

Morphofunctional renal alterations in rats induced by intrauterine hyperglycemic environment

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Abstract

Introduction: The renal development of rats begins in intrauterine life, finishing by 15 days after birth. Diabetes and other diseases during pregnancy can cause systemic changes in the offspring. We evaluated the structural and functional renal alterations of the offspring from diabetic mothers.

Material and methods: Pregnant rats were separated and 1, 7, 30 and 90 days-old (DO) pups were divided into groups according to the treatment that the mothers received: G1: control, G2: untreated diabetic and G3: insulin-treated diabetic. The kidneys from offspring at 1, 7 and 30 DO were removed for immunohistochemical and histological studies. Furthermore, blood and urine samples were collected from animals at 30 DO to determine the glomerular filtration rate (GFR) by creatinine clearance, and the animals at 90 DO were subjected to blood pressure measurement by plethysmography.

Results: Our results show an increase of PCNA⁺ glomerular cells at 7 DO and a reduction in 30 DO animals as well as increased α -smooth muscle actin (α -SMA) tubulointerstitial expression at 1 and 7 DO in animals from G2, when compared with controls. The adult offspring from G2 showed reduced GFR and increased blood pressure.

Conclusions: Maternal diabetes may have induced programming of renal damage in offspring of hyperglycemic mothers, which may have contributed to the impairment of renal function.

Key words: maternal diabetes, postnatal renal development, offspring.

Introduction

Renal development of rats begins during pregnancy and is completed in the postnatal period (about 15 days after birth) [1]. Congenital renal malformations are frequently seen in offspring from diabetic mothers [2–4], and these defects usually occur in early organogenesis [1].

Diabetes can cause changes in the number of proliferating renal cells and also increased production of collagen and other extracellular matrix (ECM) components [5–7]. Alterations in ECM composition will impair the nephrogenesis in pups of hyperglycemic mothers, because ECM formation is a key event in renal cell differentiation [8].

Many studies have indicated that renal hemodynamics are altered in clinical and experimental diabetes mellitus and changes in the renal hemodynamics play an important role in the pathogenesis of nephropathy that is commonly seen in this metabolic disorder [9–13].

The aim of the present study was to evaluate the renal effects of maternal diabetes on the offspring.

Material and methods

Animals

Female Wistar rats (2–3 per male) were caged overnight with a male. Vaginal smears were taken on the following morning. All dams were housed and fed individually. After birth, the offspring were divided into three groups: G1 (control – offspring from control mothers that received a single intraperitoneal injection of 0.9% saline); G2 (untreated diabetic – offspring from diabetic mothers that received a single intraperitoneal injection of alloxan (Sigma-Aldrich, S. Louis, MO.) – 100 mg/kg, diluted in 0.9% saline); and G3 (treated diabetic – offspring from diabetic mothers treated with insulin that received a single intraperitoneal injection of alloxan – 100 mg/kg – diluted in 0.9% saline and were treated daily with insulin until the end of the weaning period). Diabetic state was confirmed 2 days after induction by measuring blood glucose; only those animals with glycemic levels greater than or equal to 150 mg/dl were considered diabetic. The female offspring aged 1, 7, and 30 days old (DO) were anesthetized and the kidneys were removed for histological and immunohistochemical studies. Animals aged 30 DO had their kidneys removed only after functional studies. The kidneys were fixed in methacarn solution for 24 h and then rinsed in 70% ethanol. This study was submitted and approved by the Institutional Animal Care and Use Committee of the Federal University of Uberlândia (Animal Care and Use Committee approval no. 043/11).

Histological and morphometric analysis

The slices of kidneys from female offspring aged 1, 7 and 30 DO of each group were stained with Masson's trichrome, which enabled the analysis of renal tissue and determination of the area corresponding to the glomerular tufts ($n = 5$ for each group aged 30 DO). The program used to measure glomerular tuft area was Image J (Version 1.4).

Immunohistochemical analysis

Renal tissue sections were deparaffinized and incubated with primary monoclonal antibodies anti- α -smooth muscle actin (anti- α -SMA) (1 : 1000 – Sigma-Aldrich, S. Louis, MO.) overnight at 4°C and anti-proliferating cell nuclear antigen (anti-PCNA) (1 : 1000 – Sigma-Aldrich, S. Louis, MO.) for 30 min at room temperature. The sections were then subjected to further incubation with a secondary mouse anti-IgG antibody (1 : 200 – monoclonal – Vector Laboratories; Burlingame, CA). Im-

munochemical staining was detected by an avidin-biotin-peroxidase system (Vector Laboratories; Burlingame, CA), stained with DAB (3,3 diaminobenzidine) (Sigma; Israel), and the sections were counterstained with methyl green.

Immunohistochemical evaluation

Immunohistochemistry for anti- α -SMA was evaluated by analyzing the percentage of glomeruli or renal cortex labeled by assigning a score between 0 and 4, where 0 was equivalent to 0–5% staining; 1 indicated 5–25%; 2 indicated 25–50%; 3 indicated 50–75%; and 4 indicated 75–100% staining [14]. The numbers of PCNA-positive cells in each glomerulus or cortical interstitial grid field were determined in all renal cortex samples and the mean counts were calculated for each kidney.

Renal function studies

Offspring aged 30 DO (G1: $n = 15$; G2: $n = 8$; G3: $n = 4$) were placed in metabolic cages for a period of 24 h, and urine and blood samples were collected for creatinine determination. Glomerular filtration rate (GFR) was evaluated by creatinine clearance (Gold Analisa Diagnóstica Ltda; Belo Horizonte, Minas Gerais, Brazil).

Systolic blood pressure

Systolic blood pressure (SBP) was measured indirectly in offspring aged 90 DO (G1: $n = 8$; G2: $n = 7$; G3: $n = 5$) using plethysmography (PowerLab and LabChart7 Pro; ADInstruments).

Statistical analysis

Statistical significance of differences between the experimental groups was assessed using GraphPad Software Prism 5.0 (La Jolla; CA). Data were analyzed using the non-parametric Kruskal-Wallis test followed by the Dunn post-test. These data were expressed as the mean \pm standard error of the mean (SEM). In all cases, the level of significance was set at $p < 0.05$.

Results

Histological, morphometric and immunohistochemical analysis

Histological analysis showed glomeruli in different evolution phases on the renal cortex of animals aged 1 and 7 DO, while offspring aged 30 DO presented glomeruli in the final phase of differentiation. The morphometric analysis showed a smaller glomerular tuft area in the renal cortex of pups aged 30 DO ($1043.883 \pm 42.270 \mu\text{m}^2$) from G2 when compared to controls ($1501.857 \pm 36.720 \mu\text{m}^2$) of the same age. G3 animals showed a similar glo-

merular tuft area ($1546.525 \pm 96.480 \mu\text{m}^2$) to G1 animals (Figure 1).

The PCNA immunohistochemistry performed for the detection of cells undergoing proliferation revealed a reduction in the PCNA⁺ cells over the postnatal kidney development (Table I; Figures 2 and 3), as seen for α -SMA expression (Table I; Figure 4).

Immunohistochemical analysis showed that the offspring from dams exposed to diabetes without treatment presented increased α -SMA tubulointerstitial expression, predominantly in animals aged 1 (2.284 \pm 0.119) and 7 DO (2.271 \pm 0.154), when compared to controls (1 DO = 1.758 \pm 0.277; 7 DO = 1.749 \pm 0.180), while 30 DO pups of the same group (0.004 \pm 0.002) had a reduction in α -SMA glomerular expression in relation to the G1 animals (0.039 \pm 0.007) (Figures 5 and 6). Furthermore, pups from G2 aged 7 DO (4.583 \pm 0.432) exhibited an increase in the number of glomerular PCNA⁺ cells compared to the controls (3.669 \pm 0.294) (Figures 2 and 3), in addition to certain tubular dilatation, as morphological alterations, while animals aged 30 DO (1.757 \pm 0.194) had a reduction of proliferative cells, when compared to controls of the same age (3.551 \pm 0.524) (Figures 2 and 3). There were no significant differences between G1 and G3 for the immunohistochemical analyses cited above for the different ages (Figures 3, 5 and 6).

Diabetic mothers (G2) had increased urine volume and fluid intake during pregnancy, signals characteristic of diabetes type 1. Furthermore, blood glucose of these mothers in the same pe-

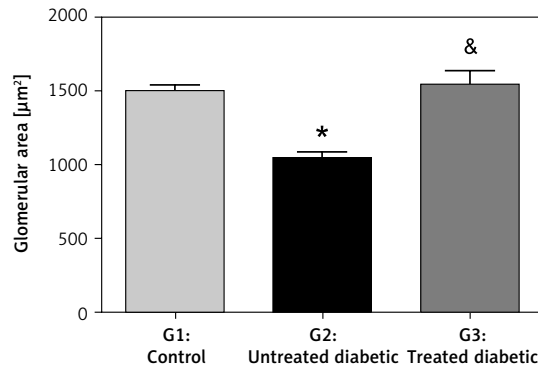


Figure 1. Morphometric analysis of glomerular tuft area in the renal cortex of G1 (light gray bar), G2 (black bar) and G3 (dark gray bar) groups at 30 days old

Data are expressed as mean \pm SEM; * p < 0.05 (vs. control); & p < 0.05 (vs. untreated diabetic).

riod was 441.40 \pm 49.14 mg/dl, while the glucose levels of dams from G1 and G3 were similar and lower than G2 (146.40 \pm 11.28 mg/dl).

Functional studies

The GFR, evaluated by creatinine clearance, was reduced in animals from G2 at 30 DO (0.422 \pm 0.113 ml/min) compared to aged-matched controls (0.912 \pm 0.058 ml/min) (Table II; Figure 7). The SBP measured indirectly by plethysmography was increased in pups from G2 (147.600 \pm 3.247 mm Hg) when compared to controls (127.200 \pm 4.777 mm Hg) (Table II; Figure 8).

Table I. Score for glomerular and tubulointerstitial α -SMA expression and number of PCNA⁺ cells per glomerulus

Groups (age in days)	PCNA ⁺ cells	Glomerular α -SMA	Tubulointerstitial α -SMA
G1:			
1	7.938 \pm 0.447	1.489 \pm 0.197	1.758 \pm 0.277
7	3.669 \pm 0.294	0.637 \pm 0.109	1.749 \pm 0.180
30	3.551 \pm 0.524 ⁺¹	0.039 \pm 0.007 ^{+1, +7}	0.104 \pm 0.040 ^{+1, +7}
G2:			
1	6.708 \pm 0.303	1.727 \pm 0.119	2.284 \pm 0.119*
7	4.583 \pm 0.432 [†]	0.702 \pm 0.119 ^{###1, #7}	2.271 \pm 0.154 ^{#1, #7, *}
30	1.654 \pm 0.182 ^{#1, #7, *}	0.004 \pm 0.002	0.247 \pm 0.031 ^{**}
G3:			
1	6.224 \pm 0.435	1.722 \pm 0.085	1.782 \pm 0.131
7	3.781 \pm 0.163 ^{&}	0.755 \pm 0.125	2.040 \pm 0.074
30	4.327 \pm 0.386 ^{&}	0.038 \pm 0.026 ^{""1}	0.247 \pm 0.031 ^{""1, ""7}

Data are expressed as mean \pm SEM; PCNA⁺: ⁺¹ p < 0.05 (vs. control at 1 day old); ^{#1} p < 0.05 (vs. untreated diabetic at 1 day old); ^{#7} p < 0.05 (vs. untreated diabetic at 7 days old); * p < 0.05 (vs. control at the same age); [&] p < 0.05 (vs. untreated diabetic); glomerular and tubulointerstitial α -SMA: ⁺¹ p < 0.05 (vs. control at 1 day old); ⁺⁷ p < 0.05 (vs. control at 7 days old); ^{###1} p < 0.001 (vs. untreated diabetic at 1 day old); ^{#1} p < 0.05 (vs. untreated diabetic at 1 day old); ^{#7} p < 0.05 (vs. untreated diabetic at 7 days old); ^{""1} p < 0.01 (vs. treated diabetic at 1 day old); ^{""7} p < 0.05 (vs. treated diabetic at 7 days old); ^{""1, ""7} p < 0.05 (vs. treated diabetic at 1 day old); ^{""1, ""7} p < 0.05 (vs. treated diabetic at 7 days old); tubulointerstitial α -SMA: ^{*} p < 0.05 (vs. control at the same age); glomerular α -SMA: ^{**} p < 0.01 (vs. control at the same age).

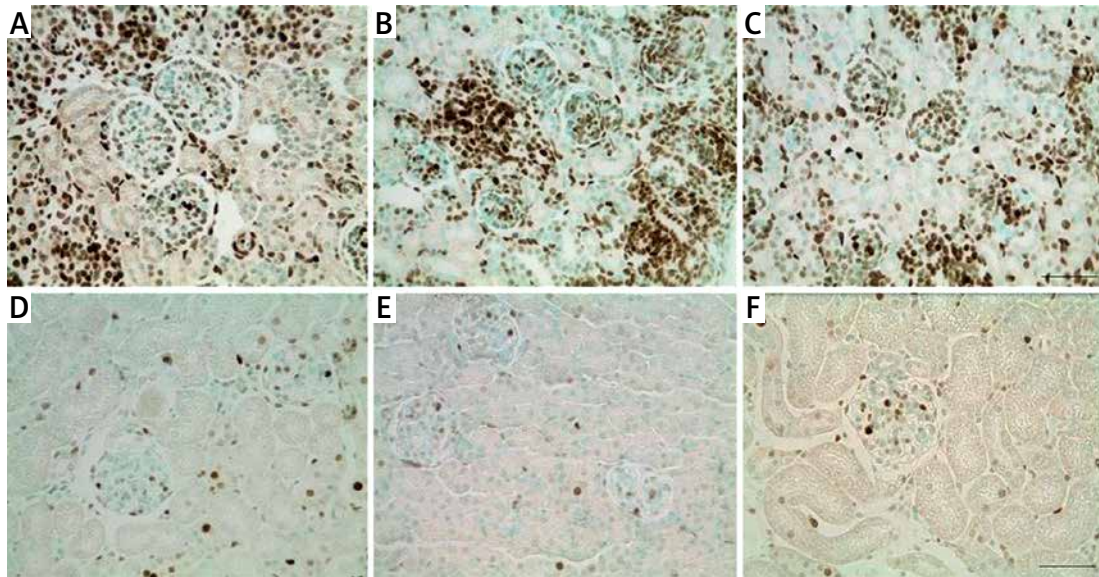


Figure 2. Immunostaining for PCNA in the renal cortex of G1 (control – A, D), G2 (untreated diabetic – B, E) and G3 (treated diabetic – C, F) groups at 7 (A–C) and 30 (D–F) days old. Note that there are more PCNA⁺ cells per glomerulus in the animals aged 7 days old than at 30 days old in G1, G2 and G3. Bar = 50 μm

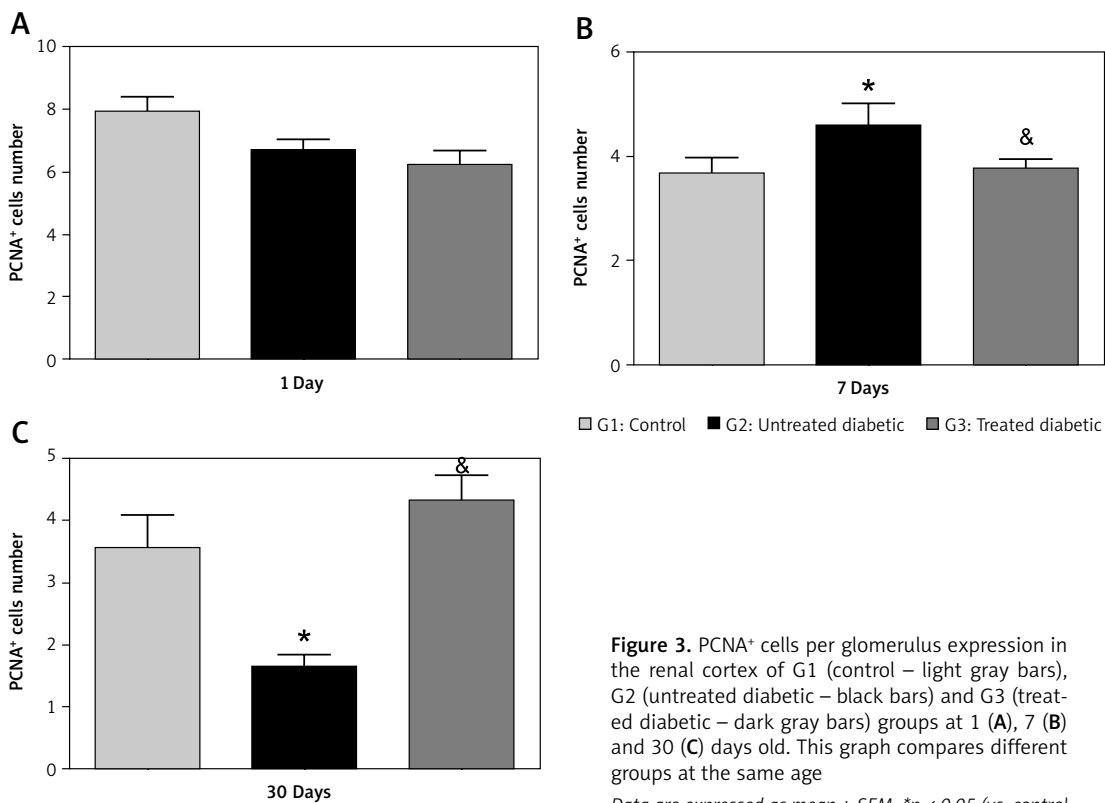


Figure 3. PCNA⁺ cells per glomerulus expression in the renal cortex of G1 (control – light gray bars), G2 (untreated diabetic – black bars) and G3 (treated diabetic – dark gray bars) groups at 1 (A), 7 (B) and 30 (C) days old. This graph compares different groups at the same age
Data are expressed as mean ± SEM; **p* < 0.05 (vs. control of same age); &**p* < 0.05 (vs. untreated diabetic).

Insulin treatment was effective in minimizing functional impairment because animals from G3 had similar data to controls, in relation to both GFR (0.865 ± 0.163 ml/min) (Table 2; Figure 7) and SBP (120.600 ± 2.637 mm Hg) (Table II; Figure 8). Animals from G3 were also similar to G1 in relation to the immunohistochemical studies, which allowed us to infer and expect a similar-

ty in the functional data from these two groups, which was actually found.

Discussion

In the developing kidney, immature glomeruli with formations in vesicles and in S (second phase of glomerular development) are predominant-

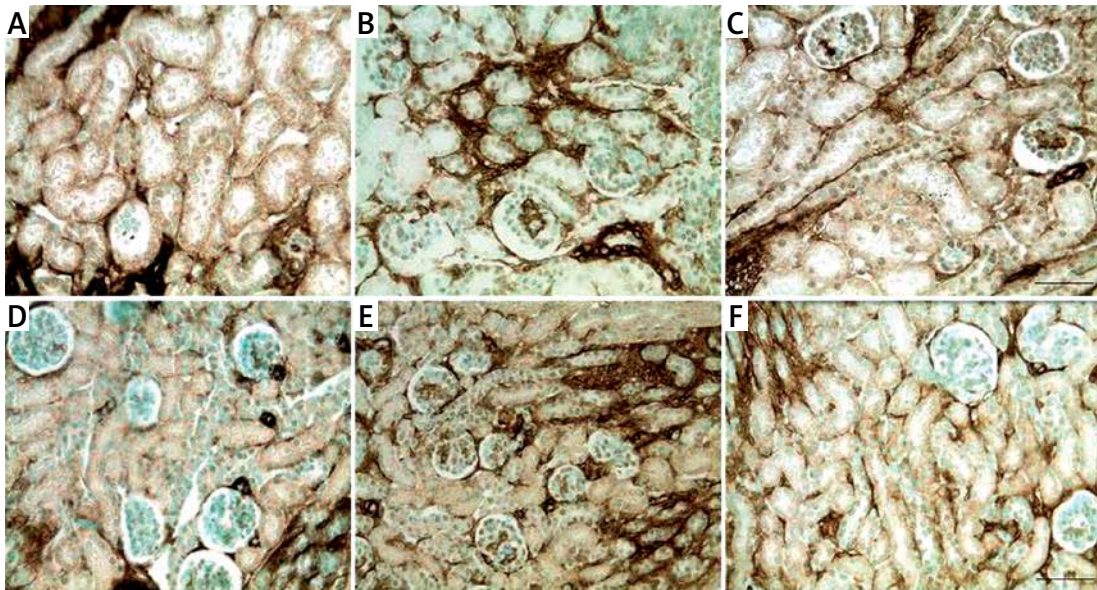


Figure 4. Immunostaining for α -SMA in the renal cortex of G1 (control – A, D), G2 (untreated diabetic – B, E) and G3 (treated diabetic – C, F) groups at 1 (A–C) and 7 (D–F) days old. Note that there is an increase in α -SMA expression in the tubulointerstitial compartment of animals from G2 at 1 and 7 days old, when compared to controls of the same age. Bar = 50 μ m

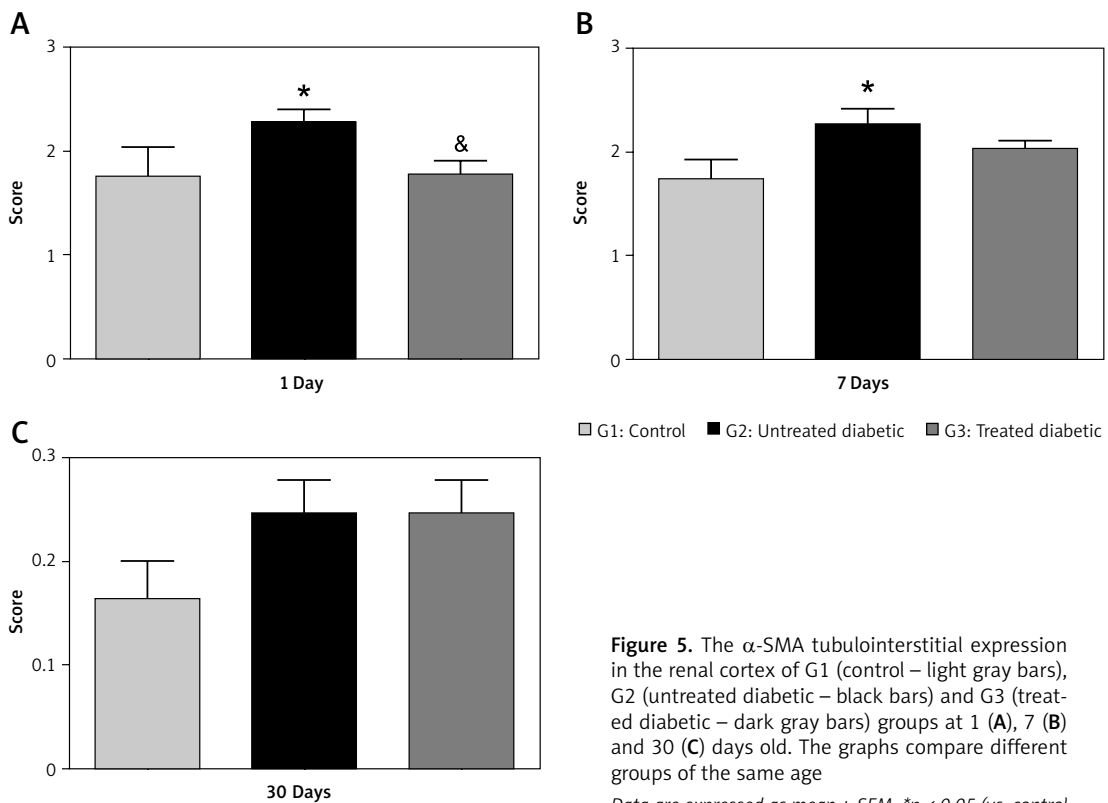


Figure 5. The α -SMA tubulointerstitial expression in the renal cortex of G1 (control – light gray bars), G2 (untreated diabetic – black bars) and G3 (treated diabetic – dark gray bars) groups at 1 (A), 7 (B) and 30 (C) days old. The graphs compare different groups of the same age

Data are expressed as mean \pm SEM; * $p < 0.05$ (vs. control at the same age).

ly found in more superficial regions of the renal cortex called the nephrogenic zone [15, 16]. Our histological study showed glomeruli in different development phases in the renal cortex of animals aged 1 and 7 DO, while animals aged 30 DO had glomeruli in the final differentiation phase.

An immunohistochemical study for α -SMA revealed a decrease in the expression of this cyto-

skeleton protein during postnatal renal development in the glomerular and the tubulointerstitial compartment while animals aged 30 DO have this protein restricted to the vessel walls, where α -SMA has a contractile function. However, offspring aged 1 and 7 DO from G2 had increased α -SMA tubulointerstitial expression when compared to controls of the same age, indicating ex-

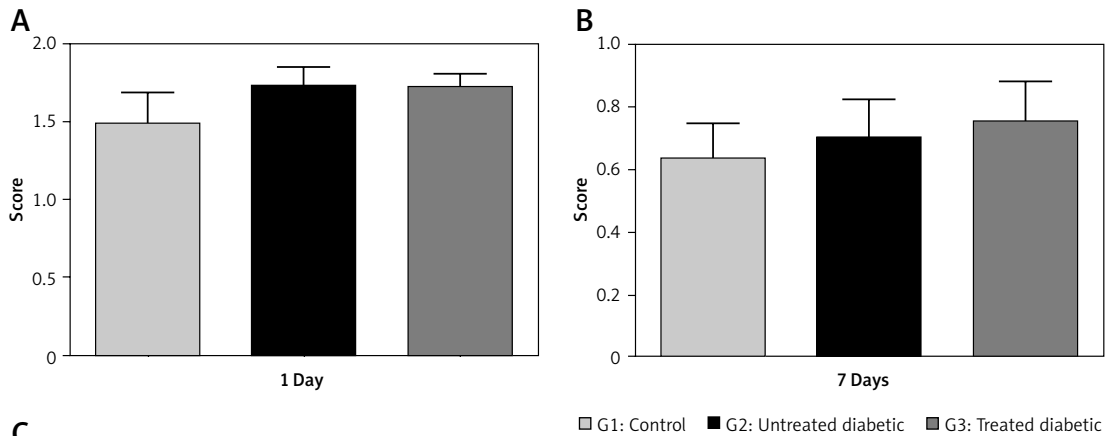


Figure 6. The α -SMA expression per glomerulus in the renal cortex of G1 (control – light gray bars), G2 (untreated diabetic – black bars) and G3 (treated diabetic – dark gray bars) groups at 1 (A), 7 (B) and 30 (C) days old. This graph compares different groups at the same age
Data are expressed as mean \pm SEM; ** $p < 0.01$ (vs. control at the same age).

Table II. Data from SBP and GFR

Groups	SBP [mm Hg]	GFR [ml/min]
G1	130.7 \pm 5.448	0.912 \pm 0.058
G2	142.7 \pm 5.592	0.422 \pm 0.113**
G3	120.0 \pm 2.637	0.865 \pm 0.163&&

Data are expressed as mean \pm SEM; ** $p < 0.01$ (vs. control); && $p < 0.01$ (vs. untreated diabetic).

acerbated mesangial cell or fibroblast activation, which is capable of causing proliferation and producing more collagen and other ECM components. In some pathological situations, the expression of this protein has been shown to increase in the renal cortex, and this gain has been implicated in the progression of kidney disease [17–21].

Our PCNA data confirmed intense cellular proliferation in the early postnatal kidney develop-

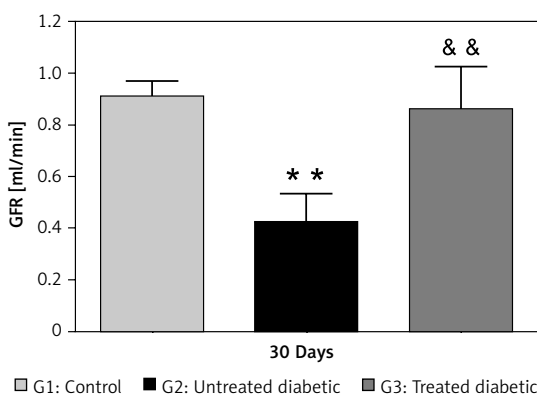


Figure 7. The GFR determination by creatinine clearance of G1 (control – light gray bar), G2 (untreated diabetic – black bar) and G3 (treated diabetic – dark gray bar) groups at 30 days old
Data are expressed as mean \pm SEM; ** $p < 0.01$ (vs. control); && $p < 0.01$ (vs. untreated diabetic).

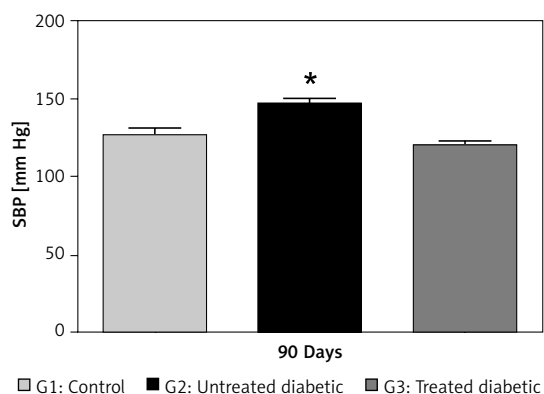


Figure 8. The SBP determination by plethysmography of G1 (control – light gray bar), G2 (untreated diabetic – black bar) and G3 (treated diabetic – dark gray bar) groups at 90 days old
* $p < 0.05$.

ment with a subsequent decrease in the renal cortex of animals aged 30 DO. However, there was a significant increase in PCNA⁺ glomerular cells in pups of 7 DO from diabetic mothers. Although we did not find any significant changes in the expression of α -SMA in the kidneys of animals aged 30 DO from G2, these animals showed a smaller number of glomerular proliferating cells, a tendency to a reduced number of glomeruli and a smaller glomerular tuft area when compared to controls. These findings are in agreement with those of Cunha *et al.* [22], who evaluated the influence of hyperglycemia in nephrogenesis of 7 and 21 DO Swiss mice treated with streptozotocin (STZ) and showed the presence of PCNA⁺ cells in both the control and the treated group in animals aged 7 DO. The authors found higher marking in the STZ-treated group, with the most intense expression found in the nucleus of tubular cells and a higher rate of immature glomeruli, indicating a delay in maturity of the renal cortex of these animals due to the administration of this drug [22, 23]. However, Magaton *et al.* [24] found no changes in α -SMA and PCNA expression in the renal cortex of offspring aged 1, 2 and 3 months from STZ-induced diabetic mothers.

As proposed by Srinivasan *et al.* [25, 26], it is possible that the characteristics of maternal diabetes are transmitted to the offspring, perpetuating certain pathological situations that appear in adulthood. In fact, our results showed that offspring of diabetic mothers showed significant changes in renal structure and function in the early postnatal renal developmental stages. In this work, the changes found included dilatation of the tubular lumen, an increase in the number of PCNA⁺ cells, expression of tubulointerstitial α -SMA and a smaller glomerular tuft area in this period, which may be responsible for the reduction in GFR and increased SBP presented by animals aged 30 and 90 DO, respectively, from diabetic mothers when compared to controls.

Wichi *et al.* found that hyperglycemia in pregnancy has long-term effects on fetal physiological programming, inducing cardiovascular diseases such as hypertension, baroreflex dysfunction and activation of the renin-angiotensin system (RAS) [27]. Tran *et al.* [28] observed that maternal diabetes impairs renal development and induces nascent nephron cell apoptosis via enhanced intrarenal RAS activation and NF- κ B signaling. Magaton and coworkers showed that intrauterine exposure to diabetes may cause decreased GFR and increased SBP in animals aged 2 months old, with no changes in the number of nephrons [24].

Our results showed that by the age of only 30 DO, pups from non-treated diabetic mothers (G2) already showed a decrease in GFR, which can be attributed to the smaller area available for fil-

tration and the observed structural alterations when compared to controls. When these animals reached 90 DO, we observed a tendency towards a reduction in the number of glomeruli, which together with falling GFR may have contributed to the increase in SBP. Chen and colleagues attributed the increase in SBP presented by offspring of diabetic mothers to increased angiotensin II (All) in the proximal tubules, which may increase the activity of the Na⁺ transporter, elevating its reabsorption, thereby leading to SBP elevation [29]. Moreover, as evidence of possible kidney damage, these animals also showed higher urinary protein excretion compared to controls, as well as decreased urinary concentration. These data demonstrate that disturbances occurring during intrauterine life may induce persistent changes in the biology of offspring in adulthood [30].

Studies have also shown that, during pregnancy, there is stimulation of the pancreatic endocrine axis in children of diabetic mothers with increased insulin concentrations in the amniotic fluid [31]. This is due to stimulation of maternal hyperglycemia with subsequent fetal hyperglycemia [32]. Excess insulin in this phase can result in children of diabetic mothers displaying increased weight and height (macrosomia) by stimulating growth, as reported for insulin-like growth factors (IGFs). The measurement of C-peptide in the amniotic fluid may provide data for the endocrine function of the pancreas; results of the study by Gollin *et al.* showed that this peptide significantly increased in diabetic pregnancies [33]. Therefore, it is possible that the maternal hyperglycemic environment added to increased insulin and All fetal levels may be involved in the changes found in our model.

The changes observed in G2 were not verified in the offspring of diabetic mothers treated with insulin (G3), and this group (G3) behaved similarly to the control (G1). Indeed, it was found in clinical practice that the instructions given to pregnant diabetic patients in order to intensively control blood glucose significantly reduced the risk of congenital malformations [3, 34–36]. Furthermore, metabolic, structural and functional abnormalities in the pups of diabetic mothers may be subject-specific according to the level of hyperglycemia presented during pregnancy/weaning and the life period studied [37].

In this regard, pregnant women are typically not screened for diabetes until 28–30 weeks gestation, and human kidney development begins at 5 weeks gestation, with the full number of nephrons reached by 36 weeks. However, Hokke *et al.* observed that glycemic control which normalized maternal glucose levels to that of control values did not prevent embryo growth restriction or a nephron deficit, which damaged renal development of offspring [38]. In other words, delayed

insulin therapy may not be effective in minimizing renal damage to offspring. Therefore, more than glycemic control, it is important that pregnant women receive monitoring from the beginning of pregnancy and, once diabetes mellitus is detected, that strict glycemic control is provided. In addition, if the diabetes was detected during gestation and glycemic control has not been done properly, it is suggested that the offspring be monitored with regard to renal function, since kidney impairment may result in hypertension in adult life, as was observed in our experiments.

Mohamed *et al.* reported that macrosomic and non-macrosomic infants of diabetic mothers had elevated levels of cord serum insulin and suppressed levels of cord serum resistin and adiponectin, hormones involved in metabolism regulation [39]. Some studies in humans have been performed with biomarkers that can be detected prior to or early in pregnancy; they might serve as risk indicators to plan possible preventive measures reducing the extent of fetal damage. Such biomarkers in amniotic fluid could include insulin and erythropoietin, whereas in cord plasma they could include IGFs, adiponectin and resistin [40].

Finally, more studies in humans are necessary to determine the renal and systemic consequences for infants who were exposed to an intrauterine hyperglycemic environment.

In conclusion, the present results allow us to conclude that maternal diabetes can induce programming of renal injury in the offspring, contributing to the impairment of nephrogenesis. This impairment was able to alter the renal function of offspring in adulthood, as evidenced by reduced GFR and increased blood pressure.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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