Morphometry: Assessing Direct and Indirect Methods of Measuring the Diameters of Tubular Structures

Morfometría: Evaluación de Métodos Directos e Indirectos para Medir los Diámetros de las Estructuras Tubulares

Assad Ali Rezigalla


SUMMARY: Knowledge of the diameter of a structure or particle is required for stereological calculations. However, there is no consensus on the methodology for its measurement. This study aims to assess the differences between direct and indirect methods of measuring diameter. It is hypothesised that kidneys were removed, fixed, processed, sectioned, and stained. The stained slides were imaged using a digital microscope. The images were processed using the ImageJ software. The diameters of the renal glomeruli and collecting tubules were measured using direct and indirect methods. The measured diameters were analysed using the SPSS software v20. The differences between the measurements were assessed using a Z-test and test of association, and P < 0.05 was considered significant. No significant differences were observed between the diameters of the glomeruli (P = 0.82) and proximal (P = 0.86) and distal (P = 0.55) convoluted tubules as measured via direct and indirect methods. There was a strong positive correlation between the diameters of glomeruli (P = 0.97) and proximal (P = 0.82) and distal (P = 0.93) convoluted tubules measured using the two methods, both of which are convenient, accurate and suitable. The P-values based on these measurements were more than 0.05. Therefore, the study hypothesis was rejected. There was no significant difference between the direct and indirect methods of measuring diameter, and the null hypothesis was rejected; thus, both methods can be applied either independently or jointly.

KEY WORDS: Measurement; Diameter; Direct; Indirect; Glomerulus; Proximal, distal; Convoluted tubules.

INTRODUCTION

Stereology is defined as ‘a methodology which provides meaningful quantitative descriptions of the geometry of real three-dimensional glob structures from measurements that are made on two-dimensional images sampled from the glob’ (Dehoff, 2000; Alic’elebic’, 2003; Ortega-Martinez et al., 2021). On the other hand, morphometry is ‘the quantitative description of a structure’ obtained through measurement; the structure can be of macroscopic or microscopic size (Baak & Oort, 2012; Mandarim-de-Lacerda & del Sol, 2017).

Both morphometry and stereology utilize the mean diameter of a structure or particle of interest as a point of reference (Bertram, 1995). However, there is no current consensus on methods for measuring the diameter or radius of rounded structures and particles (Ortega-Martinez et al.). Assessing the diameter of tubular structures is essential for certain morphological or stereological methods and provides a marker for possible structural changes (Kambham et al., 2001; Cheng et al., 2009). In renal research, for instance, the tubular diameter has been reported to be a robust biomarker for kidney viability after transplant (Andrews et al., 2002; Li et al., 2009).

The calculation of the surface area of a structure of interest depends on the diameter and vice versa, which in turn depends on the measurement method used (Truter et al., 2018; Ortega-Martinez et al.). An inaccurate evaluation of diameter would bias the estimates. In the direct method of measuring diameter, some authors only measure the diameter once, assuming the structure of interest to be circular (Saraga et al., 2014; Hemmi et al., 2015; Truter et al.; Ortega-Martinez et al.). Other authors, however, assume that the structure of interest is elliptical and used two measurements, namely, those of the minor and major axes (Leh et al., 2011; Alkharfy et al., 2015; Sukhorum et al.,...
Furthermore, some authors assume that the structures of interest in a sample have two profiles, i.e., circular and elliptical (Rangan et al., 1999; Kang et al., 2005; Ortega-Martínez et al.). In contrast to these approaches, the edges or boundaries of the structure of interest must be defined for the indirect method of measurement. The surface area can then be calculated and the diameter determined using a mathematical equation (Cheng et al., 2012). While image processing software can be used for both methods (Cheng et al., 2012; Oakley & Williams, 2015; Sukhorum et al.; Ortega-Martínez et al.), direct methods require an optical eyepiece for measurement (Johora et al., 2014).

Accordingly, this study aims to assess the difference between direct and indirect methods of measuring the diameters of structures. We hypothesise that the diameters obtained using direct and indirect methods are different. The study outcomes have important implications for determining the optimal methodology for diameter measurement.

**MATERIAL AND METHOD**

**Experimental design and animals.** Forty adult female Wistar rats weighing 200–250 g were used in the study. The animals were kept in an animal house at King Khalid University (KKU), Abha City, Kingdom of Saudi Arabia (KSA) for 2 weeks to acclimatize to the environment. The rats were kept in separate cages with a light/dark cycle of 12 h. They had free access to purified water and were fed ad libitum.

**Sample collection.** The rats were anaesthetised using chloroform. The left kidneys were used for the experiment. These were removed through a low median abdominal incision, weighed using a sensitive balance and then immediately fixed in 10 % formaldehyde (Suvarna et al., 2018). After sample collection, the animals were sacrificed using an overdose of anaesthesia.

**Tissue processing.** Selected sample blocks were processed, sectioned and stained with haematoxylin and eosin (Suvarna et al.). The stained slides were then used for optical microscopic observations and histometry.

**Correction of kidney shrinkage.** Fixation and tissue processing reportedly cause tissue shrinkage or expansion (Palaia et al., 2011; Vent et al., 2014; Tran et al., 2015; Mandarim-de-Lacerda & del Sol). The correction factors for shrinkage were calculated as the ratio between organ volume before and after fixation and the ratio between the surface area in the blocks and the slides (Aherne & Dunnill, 1987; Bach & Lock, 2012).

**Measurement of diameters.** The stained slides were imaged using a digital microscope (Labomed, LC-20) and the images processed using the ImageJ image analysis software (Fiji software version 1.53c) (Broeke et al., 2015). All images were saved in the TIFF format (Baak & Oort; Mandarim-de-Lacerda & del Sol).

**Measurement method.** After calibration of the microscope and software, the diameters were measured as follows:

(i) Direct method: The diameter was calculated as the mean of three perpendicular measurements (longest, medium, and smallest) (Cheng et al., 2012; Ortega-Martínez et al.) (Fig. 1a).

(ii) Indirect method: Using the freehand tool in the ImageJ software, the outer margin was outlined and the area was automatically calculated by the software. The resulting area represents the area of interest, measured in square units. The diameter was calculated using the following formula: , where \( d = 2r \) (A is the calculated area of interest, \( r \) is the radius, and \( d \) is the diameter (Cheng et al., 2012; Ortega-Martínez et al.) (Fig. 1A).

Both methods were applied to the same images using the ImageJ image software. The parietal layer of the glomerular capsule (Bowman’s capsule) formed the outer margin or edge of the glomeruli. Only glomeruli with vascular or urinary poles (or both) were included in the study (Mandarim-de-Lacerda & del Sol). The outer margin or edge of the collecting tubules was defined as the basement membrane of the epithelial cells that form the tubules. Only tubules with a circular or oval appearance were included in the study. The mean of all measured glomerular diameters was then calculated.

**Statistical analysis.** The data were analysed using the IBM SPSS software v20. The categorical data are presented in the form of tables and frequencies. A Z-test and test of correlation were performed on the diameters measured by direct and indirect methods. A P-value of <0.05 was considered statistically significant.

**Ethical considerations.** The study was approved by the research and ethics committee at KKU (Abha, KSA). The experimental animals were part of a project assessing the effect of hypoxia and were generously provided by Dr Ali Jadkarim (Department of Anatomy, College of Medicine, KKU).
RESULTS

**Glomeruli.** The mean glomerular diameter from the direct method of measurement was 102.89 ± 15.59 µm, whereas the corresponding value for the indirect method was 102.30 ± 16.11 µm (Table I).

The Z-test showed a non-significant difference between the results of the direct and indirect measurement methods (P = 0.81; Table II).

A significant positive correlation between the direct and indirect methods was found (r = 0.958, P = 0.00; Table III; Fig. 1B). A runs test revealed 71 points above the line, 81 below and 59 runs, with a P-value of 0.0024, indicating a significant departure from linearity.

**Collecting tubules**

**Proximal convoluted tubules.** The mean diameter of the proximal convoluted tubules measured using the direct method was 34.51 ± 4.695 µm; for the indirect method, the corresponding value was 34.61 ± 4.237 µm (Table I).

The Z-test showed no significant difference between the direct and indirect methods of measurement (P = 0.86; Table II).

A significant positive correlation was found between the direct and indirect methods (r = 0.958, P = 0.00; Table III; Fig. 1C). The runs test indicated that there were 59 points above the line, 68 below and 55 runs, with a P-value of 0.0598, which is considered a marginally non-significant departure from linearity.

**Distal convoluted tubules.** The mean diameter of the distal convoluted tubules as measured by the direct method was 31.51 ± 5.237 µm; the corresponding measurement for the indirect method was 31.92 ± 5.270 µm (Table I).

The Z-test showed a non-significant difference between the direct and indirect methods of measurement (P = 0.55; Table II).

A strong positive correlation was reported between the direct and indirect methods (r = 0.934, P = 0.00; Table III; Fig. 1d). The runs test revealed 34 points above the line, 52 below and 39 runs, with a P-value of 0.2768, which is considered a non-significant departure from linearity.
Table I. Descriptive statistics of the diameters of renal glomeruli proximal and distal convoluted tubules in microns.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glomerulus</th>
<th>PCT</th>
<th>DCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Indirect</td>
<td>Direct</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>88.6 ± 18.8</td>
<td>89.1 ± 18</td>
<td>34.51 ± 4.68</td>
</tr>
<tr>
<td>Minimum</td>
<td>50</td>
<td>47.9</td>
<td>25</td>
</tr>
<tr>
<td>Maximum</td>
<td>146.2</td>
<td>148.2</td>
<td>47</td>
</tr>
<tr>
<td>N</td>
<td>152</td>
<td>152</td>
<td>127</td>
</tr>
</tbody>
</table>

Direct and indirect references to the corresponding method of measurement; PCT = Proximal convoluted tubule; DCT = Distal convoluted tubule.

Table II. Results of the Z-test for the diameters of renal glomeruli and proximal and distal convoluted tubules.

<table>
<thead>
<tr>
<th>z-Test: Two-Sample for Means</th>
<th>Glomerulus</th>
<th>PCT</th>
<th>DCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Indirect</td>
<td>Direct</td>
</tr>
<tr>
<td>Mean</td>
<td>88.61</td>
<td>89.11</td>
<td>34.51</td>
</tr>
<tr>
<td>Known Variance</td>
<td>351.931</td>
<td>319.271</td>
<td>21.868</td>
</tr>
<tr>
<td>N</td>
<td>152</td>
<td>152</td>
<td>127</td>
</tr>
<tr>
<td>P(Z≤z) two-tailed</td>
<td>0.82</td>
<td>0.86</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Direct and indirect references to the corresponding method of measurement; PCT = Proximal convoluted tubule; DCT = Distal convoluted tubule.

Table III. Correlation values between the diameters of renal glomeruli and proximal and distal convoluted tubules were measured by direct and indirect methods.

<table>
<thead>
<tr>
<th>Correlations</th>
<th>Glomerulus</th>
<th>PCT</th>
<th>DCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Indirect</td>
<td>Direct</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>Direct</td>
<td>1</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>0.967</td>
<td>1</td>
</tr>
<tr>
<td>Sig. (1-tailed)</td>
<td>Direct</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td>Direct</td>
<td>152</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>152</td>
<td>152</td>
</tr>
</tbody>
</table>

Direct and indirect references to the corresponding method of measurement; PCT = Proximal convoluted tubule; DCT = Distal convoluted tubule.

DISCUSSION

Using image analysis software to assess the diameter of tubular structures is more convenient and accurate than using a microscope with a built-in micrometer (Kambham et al.; Cheng et al., 2012). Both direct and indirect methods can be performed using image analysis software, but the indirect approach is less reported in the literature (Cheng et al., 2012).

The average diameter values of the glomeruli obtained using the direct and indirect methods were 102.89 ± 15.59 µm and 102.30 ± 16.11 µm, respectively. The average diameter values of the proximal convoluted tubules were 34.51 ± 4.695 µm and 34.61 ± 4.237 µm for the direct and indirect methods, respectively. Finally, the average diameter values of the distal convoluted tubules were 31.51 ± 5.237 µm and 31.92 ± 5.270 µm for the direct and indirect measurement methods, respectively. The diameters of the glomeruli and renal tubules as measured using both methods are close to the reported values (Hackbarth et al., 1983; Tanner et al., 1989; Heilmann et al., 2012).

The Z-test showed no significant difference between the two methods of measurement, and measurements using both methods were positively correlated. These findings indicate no significant differences between the methods, an outcome that is supported by the fact that both methods were applied to the same samples. The findings of this study are consistent with the work of Cheng et al. (2012), who reported that both methods are feasible for measuring the diameter of tubular structures (Cheng et al., 2012).

Both direct and indirect methods of assessing the diameter of tubular structures are manual, and their accuracy depends on the observer’s skill and precision. Both methods can be very tedious and time-consuming (Li et al.). Although image processing software can perform an automatic assessment of the surface area, manual methods have potential advantages. First, through direct observation, histological structures with abnormal cytoarchitectures, such as a sloughed epithelium in renal tubules or distributed blood
capillaries, can be avoided. Second, the manual method may be preferable for determining the tubular shape of the structure of interest and allow the avoidance of tangential sections that do not show the lumen. Furthermore, in small samples such as needle biopsies, skill and direct observation are required to make reliable judgements regarding the completeness of a tubular structure or the histological appearance. On comparing the direct and indirect methods, the latter is found to have a technical shortcoming in that it may be difficult to achieve consistency both within and between studies due to variability in the number of measurements per structure of interest and their presumed profiles (i.e., circular or elliptical (Ortega-Martinez et al.).) The indirect method has the advantage of assessing particles of irregular appearance.

In conclusion, there was no significant difference between the direct and indirect methods of measuring diameter. Thus, both methods can be used on the same samples. This study is the first to provide an evidence-based analysis confirming the similarities in the results obtained using both methods.

The study limitations include the small sample size and its application to a single organ, lack of previous research on methods of measuring diameter, and funding constraints. However, the study offers a detailed description of the methods for measuring diameter demonstrates their similarity and confirms that both are reliable.

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REFERENCES
