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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

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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; Alicˇelebic´, 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*.,

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2016; Ortega-Martinez *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-Martinez *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-Martinez *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 processed for conventional histology. The kidneys were divided into small blocks (3–4 mm) and 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-Martinez *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-Martinez *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).

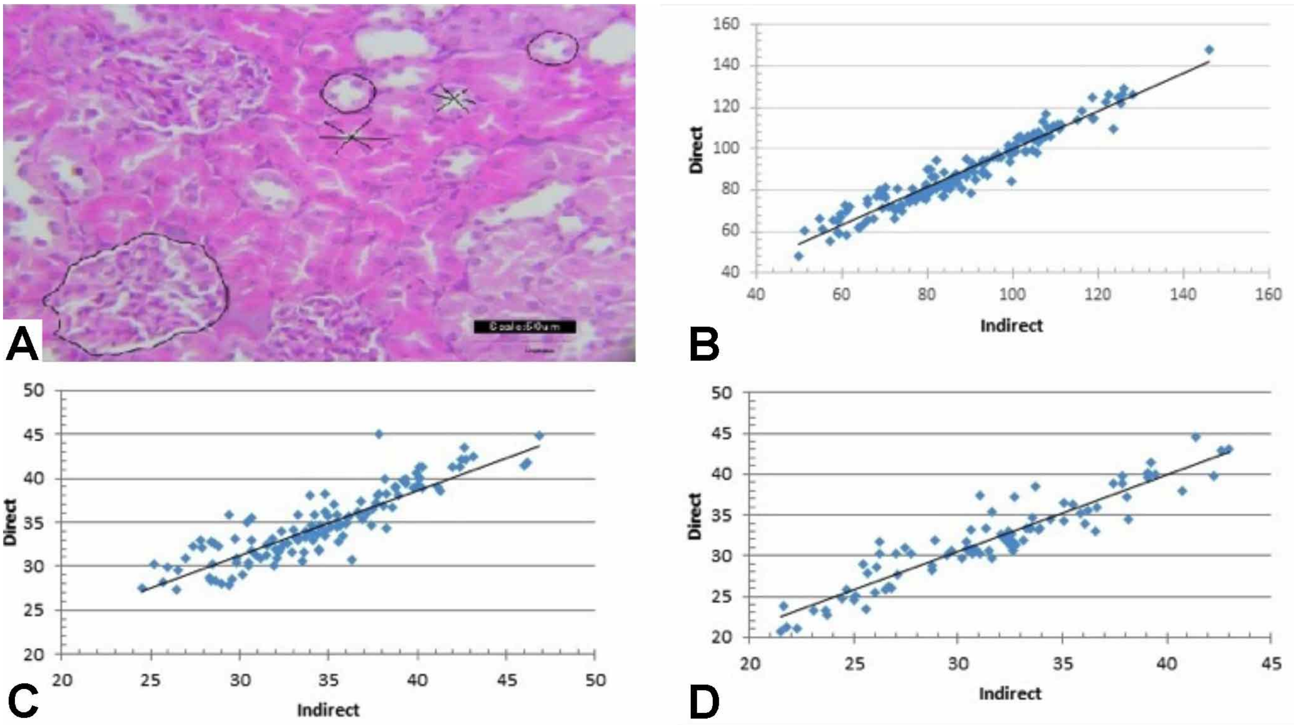

Fig. 1. Methods of direct and indirect measurement and linear correlation between the diameters measured using the direct and indirect measurement methods. 1A= methods of measurement 1B= glomerular diameter; 1C= diameter of proximal convoluted tubules; 1D= diameter of distal convoluted tubules.

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 direct 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 statics of the diameters of renal glomeruli proximal and distal convoluted tubules in microns.

Parameter	Glomerulus		PCT		DCT	
	Direct	Indirect	Direct	Indirect	Direct	Indirect
Mean±SD	88.6±18.8	89.1±18	34.51±4.68	34.61±4.24	31.51±5.237	31.92±5.27
Minimum	50	47.9	25	20	21.49	20.7
Maximum	146.2	148.2	47	45	42.98	44.6
N	152	152	127	127	87	87

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.

z-Test: Two-Sample for Means	Glomerulus		PCT		DCT	
	Direct	Indirect	Direct	Indirect	Direct	Indirect
Mean	88.61	89.11	34.51	34.611	31.511	31.921
Known Variance	351.931	319.271	21.868	17.813	21.865	17.813
N	152	152	127	127	87	87
P(Z<=z) two-tail	0.82		0.86		0.55	

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.

Correlations		Glomerulus		PCT		DCT	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
Pearson Correlation	Direct	1	0.967	1	0.822	1	0.934
	Indirect	0.967	1	0.822	1	0.934	1
Sig. (1-tailed)	Direct	0	0	.	0	.	0
	Indirect	0	.	0	.	0	.
N	Direct	152	152	127	127	87	87
	Indirect	152	152	127	127	87	87

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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REZIGALLA, A. A. Morfometría: Evaluación de métodos directos e indirectos para medir los diámetros de las estructuras tubulares. *Int. J. Morphol., 40(2)*:314-319, 2022.

RESUMEN: Se requiere el conocimiento del diámetro de una estructura o partícula para los cálculos estereológicos. Sin embargo, no existe consenso sobre la metodología para su medición. Este estudio tuvo como objetivo evaluar las diferencias entre los métodos directos e indirectos de medición del diámetro de una estructura. Riñones de ratas Wistar fueron extirpados, fijados, procesados y seccionados, y luego se tiñeron con HE. Se tomaron imágenes de las muestras teñidas usando un microscopio digital. Las imágenes fueron procesadas utilizando el software ImageJ. Los diámetros de los glomérulos renales y túbulos colectores se midieron por métodos directos e indirectos. Los diámetros medidos se analizaron utilizando el software SPSS v20. Las diferencias entre las medidas se evaluaron mediante una prueba Z y una prueba de asociación, y se consideró significativa $P < 0,05$. No se observaron diferencias significativas entre los diámetros de los glomérulos ($P = 0,82$) y túbulos contorneados proximales ($P = 0,86$) y distales ($P = 0,55$) medidos mediante métodos directos e indirectos. Hubo una fuerte correlación positiva entre los diámetros de los glomérulos ($P = 0,97$) y los túbulos contorneados proximales ($P = 0,82$) y distales ($P = 0,93$) medidos con los dos métodos, ambos convenientes, precisos y adecuados. Los valores P basados en estas mediciones fueron superiores a 0,05. Por lo tanto, se rechazó la hipótesis del estudio. No hubo diferencia significativa entre los métodos directo e indirecto de medición del diámetro, y se rechazó la hipótesis nula; por lo tanto, ambos métodos se pueden aplicar de forma independiente o conjunta.

PALABRAS CLAVE: Medición; Diámetro; Directo; Indirecto; glomérulo; proximal, distal; Túbulos contorneados.

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