RESEARCH

The Effect of Streptozotocin-Induced Diabetes on Renal Development in Different Development Periods

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Abstract: Diabetes mellitus (DM) is the most common endocrine disease worldwide and the leading cause of diabetes-related mortality and morbidity in diabetic nephropathy. This study, it was aimed to compare the fetal kidney tissues of streptozotocin-induced diabetic rats at different developmental stages with both histopathological and immunohistochemical methods. In the study, 48 Wistar albino pregnant rats weighing between 200-250gr were used. The rats were divided into two main experimental groups control and streptozocin. Then, each group was divided into 3 subgroups as 15, 17 and 19 days of development. Thus, a total of 6 experimental groups were formed. An experimental diabetes model was created in female rats by administering a single dose of streptozotocin (60 mg/kg) prepared in citrate buffer to streptozotocin groups and they were conceived. Fetuses of pregnant rats belonging to both control and streptozocin groups were then harvested at 15, 17 and 19 days of development. While the effects of diabetes on kidney development were evaluated histopathologically with Hematoxylin&Eosin, Masson trichrome and Periodic acid shift staining in fetuses of 15, 17 and 19 days of development, Urotensin II and TGF-β1 expression levels were demonstrated by immunohistochemical staining method. The histopathological findings obtained from the study showed that diabetes adversely affected kidney development on the 15th day compared to other developmental stages. In addition, the immunoreactivity intensity of Urotensin II and TGF-β1 in the kidney tissues of the fetuses of diabetic pregnant rats was higher than the control group. Our results support that Urotensin II and TGF-β play a regulatory role in the early stages of kidney development, and we believe that it will help in elucidating the molecular pathogenesis and treatment of kidney failure.

INTRODUCTION

Diabetes mellitus (DM), a metabolic disease that affects millions of individuals worldwide, is a global health issue. It's a prevalent systemic disease with well-known and devastating outcomes. The World Health Organization (WHO) predicts that 300 million people will have diabetes by 2025[1]. Significant damage occurs in diabetic tissues when glucose entry is not controlled by insulin, such as the kidney[2]. Extracellular matrix accumulation and glomerular basement membrane thickening are the most common histological abnormalities in early diabetic nephropathy. Increased production of glomerular basement membrane collagens and mesangial matrix proteins has been linked to the development of diabetic nephropathy[3]. In animal models[4-6], the link between a hyperglycemic intrauterine environment and later renal disease has been explored, with maternal hyperglycemia associated with fewer nephrons, higher blood pressure, microalbuminuria, and a poorer glomerular filtration rate in children. The offspring of pregnant mice with severe diabetes have a higher risk of congenital abnormalities and poor nephrogenesis, resulting in smaller kidneys4. Similarly, diabetic female mice's newborn offspring had 40% fewer nephrons on average than nondiabetic mice's offspring, according to Chen et al.[6].

One of the most modern and commonly used endothelial systems is urotensin. Urotensin-II protein, which elevates blood glucose levels by mobilizing glucose, may play a role in the pathogenesis of diabetic mellitus (DM)[7]. The structure of urotensin-II is a cyclic peptide (similar to somatostatin peptide). Levels have been shown to rise in diabetic patients[8]. In diabetic nephropathy, transforming growth factor-β1 (TGF-β1) has been demonstrated to have a substantial role in renal cell hypertrophy[9]. Renal urotensin-II and the urotensin-II receptor are thought to trigger TGF-β1 upregulation in diabetes, which leads to renal fibrosis and possibly diabetic nephropathy[10]. Animal investigations of a relationship between maternal diabetes during pregnancy and child kidney anatomy and development have been limited. A systematic review of animal studies has not yet been done, as far as we know. Given the growing body of evidence that many chronic diseases have their roots in prenatal circumstances, this is an extreme case. The goal of this research was to look at the involvement of TGF-β1 and Urotensin II in STZ-induced developing renal injury in diabetic rat pups, as well as any potential effects of TGF-β1 and Urotensin II on kidney injury utilizing molecular and histological approaches.

MATERIAL and METHODS

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Study design, and setting

The protocol of this study was approved by the Experimental Animals Ethics Committee of Erciyes University (Decision number; 19/004). In the study, 48 previously unmated, three-month-old, healthy Wistar albino female rats, weighing between 200-250gr, produced in Erciyes University Experimental and Clinical Research Center were used. During the experiment, rats were fed with normal pellets. They were divided into two main groups with 24 female rats in each group. Each group was divided into 3 subgroups with different developmental periods (15, 17, and 19 days) and 8 female rats in each group. Blood glucose levels were measured by glucometer 3 days after 60 mg/kg intraperitoneal (ip) STZ administration to female rats. Those with blood glucose values above 200 mg/dl were considered as diabetes and allowed to mate for pregnancy. In the study, 3 females and one male were left in the same cage overnight, and a vaginal smear was performed in the morning after spermatozoon (+) was accepted as 0.5 day-old pregnant. Pregnant rats were placed in plastic cages according to groups under the same laboratory conditions.

Groups were created as described below

Group I (Control group) (n=24): The group in which no treatment was applied to the control group pregnant rats during their pregnancy. Pregnant rats belonging to the control group were divided into 3 subgroups; 5 (n=8), 17 (n=8), and 19 (n=8) days of development.

Group II (STZ group) (n=24): Female rats were given a single dosage of STZ (60 mg/kg) formulated in citrate buffer as part of an experiment to generate an experimental diabetes model. An experimental diabetes model was created in female rats by administering a single dose of STZ (60 mg/kg) prepared in citrate buffer to rats belonging to this group. Pregnant rats belonging to the STZ group were divided into 3 subgroups; 15 (n=8), 17 (n=8), and 19 (n=8) days of development.

Pregnant rats in the control and experimental groups were anesthetized with ketamine hydrochloride (50 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) as ip on the 15th, 17th, and 19th days of their pregnancy, and their fetuses were removed by cesarean section under anesthesia was taken into a formaldehyde solution.

Histological Tissue Tracking Method

Tissues, which were kept in fixation solution for 72 hours, were washed in running tap water, and then water was removed by passing them through a series of increasing grades of alcohol. Afterward, they were passed through xylene to make the tissues transparent and kept overnight in melted paraffin. Finally, it was blocked by embedding in paraffin. For light microscopic examinations, 5µm sections were taken on a microtome (Leica RM 2155). Serial sections were taken to examine the kidney tissues of embryos at different stages of development. The sections of 5 µm thickness obtained from the embryos in the prepared paraffin blocks were kept in an oven at 60 ^{o}C for one night. Hematoxylin & Eosin, Periodic Acid Schiff (PAS), Masson Trichrome (MT) stains were applied to the sections to evaluate the kidney tissues at different developmental stages. Sections were viewed under an Olympus BX51 computer-aided light microscope.

Immunohistochemical Application

To determine the expression of Urotensin II and TGF-β, the immunohistochemical staining method with the avidin-biotin-peroxidase method was applied to the preparations. For this, paraffin blocks prepared after keeping them in 10% formaldehyde and performing routine histological tissue follow-up were used. In summary, 5µm sections from paraffin blocks were kept at 60°C overnight, first rehydrated by passing through xylene and then graded alcohol series, and then the sections were washed with Phosphate Buffer Saline (PBS). Then, it was boiled in a microwave oven at 600 W for 5 minutes with 10% citrate buffer. 3% hydrogen peroxide (H_2O_2) was applied to the sections. Slides were washed with PBS. Thermo Scientific (Lab Vision™ UltraVision™ Large Volume Detection System: anti-Polyvalent, HRP (Ready-To-Use), TA-125-HL) immunohistochemistry staining kit was used for the next steps. Ultra V Block was applied to the sections washed with PBS again for 20 minutes at room temperature. Immediately afterward, the sections were incubated at +4°C for one night by applying primary antibody to Urotensin II (LifeSpan BioSciences, LS-C403705) or TGF -β1 (Santa Cruz Biotechnology, SC-146) and incubated at room temperature for 30 minutes the next morning. After washing, the sections were incubated with the biotin-secondary antibody for 30 minutes to ensure binding to the primary antibody. After washing with PBS again, the sections treated with streptavidin peroxidase enzyme for 30 minutes were washed to make the immunoreactivities visible by washing with 3,3' P-diaminobenzidine tetrahydrochloride (DAB) (Lab vision, UltraVision detection system Large volume DAB substrate system, TA-125-HD) was treated with the peroxidase substrate in the kit for 1-10 minutes and then washed with distilled water for 5 minutes. Sections counterstained with Gill hematoxylin were washed several times with distilled water. Finally, the sections, which were passed through xylene by removing the water with increasing alcohol series, were closed with entellan and examined under a light microscope. Image J software program was used to calculate the Urotensin II and TGF-β1 immunoreactivity intensity. For this, light microscopic photographs taken from five different regions at 40X magnification of each tissue were used. The obtained data were evaluated statistically.

Statistical Analysis

PRISM (Graphpad Software Inc, Version 8.0d) program was used for statistical analysis. Raw data are presented as group means ± SEM (standard error of the mean). Immunohistochemical data were analyzed by the 2-way ANOVA method. Intergroup immunohistochemical immunoreactivity intensities were analyzed using Bonferroni's multiple comparison test. Statistical analysis was considered significant if $p<0.05$.

RESULTS

Morphological Findings

When the kidney tissues of the control and STZ group on the 15th day of development were evaluated microscopically, tubular hypertrophy was noted in the STZ group with the tubule structures in the early period of development being wider and the lumen more prominent than the control group. There was no significant difference in connective tissue content between the groups in this study (Fig.1).

Cortex and medulla segmentation were clearly distinguished in the histological evaluation of the 17th-day kidney tissues of the control group. The proximal and distal tubules and renal corpuscle structures were not fully developed, but they were prominent and numerous glomerular structures were present in the cortex. When the kidney tissues of the experimental group on the 17th day of STZ were evaluated histologically, it was observed that the tubule structures were smaller compared to the control group and there were shedding on the brush-like edges of the apical membranes of the proximal tubules, especially in PAS staining, compared to the control group. While the control group showed morphologically normal glomeruli and tubule structures, the renal glomerulus was more scattered in the 17-day-old STZ kidney tissues, and it was noted that the Bowman's space was enlarged, especially in the 17th-day experimental group, compared to the control group (Fig.2).

At STZ day 19, the lumen of the renal tubules was clearly distinguishable (Fig.3). The apical membrane areas of the tubules were stained darkly due to their microvilli contents and mitotic figures of most of the tubule cells were noted with PAS staining. It was observed that the brushy edges of the proximal convoluted tubule were reduced in the kidney sections of the experimental group on the 19th day given STZ. There was a narrowing of the lumen of some tubules and less mitotic activity of tubule cells. In addition, vacuolization of tubular epithelial cells was observed in the proximal tubules. In MT stained sections, no significant difference was observed in the areas of connective tissue in the control and STZ groups of all developmental stages.

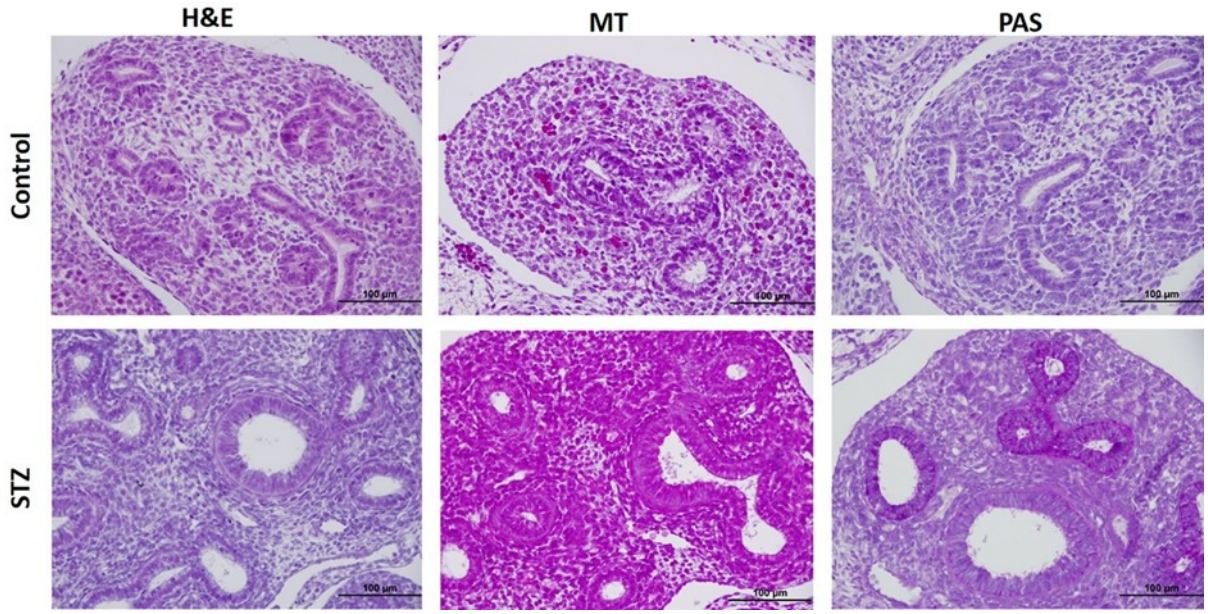

Fig. 1. Kidney sections on day 15 of development from all groups. HE; Hematoxylin&Eosin, MT; Masson trichrome, PAS; periodic acid schiff.

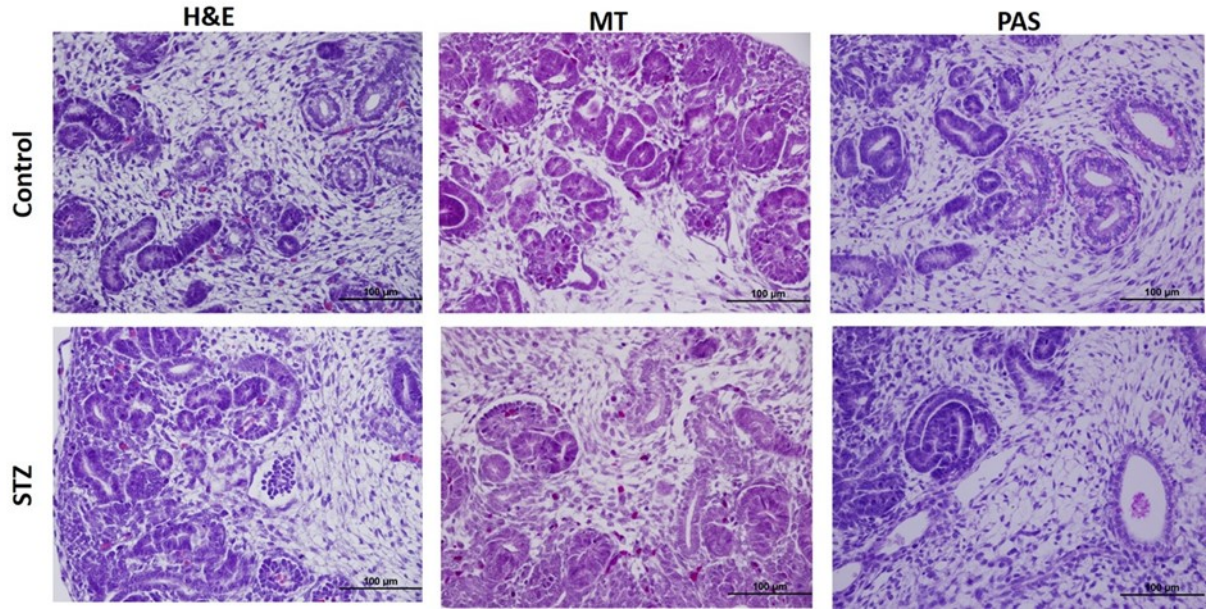

Fig. 2. Kidney sections on day 17 of development from all groups. HE; Hematoxylin&Eosin, MT; Masson trichrome, PAS; periodic acid schiff.

Immunohistochemical Findings

When the Urotensin II immunoreactivity intensity of kidney tissues of different developmental stages of the control and STZ groups were compared, there was a statistically significant increase in the STZ group on the 15th day of development ($p<0.05$, Fig.6). However, on days 17 and 19 of development, the intensity of Urotensin II immunoreactivity increased in the STZ group compared to the control group, but the result was not statistically significant ($p>0.05$). Although there was no statistically significant difference between the intensity of Urotensin II immunoreactivity in the kidney tissues of the 15th, 17th and 19th days of development in the control group, while the intensity of Urotensin II immunoreactivity was high in the early 15th day of development in the STZ group, there was a decrease in the intensity of Urotensin II immunoreactivity in the later stages of development. It was noticed that. This decrease in the intensity of immunoreactivity was statistically significant between the 15th and 17th days of STZ ($p<0.05$) (Fig.4).

When the TGF-β1 immunoreactivity intensity of the kidney tissues of the control and STZ groups were compared, it was noted

that there were significant differences between the groups (Fig.6). On the 17th day of development, there was no significant difference between the control and STZ groups in terms of TGF-β1 immunoreactivity intensity, while on the 15th day of development, the TGF-β1 immunoreactivity intensity increased statistically significantly in the STZ group compared to the control ($p<0.001$). With the progression of development, the intensity of TGF-β1 immunoreactivity on the 19th day of development was statistically significantly decreased compared to the 15th day ($p<0.001$). Similarly, the developmental period with the highest TGF-β1 immunoreactivity intensity in the STZ group was on the 15th day. On the 17th and 19th days of STZ, the intensity of TGF-β1 immunoreactivity was statistically significantly decreased compared to the 15th day ($p<0.001$) (Fig.5).

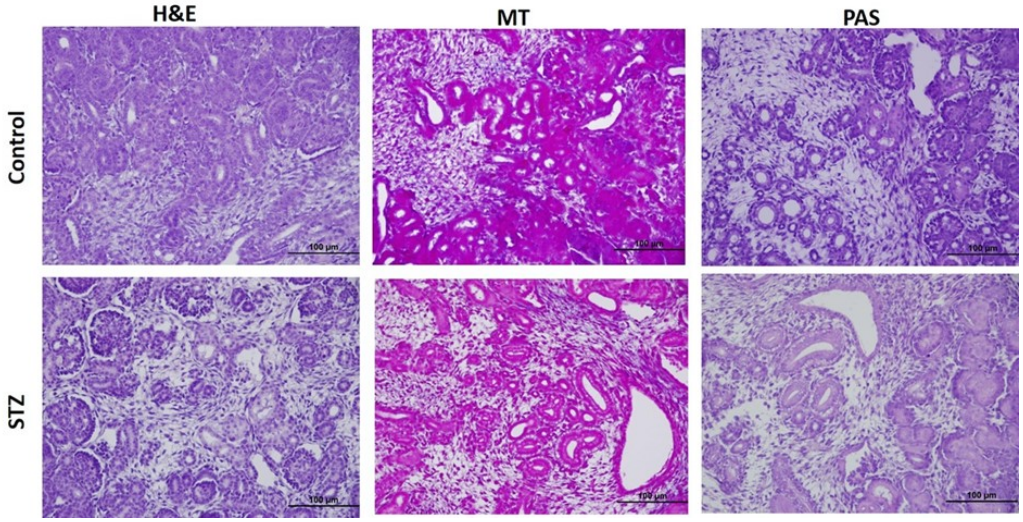

Fig. 3. Kidney sections on day 19 of development from all groups. HE; Hematoxylin&Eosin, MT; Masson trichrome, PAS; periodic acid schiff.

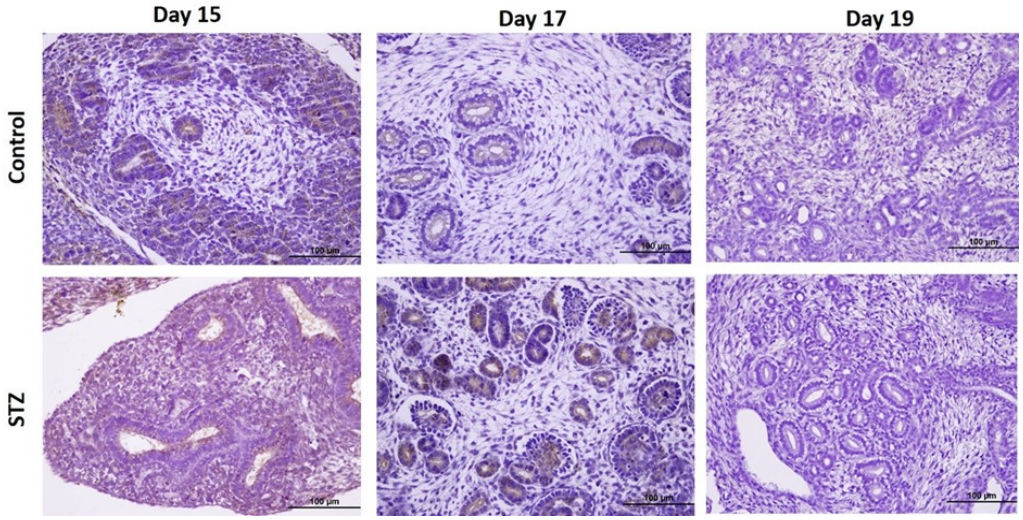

Fig. 4. Urotensin II immunoreactivity in kidney sections of 15, 17 and 19 days of development in all groups.

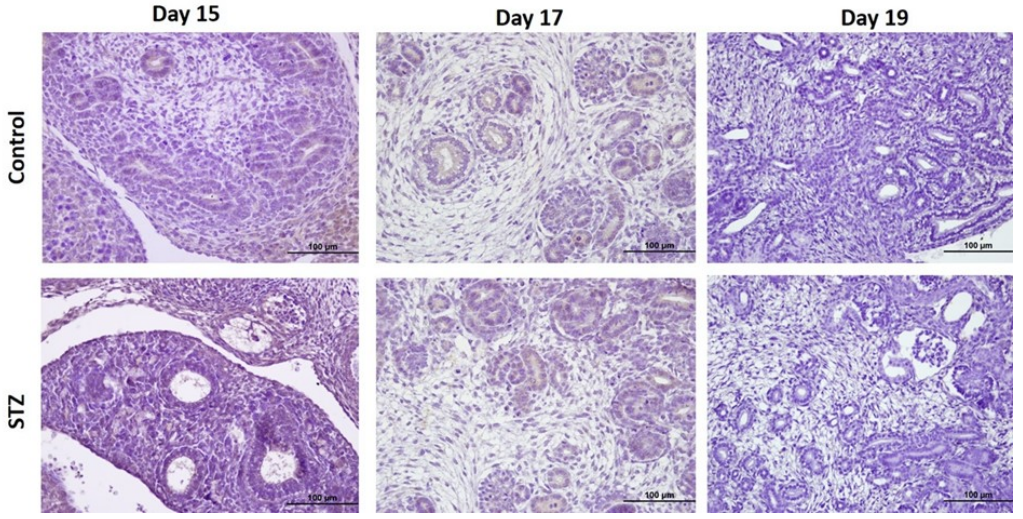

Fig. 5. The immunoreactivity intensity of TGF-β1 in kidney tissue of different developmental stages.

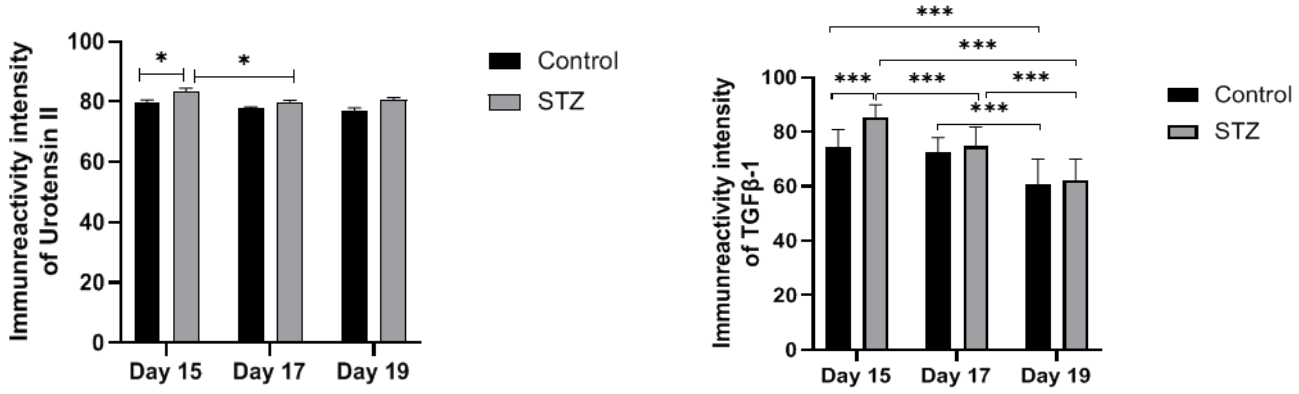

Fig.6. The graphs show immunoreactivity intensity result of Urotensin II and TGF-β1 in kidney tissue of different developmental stages.

DISCUSSION

DM is a worldwide health concern that affects millions of people. Chronic hyperglycemia depletes the activity of antioxidative defense mechanisms in diabetic individuals. In diabetes, substantial damage occurs in tissues where glucose intake is not regulated by insulin[11], such as the kidney. The present work shows that Urotensin II and TGF-β1 level are very important on induced kidney injury in offspring of diabetic rat.

DM is one of the most important causes of end-stage renal disease in developed societies. It is a disease characterized by chronic hyperglycemia, resulting from impaired insulin secretion or insulin action; It causes serious disorders in carbohydrate, protein and fat metabolism[12]. Diabetic kidney damage is used for all lesions frequently seen in the kidneys of diabetic patients[13]. It affects the glomeruli, tubules, interstitium and blood vessels in the kidney. In the early period of diabetic kidney damage; Studies have shown that histopathological changes such as hypertrophy in the glomeruli and tubules, tubular vacuolization, thickening of the glomeruli and tubular basement membranes, hyalinosis in arterioles, mesangium matrix and cell increase in the glomeruli ocur[14]. Although the structural changes in diabetic nephropathy cover all parts of the kidney, the most characteristic changes were observed in the glomeruli. The enlargement of the glomeruli is attributed to the increase in the number and volume of mesangium cells, podocyte hypertrophy and glomerular basement membrane thickening[15]. This enlargement is one of the most important reasons that lead to a decrease in the glomerular filtration rate[16]. As the mesangium expands, it narrows the lumen of the surrounding capillaries and accordingly the filtration area[17]. The filtration barrier is formed by the podocytes, the common basal lamina, and the windowed glomerular endothelium without a diaphragm. As in some kidney diseases, in diabetic nephropathy, there is a large amount of protein passage through the barrier due to damage to this filtering barrier[18]. The tubules and interstitium may show many structural changes in diabetic nephropathy, including tubular cell vacuolization and tubulointerstitial fibrosis[19]. In the past decade, fetal exposure to maternal diabetes has been identified as a risk factor for the development chronic non-communicable disease in offsprings[20]. Uncontrolled blood glucose throughout the second and third trimesters of pregnancy has also been linked to an increased risk of preterm birth, preeclampsia, macrosomia, and maternal and perinatal morbidity and mortality[21]. However, no studies have been published on the structural and functional effects of diabetes induced during pregnancy and sustained in the postpartum period, which are study's aim. In our study, histopathological examinations revealed that kidney damage developed in the offspring of the diabetic group. According to the light microscopic findings obtained from the study, hypertrophy of the tubules and thickening of the basement membranes were noted in the fetal kidney sections of the pregnant rats with DM on the 15th day of development. On the 17th day of development, it was noted that the tubule structures of the kidney tissues of the STZ group shrank when compared to the control group. The less staining intensity was observed in the brush border of the apical membranes of the proximal tubules compared to the control group in PAS staining. On the 19th day of development, the kidney microscopy of the experimental group on the 19th day given STZ was damaged compared to the control group. All these findings showed that diabetes negatively affects kidney development and may cause findings similar to diabetic nephropathy.

There are limited studies that have investigated how the Urotensin II molecule is affected on developing kidney of maternal diabetes. Majorly, the production site of Urotensin II is the kidneys. It is produced in the proximal tubule, distal tubule, and collecting duct cells in the kidneys, and its receptor is located in the kidney medulla. The present study is the first that have investigated this matter. It is known that the Urotensin system has a heightened role in people with chronic illnesses and diseases that involve acute hemodynamic modulator[22]. Urotensin II reportedly reduces glucose-induced insulin secretion in the perfused rat pancreas[23] and also known that it has a wide range of effects, including a regulatory role in the kidney in studies[24]. In fact, kidney is an important source of Urotensin II in both humans and rats[25]. High concentrations of Urotensin II have been noted in various kidney patients, including diabetic kidney[26,27]. Serum or urinary Urotensin II concentrations are higher in patients with more severe renal dysfunction, leading to the notion that Urotensin II plays a role in chronic kidney disease. Mori et al showed parallel increases in Urotensin II immunoreactivity in the kidneys of rats as kidney function decreased. These observations indicate that Urotensin II expression continues to increase as kidney function declines[28]. In the present study, it was observed that Urotensin II immunoreactivity in the kidney tissues of the offspring from the 15th day of development in STZ group significantly increased compared to the control group and was in line with previous studies. This study shows that diabetes adversely affects kidney development in developing fetuses of diabetic mothers and that increased Urotensin II expression may be associated with renal dysfunction.

TGF-β1 regulates cell proliferation, hypertrophy, apoptosis, and fibrogenesis[29] and is a major activator of processes leading to chronic progressive kidney disease. TGF-β1 levels were shown to be higher in diabetes patients' kidneys. TGF-β1 mediates a variety of cellular effects, some of which may promote diabetic nephropathy progression and others that may be adaptive[19]. Yet, most of the animal models in which TGF-β1 signaling is systemically modified suggest that, overall, this growth factor contributes to diabetic nephropathy pathophysiology. Many studies have reported that TGF-β1 expression and activation are increased in progressive forms of kidney disease[30]. According to our findings, we determined that the TGF-β1 immunoreactivity intensity showed significant changes in the kidney tissues of the STZ group compared to the control. The developmental period with the highest TGF-β1 immunoreactivity intensity was on the 15th day. With the progression of development, a significant decrease in TGF-β1 immunoreactivity was observed in both control and STZ groups.

Conclusion

In this study, it was concluded that diabetes affects kidney

development more in the early developmental period and negatively affects kidney development. In addition, we presented an overview of the signaling of Urotensin II and TGF-β1, which have a regulatory role in kidney development, and their relationship with progressive kidney disease. Our results may influence the understanding of the role of Urotensin II and TGF-β1 in the regulation of cellular function, which may mediate in elucidating the molecular pathogenesis and treatment of progressive renal failure.

Conflict of interest

The authors declare that there are no conflict of interests.

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