The Convoluted Tubules of the Nephron Must Be Considered Elliptical, and Not Circular, in Stereological Studies of the Kidney

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Keywords

Distal convoluted tubule · Kidney · Mouse · Proximal convoluted tubule

Abstract

Introduction: The diameter and area of the proximal convoluted tubule (PCT) and the distal convoluted tubule (DCT) are of the main parameters analyzed in stereological studies of the kidney. However, there is no consensus about if the PCT and DCT should be considered circular or elliptical in shape.

Objective: To analyze if there are significant differences in the diameter and area of the PCT and DCT, depending on whether they are considered circular or elliptical. **Methods:** Paraffin-embedded sections of kidneys from CD1 mice were stained with hematoxylin and eosin and examined using a light microscope. Images were captured using a camera linked to image analysis software. A short diameter (d) and a long diameter (D) were measured in both PCT and DCT. A small circular area (SCA), a large circular area (LCA), and an elliptical area (EA) were calculated with mathematical formulas that incorporate d and D values, while a program area (PA) was provided by the software. **Results:** There was a significant difference between d and D in both PCT ($F = 1.354$, Sig = 0.000) and DCT ($F = 4.989$, Sig = 0.000). Also, there were

significant differences in the tubular areas in both PCT ($F = 34.843$, Sig = 0.000) and DCT ($F = 22.390$, Sig = 0.000); circular areas were different from elliptical areas (SCA and LCA vs. EA and PA). **Conclusion:** The convoluted tubules of the nephron must not be considered circular, but rather elliptical; care should be taken every time the tubules are analyzed in stereological studies of the kidney, especially when evaluating their diameters and areas.

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Introduction

Morphometry is the term applied to the measurement of shape or structure. Stereology, sometimes used synonymously, refers to mathematical methods based on geometric probability that allow to measure three-dimensional characteristics from two-dimensional images [1].

Stereological methods have been used to examine how the kidney reacts to trauma, disease, or experimental conditions. Exact knowledge of the number, size, and distribution of the kidney components in those situations provides important information about its organization and function [2].

Fig. 1. Schematic representation of a PCT (**A**) and a DCT (**B**) showing the morphometric parameters analyzed. *d*, the short diameter, is defined as the length of the shortest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM; *D*, the large diameter, is defined as the length of the largest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM. The tubular area, defined as the space bounded by the BM (blue line), was measured in 4 ways. The SCA was calculated by using the formula for the area of a circle, $A = \pi \cdot r^2$, where *r* is one-half of *d*. The LCA was calculated by using the same formula, but replacing the *d* value by *D*. The EA was obtained by using the formula for the area of an ellipse, $A = \pi \cdot d' \cdot D'$, where *d'* and *D'* are one-half of *d* and *D*. The PA was provided by the image analysis software used. PCT, proximal convoluted tubule; DCT, distal convoluted tubule; BM, basement membrane; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.

In addition to the glomerulus, the proximal convoluted tubule (PCT) and the distal convoluted tubule (DCT) are the most widely analyzed structures in stereological kidney research. In turn, the diameter and less frequently the area are the main measurements evaluated in the PCT and DCT.

However, there is no consensus in the literature on how tubular diameter (or diameters) should be evaluated. Several authors measure only one diameter, as if the tubules were circular [3–10]. On the contrary, other authors consider that the tubules have an elliptical shape and report the short diameter (minimum, proximal), sometimes along with the large diameter (maximum, distal) [11–20]. Even more, other authors measure in the same sample some tubules as circles and others as ellipses [21, 22]. Besides, the calculation of the tubular area may depend on the way the diameter is evaluated [10, 15].

Other approaches to the study of the nephron tubules also do not consider whether they are circular or elliptical. Since the *in vivo* analysis of the function of the tubules is significantly difficult, mathematical models have been created, but they only consider tubules as circular cylinders [23, 24]. Interestingly, fractal analysis of nephron tubules in paraffin-embedded sections stained with routine histological methods has revealed that they are, in fact, fractals [25, 26]. Since a circle is a nonfractal structure

[27], tubules could have another shape. The purpose of this work was to analyze if there are significant differences in the diameter and area of PCT and DCT, depending on whether they are considered circular or elliptical in the stereological analysis.

Materials and Methods

Animals and Experimental Design

Animals used were described in previous papers, in which stereological methods were employed to examine the lung [28, 29]. Male CD1 mice were maintained in standard conditions in stainless steel cages (temperature: 18–21°C; relative humidity: 55–60%; circadian rhythm: 12 h of artificial light per day, 12 h of darkness per day). The standard chow and water were available *ad libitum*. Three animals were sacrificed at the age of 2 months by cervical dislocation. Only the right-side kidneys were processed and analyzed in order to have a more consistent control in the selection of samples. Kidneys were divided into 2 equal halves longitudinally, fixed by immersion in 10% neutral buffered formalin, and embedded in paraffin with the samples oriented with the flat surface parallel to the surface of the paraffin block to be sectioned. Of note, although some authors who analyzed the renal tubules mor-

Fig. 2. Morphometric analysis of a PCT. Representative kidney tissue section from a CD1 mouse stained with hematoxylin and eosin. The short (d) and large (D) diameters are exemplified. The area was the space delimited by the blue line. The PA is exemplified. The other 3 areas, SCA, LCA, and EA, were obtained with mathematical formulas using the values of d and D . Bar = 20 μm . G, glomerulus; BV, blood vessel; PCT, proximal convoluted tubule; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.

Fig. 3. Morphometric analysis of a DCT. Representative kidney tissue section from a CD1 mouse stained with hematoxylin and eosin. The short (d) and large (D) diameters are exemplified. The area was the space delimited by the blue line. The PA is exemplified. The other 3 areas, SCA, LCA, and EA, were obtained with mathematical formulas using the values of d and D . Bar = 20 μm . G, glomerulus; DCT, distal convoluted tubule; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.

phometrically fixed the kidneys by perfusion before inclusion in paraffin [4, 11, 22], most used the immersion method for this purpose [6, 8–10, 12–21]. Then, 5- μm thick sections were cut, deparaffinized in xylene, hydrated in a graded series of alcohol, and stained with hematoxylin and eosin (H&E) according to standard techniques.

Analysis was conducted using 3 tissue sections per animal. In each slide, 20 PCTs and 20 DCTs were examined in randomly selected microscopic fields at $\times 400$ magnification. Thus, a total of 120 tubular structures were analyzed per animal. It is noteworthy that in the literature, some authors do not specify the total number of tubules examined per subject [5, 6, 8, 9, 11, 20]. When specified, most authors report measurements in 100 tubules or fewer [3, 4, 10, 12–18, 21, 22]. Only transverse tubules with complete basement membrane (BM) were evaluated; longitudinal tubules were not considered.

Morphometry

Sections were examined with a light microscope (Primo Star; Carl Zeiss Microscopy GmbH, Oberkochen, Germany), and high-resolution color images were digitalized onto a computer screen using a AxioCam ICc1 camera (Carl Zeiss Microscopy GmbH) linked to image analysis software (Zen lite 2011; Carl Zeiss Microscopy

GmbH). All the sections were examined on coded slides by a single observer.

The parameters analyzed in both PCT and DCT are illustrated in Figures 1–3. The short diameter (d) was defined as the length of the shortest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM. The large diameter (D) was defined as the length of the largest straight line that passes through the center of the tubule and connects 2 opposite extreme points of the BM. To measure both diameters, lines d and D were manually drawn on the captured images described above, and the length of the lines was provided by the software.

The tubular area was defined as the space bounded by the BM. The small circular area (SCA) was calculated by using the formula for the area of a circle, $A = \pi \cdot r^2$, where r is one-half of d . The large circular area (LCA) was calculated by using the same formula, but replacing the d value by D .

The elliptical area (EA) was obtained by using the formula for the area of an ellipse, $A = \pi \cdot d' \cdot D'$, where d' and D' are one-half of d and D . Finally, the program area (PA) was obtained using the software mentioned above. To measure it, a line was drawn manually through the entire tubular perimeter, and the area was provided by the software.

Table 1. Diameters (μm) of the convoluted tubules

	Short diameter	Large diameter	<i>p</i> values
Proximal convoluted tubules	37.4 $\pm$ 0.5	44.0 $\pm$ 0.7	0.000
Distal convoluted tubules	32.2 $\pm$ 0.6	40.2 $\pm$ 0.9	0.000

Values are reported as mean $\pm$ 1 standard error. Data were analyzed by Student's *t* test. The results were considered statistically significant at *p* < 0.05.

Table 2. Areas (μm^2) of the convoluted tubules

	SCA	LCA	EA	PA	<i>p</i> values
Proximal convoluted tubules	1,112 $\pm$ 31	1,543 $\pm$ 47	1,300 $\pm$ 33	1,097 $\pm$ 25	0.000 ^{a, b}
Distal convoluted tubules	829 $\pm$ 29	1,313 $\pm$ 69	1,027 $\pm$ 35	976 $\pm$ 20	0.000 ^c 0.001 ^d 0.016 ^e

Values are reported as mean $\pm$ 1 standard error. Data were analyzed by a one-way ANOVA followed by a least significant difference test. SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area. The results were considered statistically significant at *p* < 0.05. ^a LCA versus SCA, EA, and PA. ^b EA versus SCA and PA. ^c LCA versus SCA, EA, and PA. ^d EA versus SCA. ^e PA versus SCA.

Statistical Analysis

The results are presented as means $\pm$ 1 standard error. Student's *t* test was used to determine statistical significance between the tubular diameters (*d* and *D*). Comparisons between the tubular areas (SCA, LCA, EA, and PA) were made by a one-way ANOVA, followed by post hoc analysis using the least significant difference (LSD) test. The significance level for all tests was set at *p* < 0.05. The data were analyzed using the SPSS for Windows software (SPSS, Inc., Chicago, IL, USA), release 21.0.

Results

The results are summarized in Tables 1 and 2. Student's *t* test showed a significant difference between the diameters analyzed in both PCT (*F* = 1.354, Sig = 0.000) and DCT (*F* = 4.989, Sig = 0.000). In the PCT (Fig. 4), *D* (44.0 $\pm$ 0.7 μm) was significantly greater in comparison with *d* (37.4 $\pm$ 0.5 μm). Similarly, in the DCT (Fig. 4), *D* (40.2 $\pm$ 0.9 μm) was significantly greater when compared to *d* (32.2 $\pm$ 0.6 μm).

The ANOVA test showed significant differences in the tubular areas analyzed in both PCT (*F* = 34.843, Sig = 0.000) and DCT (*F* = 22.390, Sig = 0.000). The LSD test revealed that in the PCT (Fig. 5), LCA (1,543 $\pm$ 47 μm^2) was significantly greater than SCA, EA, and PA (1,112 $\pm$

31, 1,300 $\pm$ 33, and 1,097 $\pm$ 25 μm^2 , respectively; all *p* values of 0.000). Also, EA was significantly greater when compared to SCA and PA (*p* values of 0.000).

The LSD test revealed that in the DCT (Fig. 5), LCA (1,313 $\pm$ 69 μm^2) was significantly greater than SCA, EA, and PA (829 $\pm$ 29, 1,027 $\pm$ 35, and 976 $\pm$ 20 μm^2 , respectively; all *p* values of 0.000). EA was significantly greater when compared to SCA (*p* value of 0.001). Finally, PA was significantly greater than SCA (*p* value of 0.016).

Discussion

The diameter and the area of PCT and DCT are some of the main parameters analyzed in stereological studies of the kidney. The results obtained in this work indicate that the accuracy and reproducibility of those analyses may depend on how the shape of the tubules is considered.

Several authors considered that the tubules have a circular shape and therefore measured only one diameter [3–10]. However, our results showed that the convoluted tubules of the kidney are elliptical in shape; we measured a short diameter (the short axis of the ellipse) and a long diameter (the long axis of the ellipse) which differed significantly from each other (Table 1; Fig. 4). Therefore, when analyzing the tubules in stereological studies of the

Fig. 4. Diameter measurements of the PCT and the DCT. In both PCT (A) and DCT (B), the large diameter was significantly greater when compared to the short diameter (**p* values of 0.000). Values are expressed as means $\pm$ 1 standard error. Data were analyzed by Student's *t* test. The results were considered statistically significant at *p* < 0.05. PCT, proximal convoluted tubule; DCT, distal convoluted tubule.

Fig. 5. Comparison between the areas analyzed in the PCT and in the DCT. In the PCT (A), the LCA was significantly greater than the SCA, the EA, and the PA (*all *p* values of 0.000). The EA was significantly greater when compared to the SCA and the PA (†*p* values of 0.000). In the DCT (B), the LCA was significantly greater than the SCA, the EA, and the PA (*all *p* values of 0.000). The EA was significantly greater when compared to the SCA (†*p* value of

0.001). Finally, the PA was significantly greater than the SCA (‡*p* value of 0.016). Values are expressed as means $\pm$ 1 standard error. Data were analyzed by a one-way ANOVA followed by a least significant difference test. The results were considered statistically significant at *p* < 0.05. PCT, proximal convoluted tubule; DCT, distal convoluted tubule; SCA, small circular area; LCA, large circular area; EA, elliptical area; PA, program area.

kidney, it should be considered that if only one diameter of the tubules is measured and a significant difference is observed, it may be due to the fact that both axes of the ellipse were actually measured, either randomly or alternatively, and not to the treatment or phenomenon analyzed per se. Furthermore, it can be difficult to reproduce

a study when the shape of the tubules is considered circular because any axis of the ellipse, or both in a random way, could be measured in the replication study.

These problems may not arise if the tubules are considered elliptical and the same axis (short or long) is evaluated in all tubules. Most authors report the value of the

short axis (short diameter) of the ellipse [12–14, 16–19], while others report the value of both axes (short and long diameters) of the ellipse [11, 15, 20]. Furthermore, care should be taken to always measure tubules as ellipses and not some tubules as ellipses and other tubules as circles in the same sample [21, 22].

The tubules certainly have an elliptical shape. We were able to calculate an EA with a formula that incorporates the values of a short axis and a long axis, and the line used to measure the area with the software (PA) clearly delimited an ellipse (Fig. 2, 3).

The SCA and the LCA differed significantly from each other in both PCT and DCT (Table 2; Fig. 5). SCA and LCA were calculated using the value of the short diameter and the value of the large diameter of the circle, respectively, and this fact would explain the difference encountered. Therefore, observations regarding accuracy and reproducibility described above for the circular diameters could also be applied to the circular area analysis.

Interestingly, significant differences were also observed between other areas evaluated. In the PCT, LCA was also significantly different from EA and PA, while EA was significantly different from SCA and PA (Table 2; Fig. 5). In the DCT, LCA was also significantly different from EA and PA, while EA and PA were significantly different from SCA (Table 2; Fig. 5). It is likely that the area of a tubule may vary not only depending on whether it is considered a circle or an ellipse (SCA and LCA vs. EA and PA) but also if the area is evaluated directly with a piece of software or with another strategy (PA vs. EA). While the use of image analysis software to assess morphometric data might be more exact in comparison with other strategies, it should be remembered that it is the researcher who ultimately provides the necessary information to the software for the correct execution of the measurements [30]. In this case, the software would be of little use if the shape of the tubules were considered circular instead of elliptical.

Finally, although recommendations have been made for the morphometric analysis of the kidney [2], there is no consensus in the literature on how to perform it regarding several aspects, such as which method for the fixation of the kidney is the correct one, the correct orientation of the samples for histological analysis, the number of structures that should be evaluated, the optimal sampling strategy, and if the nephron tubules should be considered circular or elliptical. With this work, we try to clarify the last-mentioned aspect. In conclusion, the results obtained in this work indicate that the convoluted tubules of the nephron must not be considered circular,

but rather elliptical, and care should be taken every time the tubules are analyzed in stereological studies of the kidney, especially when evaluating their diameters and areas.

Statement of Ethics

The present study was performed in accordance with the principles and procedures defined in the National Research Council Guide for the Care and Use of Laboratory Animals (8th edition) and in the Mexican Guidelines ZOO-062. Experimental protocols were approved by the Institutional Review Board and Ethics Committee of the School of Medicine of the Autonomous University of Nuevo Leon (No. PA19-00001).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

M.O.-M. and V.G.-D. performed experiments. G.J.-R. planned experiments. M.O.-M. and E.G.-A. provided the image analysis. G.J.-R., M.O.-M., and E.G.-A. wrote the manuscript. R.M.C.-F. provided the statistical analysis of the data. G.J.-R. conceived and designed the study. A.N.-G. performed the analysis and interpretation of data. All authors read and approved the manuscript.

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