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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, stereological studies were used to obtain quantitative histological data regarding the structure of the penis. ***Methods:*** In this study, 20 adult male rabbits of similar age and weight were allocated to two sham and surgery+AM groups. Both groups underwent surgery by longitudinal Ishape midline incision of the tunica albuginea on the dorsal surface of the penis. The surgery +AM group underwent PGE by AM graft. The penile length and mid circumference were measured using a Vernier caliper before and two months after the surgery. Stereological studies were used to obtain quantitative histological data regarding the structure of the penis. ***Results:*** The mean total volume and diameter of the penis increased in the surgery +AM group ($p<0.03$ and $p<0.04$, respectively).

The stereological evaluation showed a significant increase in the mean volumes of the tunica albuginea and corpora cavernosa in the surgery +AM group compared to the sham group ($p<0.01$, $p< 0.03$). Additionally, the mean volume density of the collagen bundles, muscle fibers, and cavernous sinuses and the total number of fibroblasts and smooth muscle cells increased in the surgery +AM group compared to the sham group ($p<0.01$, $p<0.01$, $p<0.03$, $p<0.01$, and $p<0.05$, respectively). No infections, bleedings, or other complications were seen. ***Conclusions:*** AM is a method that has appeared promising for material use in penile enhancement. Thus, it may be used for PGE in the future.

Keywords: Amniotic Membrane; Histopathology; Animal; Penile Girth Enhancement.

Advances in knowledge

- Amniotic membrane is a new method, which has appeared helpful for material use in penile girth enhancement.
- Amniotic membrane may be used for humman penile girth enhancement in future**.**

Application to patient care

- This study aimed to evaluate the efficacy of penile girth enhancement using amniotic membrane as a graft in a rabbit model.

Introduction

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

Penile Girth Enhancement (PGE) is carried out for cosmetic purposes and psychological causes in some patients, similar to breast enlargement amongst females.[2] Men with a small penis and patients with special urological conditions such as micropenis, Peyronie's disease, and trauma to the penis may benefit from this procedure.[3] PGE aims at improving the penile function and appearance. Nonetheless, there are no suggested guidelines and specific techniques for PGE.[4]

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

Many studies have been done regarding the impact of graft fat procedures on the penis and have indicated the effectiveness of graft fat in enhancing the penile girth. For example, Zhang et al. (2020) evaluated the effectiveness and safety of human acellular dermal matrix graft in the augmentation phalloplasty method.[8] Xu et al. (2016) also illustrated the effectiveness and safety of dermal fat graft in augmentation phalloplasty amongst men with a small penis.[9] Similarly, Leungwattanakij et al. (2006) showed the promising effect of using small intestinal submucosa on penis enlargement in a rat model.[3] In addition, Küçükçelebi et al. (2006) reported that the use of microvascular temporalis fascia strengthened the penis in humans.[7]
Considering the social progress, increase in people's awareness and sexual needs, and increasing 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 suggest its value in several medical applications. AM has also been used in many genitourinary surgeries. [10-13] In the current 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, each having different effects and techniques.[14] It is worth mentioning that 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]

Rabbit has 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 human's 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

our knowledge, no study has evaluated the efficiency of the application of AM in PGE in a rabbit model using stereological methods in order to obtain quantitative histological data. The chief advantage of stereological methods is the provision of unbiased and precise assessments. Thus, the present study aims to investigate whether PGE using AM accelerates the regeneration of various parts of the penile tissue and leads to an increase in its size.

Methods

Experimental design

A total of 20 adult male New Zealand White (Oryctolagus cuniculus) rabbits (weight: 1600–2500 grams; age: 18 weeks) were obtained from the University's Center of Comparative and Experimental Medicine. The rabbits were kept individually in cages with a 12/12-h light-dark cycle at room temperature of 22–24 °C and humidity of 50% and had access to water and food *ad libitum*. All animals were kept according to the Animal Care and Ethics Committee of the University. The rabbits were divided two sham and surgery +AM groups using simple random sampling (n=10). In the both groups, the surgery was done by a longitudinal I-shape midline incision of the tunica albuginea on the dorsal surface of the penis .The second group (surgery +AM group) underwent PGE using AM.

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

Human amniotic membrane preparation

Human AM, provided by Burn and Wound Healing Research Center, were kept in alcohol (95%) until application. (In this center, AM are provided from delivery rooms and are employed as a biological dressing in burn patients).AM were gained from women delivery no history of premature rupture of membrane, endometritis, or meconium ileus. All women were seronegative tests for human immunodeficiency virus, hepatitis types B and C, and syphilis.[17]

Surgical procedure

All rabbits were anesthetized using the intramuscular injection of ketamine (10–15 mg/kg) and xylazine (6–10 mg/kg). Supplemented doses of ketamine were administered as needed to maintain

a uniform level of anesthesia. All animals were well shaved and prepared with a povidone iodine topical antiseptic solution and were then draped with sterile sheets. After that, the penis was exposed under aseptic conditions and then, the glans was sutured with 4/0 nylon held with a mosquito clamp under gravity to stretch the penis downward.

In the both groups, the surgery was done by a longitudinal I-shape midline incision of 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 covernosal tissue in both sides of penis and was sutured with a 6-0 PDS (polydioxanone) [Figure 1].

All rabbits were housed individually and were fed with 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, hematoma, 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 to those of other rabbit categories.[18]

Penile tissue preparation

After two months, all the rabbits were sacrificed with deep anesthesia. The penis and skin sutures were removed in its entirety by dissecting along the shaft to the crura and separating each cru from its point of attachment at the ischial tuberosity. The penis was divided to 8-12 sections based on length with equal distances between the sections "T" [Figure 2 a]. The sections of each penis were processed, embedded, sectioned (4 and 25 µm), and stained (hematoxylin-eosin) [Figure 2 b].[19]

Estimation of the volumes of the penis and its components

The sections with a 4-µm thickness were used in order 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 characterized [Figure 3 a]. 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 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 12X magnification [Figure 3 b]:

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

Where $\sum p$ was the total number of points hitting the structure of interest, A(p) was the area related to every grid point, and "T" was 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[19] [Figure 4 a]:

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

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

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

Estimation of the numerical density of the fibroblasts and smooth muscle cells in the 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 utilized on 25 µm sections. The optical disector contained an Eclipse microscope with a high Numerical Aperture (NA=1.30) ×40 oil-immersion objective lens connected to a video camera that transmitted microscopic live images to a computer monitor and an electronic microcator with digital readout for estimating the number of fibroblasts by moving in the Z-direction. The numerical density (NV) of the fibroblasts and smooth muscle cells was estimated using the following formula:

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

Where "$\sum Q$" was the number of sampled fibroblasts or myocytes, "$\sum P$" was the number of disectors, $a(f)$ was the area of the frame, "h" was the height of the disector, and "t" was the mean section thickness. 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 4 b].[19]

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

Statistics and data analysis

GraphPad Prism software, version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) was applied to analyze the data. The data were compared using Mann-Whitney U test and were presented as dot plots. $P<0.05$ was considered statistically significant.

Results

The total volume, diameter, and length of the penis

The total volume, length, and diameter of the penis increased by respectively 26%, 8%, and 4% in the surgery +AM group in comparison 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 to the sham group ($p<0.03$ and $p<0.04$, respectively) [Figure 5 a and b]. However, there was no significant difference between the surgery +AM and sham groups regarding the mean length of the penis [Figure 5 c].

The volumes of the fascia, tunica albuginea, and corpora cavernosa of the penis

The mean volumes of the fascia, tunica albuginea, and corpora cavernosa increased by respectively 15%, 29%, and 40% in the surgery +AM group in comparison to the sham group. The results also 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) [Figure 5 e and f]. However, there was no significant difference between the surgery +AM and sham groups concerning the mean volume of the fascia [Figure 5 d].

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

The mean volume density of the collagen bundles, smooth muscle cells, and cavernous sinuses increased by respectively 24%, 33%, and 32% in the surgery + AM group in comparison 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) [Figure 6 a, b, and c]. However, there was no significant difference between the surgery +AM and sham groups in terms of the mean volume of the vessels (Figure 6 d).

The number of fibroblasts and smooth muscle cells of the corpora cavernosa

The mean number of fibroblasts and smooth muscle cells increased by 41% and 36%, respectively in the surgery +AM group in comparison 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) [Figure 6 e and f].

Discussion

This study aimed to determine the effectiveness of AM as a graft in 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] The utilization of an AM graft for PGE was first introduced in the present research.

In the previous studies, different techniques were described for PGE and a variety of exogenic materials were utilized in the procedures. However, no standard guidelines are available. Moreover, the employed exogenic materials have shown different degrees of success. For example, autologous fat, silicone, and hyaluronic acid gel were injected to the subcutaneous space of the penile body. Additionally, dermal fat grafts as well as a cellular dermal matrix derived from a donated human skin tissue (allograft) were used for PGE procedures.[21,22] In a prior research, dermal cellular porcine grafts were used in 69 participants and the results revealed a promising long-term outcome. After one year of follow-up, the penial circumference increased by 3.1 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, these injectable materials carried a risk of foreign body response, swelling, and penile deviation.[25] However, autologous fat grafting reduced the risk of foreign body response and was found to improve PGE.[26] On the other hand, evidence demonstrated that autologous fat transplantation would lose a large amount of its volume over time and, consequently, needed several procedures to bring about a favorable outcome.[25] In the present 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 to facilitate wound healing.[27] The mechanism of 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. In that study, PGE was performed via the bilateral incision of tunica albuginea and 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 fibers of the graft were oriented in a circular direction.[3] In the present study, the same procedure was used 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 neovascularization into the graft) and the mean number of fibroblasts and smooth muscle cells increased in the surgery +AM group, which represented good tissue acceptance.

Shakeri et al. reported the proper re-epithelialization of the urethra reconstructed with AM by transitional epithelium with cytokeratin expression in a rabbit model. However, the fistula was detected in one case (5%) and urethral strictures 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 mentioned that the use of AM might be studied for shorter defects or as a patch graft.[30] Salehipour et al. also assessed the efficacy of human AM grafting in the canine penile tunica albuginea defect. The results of histopathological examinations showed complete re-epithelialization with squamous epithelium and collagen fiber deposition. Besides, no dysplasia was detected.[8]

This study had some limitations. Firstly, the operation performed in the sham group might induce scaring, which could have affected the final PGE and make the comparison more difficult. Therefore, a group without surgical procedure (control) had to be added to the group design.

Secondly, the effects of the surgical operation on ejaculation and erection were not evaluated after PGE. The third study limitation was the increase in collagen in the penis, which could have affected the function of the penis. Therefore, anti-fibrotic drugs can be used to reduce collagen in future studies.

Conclusions

AM is a new method, which has appeared helpful for material use in PGE. Hence, it may be used for human PGE in future**.**

Authors' Contribution

AA conceptualized and designed the study. FA and AE were involved in the visualization 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.

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Conflicts of Interest

The authors declare no conflicts of interest.

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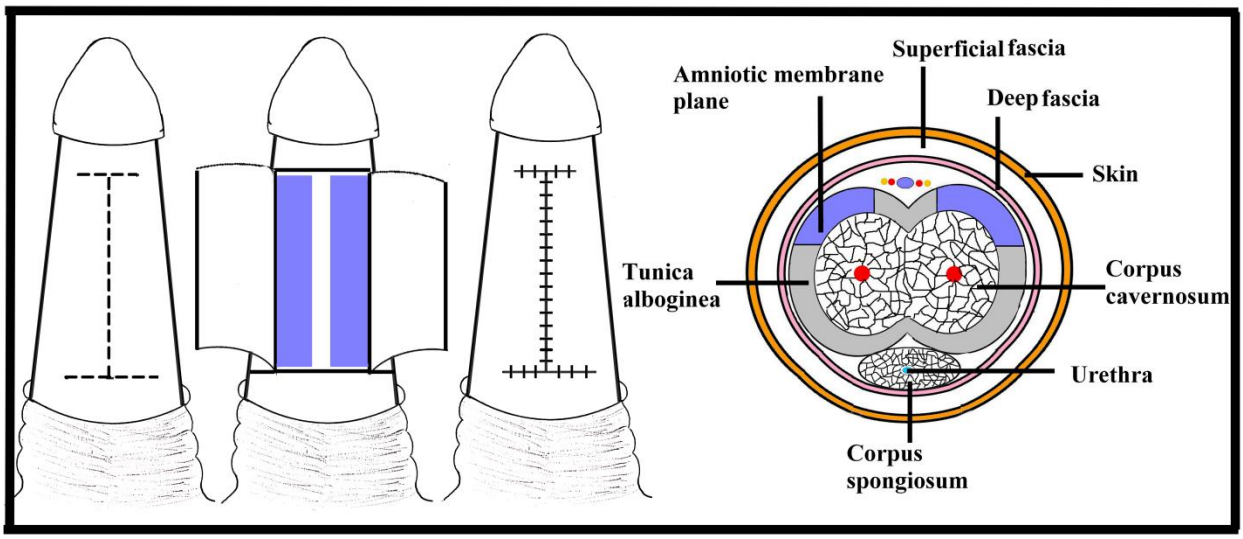


Figure 1: Surgical procedure was done by the longitudinal I-shaped midline incision of the tunica albuginea on the dorsal surface of the penis and the placement of the AM graft between the tunica albuginea and the corpus cavernosum in both right and left sides of penis.

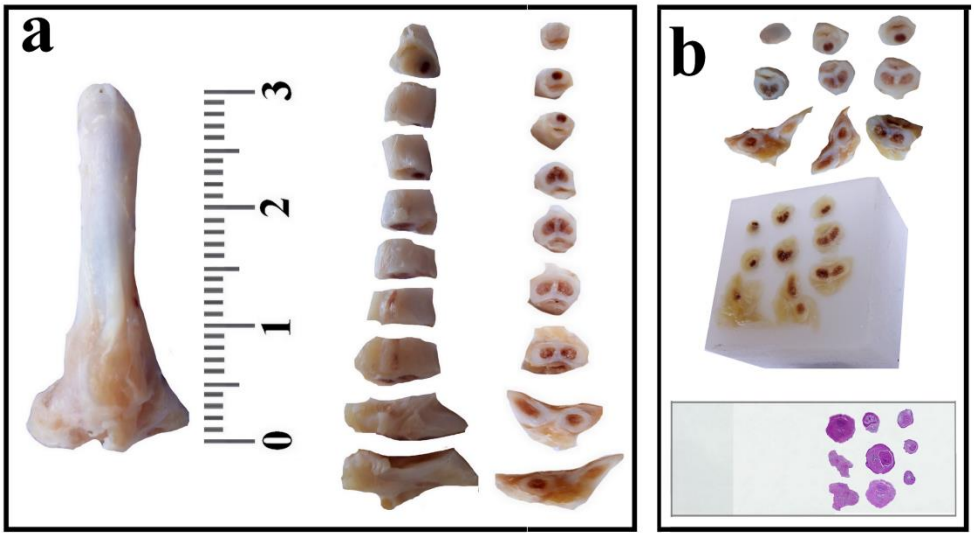
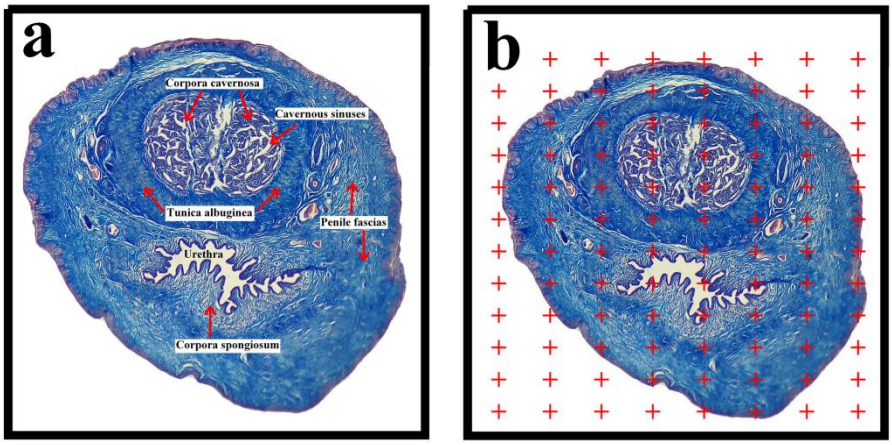


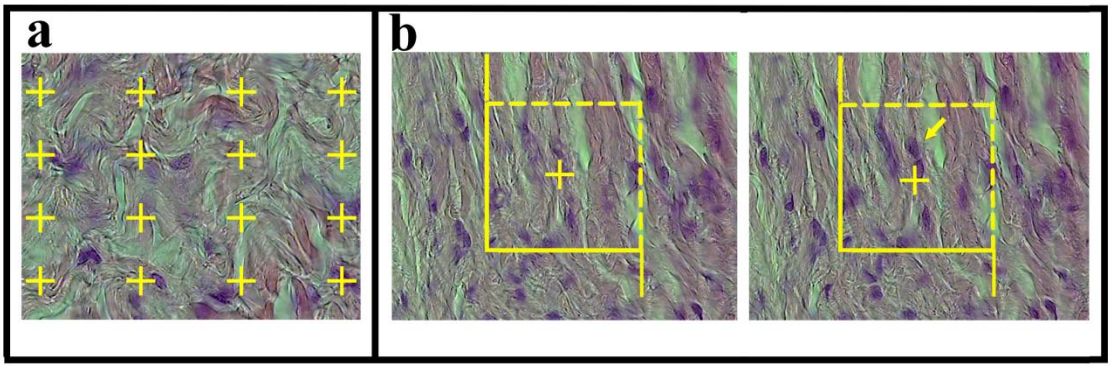
Figure 2: Processing technique. The penis was cut into 8-12 sections according to its length (a). The sections were embedded in paraffin blocks, sectioned, mounted on a slide, and stained (b).



437

438 **Figure 3:** Assessment of the rabbit penile tissue. The penile components were indicated on the
439 histological section by arrows (a). The volumes of the penis and penile components were assessed
440 by Cavalieri's technique and point-counting method (b).

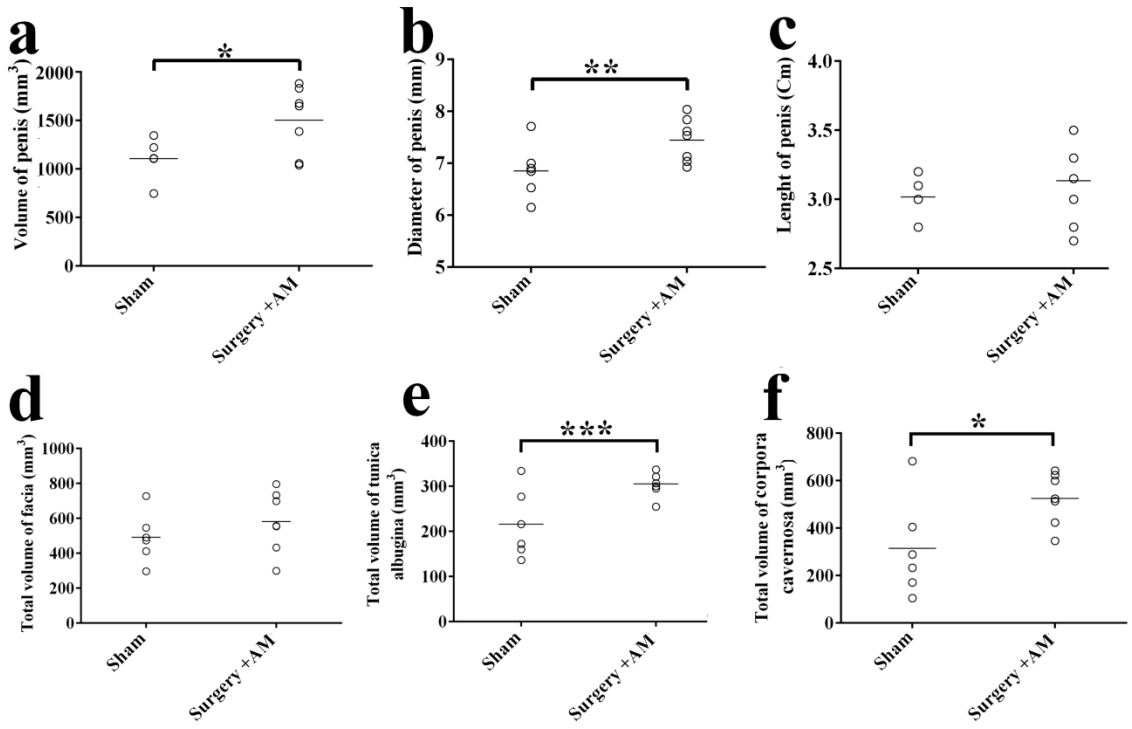
441



442

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

448



449

450 **Figure 5:** The aligned dot plots of the total volume (a), diameter (b), and length (c) of the fascia
451 (d), tunica albuginea (e), and corpora cavernosa (f) of the penis in the sham and surgery+AM
452 groups. Each dot shows an animal and the horizontal bars represent the means of the parameters.
453 The p-values and significant differences have been shown on each dot plot by stars. Statistical
454 significance was determined by Mann-Whitney U test. **P*=0.03, ***P*=0.04, ****P*=0.01.

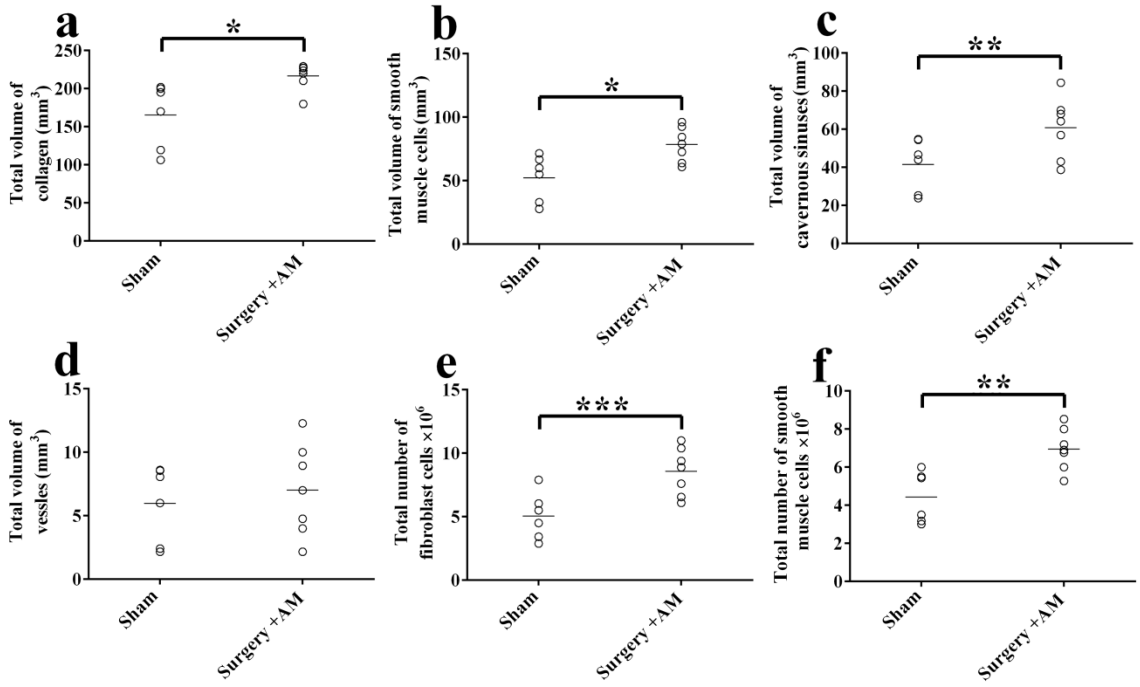


Figure 6: The aligned dot plot of the volume density of the collagen bundles (a), smooth muscle cells (b), cavernous sinuses (c), and vessels (d) and number of fibroblasts (e) and smooth muscle cells (f) of the corpora cavernosa in the sham and surgery +AM groups. Each dot represents an animal and the horizontal bars show the means of the mentioned parameters. The significant differences and p-values have been presented on each dot plot by stars. Statistical significance was determined by Mann-Whitney U test. *P=0.01, **P=0.03, ***P=0.05.