

# Stereological Evaluation of Renal Glomeruli in Offspring of Diabetic Female Rats

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## Abstract

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**Objective:** Although in vitro studies have shown that high concentrations of glucose can induce dysmorphogenesis of the embryonic kidney, the possible adverse effects of exposure to intrauterine hyperglycemia on kidney development, especially in regard to nephrogenesis, has not been evaluated.

The aim of this study is to investigate the effects of maternal diabetes on glomeruli structures of the offspring, focusing on the following parameters: glomeruli volume and number, mesangium volume, mesangial cell number and glomerular capillary volume.

**Materials and Methods:** Before mating, fifteen female Sprague Dawley rats, divided into three groups, were diabetes induced by a single intraperitoneal dose of 65 mg/kg streptozotocyn (STZ). After 30 days of breast feeding, ten offsprings from each group (two per mother) were randomly selected for kidney removal. The kidneys were weighed and their tissues were processed for light microscopy. Glomerular features were evaluated quantitatively using dissection as well as the Cavalieri method and were then compared with sham and control groups.

**Results:** At birth, the mean body weight of diabetic mothers' offspring (DO) was significantly lower than that of the control group's offspring (CO) and sham group's offspring (SO) ( $p=0.001$ ), however, the mean body weight of the 30 day-old DO was not lower than that of CO and SO ( $p>0.05$ ). The total renal volumes, cortical volumes, glomerular mean and total volumes, total mesangial volumes, total capillary volumes and total glomerular numbers were significantly lower in the DO than in CO and SO ( $p<0.05$ ). The numerical density of glomeruli and mesangial cells per glomeruli were significantly greater in DO than in CO and SO ( $p<0.05$ ).

**Conclusion:** We concluded that intrauterine hyperglycemia is accompanied by a nephron deficit which may not be compensated within the first 30 days after birth.

**Keywords:** Diabetes, Streptozotocyne, Gestational Diabetes, Diabetic Nephropathy, Histological Techniques

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## Introduction

A mother's lifestyle may have long-lasting effects on her offspring. For example smoking, alcohol consumption, and presence of diseases such as diabetes affect fetal development (1). There is a strong association between pregnancy in women with any form of diabetes and high infant mortality and morbidity rates (2). Maternal diabetes has the potential to adversely affect the development of multiple organ systems, resulting in a wide range of congenital malformations including: caudal regression, situs inversus, kidney malformations, cardiac anomalies and neural tube defects (NTDs) (3). Metabolic changes in a diabetic pregnancy affect fetal

development and fetal glucose homeostasis. Several epidemiological studies (1, 4) and animal experiments (5-7) have shown that offsprings of diabetic mothers have higher incidences of glucose intolerance, obesity, insulin resistance, non-insulin-dependent diabetes mellitus (NIDDM) and hypertension in later life. There are several hypotheses relating maternal diabetes to adulthood diseases and potential stillbirths (8). Congenital malformations are also more frequent in children of mothers with diabetes (9, 10), and are results of developmental defects occurring in early organogenesis (11, 12). They include failure of neural tube closure, caudal regression syndrom and urogenital ab-

normalities which can be as severe as renal agenesis (13), and are the main causes of perinatal mortality in offspring of diabetic mothers (14).

In humans, nephrogenesis occurs only during fetal life and stops after birth (15). In rats however, it occurs during the last third of gestation and continues for several days post birth (16). Maternal protein restrictions during the last third of gestation or during the period of nephrogenesis result in offspring which exhibit a marked reduction in glomeruli or nephron numbers and develop hypertension (17, 18). Intrauterine malnutrition due to placental dysfunction or poor maternal health, as well as maternal hyperglycemia/diabetes result in decreased nephron numbers (19). Amri et al. showed that the intrauterine glucose concentration must be closely controlled to ensure optimum metanephros development. Severe diabetes leads to a decrease in fetal pancreatic weight, an increase in pancreatic endocrine tissue and a decrease in pancreatic and circulating insulin (20, 21). Interference with third trimester fetal growth has been shown to affect nephron development. Nephron number is undoubtedly genetically determined, but there is a growing body of evidence that the number of glomeruli is additionally influenced by social and behavioral factors that effect intrauterine environment (22). Diabetic glomerulopathy is characterized by a very slow development in basement membrane (BM) accumulation, manifested as thickening of the peripheral BM, increased volume of the mesangial BM-like material (BMLM) along with mesangial expansion. The initiation of the process is probably at the onset of diabetes since the BM thickening is detectable after a few years. In the long run however, the two in concert lead to the ultimate solidification of the glomerular tuft with loss of capillary surface. The end-stage is glomerular closure with elimination of glomerular function (23). Hughson et al (22) investigated the link between birth weight and glomerular number. Using the physical disector/fractionator combination in kidney autopsies, birth weight was also found to be a strong predictor of total glomerular number and mean glomerular volume in African American, Caucasian as well as Hispanic infants (24). Accurate estimation of glomerular volume has become increasingly important because Vglom varies significantly in normal human kidneys (25), and alterations in Vglom may play a significant pathophysiological role in the development or progression of a range of nephropathies (26). It is therefore, important to determine whether exposure to the diabetic environment in utero influences nephrogenesis and final number of nephrons. In this study we estimated renal volume, absolute glomerular volume, total glomerular number, total mesangial cell number and mesangium volume in the offspring of diabetic female rats by the use of unbiased stereological methods.

## Materials and Methods

15 female Sprague-Dawley rats, with an average weight

of 200-250g were obtained from the Pasture Institute in Tehran, Iran. The animals were divided into three groups: the experimental group, the control group which was treated with citrate buffer and the sham group of untreated rats. The experimental group received a single dose of intraperitoneal 65 mg/kg streptozotocine (STZ) (Sigma-Aldrich) dissolved in 0.4 mol/l citrate buffer with pH: 4.5 (27). Presence of diabetes in the rats was glucometrically confirmed 3 days after STZ injection.

Animals with glucose levels greater than 300 mg/dl were considered manifestly diabetic and were included in the study. Diabetic females of the experimental group, as well as the non-diabetic females in the control and sham groups were housed (on female and one male in per cage) with males over night, and the appearance of a vaginal plug in the morning indicated that copulation and potential pregnancy had occurred. After birth, the offspring were housed in cages with standard conditions (temperature breast feeding and water) and water ad libitum. Ten 30 day old offspring from each group, two per mother, were then randomly selected.

## Tissue preparation

The offspring were anaesthetized with chloroform and were fixed by retrograde perfusion through the abdominal aorta with formal phosphate buffer. Right kidneys were then excised, decapsulated and immersed in the same fixative for 48 hours at room temperature. The fixed kidneys were embedded in 7% agar and sliced into 1 mm slices using a macrotome perpendicular to the longitudinal axis (28). Approximately 8-10 slices were obtained from each kidney. The slices were arranged in a number of sequences on meshed tissue processing baskets and were then processed and embedded in paraffin. The blocks were systematically sectioned at 5 micrometers, and every seventh section in order, with the first chosen randomly in the interval. They were then mounted on glass slides and stained with periodic acid-Schiff (PAS). Every selected (1st and 7th) slice underwent a stereological analysis which involved the use of Olympus Optical BH2 microscopes. To estimate the volume of cortex, medulla and the whole kidney, every first section was viewed on a Motic loop microscope at a magnification of 20× with the image being projected on a computer monitor. A fine grid of points distanced every 6 millimeters was superimposed over the visual field of the sampled sections. Point counting using Cavalieri principle was then used to estimate the volume of cortex, medulla and whole kidney using the following equation (29).

$$V = \frac{\sum P.a/p.t}{M^2} (1)$$

V is the volume of kidney,  $\sum P$  is the sum of all points

counted in sections,  $a/p$  is the area associated with each point,  $t$  is the distance between two consecutive sections in which points were counted,  $M$  is the linear magnification of the projector. The volume fraction ( $V_v$ ) of the glomeruli was obtained by use of the estimated point count of the random cross sections in the following equation:

$$V_v (\text{glom/cortex}) = \frac{\sum P_p}{\sum P_t} \quad (2)$$

$\sum P_p$  is the sum of all counted points overlying glomeruli.

$\sum P_t$  is the sum of probe points of  $n$  field.

Absolute volume of glomeruli was estimated indirectly by multiplying the volume fraction by volume of the reference space (28).

$$V_{\text{total}} (\text{glom/kid}) = V_v (\text{glom/cortex}) \cdot V_{\text{ref}} \quad (3)$$

$V_{\text{ref}}$  is the volume of cortex

The physical disector was used to estimate the numerical density of glomeruli. The first and 7th sections from each slice were chosen as reference and look-up sections respectively (29). For each pair of samples, the first section (the reference section) was examined with microscope A (Olympus Optical BH2). The resulting image was then projected onto the computer screen with the use of a Motic Moticam 2000 digital camera. The 7th slice of each section (the look-up section) was examined with microscope B (Olympus Optical BH2). Then video images of the first (reference) sections were compared to the live images of the seventh (look-up) sections. An orthogonal frame (6cm . 6cm) with certain areas of forbidden and acceptance lines was superimposed over the visual fields of sampled sections at a magnification of 100x. Counting of the glomerular profiles was performed in the first (reference) but not in the seventh (look-up) sections in areas not cut by the forbidden lines. In each kidney, about 30 to 60 frames

from all slices were chosen and glomerular profiles were counted. The estimation of numerical densities and glomerular counts were determined by the use of the following equation:

$$N_v (\text{glom/cortex}) = \frac{\sum Q}{\sum P \cdot a \cdot d \cdot 2} M^2 \quad (4)$$

$\sum Q$  is the sum of all glomeruli in all of the disector frames placed in the sections.  $\sum P$  is the number of frames placed in the sections.  $a$  is the fraction of the section area used for glomerular counting.  $d$  is the height of disector (30  $\mu\text{m}$ ),  $M$  is linear magnification. Using the numeric estimations of glomerular density and cortex volumes, the total number of glomeruli was obtained from the following equation: (30).

$$N_{\text{total}} (\text{glom}) = V (\text{cortex}) \cdot N_v (\text{glom/cortex}) \quad (5)$$

Values are expressed as Mean  $\pm$  Standard deviation. A One-Way ANOVA followed by a Tukey test were used for analysis of differences in multiple comparisons between the groups. In each case, the null hypothesis was rejected if the probability of no differences was less than 5%.

## Results

Body weights and kidney dimensions for the offspring of diabetic mothers as well as offspring of sham and control subjects are presented in Table 1.

The mean body weight of newborns of diabetic mothers was significantly lower than that of newborn offspring of control and sham subjects ( $p=0.000$ ). However, the body weight of 30 day-old offspring of diabetic mothers (DO) was not significantly lower than that in control (CO) and sham (SO) subjects ( $p=0.218$ ). The kidney weights were similar in all 3 groups ( $p=0.773$ ).

**Table 1: Comparison of the mean body weight, mean kidney weight and volume, and mean cortex and medulla volumes**

Characteristic	Diabetic mothers' offspring (n=10)	Sham (n=10)	Control (n=10)	P value	
				DO vs SO	DO vs CO
Mean body weight(g) 3 days	7.63 $\pm$ 0.21*	10.62 $\pm$ 0.73*	10.68 $\pm$ 0.75*	0.001	0.000
Mean body weight (g) 30 days	35.39 $\pm$ 1.27	39.98 $\pm$ 2.441	39.80 $\pm$ 2.16	0.266	0.299
Mean kidney weight (mg)	322.9 $\pm$ 22.27	304 $\pm$ 20	319 $\pm$ 16	0.781	0.991
Mean kidney volume (mm <sup>3</sup> )	133.66 $\pm$ 5.66*	181.26 $\pm$ 8.53*	173.41 $\pm$ 10.59*	0.001	0.007
Mean cortical volume (mm <sup>3</sup> )	91.14 $\pm$ 4.47*	135.26 $\pm$ 6.12*	131.12 $\pm$ 7.72*	0.000	0.000
Mean medulla volume (mm <sup>3</sup> )	42.81 $\pm$ 3.23	45.81 $\pm$ 3.41	42.29 $\pm$ 3.20	0.787	0.994

Results are Mean  $\pm$  Standard error; \*  $p < 0.05$  (ANOVA)

DO: offspring of diabetic mothers; SO: offspring of mothers in sham group; CO: offspring of mothers in control group.

Table 2: Comparison of volume fractions of renal cortical features between the groups.

Characteristic	Diabetic mother offspring (n=10)	Sham (n=10)	Control (n=10)	P value	
				DO vs SO	DO vs CO
Vv (glom/cortex) 0.005	0.062474 ± 0.004*	0.08235 ± 0.005*	0.09113 ± 0.007*	0.043	0.005
Vtotal (glom/kid) 0.000	5.53936 ± 0.188*	10.79032 ± 0.96*	11.2819 ± 1.063*	0.001	0.000
Vv (mes/glom)	0.4072 ± 0.0113	0.3485 ± 0.018	0.3588 ± 0.028	0.112	0.237
Vtotal (mes/kid)	2.26406 ± 0.12 *	3.47778 ± 0.47 *	4.09037 ± 0.57*	0.120	0.019
Vv (cap/glom)	0.596281 ± 0.012	0.66904 ± 0.028	0.641129 ± 0.28	0.098	0.0409
Vtotal (cap/kid)	3.293079 ± 0.09*	6.862921 ± 0.93*	7.1916 ± 0.66 *	0.003	0.002

Results are Mean ± Standard error; \*  $p < 0.05$  (ANOVA)

Vv (glom/cortex): volume density of glomerulus; Vtotal (glom/kid): total glomerular volume; Vv (mes/glom): volume density of mesangium; Vtotal (mes/kid): total mesangium volume; Vv (cap/glom): volume density of capillary; Vtotal (cap/kid): total capillary volume. DO: offspring of diabetic mothers; SO: offspring of mothers in sham group; CO: offspring of mothers in control group.

Table 3: Comparison of the numerical fractions of renal cortical features between the groups.

Characteristic	Diabetic mother offspring (n=10)	Sham (n=10)	Control (n=10)	P value	
				DO vs SO	DO vs CO
Nv (glom/cortex) 0.005	77.9170 ± 5.93*	63.9635 ± 1.17*	61.8238 ± 1.78*	0.041	0.023
Ntotal (glom/kid) 0.000	6536.72 ± 532*	8349 ± 519 *	7639.9 ± 429 *	0.046	0.030
Nv (mes/glom)	984.061 ± 54.71	592.51 ± 25.23*	596.95 ± 46.4*	0.000	0.000
Ntotal (mes/kid)	5438 ± 331	6398 ± 645	6909 ± 1048	0.588	0.330

Results are Mean ± Standard error; \*  $p < 0.05$  (ANOVA)

Nv (glom/cortex): Numerical glomerular density; Ntotal (glom/kid): total glomerular number per cortex; Nv (mc/glom): Numerical mesangial density;

Ntotal (mes/glom): total mesangial cell number per kidney. DO: offspring of diabetic mothers; SO: offspring of mothers in sham group; CO: offspring of mothers in control group.

Light-microscopic stereological data are presented in Table 2. The mean offspring kidney and cortical volumes were significantly lower in DO than in CO and SO groups ( $p < 0.005$ ), although medullary volumes were not changed in the DO group ( $p > 0.05$ ). The glomerular volume density, glomerular volume divided by cortical volume and total glomerular volume were significantly lower in DO than in CO and SO groups ( $p < 0.05$ ). Although the numerical glomerular density (glomerular number divided by cortical volume (repetition)) in DO was greater than in CO and SO groups ( $p < 0.05$ ), the total glomerular number was significantly lower in DO than in CO and SO groups ( $p = 0.055$ ). The mesangial volume density was numerically but not significantly greater in DO than in CO and SO groups, whereas the total mesangial volume was significantly lower in DO than in CO and SO groups. The offspring capillary volume density in the diabetic group was similar to that in the control and

sham subjects; however, the total capillary volume was lower in diabetic mothers' offspring. The mesangial cell density (mesangial cell numbers divided by glomerular volume) was significantly greater in DO than in CO and SO groups ( $p = 0.000$ ); however, the total numbers of mesangial cells were similar in all three groups.

## Discussion

In the present experimental study, our results showed that the body weight of diabetic mothers' offspring was reduced at birth or in neonates. However, at 30 days of life, the body weight was not significantly lower in the DO than in the control and sham groups, suggesting that the diabetic fetal environment compromised development. Previous studies also demonstrated that a diabetic pregnancy is associated with suppression in fetal growth, related to reduced protein synthesis (31). Contrary to our results, Wichi et al (32) showed that



the body weight of offspring of diabetic mothers was not reduced at birth or in neonates, but was significantly lower 30 days after birth. Both mild gestational diabetes and a reduction in litter size are associated with prenatal hyperinsulinism, (33). Thus as predicated by Barker, timing of the insult in utero may be critical in the fetal programming of adult disease (34). The experimental induction of diabetes using streptozotocine (STZ) has dose-dependent effects: low doses of STZ result in mild gestational diabetes associated with fetal macrosomia (33), whereas high doses induce insulin-deficient diabetes associated with fetal growth restriction (35).

The present study showed a reduction in kidney volume particularly in cortical zones, but not in medullary portions in the DO group. Osmand et al. also demonstrated that slowing of growth in late gestation leads to disproportion in the size of organs that develop rapidly in late gestation (e.g. the kidneys). Possibly through the effect of decreased maternal IGF, fetal insulin, insulin like growth factor (IGF), and glucose concentrations fall if a mother decreases her food intake. This leads to a slowing in growth (36).

The numerical glomerular density in DO group was significantly greater than in CO ( $p=0.041$ ) and SO ( $p=0.023$ ) groups, and also total glomerular number in DO was significantly lower than in CO and SO groups ( $p=0.046$ ). This is due to the fact that cortical volume in DO was lower than in CO and SO groups. Our findings were in agreement with the observations of others. Low glomerular numbers in diabetic mothers' offspring have been directly correlated with low birth weights. Hughson et al. (22) demonstrated a direct relationship between total glomerular number (Nglom) and birth weight in adult and fully developed postnatal kidneys of infants. A low glomerular number may be the result of imperfect nephrogenesis during kidney development and/or loss of nephrons before nephrogenesis (37). In animal models, in-utero exposure to hyperglycemia was associated with a reduction of number of nephrons in the offspring (38). In this study, the mesangial volume density was numerically, although not significantly ( $p>0.05$ ), greater in DO than in CO and SO groups. However, total mesangial volume was significantly lower in DO because the cortical volumes were lower than CO and SO groups. The increment in glomerular volume is due to an increase in mesangial matrix as well as hypertrophy of other glomerular structures (39). Previous studies on the short-term effects of diabetes on kidney morphology have suggested cortical hypertrophy accompanied by glomerular mesangial hypertrophy (23, 24), whereas long-term diabetes caused increased glomerular basement membrane thickness (23). In this study, there were significant changes in the mesangial matrix. Further increase in glomerular and mesangial volumes, as well as capillary length and surface area changes were attributed to compensatory hypertrophy due to the loss of nephrons (39).

The capillary volume densities in diabetic mothers' offspring were similar to control and sham subjects. However, total volume of capillary was lower in DO

because the cortical volume was lower in this group. Mesangial cell densities however, were significantly greater in diabetic mothers' offspring, suggesting that the increase in cell number is not simply in keeping with the degree of glomerular enlargement. There is a greater proportion of mesangial cells in the DO glomerulus. The short-term effects of diabetes have been suggested to be increased number of glomerular mesangial cells and interstitial alterations (40-42). These findings are in agreement with the observations of White et al. (43), and are in contrast to those of Steffes et al. (44), who did not find a significant increase in the absolute number of mesangial cell density. However, it must be taken into consideration that the patients in Steffes et al study were at an earlier stage of nephropathy and did not demonstrate any increase in glomerular volume.

## Conclusion

This study identifies maternal diabetes as a novel risk factor for inborn nephron deficit in addition to other factors such as fetal growth retardation which alter nephrogenesis. It points out the particular risk of a high blood glucose level during the critical period of nephron formation, particularly in early stages, and calls for a strict control of blood glucose in a diabetic pregnancy.

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