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Research Article

Increased Expressions of eNOS and iNOS Correlate with Apoptosis of Diabetic Nephropathy in Streptozotocin-induced Type 1 Diabetic Rats [1] [2]

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Abstract

The present study was designed to evaluate the effects of high level of nitric oxide (NO) production and oxidative stress (OS) on nephropathology and to identify whether NO and OS have any correlation with apoptosis seen in diabetic kidney, elucidating the underlying mechanism(s) involved in the development of nephropathology in streptozotocin (STZ)-induced diabetic rats. Expression levels of caspase 3, caspase 9, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), 8-hydroxy-2'-deoxyguanosine (8-OHdG), for the detection of oxidative damage to DNA, were examined in diabetic kidney tissues. Results of the study revealed that the levels of 8-OHdG ($P<0.005$), eNOS ($P<0.005$), iNOS ($P<0.005$), caspase 3 ($P<0.005$) and caspase 9 ($P<0.005$) were remarkably higher in diabetic kidney tissues than in controls. In addition, STZ-treated animals showed significant loss of body weight and renal enlargement. It was suggested that apoptosis, OS and increased NO levels are involved in the pathogenesis of diabetic nephropathy. The results also strongly suggested that STZ-induced apoptosis through activation of the intrinsic pathway and that might be most likely related to increased NO levels. Moreover, high NO production was not only mediated by eNOS but also by iNOS. Increased NO production may contribute to hyperfiltration and microalbuminuria in early diabetic nephropathy. Furthermore, expression of 8-OHdG might give an idea of the progress and may be essential as it has a diagnostic significance for this disease. In conclusion, we believe that eNOS and iNOS overexpressions induce diabetic nephropathy by mediating apoptosis in STZ-induced rats.

Keywords: *Diabetic nephropathy, Type 1 diabetes mellitus, Nitric oxide, Apoptosis, 8-OHdG*

Streptozotosin Kaynaklı Tip 1 Diyabetik Sıçanlarda Meydana Gelen Nefropatilerde Artan eNOS ve iNOS Sunumlarının Apoptozisle İlişkilendirilmesi

Özet

Diyabetik nefropatilerin patogenezi uzun yıllardır çalışılan fakat hala tam açıklığa kavuşmuş bir konu değildir. Bu çalışma streptozotocin (STZ) ile tetiklenmiş diyabetik sıçanlarda nitrik oksit (NO) üretiminin ve oksidatif stresin (OS) nefropatoloji/nefrodejenerasyonların üzerine olan etkilerinin ve bu faktörlerin apoptozisle bir ilişkisinin olup olmadığının araştırılması için tasarlanmıştır. Bu çalışmada kaspaz 3, kaspaz 9, endotelyal nitrik oksit sentaz (eNOS), indüklenebilir nitrik oksit sentaz (iNOS) ve 8-hydroxy-2\'-deoxyguanosine (8-OHdG) sunumları diyabetik böbrek dokularında araştırıldı. Çalışma sonuçlarında, 8-OHdG ($P<0.005$), eNOS ($P<0.005$), iNOS ($P<0.005$), kaspaz 3 ($P<0.005$) and kaspaz 9 ($P<0.005$) sunumlarının diyabetik böbrek dokularında önemli düzeyde arttığı görüldü. Bununla beraber diyabetik hayvanlarda kilo kaybı ve böbrek boyutlarında artış tespit edildi. Bu çalışmada elde edilen bulgulara göre OS ve yükselmiş NO seviyelerinin diyabetik nefropatilerin patogenezinde önemli bir rolünün olduğu düşünülmektedir. Ayrıca, STZ ile tetiklenen apoptozisin iç yolakla gerçekleştiği ve yüksek düzeyde üretilen NO'nun apoptozis düzeyini arttırdığı düşünülmektedir. Bununla beraber NO düzeylerinin artışına sadece eNOS değil iNOS'un da önemli katkı sağladığı görülmektedir. Erken diyabetik nefropatilerde NO'nun hiperfiltrasyona ve mikroalbuminüriye katkı sağladığı düşünülmektedir. 8-OHdG'nin tanısal bir öneminin olduğu ve hastalığın takibinde fikir vereceği düşünülmektedir. Sonuç olarak, diyabetik sıçan modellerinde eNOS ve iNOS'un aşırı salınımlarının apoptozisi tetikleyerek diyabetik nefropatilere neden olduğu düşünülmektedir.

Anahtar sözcükler: *Diyabetik nefropati, Tip 1 diyabetes mellitus, Nitric oksit, Apoptozis, 8-OHdG*

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INTRODUCTION

Diabetes is a metabolic disorder characterized by specific complications such as diabetic nephropathy that causes long-term dysfunction and failure in various organs and tissues [1,2]. Microvascular complications that may occur in diabetic nephropathy may result in end-stage renal disease, further requiring dialysis or transplantation [1,2]. Pathogenesis of nephropathies related to hyperglycemia is an issue that has been studied in the recent years but has still not been fully clarified.

Apoptosis occurs by activation of intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways [3]. For the formation of apoptosome complex, initiator procaspase 9 is needed [3]. In intrinsic apoptosis apoptosome complex cleaves initiator procaspase 9 and activates it. Apoptosis is induced when cleaved caspase 9 activates effector caspase 3, caspase 6 and caspase 7 [4-6]. Oxidative stress (OS) is known to induce damage to the mtDNA. If the antioxidative and the DNA damage repair systems are insufficient, cellular dysfunction and ultimately, apoptosis occur [7]. High glucose concentrations were previously defined to increase OS, causing apoptosis in HK2 cells [8].

NO synthesized from L-arginine by NOS isozyme is known to trigger apoptosis [9-11]. NO mediated cytotoxicity was first defined in macrophages and later on high concentration of NO was shown to cause apoptosis [9]. It is explained that NO causes consecutive loss in mitochondrial membrane potential and thus induces cytochrome c release to the cytosol [10,12,13].

Although there are studies regarding hyperglycemia related OS and apoptosis, contribution of NO production and OS related apoptosis in the pathogenesis of diabetic nephropathy is not clear yet. Moreover, the role of nephrodegeneration in the pathogenesis of diabetic nephropathy is not fully defined yet. The purpose of this study was to investigate the relationship between apoptosis and the severity of nephropathological changes that occur in the kidney tissues of rats in which we established type 1 diabetes mellitus. Moreover, the relationship between NO production and hyperglycemia related apoptosis in kidney was studied.

MATERIAL and METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). The experimental protocol was approved by the Committee on the Ethics of Animal Experiments at Ataturk University (Permit Number: 46-02.03/2014).

Experimental Animals

Twenty male Wistar albino rats weighing 250-300 g were randomly allotted to two experimental groups (n = 10 per group). Animals were housed in a well-ventilated and air-conditioned area provided with independently adjustable light-dark cycle (12 h light/12 h dark cycle) and temperature regulation systems. The rooms and animal cages were cleaned daily, and the animals were provided with fresh food and water ad libitum on a daily basis.

Induction of STZ Model of Diabetes

Type 1 diabetes was induced in the rats (diabetic group) by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg body weight) dissolved in 0.1 mM sodium citrate, pH 4.5, while the normal control rats (nondiabetic group) were injected with the buffer only. The development of hyperglycemia in rats was confirmed by blood glucose evaluation. Blood glucose was determined by using an automatic glucometer (ACCU-CHEK Active, Roche Diagnostics Ltd, Germany). Plasma glucose level of the animals higher than 250 mg/dL were considered hyperglycemic. This animals were selected for studies and this study covers acute period.

Necropsy and Histopathologic Examination

At the end of 20 days of experiment period, animals were sacrificed by decapitation and kidneys were quickly removed and processed for histopathology and immunohistochemistry analyses. Kidney tissue samples were fixed in 10% neutral buffered formaldehyde for 48 h and washed under tap water overnight. Following routine tissue preparation procedures, tissue samples were dehydrated through graded series of alcohol and xylene and embedded in paraffin blocks. Paraffin serial sections were cut at a thickness of 4-5 µm. Kidneys were sectioned to 4-5 µm thickness, stained with H&E, and examined under a light microscope (Olympus BX51 and DP25 digital camera, Japan).

Immunoperoxidase Examinations

Immunohistochemistry was performed to investigate eNOS, iNOS, caspase 3, caspase 9 and 8-OHdG expressions. Commercial antibodies were visualized on 4- to 5-µm-thick paraffin sections using an indirect streptavidin/biotin immunoperoxidase kit (HRP; Thermo Scientific, USA). All steps were carried out following the procedure described by Dincel and Kul, 2015 [14]. Tissue sections were incubated with the primary antibody (eNOS, iNOS, caspase 3, caspase 9 and 8-OHdG) for 60 min. Finally, sections were incubated in aminoethyl carbazole chromogen (Thermo Scientific, USA) for 5-10 min to induce the color reaction. Mayer's hematoxylin was applied as a counterstain for 30 sec. As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted.

Quantitative Histomorphometric Analysis and Statistics

The density of positive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK), connected to a Lecia DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.) was used according to the procedure described by Dincel and Atmaca [15]. The pictures of five random fields selected and consecutive 20x objective microscopic fields were captured by the Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions) at a setting identical to the image system. For the quantification of mean was quantified as the eNOS-, iNOS-, caspase 3-, caspase 9- and 8-OHdG-positive area/total area were measured and calculated by Leica Qwin Plus on the pictures. Data were statistically described in terms of mean and standard deviation (mean±SD) for area %. For evaluating the non-parametric data, Mann-Whitney U-test was performed to compare eNOS, iNOS, caspase 3, caspase 9 and 8-OHdG immunoreactive cells and immunopositively stained areas in the diabetic animals versus the healthy controls. A *P* value of <0.05 was considered significant. The data were presented as means ± SD. All statistical analyses and graphs were prepared using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California, USA).

RESULTS

Histopathologic Findings

Hematoxylin and eosin (H&E)-stained kidney sections from control group animals exhibited normal architecture *(Fig. 1A)*. The focal endocapillary hypercellularity in glomeruli and calcification on tubules were observed in diabetic nephropathy group *(Fig. 1A)*. Moderate to severe lymphocyte infiltration with tubular degeneration/necrosis were detected in kidney from diabetic nephropathy group *(Fig. 1A)*. In addition the focal mesangial hypercellularity and focal microcystic dilations of tubules were shown in kidney. Morover, the number of atrophic tubules and acute renal hyperemia were also observed in kidney from diabetic nephropathy group *(Fig. 1B)*.

Immunoperoxidase Findings

In this study, eNOS and iNOS, caspase 3 and caspase 9 and 8-OHdG (cytoplasmic) expressions in the kidney were higher in diabetic nephropathy group than in healthy control animals ($P<0.005$). Statistical analysis of the data on eNOS, iNOS, caspase 3, caspase 9 and 8-OHdG expressions in the kidney, measured by immunostaining in all the groups, are listed in *Table 1*.

8-hydroxy-2′-deoxyguanosine (8-OHdG) Expression

Fairly weak immunoreactivity for 8-hydroxy-2′-deoxyguanosine (8-OHdG) was observed in some cortical tubules *(Fig. 2A)* and medulla cells in healthy control animals *(Fig. 2B)*. Increased 8-OHdG expression was observed only cytoplasmic compartment of cortical tubules, medulla, tubular capillaries and interstitial vessels *(Fig. 2C,D)*. Cytoplasmic immunoreaction was localized in some degenerative/necrotic cortical tubules cells *(Fig. 2C)*. 8-OHdG expressions in the kidney were

Table 1. *Immunoperoxidase test results and statistical data*
Tablo 1. *İmmünopereksidaz test sonuçları ve istatistiksel verileri*

Animals	N	eNOS		P <	iNOS		P <	Caspase 3		P <	Caspase 9		P <	8-OHdG		P <
		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd	
Control animals	10	1.970	0.323	0.001	1.975	0.284	0.001	1.146	0.481	0.001	1.520	0.345	0.001	2.534	0.335	0.001
STZ-treated animals	10	2.939	0.527		2.784	0.390		4.207	0.955		4.182	0.983		4.722	0.318	

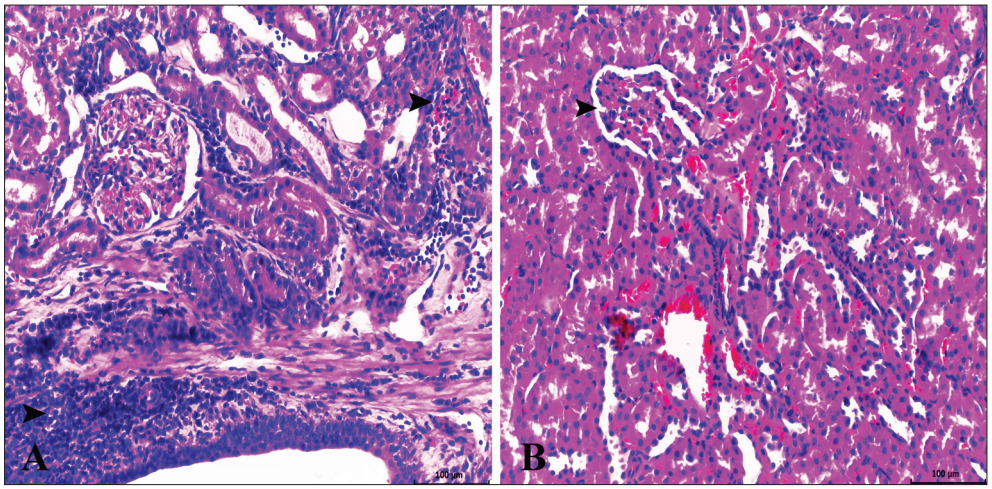


Fig 1. The focal mesangial hypercellularity in glomeruli, moderate to severe lymphocyte infiltration *(arrowheads)* (A) and atrophic/degenerative glomeruli *(arrowhead)*, H&E (B)

Şekil 1. Glomeruluslarda fokal mezanşial hücresellik artışı, orta düzeyden şiddetliye dönen lenfosit infiltrasyonları *(okbaşları)* (A) ve atrofik/dejeneratif glomerulus *(okbaşı)*, H&E (B)

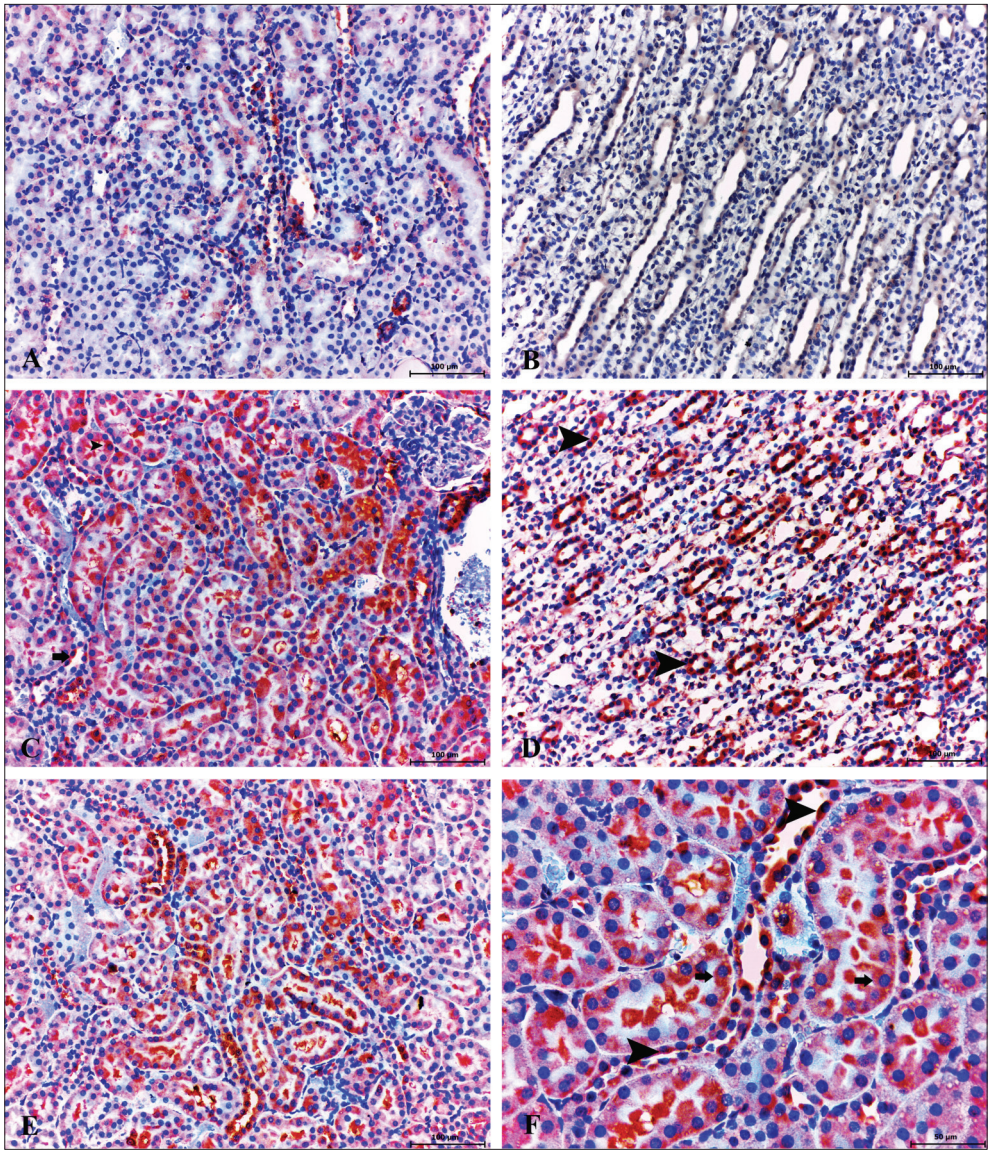
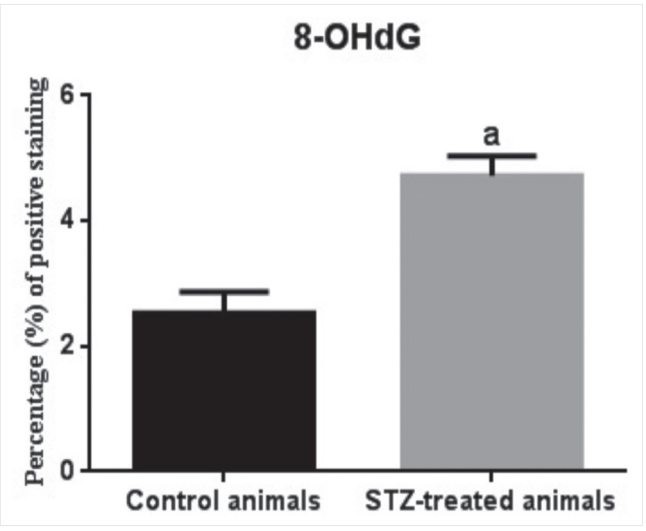


Fig 2. Healthy control group; low expression of 8-OHdG in macula densa other tubular segments and collecting ducts. (A,B) Increased cytoplasmic expression of 8-OHdG in endothelial cells, distal *(arrow)* and proximal *(arrowhead)* convoluted tubules (C), collecting ducts *(arrowheads)* (D), necrotic/degenerative distal *(arrowheads)* and proximal *(arrows)* convoluted tubules. (E,F), ABC technique (anti-8-OHdG)

Şekil2. Sağlıklı kontrol grup, makula densa, diğer tubular segmentlerdeki ve toplayıcı kanallardaki zayıf 8-OHdG sunumları. (A,B) Endotel hücrelerde, distal *(ok)* ve proksimal *(okbaşı)* konvolüt tubullerde (C), toplayıcı kanallarda *(okbaşları)* (D), Nekrotik/dejeneratif distal *(okbaşları)* ve proksimal *(oklar)* konvolüt tubüllerde artmış sitoplazmik 8-OHdG sunumları. (E,F), ABC teknik (anti-8-OHdG)

Fig 3. Comparison of 8-OHdG immunopositivity. Statistical difference is indicated as letters. "a" represent values statistically higher than control group

Şekil 3. 8-OHdG immünopozitifliklerin karşılaştırılması. İstatistiksel farklar 'a' ile gösterilmiştir. 'a' istatistiksel olarak kontrol grubundan yüksek olduğunu vurgulamaktadır



statistically higher in in diabetic nephropathy group than in healthy control animals ($P<0.005$) *(Fig. 3)*.

The most conspicuous finding of the present study was that 8-OHdG expression was markedly increased in

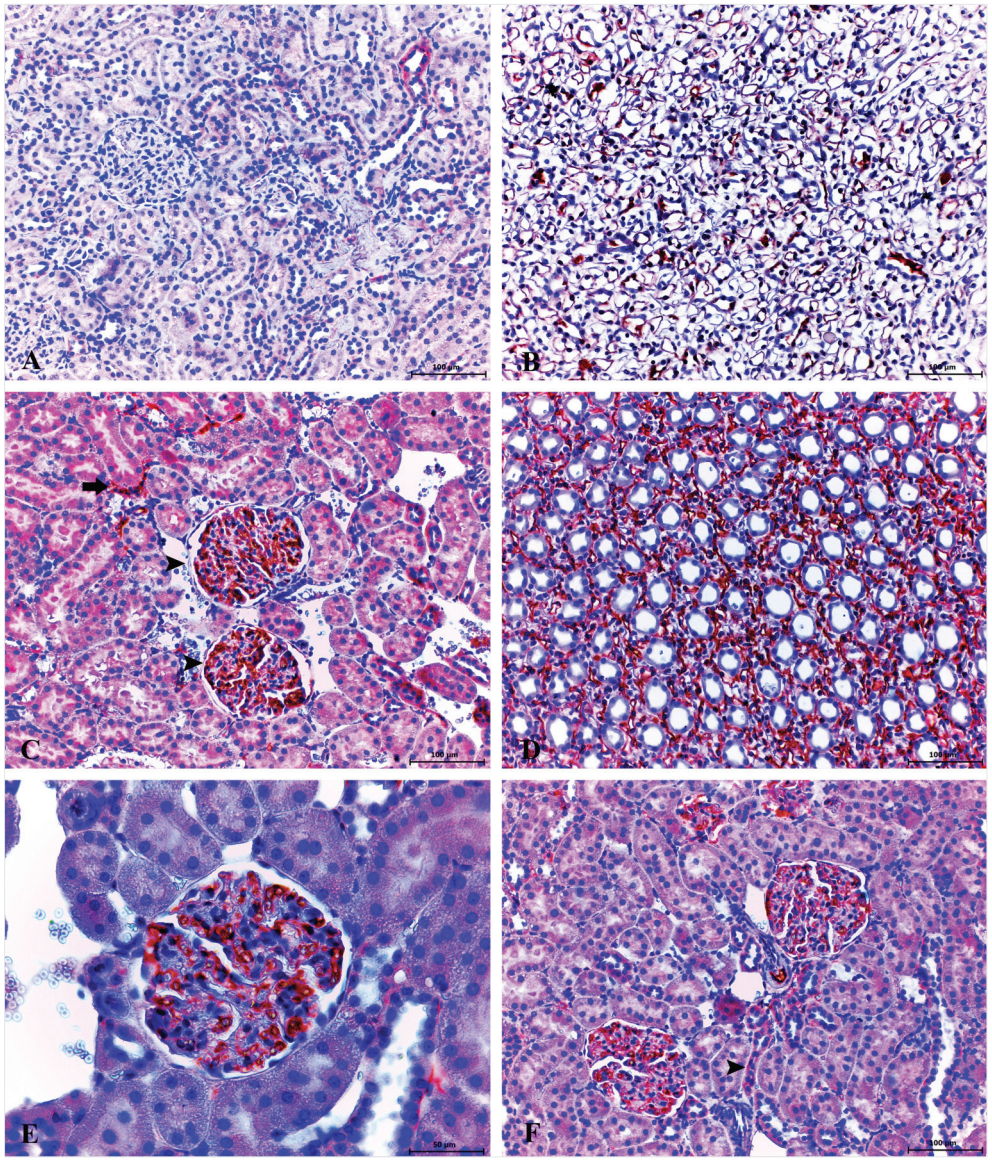


Fig 4. Healthy control group; very slight expression of eNOS in distal/proximal convoluted tubules and glomerulus (A,B) Strong expression of eNOS in glomerulus *(arrowheads)*, distal convoluted tubules *(arrow)* (C), medullary endothelial cells (D), normal/atrophic glomerulus, distal convoluted tubules *(arrowhead)* (E,F). ABC technique (anti-eNOS)

Şekil 4. Sağlıklı kontrol grup; distal/proksimal konvolüt tubuller ve glomeruluslarda zayıf eNOS sunumları (A,B) Glomerus *(okbaşları)* ve distal konvolüt tubullerde *(ok)* (C), medullar endotelyal hücrelerde (D), normal/atrofik glomerulus and distal konvolüt tubullerde *(okbaşı)* güçlü eNOS sunumları (E,F). ABC teknik (anti-eNOS)

proximal and distal convoluted tubules and medullary cells *(Fig. 2E,F)*.

This study showed that 8-OHdG, pivotal marker for measuring the effect of endogenous oxidative damage to DNA, was a good biomarker for risk assessment of this disease. In addition to this biomarker might used to estimate the DNA damage in humans/animals after exposure to hyperglycemia.

Endothelial Nitric Oxide Synthases (eNOS) and Inducible Nitric Oxide Synthases (iNOS) Expressions

Weak immunoreactivity for eNOS and iNOS was observed in some cortical tubules and medulla cells in healthy control animals *(Fig. 4A,B) (Fig. 5A)*. eNOS expressions increased significantly in the vascular endothelial and capillaries of the glomerulus and some necrotic/degenerative glomerulus *(Fig. 4C,D,E,F),* which was also significantly higher in the diabetic group than the levels in the healthy control group ($P<0.005$) *(Fig. 6)*. Importantly, strong eNOS expression markedly increased in distal convoluted tubules in diabetic kidney tissues. iNOS expressions especially increased significantly in the capillaries of the glomerulus, cortical, medullar tubules and some necrotic/degenerative glomerulus *(Fig. 5B,C,D),* which was also significantly higher than the levels in the healthy control group ($P<0.005$) *(Fig. 6)*. Another conspicuous finding of the present study was that strong iNOS expression markedly increased in the infiltrating mononuclear cells and proximal convoluted tubules in diabetic kidney tissues.

Caspase 3 and Caspase 9 Expressions

Fairly weak immunoreactivity for caspase 3 and caspase 9 was observed in some cortical tubules and medulla cells in healthy control animals *(Fig. 7A,B) (Fig. 8A,B)*. Strong caspase 3 and caspase 9 immunoreactivity is observed in glomerular capillaries *(Fig. 7F) (Fig. 8C,F)* tubular capillaries

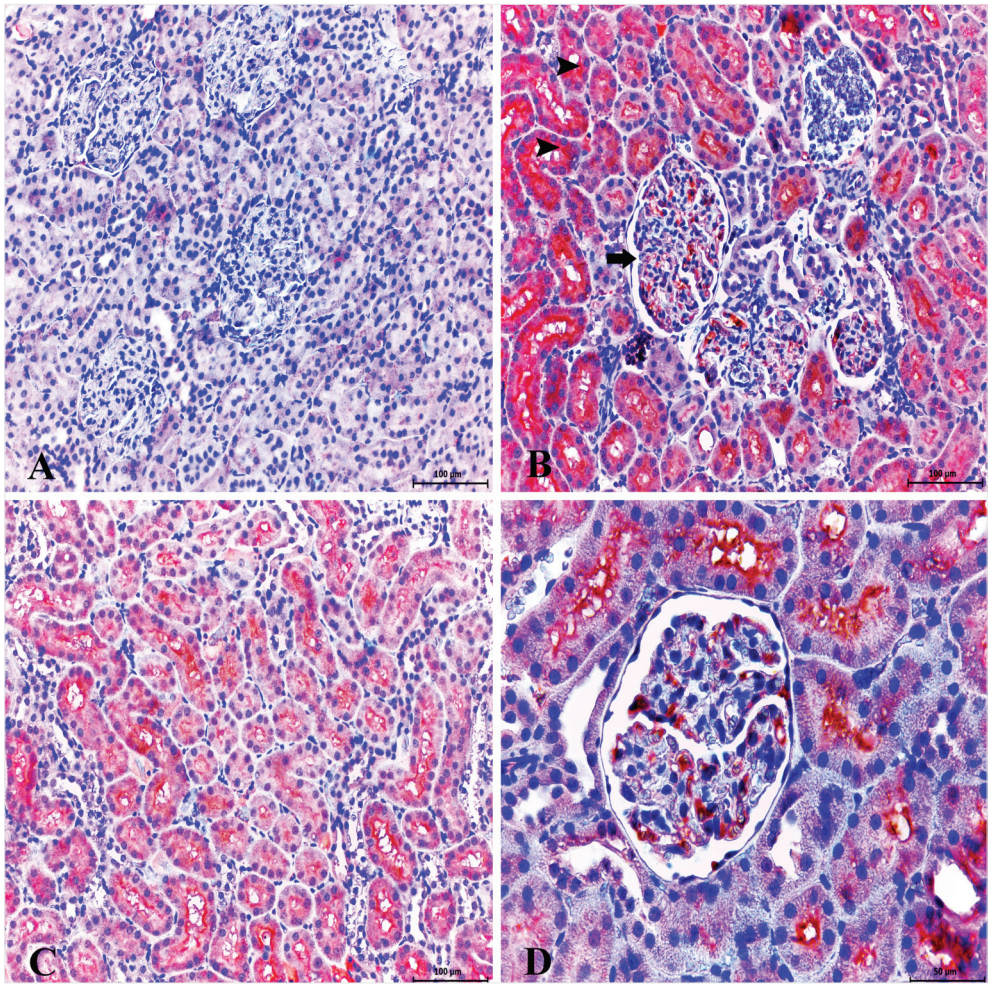
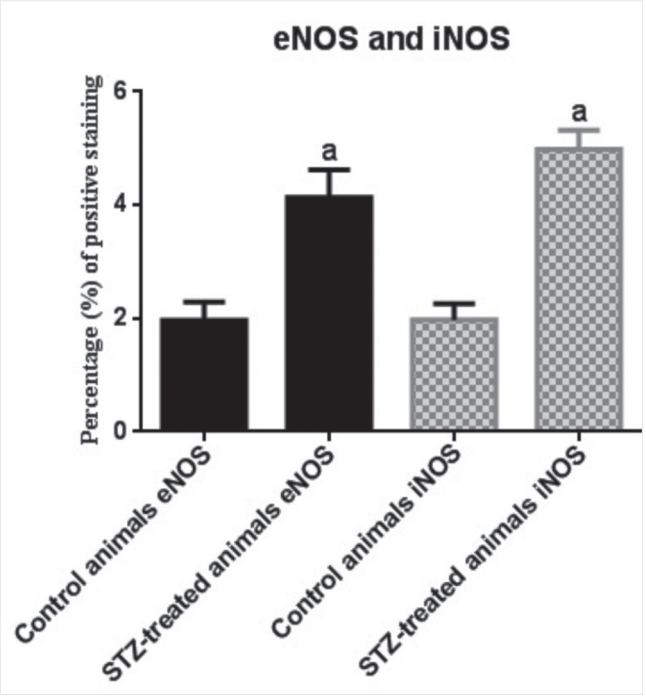


Fig 5. Healthy control group; very slight expression of iNOS in distal/ proximal convoluted tubules (A) Strong expression of iNOS in glomerulus *(arrow)* and proximal convoluted tubules *(arrowheads)* (B,C,D). ABC technique (anti-iNOS)

Şekil 5. Sağlıklı kontrol grup; distal/ proksimal konvolüt tubüllerde hafif iNOS sunumları (A) Glomerulus *(ok)* ve proksimal konvolüt tubüllerde *(okbaşları)* güçlü iNOS sunumları (B,C,D). ABC teknik (anti-iNOS)

Fig 6. Comparison of eNOS and iNOS immunopositivity. Statistical difference is indicated as letters. "a" represent values statistically higher than control group

Şekil 6. eNOS ve iNOS immünopozitifliklerin karşılaştırılması. İstatistiksel farklar 'a' ile gösterilmiştir. 'a' istatistiksel olarak kontrol grubundan yüksek olduğunu vurgulamaktadır



(Fig. 7D,E) (Fig. 8D,E) medulla and cortical tubules *(Fig. 7C) (Fig. 8D,F),* which was also significantly higher than the levels in the healthy control group ($P<0.005$) *(Fig. 9)*. Importantly, strong caspase 3 and caspase 9 expressions

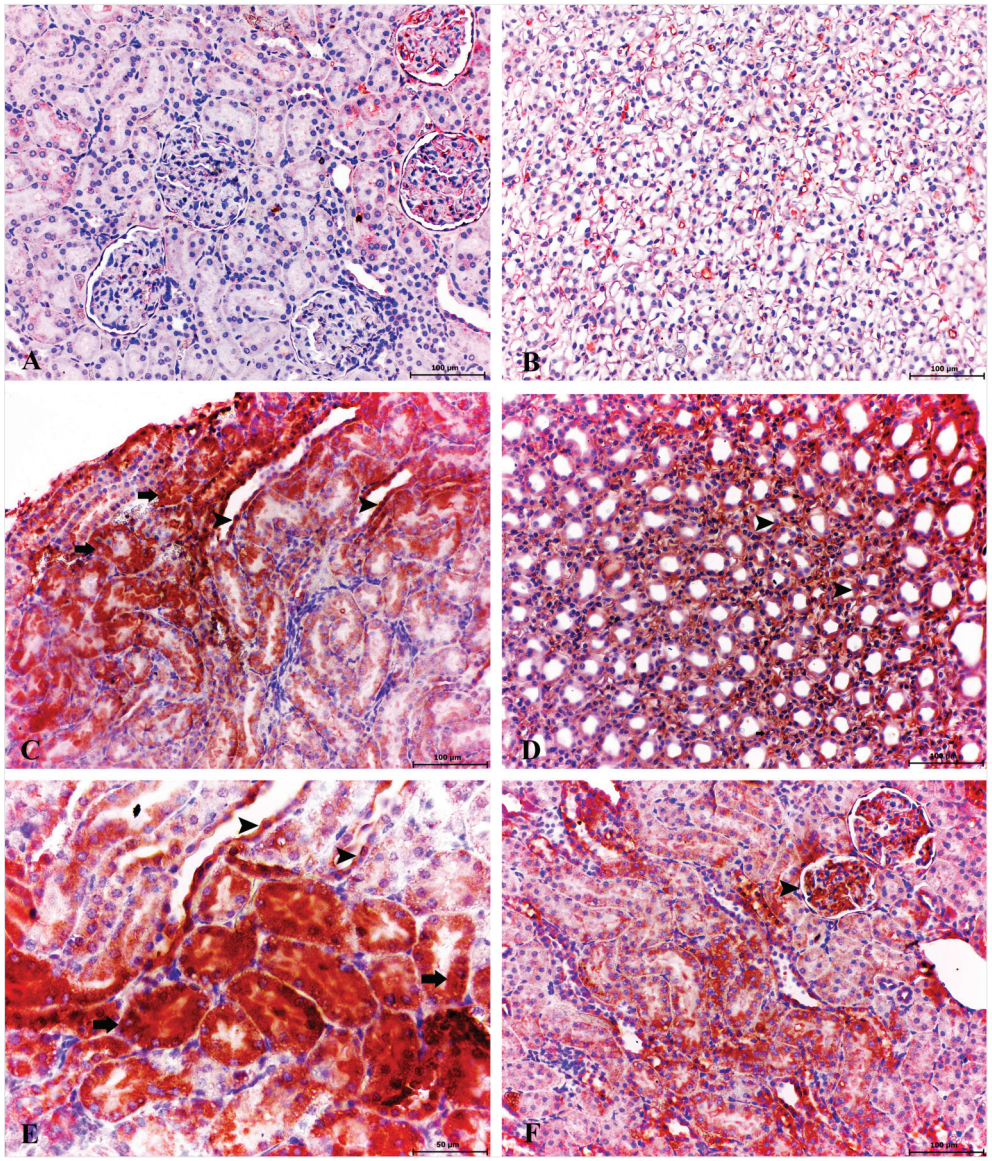


Fig 7. Healthy control group; very slight expression of caspase 3 in proximal convoluted tubules, glomerulus (A) collecting ducts (B) Strong expression of caspase 3 in distal *(arrowheads)* and proximal *(arrows)* convoluted tubules, collecting ducts *(arrow-heads)*, endothelial cells *(arrow)* (C,D,E) and glomerulus *(arrowhead)* (F). ABC technique (anti-caspase 3)

Şekil 7. Sağlıklı kontrol grup; glomerulus, proksimal konvolüt tubüllerde (A) ve toplayıcı kanallarda (B) zayıf kaspaz 3 sunumları. Distal *(okbaşları)* ve proksimal *(oklar)* konvolüttubullerde,toplayıcıkanallar *(okbaşları)*, endotel hücrelerde *(ok)* (C,D,E) ve glomeruluslarda *(okbaşı)* güçlü kaspaz 3 sunumları. ABC teknik (anti-Kaspaz 3)

markedly increased in proximal and distal convoluted tubules in diabetic kidney tissues.

DISCUSSION

There are many unanswered questions regarding the pathogenesis of hyperglycemia related nephropathies. In this study, we show that NO has severe immunopathological roles and it is not only synthesized by eNOS but also by iNOS. Moreover, it was shown that hyperglycemia induces apoptosis in diabetic kidneys and the greatest contribution to this is provided by high level of NO production and OS.

NO at physiological concentrations inhibits formation of apoptosome complex by blocking cytochrome c release in double membrane of mitochondria [16]. Thus, intrinsic apoptotic pathway activation is prevented. On the other hand, above physiological limits NO was shown to trigger apoptosis [11,17,18]. Moreover, NO plays a role in the modulation of the systemic and the renal circulation [19-21]. Therefore, it is very important that NO is kept at physiological limits for the continuity of cellular life. In this study, NO at very high concentrations was shown to be a very important factor in the induction of intrinsic apoptotic pathways. eNOS and iNOS from endothelial, tubular, mesenchymal cells and infiltrating mononuclear cells are responsible for producing pathological levels of NO. According to this situation, apoptosis that is induced by NO and OS was determined as an important factor in the pathogenesis of diabetic nephropathy related degeneration, necrosis and atrophy.

When intrinsic apoptosis pathways are activated, formation of apoptosome complex triggers activation of initiator caspase 9 [3]. In some of our diabetic animals we observed severe caspase 9 activation in kidney while caspase 3 activation was found at control levels. This situation suggests that in these animals apoptosis is triggered through intrinsic pathways. Interestingly,

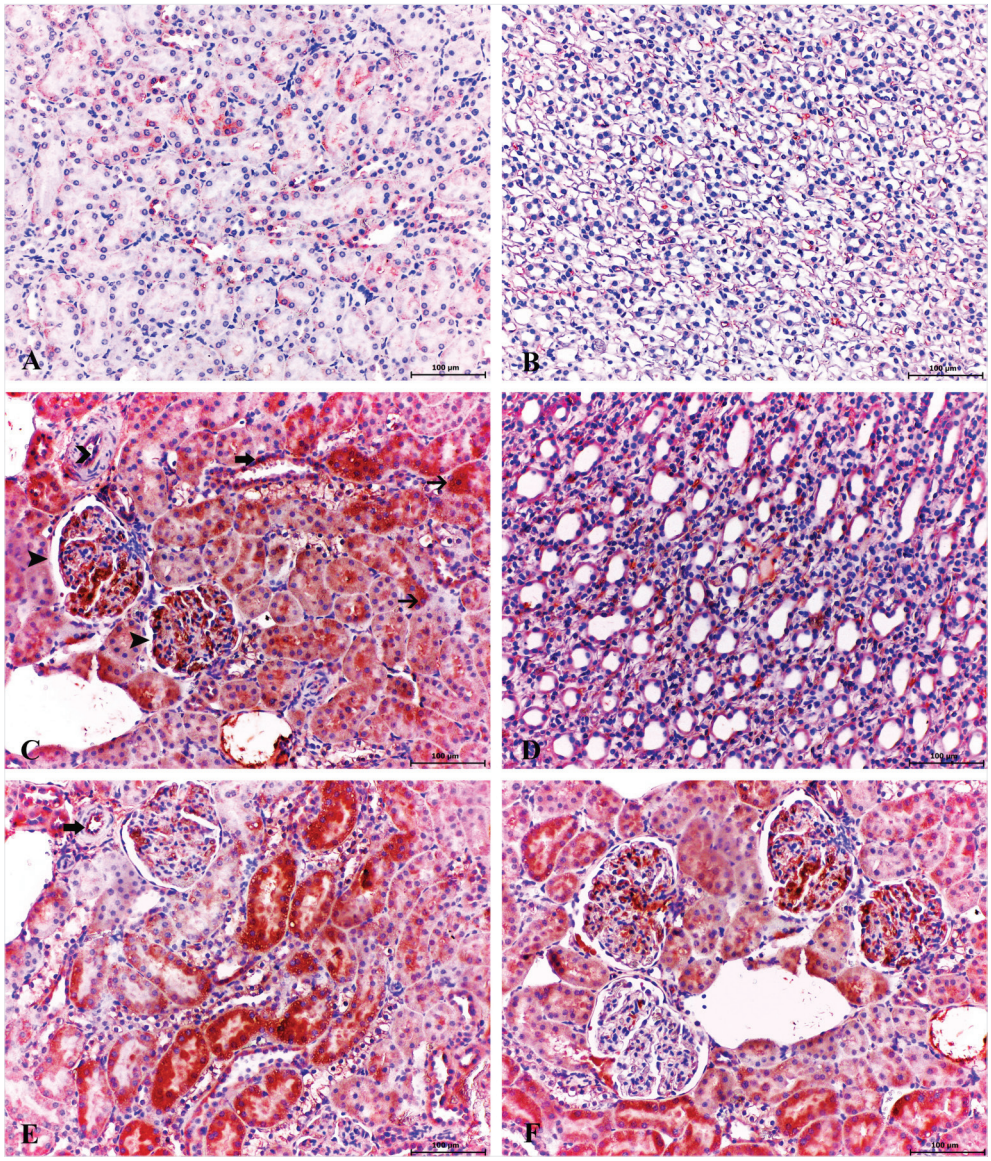
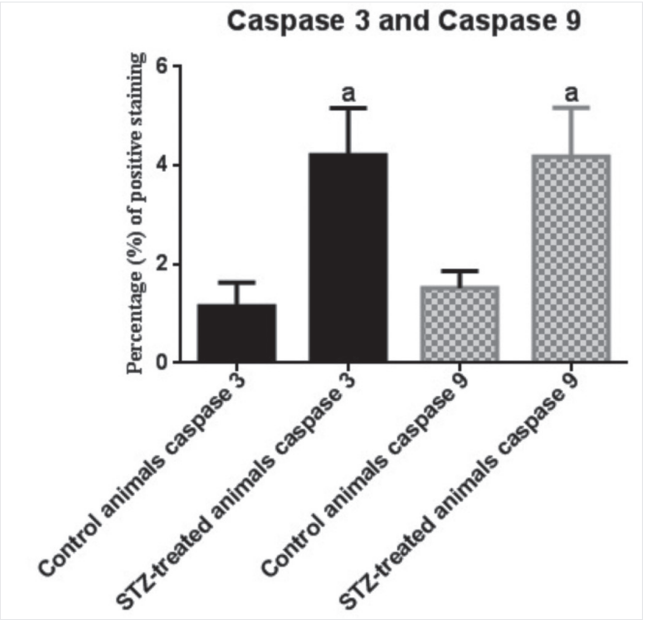


Fig 8. Healthy control group; very slight expression of caspase 9 in distal, proximal convoluted tubules and collecting ducts (A,B) Strong expression of caspase 9 in glomerulus *(arrowheads),* distal *(arrow)*/proximal *(thin arrow)* convoluted tubules, endothelial cells *(thin arrowhead)* (C), collecting ducts (D), endothelial cells *(arrow)* and convoluted tubules (E,F). ABC technique (anti-caspase 9)

Şekil 8. Sağlıklı kontrol grup; distal, proksimal konvolüt tubullerde ve toplayıcı kanallarda zayıf kaspaz 9 sunumları (A,B) Glomerulus *(okbaşları),* distal *(ok)*/proksimal *(ince ok)* konvolüt tubuller ve endotel hücrelerde *(ince okbaşı)* (C), toplayıcı kanallarda (D), endotelyal hücreler *(ok)* ve konvolüt tubullerde güçlü kaspaz 9 sunumları (E,F). ABC teknik (anti-Kaspaz 9)

Fig 9. Comparison of caspase 3 and caspase 9 immunopositivity. Statistical difference is indicated as letters. "a" represent values statistically higher than control group

Şekil 9. Kaspaz 3 ve kaspaz 9 immünopozitifliklerin karşılaştırılması. İstatistiksel farklar 'a' ile gösterilmiştir. 'a' istatistiksel olarak kontrol grubundan yüksek olduğunu vurgulamaktadır



in some cases, we detected severe levels of caspase 3 activation while caspase 9 activation was at control levels, suggesting that apoptosis observed in these animals are at late stage. This difference in the activity of caspases may be explained by physiological differences of the animals. In this kind of investigations eventhough animals used are identical (species, age, weight and gender), this negative correlation between caspase 9 and caspase 3 has to be taken into consideration when the results are evaluated.

NO increases fluid shear stress and the greatest contributor to this is eNOS [22,23]. There are also studies showing that OS also increases fluid shear stress [24,25]. We think that one of the most important finding of this study is that severe eNOS and iNOS expressions and oxidative DNA damage significantly contribute to occurrence of fluid shear stress in diabetic nephropathy. Therefore, it is very probable that in order to prevent fluid shear stress, therapies with anti-oxidants and inhibition of NO may be applied together and this may play a key role in the prevention of nephropathology that may occur.

Studies made on diabetic nephropathy show that apoptosis and nephropathology that occur in kidney are highly complex processes. In this study, we observed that in diabetic nephropathy eNOS and iNOS expression pathologically increases, resulting in high levels of NO production that eventually damages mitochondria and triggers intrinsic apoptotic pathway. Hyperglycemia ethiologically does induce degeneration in kidneys of animals and humans but here we think that severe NO production also presents a major contribution to this pathological process. We believe that ROS/reactive nitrogen species that are produced from activated infiltrating mononuclear and other renal cells and induction of eNOS and iNOS expression in endothelial, mesenchymal and tubular cells cause generation of large amounts of NO that causes OS and finally results in apoptosis. In summary, nephropathology seen in diabetes are not only caused by hyperglycemia, severely expressed proinflammatory cytokines or high levels of NO, but also as we show here are caused by apoptosis and OS.

Conflict of Interests

The authors report no conflict of interests.

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