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Novel Application of Micro-Computerized Tomography for Morphologic Characterization of the Murine Penis

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Abstract

Background: The murine penis model has enriched our understanding of anomalous penile development. The morphologic characterization of the murine penis using conventional serial sectioning methods is labor intensive and prone to errors.

Aim: To develop a novel application of micro-computerized tomography (micro-CT) with iodine staining for rapid, non-destructive morphologic study of murine penis structure.

Methods: Penises were dissected from 10 adult wild-type mice and imaged using micro-CT with iodine staining. Images were acquired at 5- μ m spatial resolution on a Bruker SkyScan 1272 micro-CT system. After images were acquired, the specimens were washed of any remaining iodine and embedded in paraffin for conventional histologic examination. Histologic and micro-CT measurements for all specimens were made by 2 independent observers.

Outcomes: Measurements of penile structures were made on virtual micro-CT sections and histologic slides.

Results: The Lin concordance correlation coefficient demonstrated almost perfect strength of agreement for interobserver variability for histologic section (0.9995, 95% CI = 0.9990–0.9997)

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and micro-CT section (0.9982, 95% CI = 0.9963—0.9991) measurements. Bland-Altman analysis for agreement between the 2 modalities of measurement demonstrated mean differences of -0.029 , 0.022 , and -0.068 mm for male urogenital mating protuberance, baculum, and penile glans length, respectively. There did not appear to be a bias for overestimation or underestimation of measured lengths and limits of agreement were narrow.

Clinical Translation: The enhanced ability offered by micro-CT to phenotype the murine penis has the potential to improve translational studies examining the molecular pathways contributing to anomalous penile development.

Strengths and Limitations: The present study describes the first reported use of micro-CT with iodine staining for imaging the murine penis. Producing repeated histologic sections of identical orientation was limited by inherent imperfections in mounting and tissue sectioning, but this was compensated for by using micro-CT reconstructions to identify matching virtual sections.

Conclusion: This study demonstrates the successful use of micro-CT with iodine staining, which has the potential for submicron spatial resolution, as a non-destructive method of characterizing murine penile morphology.

Keywords

Micro-Computed Tomography; Mouse Model; Hypospadias; Penile Anomalies

INTRODUCTION

Hypospadias, the most common manifestation of congenital penile anomalies, occurs in approximately 1 of 200 live male births.¹ The correction of severe and even sometimes mild forms of hypospadias can pose a significant challenge for surgeons.^{2,3} Surgical complications, which require additional procedures, can lead to functional, psychologic, and esthetic problems that contribute to health care expenditures and patient suffering. There is growing interest in identifying genetic and environmental factors contributing to the development of hypospadias, with the overarching goal of preventing its occurrence.⁴ Notably, Cunha et al⁵ made significant advances in understanding the similarities and differences in urethral development and hypospadias formation in the murine model compared with humans. By creating diethylstilbestrol- or flutamide-induced hypospadias in the murine model, their work has characterized the morphologic criteria specific to this malformation in the mouse and rat.

Current methods for evaluation of penile anomalies in the murine model include macrophotography or scanning electron microscopy and techniques involving 3-dimensional (3D) visualization of the penile specimen.⁵ Although macrophotography and scanning electron microscopy allow for examination of the outer surface, they do not allow for morphologic assessment of internal penile structures. 3D reconstruction of specimens can be achieved using destructive (serial sectioning) and non-destructive (whole-volume imaging) approaches.⁶ Because conventional histology and serial sectioning are labor intensive and prone to artifacts and error, non-destructive whole-volume imaging using optical projection tomography, micro-computerized tomography (micro-CT), and micro-magnetic resonance imaging (micro-MRI) for small specimens has seen increasing use. Despite the ability to

achieve resolution on the order of a few microns with optical projection tomography, it is limited by sample thicknesses greater than 10 mm and opaque tissues.⁷ Micro-MRI is well suited for soft tissue imaging but is limited by lengthy acquisition times and a relatively low spatial resolution of 25 μm .

Micro-CT was first described for preclinical imaging in small animals in 1980 and has rapidly gained popularity in the past 2 decades.⁸ Compared with the ubiquitous hospital-based CT scanner, which can achieve resolutions as high as 40 μm , micro-CT scanners can achieve spatial resolution to the submicron level.⁹ Image acquisition times are faster than those for micro-MRI. Because soft tissues yield little to no contrast when imaged with CT, initial usage in preclinical imaging was limited to highly radiopaque specimens, such as fossils and calcified bones. However, the recent introduction of staining methods for contrast-enhanced imaging has allowed for improved soft tissue visualization.

To address the shortcomings of conventional histology and serial sectioning, we report the novel use of micro-CT with iodine staining in characterizing murine penile morphology. Furthermore, we demonstrate that the ability to perform serial sectioning with histology is preserved even after processing for micro-CT.

METHODS

Experimental Design

The study was carried out in accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal research was conducted under the oversight of the Baylor College of Medicine institutional animal care and use committee approved protocols.

Sample Preparation

Penises were dissected from C57BL6/J adult wild-type mice and fixed overnight in 10% formalin. The fixed tissue was washed twice with 70% ethanol, followed by dehydration and storage in 70% ethanol. 24 hours before micro-CT scanning, iodine staining was achieved by submerging the samples in a 0.1 N iodine solution (Sigma-Aldrich, St Louis, MO, USA) on a shaker at 4°C. Before scanning, the samples were rinsed with 1 $\times$ phosphate buffered saline and suspended in a sample tube with 1% agarose.

Micro-CT Image Acquisition and Reconstruction

Scans were acquired at the Optical Imaging and Vital Microscopy Core at Baylor College of Medicine using the Bruker SkyScan 1272 micro-CT system (Bruker, Kontich, Belgium). The x-ray source was set at 60 kV and 166 μA with a 0.5-mm aluminum attenuation filter. Sequential projection images at $2,016 \times 1,344$ pixels and 5- μm resolution were obtained as the sample was rotated 180° at 0.3° increments in the anteroposterior axis. Acquisition time for each specimen was 120 minutes and batch acquisition was feasible with up to 6 specimens suspended in a single sample tube.

The raw projection image files were reconstructed using the Fledkamp cone-beam algorithm in NRecon Reconstruction (Bruker) software.¹⁰ Reconstructed images were cropped and

processed using HARP (Harwell Automated Reconstruction Processor, Medical Research Council Harwell, Harwell, Oxfordshire, UK) software to create nearly raw raster data files, a file format designed for scientific visualization and image processing. 3D volume rendering and virtual sectioning in the coronal, sagittal, and transverse planes were achieved using 3D Slicer (<https://www.slicer.org>), an open-source platform for image processing and visualization. Measurements in 3D Slicer were made by 2 independent observers after identifying the virtual section that matched with the corresponding histologic slide.

Histology

After image acquisition, the samples were removed from agarose gel and placed in 10% sodium thiosulfate pentahydrate (Sigma-Aldrich) for 4 hours while shaking at room temperature. This reduced the iodine to water-soluble iodide, which removed the iodine staining from the specimen. The samples were washed in 70% ethanol and demineralized in 3% nitric acid (EMD Millipore, Billerica, MA, USA) at room temperature overnight. Demineralization of the baculum facilitates serial sectioning. The following day, the samples were serially dehydrated and embedded in paraffin. 7- μ m serial sections were created using a rotary microtome, mounted, and stained using hematoxylin and eosin. The slides were examined under a SZX-1000 microscope (Olympus, Center Valley, PA, USA) and digital images were captured using a DP72 (Olympus) camera unit. Measurements on the digital slides were made by 2 independent observers using ImageJ (National Institutes of Health; <https://imagej.net>), an open-source image processing software.

Statistical Analysis

Collected measurements were summarized as mean and SD. Agreement between histologic and micro-CT measurement techniques was evaluated using Bland-Altman plots.¹¹ Bias was defined by the central line (mean) and the limits of agreement were demarcated by the outer lines (± 2 SD). Interobserver reliability was assessed using the Lin concordance correlation coefficient. Values higher than 0.99 are considered almost perfect strength of agreement.¹² Statistical analysis was completed using Prism 7 (GraphPad Software, La Jolla, CA, USA).

RESULTS

10 mouse penises were dissected from C57BL/6J adult wild-type mice. Measurements of the male urogenital mating protuberance (MUMP), baculum, and penile glans lengths with conventional histologic and micro-CT sectioning are presented in scatterplot form in Figure 1. The Lin concordance correlation coefficient, a measure of interobserver agreement, was 0.9995 (95% CI = 0.9990–0.9997) for histologic sections and 0.9982 (95% CI = 0.9963–0.9991) for micro-CT sections. Representative sections acquired and measured on histologic and micro-CT sections are presented in Figure 2. Bland-Altman plots for each measured landmark are presented in Figure 3. The mean difference between histologic and micro-CT measurements for MUMP length was -0.029 mm with limits of agreement spanning from -0.15 to 0.096 mm. The mean difference for the baculum length was 0.022 mm with limits of agreement spanning from -0.11 to 0.15 mm. The mean difference for penile glans length was -0.068 mm with limits of agreement spanning from -0.27 to 0.13 mm. Because the measurements appeared equally scattered above and below the line of bias, there did not

appear to be a bias toward overestimation or underestimation of measured lengths. Furthermore, the limits of agreement were narrow and all measurements were within these boundaries.

DISCUSSION

Because significant differences exist in the structure and development of the human vs murine penis, a careful understanding of these processes is necessary for translational research, especially for molecular controls of penile development and birth defects. Although the features of human hypospadias are immediately recognizable on physical examination (eg, malpositioned urethra meatus, dorsal hooded foreskin, and ventral curvature), the findings associated with mouse hypospadias are much subtler.^{5, 13} The human urethral meatus and penile urethra are formed from a urethral plate, whereas in mice the urethral plate contributes only to penile urethra formation.^{5,13,14} Formation of the urethral meatus in the mouse results from the fusion of the MUMP and MUMP ridge. Disruption to this process results in abnormalities of the MUMP (Figure 4), absence of a ventral cleft, and a frenula attachment to the inner portion of the external prepuce. Furthermore, the corpora cavernosa urethrae (homologous to the human corpus spongiosum) are hypoplastic.^{5,13}

Although anomalies in urethral meatus formation can be seen at macrophotography or scanning electron microscopy, characterization of the MUMP, corpora cavernosa urethrae, and baculum require serial sectioning. Unfortunately, inherent difficulties with orienting the specimen within a paraffin block and obtaining consistently satisfactory sections with the microtome limit reliable morphometric analysis. For these reasons, the appeal of whole-volume imaging for morphometric characterization of the murine penis is only natural. Although optical projection tomography is useful for characterizing embryonic genital tubercles, its inability to image thicker and opaque tissues renders it impractical for imaging the adult murine penis. Imaging the murine penis when it is fully mature beyond 30 days of age (puberty) is vital, because many features identified on the embryonic or neonatal genital tubercle might be mistaken for hypospadias.⁵ Micro-CT is well suited for this purpose, because it is non-destructive to the specimen and allows for virtually limitless sectioning in any desired plane (Figure 5).

Early propagation of micro-CT in preclinical research revolved around bone imaging, because of its high contrast in imaging mineralized tissues.¹⁶ The ability to characterize bone with micro-CT has led to volumes of research in orthopedics, endocrinology, and regenerative medicine.¹⁷⁻¹⁹ In the discipline of urology, its use in the study of kidney stones has led to further understanding of stone structure, mineral composition, and the role of Randall plaques.²⁰ The introduction of contrast agents, a concept borrowed from CT in clinical medicine, paved the way for imaging tissues other than bone.^{6,21} Although effective, the use of osmium tetroxide staining has been supplanted by inorganic iodine owing to easier handling and less toxicity. Vascular contrast agents also are available and are used to study renal cortical vasculature and perfusion of glomeruli in mice.²² The pairing of gating to micro-CT (image acquisition only during specific respiratory or cardiac phases) has allowed for in vivo high-resolution imaging of the heart and lungs in the murine model.

The primary limitation of micro-CT use in preclinical imaging is the associated radiation dose.²³ In the setting of in vivo imaging, high doses can result in acute and delayed toxic effects. Repeat studies can result in high cumulative doses. In longitudinal studies in which repeated imaging is required, micro-MRI might be better suited. However, with each new iteration of micro-CT systems, improved detectors and imaging protocols lead to lower radiation doses. Select centers have the ability to perform micro-CT in vivo but are currently limited to lower resolutions secondary to biological motion and the toxic effects of ionizing radiation required for higher-resolution images.²⁴ In certain soft tissues, micro-MRI can still yield superior contrast visualization compared with contrast-enhanced micro-CT. For these reasons, micro-MRI can be considered a complementary technology. In the present study of murine penis morphometric analysis, specimens are ex vivo because the size and gantry design of the micro-CT scanner used does not accommodate in vivo specimens. Despite this limitation, rapid and high-resolution imaging in vitro for phenotyping the murine penis holds tremendous potential for studying knockout murine models for candidate genes in genitourinary development.

We applied the principle of iodine staining to enhance examination of the murine penis with micro-CT. To the best of our knowledge, this represents the first report of micro-CT use in the morphologic analysis of the murine penis. Measurements made by 2 independent observers demonstrated almost perfect strength of agreement. The data presented show excellent agreement between measurements obtained through virtual sectioning at micro-CT and conventional histologic sectioning. The low values of mean discrepancy between histologic and micro-CT examination would not appear to be clinically significant. Although we were limited by invariably imperfect sections at histology, we made every attempt to simulate the identical section when making measurements at micro-CT. Current established methods for characterizing the murine penis have yielded knowledge of penile development and anomalies and the role of the baculum in reproductive success. The added benefits of detail and speed conferred with micro-CT examination have the potential to add to these investigations.

CONCLUSIONS

Micro-CT provides a reliable and non-destructive method for characterizing murine penis morphology. Subsequent embedment of the sample for serial sectioning or histologic examination also is feasible.

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REFERENCES

1. Mai CT, Isenburg J, Langlois PH, et al. Population-based birth defects data in the United States, 2008 to 2012: presentation of state-specific data and descriptive brief on variability of prevalence. *Birth Defects Res A Clin Mol Teratol* 2015; 103:972–993. [PubMed: 26611917]
2. Stanasel I, Le HK, Bilgutay A, et al. Complications following staged hypospadias repair using transposed preputial skin flaps. *J Urol* 2015;194:512–516. [PubMed: 25701546]

3. McNamara ER, Schaeffer AJ, Logvinenko T, et al. Management of proximal hypospadias with 2-stage repair: 20-year experience. *J Urol* 2015;194:1080–1085. [PubMed: 25963188]
4. Bouty A, Ayers KL, Pask A, et al. The genetic and environmental factors underlying hypospadias. *Sex Dev* 2015; 9:239–259. [PubMed: 26613581]
5. Cunha GR, Sinclair A, Risbridger G, et al. Current understanding of hypospadias: relevance of animal models. *Nat Rev Urol* 2015;12:271–280. [PubMed: 25850792]
6. Metscher BD. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol* 2009;9:11. [PubMed: 19545439]
7. Sharpe JOptical projection tomography as a new tool for studying embryo anatomy. *J Anat* 2003;202:175–181. [PubMed: 12647867]
8. Kujoory MA, Hillman BJ, Barrett HH. High-resolution computed tomography of the normal rat nephrogram. *Invest Radiol* 1980;15:148–154. [PubMed: 7372425]
9. Shearer T, Bradley RS, Hidalgo-Bastida LA, et al. Three-dimensional visualisation of soft biological structures by X-ray computed micro-tomography. *J Cell Sci* 2016; 129:2483–2492. [PubMed: 27278017]
10. Feldkamp LA, Davis LC, Kress JW. Practical cone-beam algorithm. *J Opt Soc Am A* 1984;1:612.
11. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–310. [PubMed: 2868172]
12. Lin LI. A concordance correlation coefficient to evaluate reproducibility. *Biometrics* 1989;45:255–268. [PubMed: 2720055]
13. Sinclair AW, Cao M, Shen J, et al. Mouse hypospadias: a critical examination and definition. *Differentiation* 2016;92:306–317. [PubMed: 27068029]
14. Li Y, Sinclair A, Cao M, et al. Canalization of the urethral plate precedes fusion of the urethral folds during male penile urethral development: the double zipper hypothesis. *J Urol* 2015; 193:1353–1359. [PubMed: 25286011]
15. Tannour-Louet M, Han S, Corbett ST, et al. Identification of de novo copy number variants associated with human disorders of sexual development. *PLoS One* 2010;5:e15392. [PubMed: 21048976]
16. Feldkamp LA, Goldstein SA, Parfitt AM, et al. The direct examination of three-dimensional bone architecture in vitro by computed tomography. *J Bone Miner Res* 1989;4:3–11. [PubMed: 2718776]
17. Waarsing JH, Day JS, Weinans H. Longitudinal micro-CT scans to evaluate bone architecture. *J Musculoskelet Neuronal Interact* 2005;5:310–312. [PubMed: 16340117]
18. Laib A, Kumer JL, Majumdar S, et al. The temporal changes of trabecular architecture in ovariectomized rats assessed by MicroCT. *Osteoporos Int* 2001;12:936–941. [PubMed: 11804020]
19. Ho ST, Huttmacher DW. A comparison of micro CT with other techniques used in the characterization of scaffolds. *Biomaterials* 2006;27:1362–1376. [PubMed: 16174523]
20. Williams JC, Lingeman JE, Coe FL, et al. Micro-CT imaging of Randall's plaques. *Urolithiasis* 2014;43:13–17.
21. Johnson JT, Hansen MS, Wu I, et al. Virtual histology of transgenic mouse embryos for high-throughput phenotyping. *PLoS Genet* 2006;2:e61. [PubMed: 16683035]
22. Perrien DS, Saleh MA, Takahashi K, et al. Novel methods for microCT-based analyses of vasculature in the renal cortex reveal a loss of perfusable arterioles and glomeruli in eNOS^{-/-} mice. *BMC Nephrol* 2016;17:24. [PubMed: 26936597]
23. Schambach SJ, Bag S, Schilling L, et al. Application of micro-CT in small animal imaging. *Methods* 2010;50:2–13. [PubMed: 19706326]
24. Badea CT, Drangova M, Holdsworth DW, et al. In vivo small animal imaging using micro-CT and digital subtraction angiography. *Phys Med Biol* 2008;53:R319–R350. [PubMed: 18758005]

Figure 1.

Inter-rater agreement between observers as rated by the Lin concordance correlation coefficient was 0.9995 (95% CI = 0.9990—0.9997) for histologic section (**a**) and 0.9982 (95% CI = 0.9963—0.9991) for micro-computed tomographic section (**b**). MUMP = male urogenital mating protuberance.

Figure 2. Representative sections with measurements made on histologic (a) and micro-computed tomographic (b) sections. BL = baculum length; ML = male urogenital mating protuberance length; PL = penile glans length. Figure 2 is available in color at www.jsm.jsexmed.org.

Figure 3.
Bland-Altman plots. BL = baculum length; ML = male urogenital mating protuberance length; PL = penile glans length.

Figure 4.

Representative coronal micro-computed tomographic sections from a WT and Kank-null mouse (a candidate gene for genitourinary development).¹⁵ The normal bifid configuration of the male urogenital mating protuberance, seen in the WT section, is absent in the Kank-null mouse. WT = wild type. Figure 4 is available in color at www.jsm.jsexmed.org.

Figure 5. Sagittal, coronal, and axial sections acquired by histologic section (a) and micro-computed tomography (b). Anatomic land-marks are labeled. MUMP = male urogenital mating protuberance. Figure 5 is available in color at www.jsm.jsexmed.org.