A Stereological and Electron Microscopic Study of the Development of the Nephron in Prenatal and Postnatal Rats

Prenatal ve Postnatal Ratlarda Nefronun Gelişimi Üzerine Elektron Mikroskopik ve Stereolojik bir Çalışma

Nejdet Şimşek[1], Berrrin Z. Altunkaynak[2], Deniz Ünal[2], Serpil Can[3], Ismail Malkoç[4], Bünyami Ünal[2]

[1]Atatürk University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Erzurum, Turkey
[2]Atatürk University, Faculty of Medicine, Department of Histology and Embryology, Erzurum, Turkey
[3]Atatürk University, Faculty of Medicine, Department of Physiology, Erzurum, Turkey
[4]Atatürk University, Faculty of Medicine, Department of Anatomy, Erzurum, Turkey

Correspondence to: Nejdet Şimşek, Atatürk University, Faculty of Veterinary Medicine, Department of Histology and Embryology, 25250, Erzurum, Turkey. Phone: +90.442.6314193, Fax: +90.442.6314188, e.mail: nsimsek-58@hotmail.com

Abstract

Objective. To describe the morphometric and ultra-structural features of the kidney in fetal (20-day-old), newborn (7-day-old) and adult (180-day-old) rats.

Materials and Methods. Kidneys from all animals were excised, fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% phosphate-buffered osmium tetroxide, and examined by stereological and light and electron microscopic methods.

Results. Fetal kidneys displayed kidney corpuscles, glomeruli, and proximal and collective tubules at multiple developmental stages. Glomeruli in the outer surfaces of the kidney were less mature than those in the inner surface. Kidney corpuscles were made up of parietal cells and podocytes without feet. Kidneys from newborn rats were almost completely developed, while kidneys in adult rats were fully developed. Under stereological examination, the percent volume of cortex in fetal kidneys (86.19%) was higher than in newborn (53.77%) or adult rats (76.78%). Compared to both newborn and fetal rats, adult rats displayed the highest total volumes of distal and proximal tubules, but lower mean glomerular or Bowman's capsule volumes. On the other hand, the total number of glomeruli was increased in adult rats (32,600) as compared to newborn (17,896) and fetal (11,650) rats.

Conclusion. These data suggest that development of metanephric kidneys is not yet complete by gestational day 20, but is almost complete at postnatal week one. Furthermore, the developmental stage of the kidney, as determined by stereology, correlates well with the age of the rat.

Keywords: Kidney, Stereology, Prenatal and postnatal development

Özet

Amaç. *Bu çalışma, fetüs (20 günlük), yeni doğmuş (7 günlük) ve erişkin (180 günlük) ratlarda böbreklerin morfometrik ve ultrastrukturel yapısını belirmek amacıyla yapıldı.*

Gereç ve Yöntem. *Hayvanlardan alınan böbrekler % 3'lük gluteraldehit ve 0,1 M Fosfat tamponu ile tespit edildikten sonra post fikzasyon için % 1'lik tamponlu ozmium tetroksitle tespit edilerek stereolojik, ışık ve elektron mikroskobik olarak incelendi..*

Bulgular. *Elde edilen sonuçlara göre fetüs böbreklerinin glomerulus, böbrek cisimcikleri, proksimal, distal ve toplayıcı borucuklarının farklı gelişme aşamalarında olduğu gözlemlendi. Fetüslerde böbreklerin kapsülüne yakın olan dış yüzlerindeki glomerulusların iç yüzdekilerden daha çok geliştiği, böbrek cisimsiklerinin pariyetal hücreleri ve sitoplazmik uzantısız podositler içerdiği, erişkin ve yeni doğmuş ratlarda ise böbreklerin oldukça gelişmiş yapıda görüldüğü saptandı. Stereolojik incelemelerde fötal böbrek korteks oranı (% 86,19) yeni doğanlara (% 53,77) ve erişkinlere (% 76,78) göre daha yüksekti. Yeni doğan ve fetüslere göre erişkinlerde distal ve proksimal tubullerin total hacmi daha yüksek iken, Bowman kapsülü ve glomerulusun ortalama hacminin azaldığı tepit edildi. Diğer yandan, erişkinlerde glomerulusların total hacminin (32600) fetüs (11650) ve yeni doğanlardan (17896) daha fazla bulunduğu da saptandı.*

Sonuç. *Elde edilen sonuçlara göre, metanefrik böbreklerin gelişimi gebeliğin 20. gününde henüz tamamlanmamışken, doğumdan sonraki 1. haftada hemen hemen gelişmenin sonlandığı belirlenmiştir. Ayrıca, böbrek gelişiminin stereolojik verilerinin ratların yaşları ile ilgili değişebileceği de tespit edilmiştir.*

Anahtar Kelimeler: *Böbrek, Stereoloji, Prenatal ve postnatal gelişim*

Introduction

The embryonic pronephric and mesonephric tissues originate from mesodermal precursor cells during gastrulation, while the metanephric kidney is derived from the nephrogenic chord, which arises from metanephric tissue and the ureteric bud. The nephron, the functional unit of the kidney, develops from the metanephric mesenchyme, but the collecting ducts, calyces and renal pelvis derive from branches off the meso- nephric duct [1]. The four stages of nephron development have been described in rats [2], mice [3] and hamsters [4]. In the first stage, ellipsoidal vesicles with extended lumina and basement membranes are observed, and in stage two, S-shaped bodies are seen, which contain the capillaries, collecting tubules, proximal tubules and glomerular anlagen found on the kidney surface [2,3,5]. In stage four, the glomeruli are larger, the parietal layer of Bowman's capsule contains simple squamous cells, proximal tubule cells have developed brush borders, and the longer Henle loops contain simple squamous cells [4,6]. Nephrogenesis is complete by the 36th week of gestation in humans, whereas, in the rat, it continues until approximately postnatal day 10 [7,8]. In the 10-day-old rat, juxtamedullary nephrons complete their development before cortical nephrons do. In the juxtaglomerular apparatus of the fetal rat kidney (19-day-old), very few granulated smooth muscle cells are observed; however, the number of granulated cells abruptly increases in the newborn and is followed by a three-fold decrease on postnatal day 2 [7].

The development of the mammalian kidney has been studied in vivo and in vitro using immunohistochemical and light and electron microscopy techniques [1,9,10]. The majority of these studies have focused on morphometric analysis of the nephron development using an eye piece micrometer. In recent years, however, unbiased stereological methods for counting glomeruli have been developed [11-13]. Methods for stereological counting are used for quantitative analysis of three-dimensional structures [11] and can estimate cell and/or organelle number and size at the microscopic level, yielding very important data about the anatomical and histological structure of the tissues in question [13-15]. In the present study, we report a detailed analysis of the stereological and light and electron microscopic changes that occur during prenatal (20th day) and postnatal (7th and 180th day) development of the rat kidney.

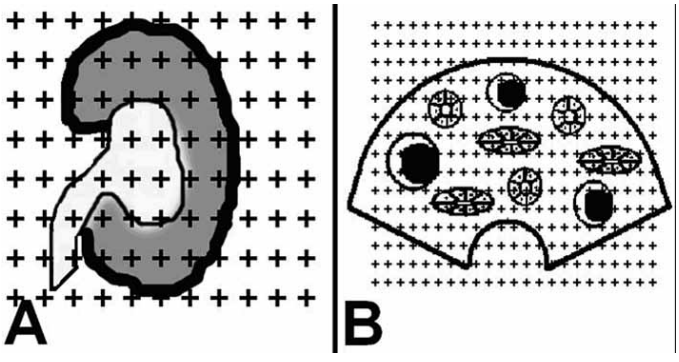

Fig. 1 — Stereological analysis procedures applying the Cavalieri method to kidney samples.

Materials and Methods

Experimental Procedure and Animals:

In this study, fetal (prenatal day 20), newborn (7 day old) and adult (180 day old) rats were used. For this investigation, female and male Sprague-Dawley rats, weighing 250-300 g, were mated in the laboratory of The Experimental Research and Application Center (Atatürk University, Erzurum, Turkey) overnight. The morning on which sperm-positive smears were obtained was declared gestation day 1. Pregnant females (n=12) were housed individually under standard conditions (12:12 hr light-dark cycle at 22±2°C) and offered food and water ad libitum. All pregnant rats were randomly allocated into two groups of equal size (n=6). Pregnancy in the first group was ended on the 20th day. Rats were killed under ether anesthesia, and fetuses were removed (n=10). Newborn rats (n=10) were obtained from the second pregnant group. A third cohort of adult rats made up an adult group (n=10). In all groups, gender was not considered. The kidneys of all fetal, newborn and adult rats were handled and prepared for histological and morphometric measurements. Experimental protocols were approved by the Local Animal Care Committee of Atatürk University.

Stereological Procedures and Electron Microscopy:

Kidney samples were removed from the fetal, newborn and adult rats. These tissues were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% phosphate-buffered osmium tetroxide, dehydrated in a graded acetone series, and washed in propylene oxide for electron microscopic examination. After dehydration, specimens were embedded in fresh Araldite CY 212 (Agar, Cambridge, UK). Sections were cut using an ultramicrotome (Nova LKB Bromma, Sweden). First, each araldite-embedded tissue block was cut into 1-µm sections. These semi-thin sections were stained with toluidine blue for light microscopic and stereological examination. Second, tissue samples were cut into 70-80 nm section-thicknesses for histological evaluation at the ultra-structural level. These thin sections were stained in 2% uranyl acetate and 0.4% lead citrate and examined under a Jeol 100 SX transmission electron microscope (Jeol, Tokyo, Japan).

Volume of Kidney Cortex and Medulla, and Volume of Glomeruli and Tubules:

We applied the Cavalieri method to 15-20 tissue sections, in order to estimate the absolute volume and fraction of whole kidney volume for the cortex, medulla, glomeruli, and distal and proximal tubules. To accomplish this, two different point-counting test grids were used (Figure 1). The point-counting grids were designed with a point density which was high enough for ≥1000 points to overlay each area of interest (i.e., renal cortex, medulla, glomeruli, and tubules) and also sufficient to obtain a statistically significant coefficient of error (CE). The coefficient of error and coefficient of variation (CV) were estimated according to

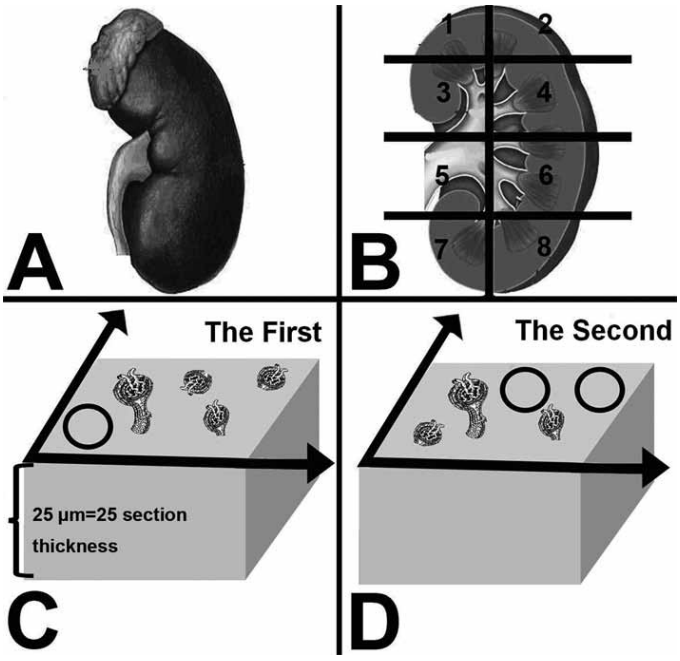

Fig. 2 — Physical dissector procedures for stereological counting.

Gundersen and Jensen's formula [16,17]. Two different point-counting grids were created, one with densely scattered and the other with sparsely scattered points (Figure 1). Grids with a systematic array were randomly placed over an image of the section in question, and all points at which the grid overlaid subjects of interest were counted. Grids with sparsely scattered points were used to estimate the volume fraction of the cortex and medulla (Figure 1a), and grids containing densely scattered points were used to estimate the volume fraction of the glomeruli, Bowman's space, and tubules (Figure 1b). Sampled areas were chosen in a systematic manner using a motorized stage but were otherwise identified at random. We counted the total number of points superimposed on each area of interest.

Subsequently, the volumes of the major structures in each section were estimated using the following equation: Volume (V) = t x a (p) x ΣP, where V is the volume of the object of interest (kidney, glomerulus, or tubules) in one section plane, t is the section thickness, a/p is the inter-point area, and ΣP is the total number of points overlying the object of interest in that section. This formula was applied to the remaining sections, and the total volume was estimated using the following equation: Total Volume = V1+ V2+...+ Vn.

Volume fractions for each region were estimated by dividing the total points superimposed on the region of interest by the sum of points superimposed on the whole kidney: i.e., volume fraction region/kidney = Pregion / Pkidney, where P is the number of points within the region of interest or within that section of kidney.

Density and Total Number of Glomeruli:

For stereological examination, kidneys were uniformly and randomly sampled (Figures 2a, b). As preparation for electron microscopy, the kidney specimens were cut into serial semi-thin sections. Selection of the physical dissector pairs was performed as described by Sterio [18]. Based on results from a pilot study (data not shown), the first section chosen and the adjacent section, together called a dissector pair, were separated by a 25-µm distance (25-µm section thickness: 25-µm distance) that was subsequently used as the rule for physical dissection. This rule required the distance between section pairs to be 30-40% of the projected average height of the object of interest (Figures 2c, d). In this way, 15-20 section pairs were identified and evaluated. This number is within an acceptable range for stereological ana lysis [14,15]. Two consecutive sections were mounted on each slide. Photographs of adjacent sections were taken with a digital camera at 400x magnification. An unbiased counting frame was placed directly onto the screen of the PC over the reference section and the section under analysis, and counting was performed using the dissector counting method [14]. The bottom and left-hand edges of the counting frame and the extension lines were considered to be exclusion lines. The top-right edges of the frame were considered to be inclusion lines, as any particle that hit these lines or was located inside the frame was counted as a dissector particle (Figures 2c, d). The size of the unbiased counting frame was adjusted to include ~600 glomeruli from each sample.

The dimensions of the counting frame on the PC screen were 200 mm x 200 mm, and the real dimensions of this counting frame (100 mm2) were estimated by the following formula: real dimension = screen size of frame / total magnification of microscope.

All glomeruli seen in the reference section but not in the section under study were counted [15]. The mean numerical den-

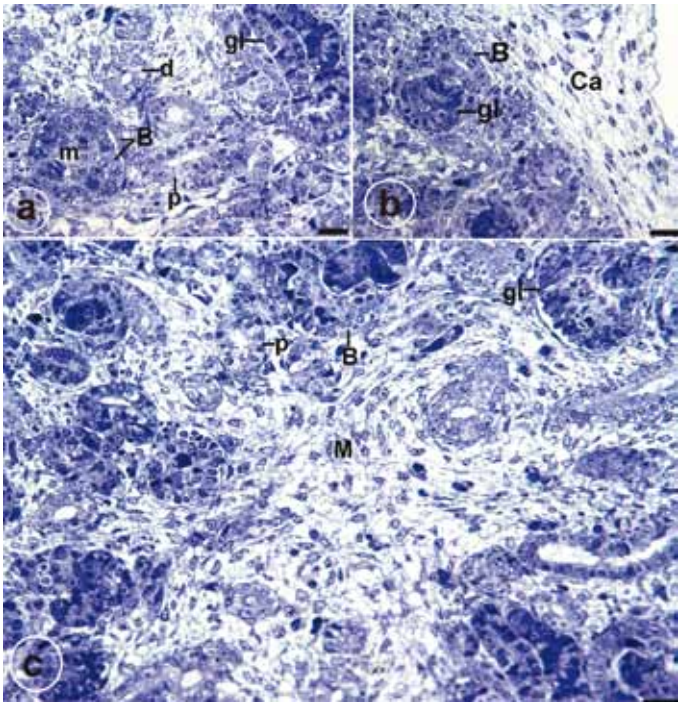

Fig. 3 — Semi-thin sections from a day-20 fetal rat kidney. The kidney capsule (Ca), glomerulus (gl), Bowman's capsule (B), mesangium (m), distal tubule (d), proximal tubule (p), and medulla (M) are marked. Scale bars: 25 µm.

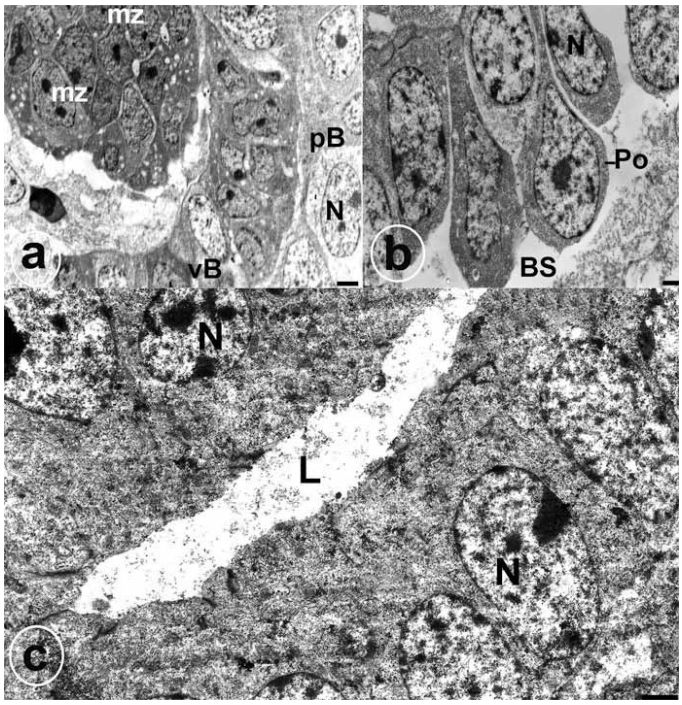

Fig. 4 — Proximal tubules and podocytes in day-20 fetal rat kidney. The nucleus (N), lumen (L), mesangial cells (mz), podocytes(Po), urinary space, (vB), visceral layer of Bowman's capsule (BS), and parietal layer of Bowman's capsule (pB) are indicated. Scale bars: 1.5 µm (4a, 4b); 2 µm (4c).

sity of glomeruli [Nvglomeruli] per mm3 was estimated using the following formula: Nvglomeruli = ∑Q glomeruli / t x A, where Qglomeruli was the total number of glomeruli counted in the reference section, t was the mean section thickness (1µm), and A was the area of the unbiased counting frame.

Thus, the total number of glomeruli (TNglomeruli) in a whole rat kidney was estimated by the following equation: TNglomeruli = Nvglomeruli x kidney volume, where Nvglomeruli was the numerical density of glomeruli per mm3, TNglomeruli was the total number of glomeruli in the whole kidney calculated using Nvglomeruli , and kidney volume results were estimated using the Cavalieri method.

Finally, histological examinations were carried out on images of the same sections.

Statistical Analysis:

Mean±standard error values were calculated for each group to compare inter-group differences. Differences in volumetric data, numerical density, total number, and glomerular height were analyzed separately using Student's t-test. P-values <0.05 were considered to be significant. All statistical calculations were performed using SPSS 11.0 software for Windows.

Results

Histological Results:

By light and electron microscopy, the structure of the fetal kidneys appeared to be moderately different from kidneys of newborn rats. The outer surfaces of the fetal kidneys were surrounded with loose shoving connective tissue. Below the renal capsule, there was a nephrogenic mesenchymal region containing glomeruli in different developmental stages, as well as immature nephrons (Figure 3). Mature glomeruli on the inner surfaces were observed to be further developed than those on the outer surface of the renal cortex. The kidney corpuscles consisted of numerous parietal podocytes without feet, located inside glomeruli in the inner zone of the kidney cortex. Moreover, both the inner and outer surface of the renal cortex contained numerous properly-formed proximal tubules without brush borders but with a regular lumen lined with cuboidal epithelium (Figure 4), as well as a few distal tubules containing a lumen lined with flat epithelium. The interstitial and medullar areas contained loose-appearing tissue and numerous oval nucleus cells. In the newborn rats, kidney development was almost complete. However, in the outer zone of the cortex, we observed a few still-developing glomeruli, as well as proximal tubules containing whole, undeveloped brush-border membranes (Figure 5). Nephrons had a glomerular capsule with a single layer of cuboidal epithelium, and cuboidal podocytes consisted of only a few roots. The proximal tubules of the newborn rats contained irregular nuclei, highly electron-dense cytoplasm, numerous polyribosomes, occasionally granular endoplasmic reticula, and a brush border surrounding small lumina. On the other hand, in the 180-day-old postnatal rats, the kidneys were characterized by mature glomeruli, flat podocytes, proximal and distal tubules in the renal cortex, and collecting duct tubules, loops of Henle, and papillary ducts in the medullar area.

Stereological Results:

All stereological results from fetal, newborn and adult rats are summarized in Table 1. In stereological examinations of the fetal, newborn and adult rat kidneys, the average kidney volume was determined to be 6.3, 134.54 and 1680 mm3, respectively.

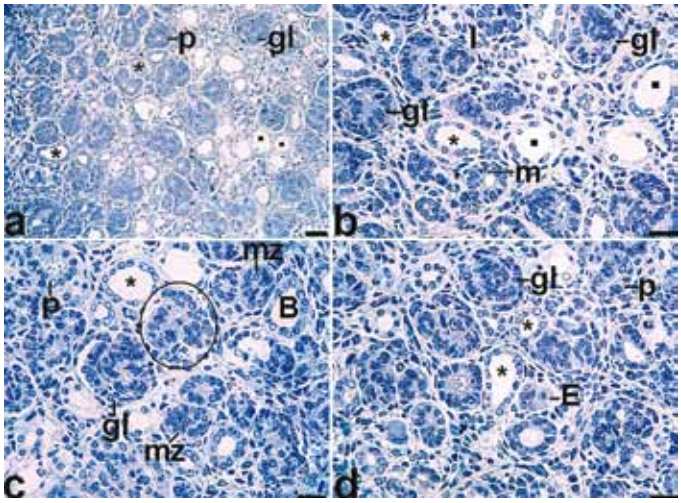

Fig. 5 — Semi-thin sections of kidneys from 7-day-old rats; structures are marked as follows: glomerulus (gl), distal tubule (asterisk), connective tubule (square), extramesangial cells (E), mesangium (mz), Bowman's capsule (B), proximal tubule (p), mitose (m), interstitium (I), a developing glomerulus (circle). Scale bars: 30 µm.

The percentage rate of the cortex in fetal kidneys (85.71%) was higher than in newborn (53.77%) or adult rats (76.78%). The total volume of the distal and proximal tubules and total number and volume of the glomeruli were significantly higher ($P<0.05$) in adult compared to newborn rats, and in newborn compared to fetal rats (Table 1). The total number of glomeruli was significantly increased ($P<0.05$), although the mean volume of Bowman's capsule was significantly decreased in adult rats compared to newborn or fetal rats (Table 1).

Discussion

The unbiased stereological counting method is used to estimate the number and volume of cells in a kidney at the microscopic level [11,13,19]. In this study, we have used stereological counting and electron microscopy to compare the kidneys of 20-day-old prenatal and 7- and 180-day-old postnatal rats. The development of nephrons in the cortical area begins at gestational week five in humans [20], at prenatal days 11 and 12 in mice and rats [21], and at prenatal days 12 and 20 in rabbits [22], and is completed at gestational week 36 in humans [20,23] and postnatal days 7 and 10 in mice and rats [21]. In this study we have found that the glomeruli and proximal and distal tubules are still developing at prenatal day 20 in the rat. In newborn rats, we observed completed kidney development at the ends of the proximal and distal tubules, but some immature glomeruli appeared in the inner surfaces of the renal cortex. Using stereological methods, we found that the average volume of the cortical and medullar kidney are significantly increased in adult rats compared with prenatal and newborn animals. We therefore conclude that the total number of glomeruli and the volumes of the distal and proximal tubules increase between the 7th and 180th postnatal days in the rat kidney.

Curto et al. [23] have reported that the epithelial cells of early renal vesicles have large nuclei, a well-defined basement membrane, tight tubular junctions and an undeveloped brush-border membrane at 13 days of gestation in mice, whereas, at 15 days (mice) [24] or 18 days (rabbit), well-defined proximal tubular epithelial cells with a more extensive brush-border membrane are present [9,24,25]. In our study of the prenatal rat kidney, we also observed most of the features reported by Curto et al. [23]. However, both the inner and outer surfaces of the cortex featured loose-appearing connective tissue, numerous proximal tubules without brush borders, a few distal tubules containing flat epithelium, glomeruli at different stages of maturation, and immature nephrons.

The developmental stages of the nephron which feature renal vesicles and comma- and S-shaped bodies can be visualized until postnatal day 5 [6,8], but kidneys from 6- to 9-day-old rats contain nephrons at developmental stages III and IV [26]. During the postnatal development of the kidney, immature nephrons can be observed on the outer surface of the renal cortex, while mature nephrons are found close to the renal medulla [2]. From postnatal days 16 to 26, the greatest number of mature nephrons can be found in the juxtamedullary region, whereas the external region of the renal cortex contains the fewest mature nephrons [26]. In this study, we observed ongoing development of the glomeruli and proximal and distal tubules in the newborn, whereas we saw fully developed nephrons in the adult kidney. Furthermore, the total volume of the distal and proximal tubules (0.693 and 1.512 mm3) in prenatal rats was significantly smaller than in newborn (14.74 and 32.35 mm3 (distal and proximal tubules)) or adult rat kidneys (230 and 401.25 mm3 (distal and proximal tubules)).

Scanning or transmission electron microscopic studies have reported irregularly shaped areas of Bowman's capsule lined with parietal podocytes, which are generally around the vascular pole [10]. Parietal podocytes located within the extraglomeru-

Table 1. Stereological analysis of the kidney in fetal, newborn and adult rats (n=10)

Estimate	Groups		
	Fetuses	Newborns	Adults
Mean volume of kidney (mm^3)	6.3 ± 0.47^c	134.54 ± 24^b	1680 ± 235^a
Mean volume of cortex (mm^3)	5.43 ± 0.07^c	72.35 ± 13^b	1290 ± 105^a
Mean volume of medulla (mm^3)	0.87 ± 0.09^c	62.19 ± 2.63^b	390 ± 12.54^a
Total volume of distal tubules (mm^3)	0.693 ± 0.005^c	14.74 ± 1.10^b	230 ± 1.43^a
Total volume of proximal tubules (mm^3)	1.512 ± 0.25^c	32.35 ± 3.17^b	401.25 ± 32.65^a
Mean numerical density of glomeruli (glomeruli/mm^3)	1850 ± 70^a	133.5 ± 13^b	19.40 ± 0.7^c
Total number of glomeruli	11650 ± 634^c	17896 ± 519^b	32600 ± 420^a
Total volume of glomeruli (mm^3)	34.1 ± 0.07^a	34.2 ± 0.03^a	23.2 ± 0.09^b
Mean volume of glomeruli (10^{-3} mm^3)	2.930 ± 0.005^a	1.913 ± 0.001^b	0.712 ± 0.004^c
Mean volume of Bowman's capsule (10^{-6} mm^3)	0.301 ± 0.002^a	0.137 ± 0.006^b	0.075 ± 0.003^c

Different superscripts a,b,c in the same line indicate significant differences between groups ($P<0.05$).

lar mesangium are surrounded by juxtaglomerular arterioles [1] and have desmosomes and zonula adherens-like junctions on la teral cell surfaces and long microprojections and infrequent cilia on apical cell surfaces [27]. In fetal nephrons, the visceral layer of the glomerular capsule consists of a single layer of cuboidal epithelium [28]. These visceral epithelial cells contain numerous free polyribosomes and an electron-dense cytoplasm, in contrast to the parietal layer, which appears to be less electron-dense, with scattered ribosomes and small mitochondria [27,29]. These studies agree with our light and ultra-structural results, although we did not observe long microprojections or cilia on the apical surfaces of podocytes in fetal nephrons. In our study, the mean volume of Bowman's capsule in adult rat kidneys (0.075x10-6 mm3) was significantly decreased compared to newborn (0.137x10-6 mm3) and fetal rats (0.301x10-6 mm3). Thus, it is possible that a smaller volume of visceral and parietal layers is made up of flat epithelium.

The number and size of glomeruli and proximal and distal tubules in a kidney may change according to functional renal disorders, species, gender and aging [19]. Hricak et al. [28] reported that the glomeruli accounted for 18% of the cortical vo lume in the neonatal human kidney, but occupied only 8.6% of the cortical volume in the adult. Using stereological methods, some researchers have reported total numbers of glomeruli as 589,000 in dogs [30], 559,000 in sheep [31], 195,000 in rabbits [32], 28,500-28,731 in Wistar-Kyoto rats [33,34] and 31,764-35,400 in Sprague-Dawley rats [19,23]. The glomerular volume in rats (0.6x10-3) [19] was higher than in dogs (0.12x10-3) [30] and lower than in sheep (0.44x10-3) [31,32]. In this study, the total numbers of glomeruli in fetal, newborn and adult rats were determined to be 11,650, 17,890 and 32,600, respectively. Our estimate for the total numbers of glomeruli in the adult kidney is similar to estimates obtained by Bertram et al. [19] and Schreuder et al. [32]. The glomerular volume (2.930x10-3 mm3) in the fetus accounted for 0.05% of the cortical volume, but was lower (1.913x10-3 mm3) in newborn and (0.712x10-3 mm3) adult rat kidneys (0.002% and 0.00005%, respectively).

In conclusion, we have observed several developmental stages of nephrons in prenatal and postnatal rat kidneys, using light and electron microscopy. Furthermore, using an unbiased stereological technique, we have shown that the average kidney volume, total volume of the distal and proximal tubules, and total number of glomeruli in the rat kidney increase according to age.

Conflict interest statement The authors declare that they have no conflict of interest to the publication of this article.

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