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Original Research Paper

COMPARISON OF PROFILE COUNTING AND DIAMETER MEASUREMENT FOR ESTIMATING THE LENGTH DENSITY OF SEMINIFEROUS TUBULES OR EPIDIDYMAL DUCT

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ABSTRACT

Transverse testicular sections are often used for histopathological studies and length of seminiferous tubules in the testis is often estimated by the tubule volume divided by the tubules' cross-sectional area, with the area being obtained by measuring the (short axial) diameters of the round (or elliptical) tubule profiles. But unbiased stereological length estimation is best based on counting of tubule profiles on isotropic sections. To determine the bias of length estimation with the diameter measurement, (i) transverse sections (methacrylate embedded) were obtained from a testis of each normal mature male Sprague-Dawley rat (10 rats in total) and isotropic sections obtained (with the orientator) from the other testis of the rat, and (ii) the tubule length densities were estimated with both methods of profile counting and diameter measurement. The results demonstrated that the method of diameter measurement on transverse or isotropic sections overestimated the length density by 20%-25% compared with profile counting on isotropic sections, probably mainly because of underestimation of the tubules' cross-sectional area estimated from the short-axial diameters. Length density of the epididymal duct in the epididymis was also estimated with profile counting in the present study, using transverse and isotropic sections for comparison, and a significant statistical difference was not found between the results obtained with the transverse and isotropic sections.

Keywords: epididymal duct, isotropic sections, length density, seminiferous tubules, stereology.

INTRODUCTION

The testis is densely packed with seminiferous tubules that are predominantly parallel to the long testicular axis (Fig. 1), thus transverse testicular sections perpendicular to the axis, which facilitate staging of tubules and recognition of spermatogenic cells, are traditionally used for histopathological studies (Clermont & Huckins, 1961; Wreford, 1995). Moreover, the tubules are cylindrical in shape and many round profiles of the tubules can be seen on such sections (Fig. 2), therefore the tubule length was often estimated with the method of diameter measurement: tubular length equal to the tubular volume divided by the tubules' cross-sectional area that was estimated with measurement of round or elliptical tubule profiles' (short axial) diameters (Zhengwei *et al.*, 1997; Zhang *et al.*, 2002; Yang, 2012; Ma *et al.*, 2016). The length of epididymal duct densely packed in the epididymis can also be estimated with this method (Wen & Yang, 2000) although it is apparently less suitable since the epididymal duct is less regular in orientation and of a large variation in diameters (Fig. 2) (Guo *et al.*, 2019).

However, the diameter measurement method is a biased, model-based method, subject to bias from the assumption of cylindrical shape. It has been well recognized that length of tubule-like structures is best estimated unbiasedly with counting of profiles (transections of the structures) on isotropic (uniform random in terms of orientation in 3D space) sections (Gundersen *et al.*, 1988; Mattfeldt *et al.*, 1990; Nyengaard, 1999; Gundersen, 2002; Yang, 2012).

We recently showed with isotropic sections that the method of diameter measurement, compared with that of profile counting, overestimated the length of renal tubules by as much as 43% (Wang *et al.*, 2020). The present study was therefore designed to see how much bias would result from the method of diameter measurement in the length estimation of seminiferous tubules or epididymal duct. For efficient comparison between methods, bilateral testes and epididymides were utilized, with

(i) isotropic sections being obtained from organs on one side and transverse sections from the organs on the other side, and (ii) both diameter measurement and profile counting being performed on isotropic or transverse sections for the estimation of seminiferous tubule length density and profile counting being performed for the estimation of epididymal duct length density.

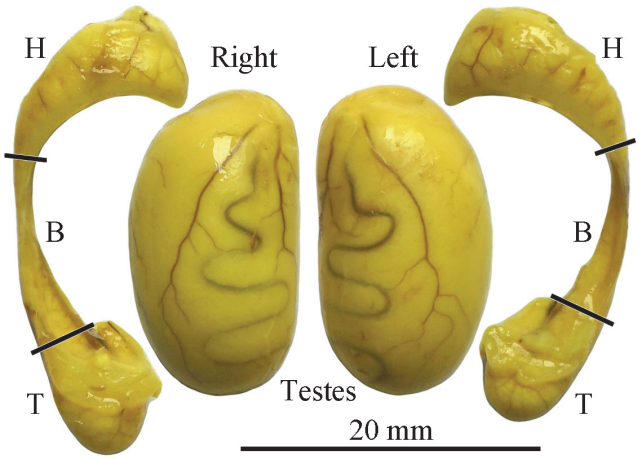

Fig. 1. *Lateral view of the bilateral testes and epididymides (after fixation in Bouin's solution of yellow color) from a normal mature male Sprague-Dawley rat. The Y-axis of this figure is approximately parallel to the long axis of either testis. Either epididymis is divided into 3 parts - epididymal head (H), epididymal body (B) and epididymal tail (T) - by 2 straight cutting lines, which are approximately perpendicular to the curvy long axis of either epididymis.*

MATERIALS AND METHODS

Animals and Organs

Ten normal male Sprague-Dawley rats, weighing 249-273 grams and aged approximately 3 months, were obtained as a part of our previous project (Guo *et al.*, 2019 & 2024). On removal from the abdominal cavity after euthanasia with intraperitoneal injection of pentobarbital sodium (50 mg/kg), the bilateral testes and epididymides from each rat were immersion fixed in Bouin's fluid for 48 hours and then stored in 70% ethanol before preparation of sections. One testis and the ipsilateral epididymal head (caput) and tail (cauda) on one side (left or right alternately chosen) of each rat were used to obtain isotropic sections of the organs; the other testis and epididymal head/tail on the other side of the rat used to obtain transverse sections.

Dissected from the surrounding tissue, each organ (Fig. 1) was weighed and its density (g/cm^3) measured to calculate its volume (Yang, 2012; Guo *et al.*, 2019). The boundaries of the epididymal head and tail were defined by 2 straight cutting lines (Fig. 1): one at the vasal end of the epididymal tail and the other at the middle of the epididymal head and body (Xu *et al.*, 2022).

Preparation of Isotropic Sections

Isotropic sections were obtained with the orientator, a practical method of determining isotropic random planes (Mattfeldt *et al.*, 1990). Briefly, each testis was first cut into 2 halves (half-testes) via a transverse cut through the middle of testis; then each half-testis, epididy-mal head or epididymal tail was cut into 3-6 parallel tissue blocks along isotropic planes, with 2 systematic (equally-spaced or alternate) blocks being sampled and embedded and one intact tissue section being cut (along isotropic planes) and stained from each block for measurement.

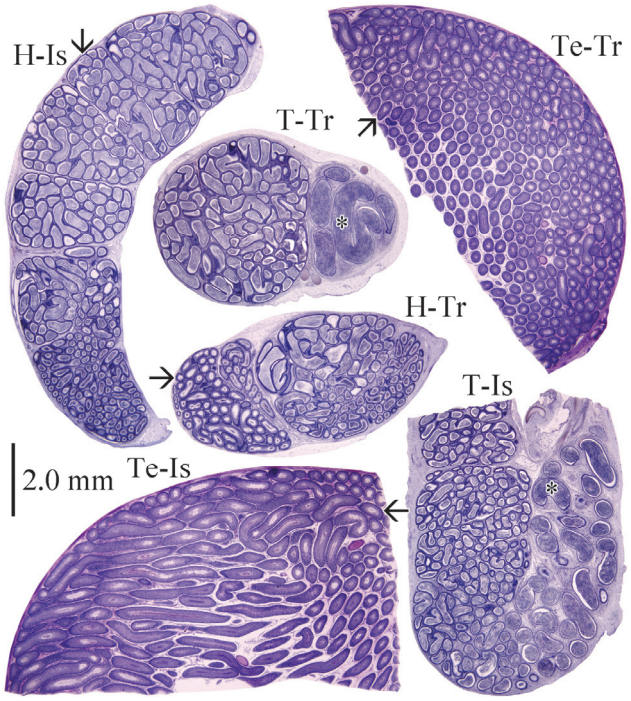

Fig. 2. *Some transverse (Tr) and isotropic (Is) sections of the testes (Te), epididymal heads (H) and epididymal tails (T). The isotropic sections shown in this figure were roughly perpendicular to the transverse sections of the testes or epididymides. The testicular sections were stained with periodic acid-Schiff's reagent and hematoxylin and the epididymal sections stained with hematoxylin. ↗/←, an elliptical seminiferous tubule profile (with a lumen) near an edge of the testicular section; ↓/→, an epididymal duct profile, filled with 2 masses of spermatozoa (↓, C-shaped duct profile) or almost devoid of spermatozoal mass (→, circular duct profile with a lumen), near the boundary of the epididymal section; *, in the lumen of a large epididymal duct profile (C-shaped) filled with sperm mass.*

Isotropic sectional planes for each half-testis were determined as we previously described (Wang *et al.*, 2020; Wang *et al.*, 2021). First a vertical cut (the first cut shown in T1 of Fig. 3) was performed through the middle of each half-testis placed on an angular board (horizontal plane); then isotropic cuts (second cuts shown in T2 of Fig. 3) were performed through the tissue (half of the half-testis), resulting in 4 parallel tissue blocks with isotropic cut-surfaces (planes).

For determination of isotropic epididymal sectional planes, each epididymal head or tail was first embedded, temporarily, in a cylindrical block of agar, with the 2 ends of the head or tail being placed at the 2 ends of the agar block. After random (blinded) rolling and proper aligning of the agar block on the angular board, isotropic cuts were performed through the agar block using a row of equally-spaced Leica knives (normally used for cutting paraffin sections), see E2 of Fig. 3, the cuts resulting in 3-6 parallel tissue blocks with isotropic cut-surfaces (planes). Note, the random rolling of the agar block corresponded to the first imaginary vertical cut shown in E1 of Fig. 3.

Non-independent uniform random numbers (between 0 and 1) needed for determination of isotropic directions (cuts) with Excel equations (see the legend of Fig. 3) were predetermined in a systematic manner as following: 0.025 (for one half-testis and the epididymal head of animal 1) and 0.525 (for the other half-testis and the epididymal tail of animal 1), 0.575 (half-testis and epididymal head of animal 2) and 0.075 (other half-testis and epididymal tail of animal 2) ... 0.425 (half-testis and epididymal head of animal 9) and 0.925 (other half-testis and epididymal tail of animal 9), and 0.975 (half-testis and epididymal head of animal 10) and 0.475 (other half-testis and epididymal tail of animal 10). Thus, the orientator used in the present study is a systematic version of the orientator (Mattfeldt *et al.*, 1990).

As we previously reported (Wen & Yang, 2000; Zhang *et al.*, 2002; Ma *et al.*, 2016; Guo *et al.*, 2019), tissue blocks (4 per testis and 2 per epididymal head or tail) were embedded in hydroxyethyl methacrylate (Historesin by Leica Microsystems Nussloch GmbH, Germany) and sections were cut with a semi-automatic microtome (RM2235, Leica Biosystems Nussloch GmbH, Germany). One intact section (thickness set by the microtome 10 μm) was obtained from each block and stained with periodic acid-Schiff's reagent and hematoxylin (testicular sections) or hematoxylin alone (epididymal sections) for stereological measurement. Care was taken in embedding and sectioning to ensure that the final sections were parallel to the isotropic cutting planes determined as above.

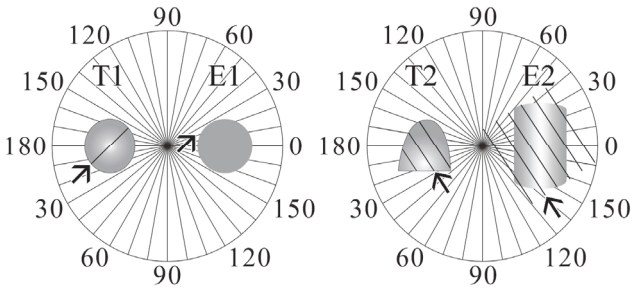

Fig. 3. *Schematic illustration showing the method (orientator) of obtaining isotropic sections. Blocks (T1, E1, T2 and E2) are placed on a flat board of uniform angles (0°−180°). T1: a half testis; T2: a half of T1. E1: a cylindrical block of agar (base on the board) embedding an epididymal head or tail (unseen); E2: E1 rotated with the side surface on the board. ↗: the first (vertical) cut (perpendicular to the board) along a uniform random angle (45° shown in the figure) determined by an Excel (Microsoft Office software) equation "=RAND()*180"; ↖: the second (isotropic) cut (perpendicular to the board) along a weighted random angle (123° shown in the figure) determined by an Excel equation "=acos(1-2*RAND())*180/PI()". Note, (i) "RAND()" is an independent, uniform random number between 0 and 1; (ii) the first cut of E1 is an imaginary cut, without cutting the block; (iii) the block (left after the first cut) for the second cut (T2 or E2) should be rotated in such a way that the "cutting line" – intersection between the first cut and the board – is parallel to the 0°−180° line on the board; (iv) testicular or epididymal sections cut along (parallel to) the second cut-surface (↖) are isotropic sections.*

Preparation of Transverse Sections

Each testis was cut into 6 slices and 2 slices (the 1st and 4th, the 2nd and 5th or the 3rd and 6th slices) were systematically sampled, with each sampled slice being further cut into 2 halves (semi-circular blocks); each epididymal head or tail was cut into 4 blocks and 2 systematic blocks (the 1st and 3rd or the 2nd and 4th blocks) were sampled. Each testicular or epididymal block was embedded and one intact section was obtained from each block as described above. Care was taken in the processing to ensure that the final sections obtained were transverse sections perpendicular to the respective organ's long axis (Fig. 1).

Length Density of Seminiferous Tubules

Each testicular section was observed on a computer screen (full-screen magnification × 255) through a × 10 objective lens (Uplan FL N, numerical aperture 0.30) of an Olympus BX53 microscope equipped with a stereology system (newCAST, Visiopharm, Denmark). Equally spaced fields of view were systematically

sampled on the whole section, the distance between fields along the x- or y-axis being set at 1.5 mm. A Gundersen's forbidden-line frame – the unbiased frame for uniform counting or sampling of profiles (Gundersen *et al.*, 1977; Wang *et al.*, 2021), 600 × 450 μm, was superimposed on the lower left part of the field for counting of tubule profiles (below) according to the unbiased counting rule. 2 × 2 evenly distributed test-points (centers of cross-shaped lines) were also superimposed on the field and counted for estimation of (i) the volume fraction of the tubules in the testis and (ii) the area of frames used for the profile counting (Gundersen *et al.*, 1988; Yang, 2012). There were some fields near the artificial edge of sections where there were some incomplete tubule profiles and it was difficult to determine whether they should be counted or not. These fields were not used for stereological measurement.

A tubule profile was defined as one that was encircled by the continuous basement membrane of the tubule (Fig. 2) and the number (1 mostly) of tubule lumens (encircled by the tubule wall - seminiferous epithelium) was regarded as the number of tubule profiles (Wang *et al.*, 2020; Wang *et al.*, 2021).

Of the tubule profiles which were counted, the single-luminal (with one lumen) and approximately circular or elliptical profiles were further measured: the diameter (for each circular profile) or the short axial diameter (for each elliptical profile) was additionally measured as a diameter of the tubule therein (Zhengwei *et al.*, 1997; Zhang *et al.*, 2002; Yang, 2012; Ma *et al.*, 2016; Wang *et al.*, 2020; Wang *et al.*, 2021).

With the method of profile counting, the length density of the tubules for each testis (length per volume of testis) was calculated by: multiplying the number of tubule profiles per area of sections (frames) by constant 2 (Gundersen *et al.*, 1988; Nyengaard, 1999; Gundersen, 2002; Yang, 2012), *i.e.* 2 × total number of profiles counted / total area of frames used for the counting on the sections from the testis. With the method of diameter measurement, the length density of the tubules for each testis was calculated by: dividing the volume fraction of the tubules by the cross-sectional area of the tubules. The cross-sectional area was calculated by: multiplying the average of the squared diameters by constant (π/4) (Zhang *et al.*, 2002; Yang, 2012; Wang *et al.*, 2020; Wang *et al.*, 2021).

Length Density of Epididymal Duct

Each complete epididymal section, observed on the computer screen through a × 1.25 objective lens (Plan-Apo N, numerical aperture 0.04) of the Olympus BX53 microscope equipped with a CCD (DP73), was photographed as a TIF file (4800 × 3600 pixels, 34-50 MB). The section image was then observed and measured using an Adobe Photoshop 8.0.1 software, with software-generated vertical and horizontal lines (distance between adjacent vertical or horizontal lines being 1.07 mm) superimposed on the image. All duct profiles (below) on each section were counted; test-points (intersections between vertical and horizontal lines) were counted to estimate area of the section (Xiang *et al.*, 2018).

A duct profile was defined as one encircled by the basal part (a layer of darkly stained nuclei) of the duct epithelium (Fig. 2), *i.e.* each profile contained a lumen (mostly filled with a mass of spermatozoa) and/or some apical part (brighter cytoplasm) of the duct epithelium. Nine of the 40 isotropic sections and 2 of the 40 transverse sections had an artificial edge because of sectioning (e.g. T-Is in Fig. 2) and each incomplete duct profile near the edge was counted as half a profile.

The length density of the epididymal duct in each epididymal head or tail (length per volume of the head or tail) was calculated by: 2 × total number of profiles / total area of sections (above).

Error of Length Density Estimation

Four sections from each testis (2 sections from each half-testis) or 2 sections from each epididymal head/tail constitute a systematic sample for the testis or head/tail. Separately calculating the length density based on measurement of the 2 testicular sections from each half-testis or measurement of each epididymal section, we obtained 2 sub-sample length densities (x_1 and x_2) for each testis or head/tail. Then we calculated an approximate sampling error (within-organ coefficient of error) of the length density estimation for each testis or head/tail by (Yang *et al.*, 2000): $(1/2)^{1/2} \times | x_1 - x_2| / (x_1 + x_2)$.

Roundness of Seminiferous Tubules

To have some objective evidence on whether the model-based method of diameter measurement is suitable for the length estimation of seminiferous tubules, 2 transverse sections from each testis (1 from each half-testis) were randomly chosen, in the sub-group of testes for transverse sections, for re-observation with the × 10 objective lens on the screen as described above. This time, we measured both the short and long axial diameters (perpendicular to each other) of each single luminal, circular/elliptical seminiferous tubule profile that was sampled with the forbidden-line frame. The ratio of the 2 diameters was calculated to reflect the roundness of the seminiferous tubules.

Statistics

Comparison of length densities (estimated with profile counting or diameter measurement) or related results between transverse sections on one side and isotropic sections on the other side (*i.e.* between the bilateral organs) was made using the paired *t*-test, and so was the within-testis comparison of length densities between profile counting and diameter measurement on the transverse or isotropic sections (Table 1). The statistical significance of difference was set at $P < 0.05$.

Normality tests were performed for all the data sets, for justification of the use of paired t-tests. Only the test for testicular volumes (Table 1) failed, thus a signed rank test was also tried for the data, with the *P* values being 0.18 (paired *t*-test) and 0.11 (signed rank test), respectively.

RESULTS

General Results

The histology of the organs in the present study was essentially comparable to what we described previously for the normal mature Sprague-Dawley rat testis and epididymis (Ma *et al.*, 2016; Guo *et al.*, 2019). Apparently, seminiferous tubule profiles are mostly round or elliptical on transverse sections and more tubule profiles are irregular in shape on isotropic sections; the epididymal duct profiles are irregular in shape on both transverse and isotropic sections (Fig. 2). The ratio of the volume of the left testis, epididymal head or epididymal tail to that of the contralateral right organ of the same animal was 0.997 (coefficient of variation 2.3%, n = 10), 0.981 (6.3%), or 0.990 (5.2%) on average, respectively, without significance of differences between the bilateral organs (paired *t*-test: $P = 0.71$ for testes and $P = 0.19$-0.37 for epididymides).

In the sub-group with isotropic sections, the mean volume of the 10 ipsilateral testes or epididymides (5 on the left side and 5 on the right side) from the 10 animals, estimated with weights and densities of the fresh organs stored in 70% ethanol (Yang, 2012; Guo *et al.*, 2019), was 1354 (coefficient of variation, 4.9%) mm^3 for testes or 407 (23.6%) mm^3 for epididymides, respectively. The individual difference (*i.e.* between-animal variation) or bilateral difference (within-animal variation, between left and right) of testicular volumes was small (Table 1). The bilateral difference of epididymal volumes was also small although their individual difference was evident (Table 1). The epididymal body, not studied in the present research, accounted for 10.6% (15.6%) of the total

Table 1: *Length densities (obtained with different methods) and related results.*

	Isotropic sections	Transverse sections	Ratio (Trans/Iso)
Length density (mm/mm^3), seminiferous tubules			
Profile counting	8.22 (27.4%) $^{*,\#}$	10.55 (13.7%)	1.381 (33.8%)
[Within-organ coefficient of error]	[11.3% (62.4%)]	[8.7% (87.2%)]	---
Diameter measurement	9.87 (8.1%)	10.27 (7.7%)	1.045 (8.1%)
[Within-organ coefficient of error]	[4.0% (75.9%)]	[3.2% (63.6%)]	---
Length density (mm/mm^3), epididymal duct			
Profile counting (epididymal head)	12.09 (18.2%)	11.92 (37.6%)	1.001 (32.3%)
[Within-organ coefficient of error]	[17.7% (43.0%)]	[22.6% (20.7%)]	---
Profile counting (epididymal tail)	7.77 (28.5%)	6.95 (14.3%)	0.930 (19.3%)
[Within-organ coefficient of error]	[13.5% (61.0%)]	[7.7% (40.1%)]	---
Related results			
Volume of testis (mm^3)	1354 (4.9%)	1341 (4.9%)	0.991 (2.1%)
V_V of seminiferous tubules in the testis (%)	71.1 (5.4%)	71.6 (4.3%)	1.009 (6.3%)
Mean D of seminiferous tubule profiles (μm)	302 (4.1%)	297 (4.1%)	0.984 (4.5%)
Mean D^2 of seminiferous tubule profiles (μm^2)	92216 (8.4%)	89121 (7.9%)	0.970 (8.7%)
Volume of epididymal head (mm^3)	189 (17.6%)	189 (18.2%)	1.000 (6.7%)
Volume of epididymal tail (mm^3)	171 (30.0%)	169 (29.0%)	0.990 (5.2%)

Data: mean (coefficient of variation) of length densities or related results and their ratios obtained from 10 organs, with the within-organ coefficient of error of the length density estimation in square brackets. V_V: volume fraction; D: single luminal, round or elliptical tubule profile's (short axial) diameter. **Design:** testes and epididymides were obtained from 10 normal mature male Sprague-Dawley rats; methacrylate-embedded isotropic sections were obtained from one testis and the ipsilateral epididymal head and tail on one side (left or right, alternately chosen) of each rat and transverse sections (perpendicular to the respective organ's long axis) obtained on the other side of the rat. **Methods:** length density (length per volume of organ) of the seminiferous tubules or epididymal duct was estimated with the stereological method of profile counting (based on the number of tubule/duct profiles per area of section) or diameter measurement (based on the tubules' volume fraction and cross-sectional area that was estimated with the diameters). **Ratio (Trans/Iso)**: ratio of the result obtained on the side with transverse sections to that on the other side with isotropic sections. **Statistical comparison** (paired *t*-test): $P = 0.014$ between isotropic and transverse sections (same line)*, $P = 0.046$ between profile counting and diameter measurement (same column)$^{\#}$, and $P = 0.15$-0.99 for other same line or column data sets.

epididymal volume on average in the sub-group of epididymides for transverse sections and 11.2% (24.7%) in the sub-group for isotropic sections.

With profile counting for length estimation, the average numbers of seminiferous tubule profiles counted per testis were 104 (14.8%) and 78 (32.8%) in the sub-groups of transverse and isotropic sections, respectively, with the numbers of multi-luminal (all 2-luminal) profiles accounting for 8.5% (21.8%) and 16.4% (35.0%) of the total profile numbers in the respective sub-groups. The number of fields not used for counting due to the section edge effect was less than 9%.

The average numbers of epididymal duct profiles counted were 200 (27.2%) per epididymal head and 158 (12.4%) per epididymal tail in the sub-group with transverse sections and 306 (37.3%) and 217 (25.1%) in the sub-group with isotropic sections. Three of the 40 transverse epididymal sections and 9 of the 40 isotropic epididymal sections had incomplete duct profiles along the artificial section edge, the number of such profiles per epididymal head/tail being 0.56 on average.

With diameter measurement for seminiferous tubule length estimation, the average numbers of short axial diameters measured per testis were 80.4 (17.2%) and 40.7 (37.7%) in the sub-groups with transverse and isotropic sections, respectively.

Volume Fraction, Diameter and Roundness of Seminiferous Tubules

There were no significant differences in the volume fraction, mean diameter or mean squared diameters between results from transverse and isotropic sections (Table 1). In particular, both the bilateral and individual differences of the volume fraction or mean diameter were small, as can be seen by the coefficients of variation (4.1%-5.4%) of the results and the coefficients of variation (4.5%-6.3%) of the ratios (Table 1).

An average of 28.6 (20.3%) tubule profiles per testis were sampled for estimation of their roundness on transverse sections. The mean short axial diameters were 297 (3.9%) μm and the mean short-to-long axial diameter ratios were 0.730 (2.7%). The percentages of the tubule profiles whose short-to-long axial diameter ratios were larger than 0.95, 0.90 and 0.85 were 4.5% (73.8%), 11.6% (54.0%) and 19.4% (39.8%), respectively.

Length Density of Seminiferous Tubules

The mean length density estimated with diameter measurement on transverse sections was 25% [= (10.27/8.22) - 1] larger than that estimated with profile counting on isotropic sections (Table 1); the ratio of the former to the latter results (results from the bilateral organs) was 1.36 (34.1%) on average; and the *P* value of the paired *t*-test for comparison between the 2 results was < 0.05. Since the number of orientations (directions) of isotropic sections was limited for each testis, it is more reliable to compare the mean results for evaluation of the overall bias. That is, it is more reliable to conclude that diameter measurement on transverse sections overestimated the length density by 25% (not 36%) compared with profile counting on isotropic sections.

Using the same method of profile counting, use of transverse sections overestimated the length density by 28% compared with isotropic sections; using the same method of diameter measurement, results with both sections were not significantly different (Table 1).

Using the same isotropic sections, diameter measurement overestimated the length density by 20% [= (9.87/8.22) - 1] compared with profile counting. Using the same transverse sections, results with both methods were similar (Table 1).

Length Density of Epididymal Duct

For the epididymal head or tail, the length density estimated with transverse sections was not significantly different from that with isotropic sections (Table 1).

Regarding the sampling of epididymal heads and tails as a stratified sampling (Yang, 2012), we obtained the overall length density of the epididymal duct in the "epididymal head and tail" (*i.e.* head and tail being regarded as one "organ") based on to their relative volumes (weights in statistical terms): 10.02 (16.3%) mm/mm^3 in the sub-group of organs for isotropic sections.

Error of Length Density Estimation

In the length density estimation of seminiferous tubules, the coefficients of variation (individual difference) with diameter measurement (~8%) on transverse and isotropic sections were smaller than those with profile counting (14%-27%); in terms of the coefficients of error (within-organ sampling error), the difference (3%-4% with diameter measurement versus 9%-11% with profile counting) was more evident (Table 1).

In the length density estimation of epididymal duct with profile counting, the variation was 14%-38% while the error was 8%-23% (Table 1).

DISCUSSION

Bias, *i.e.* underestimation or overestimation of the true result for an organ or a group of organs, may result from the use of biased samples or methods. Generally it

is not advisable to use biased methods in stereological studies since the bias cannot be estimated if not compared with unbiased methods and the increase of sample sizes does not correct the bias.

Bias: Seminiferous Tubules

It is basic geometry: the height or length of a cylinder = the cylindrical volume / the base or cross-sectional area. Use of this idea for the length estimation of seminiferous tubules can be traced back to 1925, but it was practically difficult to determine the cross-sectional area, see the review by Aherne & Dunnill (1982). Haynes (1964) tried to solve the problem by measuring and squaring the diameters of round profiles in different size classes and giving each class of profiles a weight (coefficient assigned to calculate the mean of squared diameters) based on point counting. But the idea was biased because the weights based on points were profile area weighted, not profile number weighted and therefore not tubule length weighted (Yang, 2012). We further developed the method of diameter measurement (Zhengwei *et al.*, 1997; Yang, 2012) as described in the present study. The key points in our method are uniform sampling (number-weighed sampling on sections) of profiles using Gundersen's frame, measuring the short-axial diameters, and squaring and averaging all measured diameters to calculate the cross-sectional area. We have been using this model-based method to estimate the size and length of seminiferous tubules or renal tubules (e.g. Zhengwei *et al.*, 1997; Zhang *et al.*, 2002; Ma *et al.*, 2016; Wang *et al.*, 2020; Wang *et al.*, 2021). Like all model-based approaches, however, the method is essentially not bias-free. As demonstrated in the present study, the method of diameter measurement on transverse or isotropic sections overestimated the length density by as much as 20%-25% compared with the unbiased estimate by means of profile counting on isotropic sections.

Part of the bias (overestimation) might arise from sampling of profiles for diameter measurement. For example, more profiles suitable for measurement might be formed from thinner, straighter parts of the tubules with transverse sections. But considering the relatively small variation of tubule diameters, this might not be a major source of bias. The bias might mainly result from assumption of the tubules as "round cylinders". Judging by the tubular shapes and roundness on transverse sections, the tubules may in fact be more of "flattened cylinders". For a "flattened cylinder" with an elliptical base whose short-to-long diameter ratio is 0.85, its true base (cross-sectional) area is ($= \pi \times 0.85 \times 1.0$ u^2). If the cross-sectional area is estimated by the short-axial diameters of the transections (elliptical profiles) of the "flattened cylinder", the result would be approximately ($\pi \times 0.85 \times 0.85$ u^2), underestimated by 15%, which would result in an overestimation of the cylinder's length by 18%. The flattened shape of the tubules may be explained by the fact that the tubules are crowded in the testis. Using isotropic sections, the method of diameter measurement overestimated the length of renal tubules, which appear more crowded in the kidney (Wang *et al.*, 2020; Wang *et al.*, 2021), by 43%, perhaps indicating a more flattened shape of the renal tubules.

Bias: Epididymal Duct

The present study demonstrated that there was no significant difference between transverse and isotropic sections in the length density of the epididymal duct estimated with profile counting. And the difference, if there was, seemed to be an underestimation of less than 10% with transverse sections. This suggests that the highly coiled epididymal duct in the epididymis is largely isotropic (in orientations) on the whole (considering all parts of the duct), thus it is advisable in practice to utilize transverse epididymal sections, rather than isotropic sections whose preparation is much more difficult or inconvenient (Fig. 3), to estimate the duct length, among other parameters.

Considering the large size variation and the curvy shape or isotropic orientation of the epididymal duct (Fig. 2), the method of diameter measurement is not suitable for estimation of the duct length density. At least it is hard to decide which profiles to sample for measurement. Using similar epididymal samples, we once obtained the length density of epididymal duct with diameter measurement on transverse sections (Wen & Yang, 2000) and the result (~18 mm/mm^3) suggested an overestimation of as much as 80% compared with the result obtained in the present study with profile counting on isotropic sections. In this epididymal case, a considerable part of the bias might be contributed by sampling of profiles: the regularly shaped (single-luminal, round or elliptical) duct profiles that we sampled for diameter measurement tended to be smaller profiles.

Sampling Error

In spite of overestimation of seminiferous tubule length, the method of diameter measurement has a much smaller sampling error compared to the method of profile counting, which is apparently due to the much smaller variation in the sizes (diameters) of the tubules than in the number of tubule profiles (per area). So when transverse testicular sections are used, it is advisable to (i) measure the short axial diameters, which well reflect the tubule sizes and even the spermatogenic status (Zhang *et al.*, 2002; Ma *et al.*, 2016), and (ii) estimate

the tubule length, if the bias can be tolerated, with the measured diameters as described in the present study.

More epididymal duct profiles were counted per epididymal head or tail, compared with seminiferous tubule profiles counted per testis, but the error of the duct length density estimation appeared to be larger than the tubule length density estimation. This might be due to a more variable number of duct profiles (per area of section) at different regions of the epididymis (Fig. 2), in which there was a gradual increase in the duct diameters from its testicular end to its vasal end (Guo *et al.*, 2019). The implication is that more epididymal sections are needed for more accurate estimation of the duct length density.

Paired Design

The present study was of an optimized paired design: the same sections were used to compare profile counting and diameter measurement; the paired organs used to compare transverse sections and isotropic sections were of a small bilateral difference, thus the effect of individual difference on the comparison was minimized (Xu *et al.*, 2022).

Total Length per Organ

Although it is irrelevant to comparison of stereological methods in the present study, it is worth mentioning that, like all density or ratio estimates, length density (length per volume of organ) estimated is often used for further estimation of total length (per organ) to avoid the serious problem of reference trap in practical studies (Yang, 2012; Guo *et al.*, 2024). The principle is simple: the total amount is the product of the density and the organ volume. But the density is estimated after a series of tissue processing procedures (e.g. embedding of tissue blocks and preparation of sections) and after the tissue processing the volume of organ may change. Readers interested in the correction of stereological results for the "fresh" organ before tissue processing may refer to the recent work by Guo *et al.* (2024), in which new concepts of actual and virtual volumes of the organ after processing are proposed for the correction.

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