The Distribution of Preputial Vessels at Different Severity of Rat Congenital Hypospadias Model

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Abstract

Background To reconstruct and analyze the distribution of preputial vessels using micro-computerized tomography (micro-CT) in the finasteride induced congenital hypospadias rat model. Materials and methods Pregnant rats were exposed to finasteride from gestational days 12 to 17. According to the position of the urethral meatus, the pups were divided into normal, mild hypospadias and severe hypospadias groups. Six months after birth, the preputial blood vessels were observed by vascular perfusion with Microfil (a silicone-based polymer) and scanned by micro-CT. CTvox and NRecon were used to reconstruct 3-dimensional(3D) images. A pathological analysis of the specimen was taken to determine the position of Microfil. Results The normal group and the mild hypospadias group had similar preputial image characteristics. At the junction of the inner and outer prepuce, the deep layer vessels of the superficial fascia were transverse distributed and formed a vascular ring-like structure. Among the severe hypospadias group, five had sufficient blood circulation while six had insufficient blood circulation. In the sufficient blood circulation type, the ring-like vessels were found at the junction of the inner and outer prepuce similar to the normal and mild hypospadias group. However, only a small amount of capillary supply to this area in the insufficient type. Conclusion Compares to the traditional techniques, micro-CT improves understanding the 3D structure of the hypospadias preputial vascular. The junction of the inner and outer prepuce with rich blood circulation was suitable to be a vascular pedicle flap.

Background

The incidence of hypospadias is approximately 1:200~1:300 male births, which has doubled over the past three decades. The preputial skin is the major grafting material to the urethroplasty[1,2]. However, the postoperative complications were about 5%-25%[3], include urethral fistula, stricture and diverticula. The occurrences of urethral fistula and stricture are correlated to the blood supply of molding material for urethral reconstruction[4]. To create a more reasonable preputial vessel pedicle flap design to minimize the disruption of blood circulation, anatomical study is required to have better understanding on the distribution of preputial vasculature.

The anatomic characteristic of blood supply is difficult to detect, because the capillaries of prepuce are the terminal branch and measured in micron [5]. Micro-CT scanners reach spatial resolution in submicron level [6-9]. So far little research has been done in the study of preputial vascular distribution of patients with hypospadias. Several literatures have described the anatomy of hypospadias preputial vessels using methods such as trans-illumination, microscopic observation and 3D computer reconstruction[10-12]. Although these approaches have described the vascular anatomy, the preputial vascular networks are still difficult to quantify in 3D.

The experiment produced the rat model of hypospadias successfully by injected 5-alpha-reductase inhibitors finasteride to the pregnant rats [13-16]. The micro-CT, which was widely used to detect the anatomy of the animal, was used to scan the rat hypospadias penile specimens to produce the 3D images of the preputial vessels [17].
Methods

Hypospadias rat model

The animal ethics committee's approval was obtained by the Institutional Animal Care and Use Committee of Beijing Children's Hospital, Capital Medical University prior to the experiment. Total 6 pregnant Wista rats were obtain from Animal Experimental Center (Capital Medical University, China) were used in this experiment. The pregnant rats were divided into experimental group (n=4) and control group (n=2). The experimental group received daily finasteride (Yuan Sen pharmaceutical Co . Ltd, Hebei, China) injection of 40mg/kg to the subcutaneous of the abdominal wall, from gestational days (GDs) 12 to 17. The control group was injected with same volumes of normal saline solution only. After delivery (GD22-24), mothers and the newborn rats were fed in one cage. On day 28 after delivery, hypospadias rat models were selected from the newborn rats. For testing purpose, rat models with following features were used in experiment: deficient foreskin ventrally with dorsal hood; abnormally located meatus; vary degrees of ventral penile curvature.

Silicone rubber compound infusion

Microfil infusion to rat models was started at the age of 6 months. Urethane was given to the rat models through intraperitoneal injection of 6mL/kg to induce general anesthesia. The abdominal wall was opened using midline incision. The abdominal aorta and the inferior vena cava were isolated and ligated. 24G indwelling needle was inserted to the abdominal aorta at the distal of the ligation point as an input channel. Cutting a 2mm hole at the inferior vena cava wall as output channel. Connecting the micro-pump (Product model:SDS-MP09, Shan De Shi medical company, Beijing, China), then input 50ml 40°C heparin saline (50U/ml) at 5ml/min to dilate blood vessels and anticoagulation. Microfil (MV-122 compound 4ml, diluent solution10ml, curing agent 0.7ml, Circulation Tech Company, America) at 2 mL/min added to replace heparin when the viscera turned pale. The perfusion was finished until the viscera became yellow. The specimens were stored at 25°C to clotting the Microfil, then the penile specimen was cut and stored in 4°C environment. The specimens were stored in 10% formalin. The animals were sacrificed with blood loss and perfusion, which is performed under general anesthesia.

Micro-CT scan

The Bruker SkyScan 1172 micro-CT system (Bruker, Kontich, Belgium) was used. Technical protocol of the Scanning: voltage of 59kV and current of 167μA with a 0.5-mm aluminum attenuation filter. 6.88-μm resolution were obtained. Acquisition time for each specimen was 120 minutes. The raw projection image files were reconstructed using the Fledkamp cone-beam algorithm in NRecon Reconstruction and CTvox software [18].

Pathological section
After image acquisition, the samples were embedded in paraffin and horizontally sectioned (7 mm of thickness). Samples were stained with hematoxylin-eosin (HE), rinsed in distilled water and mounted. Sections were evaluated and the Microfil particles were detected using light microscopy.

**Results**

**The morphology of the hypospadias model penis**

Normal group: a normally located meatus. The prepuce covered the penis completely. No penile ventral curvature was found (Fig. 1A).

Mild hypospadias model: meatus was located at the ventral of the penile shaft (Fig. 1C). There was a defect in the ventral prepuce. The dorsal prepuce was split into a "V" shape (Fig. 1B). The glans was observed easily in the normal position. The penile ventral curvature was not obviously.

Severe hypospadias model: meatus was located at the perineum. The appearance of the prepuce and glans was similar to that of the mild model. The fibrosis, which may induce penile ventral curvature, was developed on both sides of the ectopic urethral meatus (Fig. 1D).

**Establishment of hypospadias model**

The control group rats gave birth to 24 fetuses, including 10 females and 14 males, all male offspring had normal penis without hypospadias. The experimental group gave birth to 41 fetuses, including 17 females and 24 males. Totally, 16 hypospadias offspring were included. According to the location of the meatus, the 16 hypospadias pups were further divided into mild group (n=3) and severe group (n=13).

**Penis Images**

Tissues of different density can be observed easily by adjusting the CT threshold value. The anatomy of the penile and preputial dartos vessels, penile dorsal vessels, corpora cavernosa, corpus spongiosum, baculum was visualized clearly. (Fig. 2)

**Normal rat preputial blood vessels**

The deep layer vessels of the superficial fascia, which was originated from the external pudendal artery, were axially distributed bilaterally. At the junction of the inner and outer prepuce, the bilateral deep layer vessels showed a transverse distribution and form a vascular ring-like structure. The ring-like structure continued to send parallel terminal vessels to the inner preputial skin (Fig.3B). These terminal vessels formed numerous reticular lateral branches. The superficial layer vessels of the superficial fascia supplied blood to the penile skin. The deep and superficial layers vessels can be observed clearly by adjusting the viewing angle in 3D. There was no obvious relationship between the superficial and deep vessels of prepuce. The diameter of the superficial vessel was thinner than the deep one, but the superficial one had greater number of blood vessel and branches (Fig.3).
Hypospadias model preputial blood vessels

Mild hypospadias: The distribution and the morphology of the deep and the superficial layer vessels were similar to the normal specimen. At the junction of the inner and outer prepuce, the bilateral deep layer vessels were also converged to form a vascular ring (Fig.4A).

Severe hypospadias: We classified the severe hypospadias into good blood circulation type and poor blood circulation type according to the characteristic of the deep layer vessels at the junction of inner and outer prepuce. For the good blood circulation type (Fig.4B), with the absent of ventral prepuce, the deep layer vessels converged to form a half ring structure at the dorsal of the prepuce. For the poor circulation supply type (Fig.4C), the vascular ring was displaced by a network distribution.

Pathology

Microfil particles can be detected in the arteries, capillary networks and veins (Fig 5). The diameter of Microfil was smaller than capillaries, so Microfil can fill the entire vascular system. No Microfil leakage was observed outside the blood vessel wall to the surrounding tissue, so Microfil was an ideal angiographic reagent.

Discussion

Hypospadias is one of the most common congenital defects in pediatric urology. It is caused by anterior urethral maldevelopment, so the ectopic urethral meatus may be located anywhere from the tip of glans to perineum. The penile shaft is often accompanied by ventral curvature and the prepuce accumulate at the dorsum of the glans. Urethroplasty is the only method to treat hypospadias. Today, there are more than 300 surgical techniques to treat this disease, but every technique has some corresponding surgical complications, which is most likely in urethral fistula. So far, there are no common standard surgical techniques has been approved by all pediatric urologists [19-21].

In our center, tubularized preputial island flaps are the most commonly used technique of single-stage urethroplasty for hypospadias accompanied by penile curvature. The most common short-term complications are urethral fistula and stricture. It is reported that preputial vascular pedicle flap ischemia and necrosis are the reason for fistula. The exact relationship between the postoperative complications and the structure of the patients’ preputial blood vessels is unclear [10]. To figure out this problem, we utilized micro-CT for 3D analysis of morphologic characteristic on the rat model of hypospadias.

Preputial skin is the ideal molding materials for urethroplasty. In order to get a preputial pedicle flap with an adequate blood supply, we need to understand the distribution of preputial vessels. The prepuce vessels are the terminal branch, the capillaries are extremely narrow, thus knowledge about the vessels is limited. Much work has been done to study prepuce vessels, such as transillumination with endoscopic cold light source, microscopic observation of gelation and India ink perfusion, 3D reconstruction of
histological sections [10-12]. In this study, an advanced technology, micro-CT scanning, is used to analysis the distribution of preputial vessels. Micro-CT is an innovative non-invasive and high-resolution imaging technology. O’Neill et al found the results of virtual sectioning at micro-CT and conventional histologic sectioning are highly correlated [18]. The CTvoxBruker software can be used to observe the samples by transverse, sagittal, and coronal section image, and established 2- or 3-dimensional reconstruction on interesting parts. The highest resolution has reached 0.5 μm, while the mean diameter of capillary vessels is 6 to 9 μm, so micro-CT can be used to micro vessels scanning[22].

There are a variety of choices for preputial vessel pedicle ap in hypospadias surgery. Although all flaps are reasonably designed to preserve sufficient blood supply for a better survival, the complication did not reduce significantly. Today, blood supply of the penis is divided into three systems: 1. the dorsal penile vessels which provides the blood supply to glans, urethra and inner prepuce; 2. the cavernosa artery supply the blood to the deep soft tissues, especially to the corpora cavernosa and corpus spongiosum; 3. the superficial fascia vessels originated from the descending branch of the external pudendal arteries which provide the blood supply to the penile skin and prepuce. The superficial and deep layers of the superficial fascia are easy to dissociate. The junction of the inner and outer prepuce where has abundant blood supply and is suitable to make a preputial vessel pedicle. Four main types of deep layer vessels have been described: single branch predominant type (41%), two branches predominant type (25%), arching H-type (12.5%), or net-like type (21%). The qualities of the vascular pedicle and the urethral plate are the two important surgical variables [23].

Tubularized preputial island flaps are the ideal single-stage repairs for the proximal hypospadias. For the patients with severe hypospadias and severe ventral curvature, two-stage operation is still meaningful [24]. Clinical data indicates that penile curvature corrected in the first phase, urethroplasty is done in second phase to reduce the incidence of complications. Two-stage surgery reduce the difficulty of operation somewhat, on the other hand it increases surgical operation times and prolonged treatment time [25]. This experiment confirmed that in the normal group and the mild hypospadias group, the blood vessels at the junction of the inner and outer prepuce had a wider caliber and formed a vascular ring. While in the group with severe hypospadias, the blood vessels at the junction of the inner and outer prepuce had a relatively smaller caliber displayed a net-like distribution and could not form a clear and complete vascular ring. These results may explain patients with severe hypospadias are more likely to suffer from postoperative complications than the ones with mild hypospadias.

We divided the severe hypospadias group into two types based on their vascular morphology: The first type has a sufficient blood supply, although the blood vessels at the junction of the inner and outer prepuce could not form a vascular ring completely due to the ventral prepuce defect, there is a semi-circular vessel structure, and the semi-vascular ring diverge small branches into the inner preputial skin to ensure an efficient supply of blood; For the second type, there is no semi-vascular ring to support the
efficient blood circulation, the vessels are netlike distributed and contains mainly capillaries so there is no sufficient supply of blood to the preputial vessel pedicle flap.

This result may explain that patients with severe hypospadias achieved satisfactory results without postoperative complications after one stage surgery because their blood vessels at the junction of inner and outer prepuce provide a sufficient amount of blood for a better survival of the flap. At the same time, we conclude that patients of severe hypospadias with poor vessels structures are highly recommended to underwent two-stage surgery to reduce postoperative complications.

The limitation of this study is that the human penile anatomy is different from penile anatomy of rats. This study may not represent the anatomy of the humans. The next step, the clinical experiment will be used to detect the exact relationship between the postoperative complication and the anatomy of the preputial vessels.

**Conclusions**

Compared to traditional techniques, the application of Microfil and contrasted micro-CT scanning improve our understanding on the anatomy of the hypospadias, especially the preputial vascular structure in three-dimensional. The junction of the inner and outer prepuce with sufficient blood circulation would be a suitable vessel pedicle flap.

**Abbreviations**

Micro-CT: micro-computerized tomography; 3D: 3-dimentional; GDs: gestational days; HE: hematoxylin-eosin

**Declarations**

**Ethical approval and consent to participate**

All procedures in this study were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.
Competing interests
The authors declare that they have no competing interest.

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Authors’ contributions
DFL and PL conceived of the study, and performed the experiments and wrote the manuscript. GNW carried out the acquisition of data, or analysis and interpretation of data. WPZ participated in the design of the study and provided technical advices. NS participated in the design of the study, in discussions and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

A The normal penis appearance. B The "V" shape prepuce of the hypospadias model. C Mild hypospadias model. D The severe hypospadias model. Arrows show the ectopic urethral meatus.
Figure 2

A Transverse section image at the level penile shaft showing (1) the bilateral corpora cavernosa connected with each other at the midline, (2) the corpus spongiosum was located on the ventral side and relatively smaller than the corpora cavernosa, (3) the dorsal penile vessels, (4) the two deep layer vessels of the superficial fascia were axially distributed at the lateral of the penile shaft in the area between 9- to 8-o’clock and 3- to 4-o’clock positions. (5) Innumerable superficial layer vessels of the superficial fascia were distributed at the surrounding of the penis to supply blood to the skin. B Transverse section image at the level of the glans showing (1) the corpora cavernosa separated by the (2) baculum, (3) showing the dorsal penile vessels. (4) The deep layer vessels showed a transverse distribution and formed a ‘ring-like structure’ at the junction of the inner and outer prepuce. The ‘ring-like structure’ was oriented from bilateral (5) the deep layer vessel of the superficial fascia.
Figure 3

A The penis and preputial appearance in the nature position. (1) The deep layer of the penile dartos vessels go along with penile shaft at the dorsolateral position. At the junction of the inner and outer prepuce, the vessel transversely oriented terminal bifurcations anastomose with similar vessels of contralateral side and form a (2) vascular ring-like structure. (3) The superficial layer of the dartos vessels. (4) the corpora cavernosa, (5) the corpus spongiosum. B Reflecting the prepuce to the level of penopubic junction. (1) The bilateral deep layer of the penile dartos vessels converged and formed (2) the vascular ring, and then send (3) some parallel terminal blood vessels to the inner preputial dartos, these terminal blood vessels (4) anastomosis with each other. (5) The dorsal penile vessels.
Figure 4

The 3D images reconstructed by the micro-CT. (A) Mild hypospadias. (B) Severe hypospadias with good blood circulation. (C) Severe hypospadias with poor blood circulation. A The terminal branches originated from the deep layer of the penile dartos vessels formed an intact vascular ring and covered the glans. B Because of the ventral part prepuce defected, the deep layer vessels converge to form a half ring structure at the dorsal of the glans. C The severe hypospadias with insufficient blood circulation, which was absent of dominant vessels, the vascular ring was replaced by a network structure.

Figure 5
A Prepucie paraffin section HE×100 B Prepucie paraffin section HE×200 arrows show the Microfil particle.

**Supplementary Files**

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- supplement1.pdf