.05$.

Results

The total volume, length and diameter of the penis increased by 26%, 8% and 4%, respectively, in the surgery+AM group compared to the sham group. There was also a significant increase in the mean volume and diameter of the penis in the surgery+AM group compared with the sham group ($P < 0.03$ and $P < 0.04$, respectively) [Figures 5A and B]. However, there was no significant difference between the surgery+AM and sham groups in terms of the mean length of the penis [Figure 5C].

The mean volumes of the fascia, tunica albuginea and corpora cavernosa increased by 15%, 29% and 40%, respectively, in the surgery+AM group compared to the sham group. No significant difference was observed between the surgery+AM and sham groups in terms of the mean volume of the fascia [Figure 5D]. However, the results revealed a significant increase in the mean volumes of tunica albuginea and corpora cavernosa in the surgery+AM group compared to the sham group ($P < 0.01$ and $P < 0.03$, respectively) [Figures 5E and F].

The mean volume density of the collagen bundles, smooth muscle cells and cavernous sinuses increased by 24%, 33% and 32%, respectively, in the surgery+AM group compared to the sham group. The results indicated a significant increase in the mean volume density of the collagen bundles, smooth muscle cells and cavernous sinuses in the surgery+AM group compared to the sham group ($P < 0.01$, $P < 0.01$ and $P < 0.03$, respectively) [Figures 6A, B and C]. However, no significant difference was observed between the surgery+AM and sham groups in terms of the mean volume of the vessels [Figure 6D].

The mean number of fibroblasts and smooth muscle cells increased by 41% and 36%, respectively, in

the surgery+AM group compared to the sham group. There was also a significant increase in the mean number of fibroblasts and smooth muscle cells in the surgery+AM group compared to the sham group (P <0.01 and P <0.05, respectively) [Figures 6E and F].

Discussion

This study aimed to determine the effectiveness of the use of AM as a graft for PGE in a rabbit model. The results revealed a significant increase in the diameter and volume of the penile corpora cavernosa and the number of fibroblasts and smooth muscle cells in the corpora cavernosa in the animals that had undergone PGE surgical procedures.

Penile enlargement is usually done by auto tissue transplantation, cell injection or implantation of artificial or natural materials.[8] Autologous tissue transplantation from the adjacent tissues is one of the most common surgeries performed for PGE. Autologous fat tissue has also been recently used for PGE.[8,21] However, an AM graft for PGE was first utilised in this study.

In earlier studies, different techniques have been described for PGE and a variety of exogenic materials were utilised in the procedures, although no standard guidelines were available.[8,21] Moreover, the exogenic materials that were employed in the procedures have shown different degrees of success. For example, autologous fat, silicone and hyaluronic acid gel were injected into the subcutaneous space of the penile body. Additionally, dermal fat grafts as well as acellular dermal matrix derived from a donated human skin tissue (allograft) were used for PGE procedures.[21,22] In a prior study, dermal cellular porcine grafts were used in 69 participants and the results revealed a promising long-term result. After one year of follow-up, the penial circumference increased by 3.1 cm and 2.4 cm during flaccidity and erection, respectively.[23] However, the use of Pelvicol acellular matrix for PGE was not suitable due to the high rate of complications.[24] Overall, the use of these injectable materials involves a risk of foreign body response, swelling and penile deviation.[25] However, autologous fat grafting has been found to reduce the risk of foreign body response and was found to improve PGE.[26] On the other hand, evidence has demonstrated that autologous fat used in transplantation loses a large amount of its volume over time and, consequently, several procedures were required for a favourable result.[25] In the current study, swelling and penile deviation were not observed in the experimental groups.

AM is composed of connective tissue with a significant collagen and extracellular matrix structure. The inner surface is enclosed by a single-layer cubical epithelium, which is avascular, has anti-scarring, anti-inflammatory and antiangiogenic properties and contains several growth factors. Moreover, it has been reported to possess the exclusive quality of avoiding graft versus host disease and for facilitating wound healing.[27] The mechanism of the action of AM has been thought to be related to the rich biological construct of the amnion and chorion membranes, which include layers of basement membranes and a variety of intrinsic factors that play a vital role in cell proliferation and differentiation. It has also been reported that the AM epithelial cells secrete angiogenic factors.[28] These properties make human AM an ideal tissue graft for reconstruction in different tissues. Additionally, AM is resistant to rejection and is easy to obtain, derive and store.[27] Leungwattanakij *et al.* studied penile reconstruction using small intestinal submucosa in 20 rats.[3] In that study, PGE was performed by making a bilateral incision in tunica albuginea where the plane of dissection was between the tunica albuginea and the cavernous tissue. The tunica defect was covered with a piece of small intestinal submucosa. The histological study showed moderate amounts of fibrosis under the graft and the elastic fibres of the graft were oriented in a circular direction.[3] In the current study, the same procedure was followed and the histological study revealed a significant increase in the mean volumes of the tunica albuginea and corpora cavernosa in the surgery+AM group. Additionally, the mean volume density of the collagen bundles, smooth muscle cells, cavernous sinuses and vessels (indicating neovascularisation into the graft) and the mean number of fibroblasts and smooth muscle cells increased in the surgery+AM group, which indicates good tissue acceptance.

Shakeri *et al.*'s work involved the proper re-epithelialisation of the urethra by using the AM method, which was reconstructed using transitional epithelium with cytokeratin expression in a rabbit model. However, fistula was detected in one case (5%) and urethral structures were seen in two cases (10%).[29] In another study, Salehipour *et al.* evaluated the use of human AM in the reconstruction of long ureteral defects in a dog model and concluded that AM was not useful for long urethral defects (3 cm). They stated that the use of AM could be studied for shorter defects or as a patch graft.[30] The authors of that study also assessed the efficacy of human AM grafting in penile tunica albuginea defect in canines. The results of histopathological examinations showed complete re-epithelialisation with squamous epithelium and collagen fibre deposition, but no dysplasia was detected.[31]

This study has some limitations. First, the surgery performed in the sham group could induce scarring, which may affect the final PGE and make comparison more difficult. Therefore, a group that does not undergo surgical procedure (control) should be added to the group design. Second, the effects of the surgical operation on ejaculation and erection were not evaluated after PGE. Finally, an increase in the collagen in the penis was observed, which could affect the function of the penis. Therefore, anti-fibrotic drugs can be used to reduce collagen in future studies.

Conclusion

The findings of this stereological study showed that PGE using AM increased the volume of the tunica albuginea, cavernous bodies, collagen fibres, muscle fibres, cavernous sinuses and the number of fibroblasts and smooth muscle cells of the rabbit penis two months after surgery. Finally, AM increased the volume and diameter of the penis. AM is a new method that could be helpful for material use in PGE. Hence, it may be considered for human PGE in the future.

AUTHORS' CONTRIBUTION

AA conceptualised and designed the study. FA and AE were involved in the visualisation and investigation. ST collected the data. SK-D and ST drafted the manuscript. SK-D was involved in the validation, review and editing of the manuscript. AA supervised the work. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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