Full Length Research Paper

Renal ischemia /reperfusion injury in type II DM: Possible role of proinflammatory cytokines, apoptosis, and nitric oxide

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Diabetes mellitus (DM) especially type II is a major health problem and diabetic nephropathy is the main cause of end stage renal disease (ESRD). Renal ischemia/reperfusion (I/R) injury is common in diabetic patients. Recent studies reported increased vulnerability of kidney to I/R injury in diabetic rats. Mechanisms behind this increased vulnerability not fully understood. The present study investigated the effect of acute ischemia for 45 min on proinflammatory cytokines, apoptotic markers, and nitric oxide (NO) in a rat model of type II diabetes. Sixty male Sprague Dawley rats were divided into 4 groups (n = 15, each); Group I: Normal rats, Group II: Normal rats underwent left renal ischemia for 45 min, Group III: diabetic rats without renal ischemia, Group IV: diabetic rats underwent left renal ischemia for 45 min. Blood and kidney samples were taken 24 h after ischemia. Serum glucose, fructosamine, creatinine, TNF , as well as the expression of TGF , NFkappaB, iNOS, survivin, and Bcl2 in kidney tissue was measured. Type II DM caused significant increase in serum glucose, fructosamine, creatinine, and TNF and expression of TGF , NF_kB and iNOS in renal tissue (P < 0.001) . Also, DM caused significant increase in apoptotic cell death with increase in Bcl-2 expression and decreased survivin in kidney. 45 min ischemia in diabetic rats caused more significant increase in serum TNF-and expression of TGF - , NF-kappa B and iNOS (P < 0.001). Also; there was a positive correlation between blood glucose and TNF , TGF , NF_kB and iNOS with negative correlation with survivin (P <0.01). Type II DM render the kidney more susceptible to ischemic injury. Proinflammatory cytokines TNF- , TGF , and NF B and iNOS as well as Bcl2 and survivin may contribute to the enhanced renal ischemic injury in type II DM. Also, hyperglycaemia may be involved in hypersensitivity of kidney to ischemic injury in DM.

Key words: Diabetes, kidney, ischemia, apoptosis, TNFalpha, TGFbeta, NFkB, iNOS, Bcl-2, surviving.

INTRODUCTION

Renal ischemia/reperfusion (I/R) injury is a syndrome that develops following a sudden transient drop in blood flow to the kidney (Bonventre and Weinberg, 2003). Renal I/R injury are a relatively common cause of acute renal failure (ARF) (Rippe et al., 2006). The pathogenesis of renal I/R injury involves the release of proinflammatory cytokines such as tumor necrosis factor- (TNF-), transforming growth factor- (TGF-) and nuclear factor-B (NF -B), and reactive oxygen species (Kher et al., 2005). Also, nitric oxide (NO) plays an important role in mediating cell damage during I/R injury (Reid and Geller, 2003). Apoptosis might contribute to the structural changes that occur in the ischemic kidney (Verzola et al., 2002).

Diabetes mellitus, especially type II diabetes, with its

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complications is a very important public health issue. A paradigmatic example of diabetic complications is diabetic nephropathy (DN), the main cause of end-stage renal disease, and a medical catastrophe of worldwide dimensions (Yach et al., 2004; Schena and Gesualdo, 2005). A recent study demonstrated a higher incidence of nephropathy in DM type-II compared to DM type-I patients (Yokoyama et al., 2000) . At present, 20 - 30% of type II diabetic patients develop evidence of DN (Sasso et al., 2006) . Mechanisms behind the injury in diabetic nephropathy are not fully understood despite intense research.

Diabetic patients may need renal transplantation later in life due to diabetic nephropathy, and I/R injury is one of the dangerous complications of this procedure. The combination of both diabetes and renal ischemia plays a major role in the development of DN (Melin et al., 2003). Hyperglycemia in DM is a contributing factor in the development of renal ischemia. DM and hyperglycaemia also worsens the outcome in renal transplantation (Thomas et al., 2001). Conversely, I/R combined with hyperglycaemia could also be important in the development of diabetic nephropathy. Although, several animal models reported an increased susceptibility to renal I/R injury in diabetic rats (Melin et al., 1997, 2002), the exact mechanisms behind this increased sensitivity to renal I/R during type II DM are still poorly understood. Also, the nature of relation between blood glucose and renal injury is not fully investigated. So, the aim of this study was to investigate the role of proinflammatory cytokines e.g. (TNF-), (TGF-) and (NF -B), apoptotic markers (survivin and Bcl- 2), and NO in pathogenesis of increased susceptibility to renal I/R injury in diabetic rats and to correlate these parameters to blood glucose in a rat model of type 2 DM.

MATERIAL AND METHODS

Experimental animals

Sprague-dawley rats (130 - 180 g; 3 months) were used. All rats had free access to standard chow diet and tap water. They were housed in metabolic cages at 20 - 25°C with a 12:12 h light/dark cycle. Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the institutions' animals welfare regulatory committee. Chemicals were purchased from sigma (St. Louis, Mo., USA), if not stated otherwise.

Experimental model

Induction of diabetes

Type II diabetes was induced by feeding rats on high fat diet (containing 22.5% commercially available hydrogenated vegetable oil, 22.5% milk powder, 51.5% soybean ground, 2% corn starch, 1% sucrose and 0.5% vitamins and minerals) for 2 weeks then injection of low dose of STZ (35 mg/kg) via tail vein. Two days after the injection of STZ hyperglycemic state was first tested by

detection of glucosuria using urine glucose strips. Rats with two successive blood glucose levels more than 350 mg/dl were used in this study (Reed et al., 2000).

Model of renal ischemia

Rats were anesthetized by intraperitoneal injection of sodium thiopental (12 mg/100 gm). After anesthesia, the animal was fixed in supine position on the operating table and the abdominal skin was shaved and sterilized with 70% ethyl alcohol. Then, a midline incision was made, and the left renal artery was located and dissected free from its surrounding structures. The left renal artery was clamped for 45 min using a non-traumatic vascular clamp (Bulldog clamp). Then, the edges of the abdominal incision were approximated to each other and covered by a piece of gauze soaked with warm isotonic saline (37°C) to prevent undue loss of body fluids. 5 min before reperfusion or removal of clamp, the right kidney was removed. After removal of the vascular clamp on the left renal artery, the abdomen was properly irrigated with isotonic saline then the abdominal incision was closed by continuous stitches using vicryl 2/0 sutures.

Experimental design

Sixty rats were divided into 4 groups (n = 15, each); Group I: Normal rats, served as normal control group (NC) . Group II: Normal rats underwent left renal ischemia, served as normal ischemic group (NI). Group III: diabetic rats, served as diabetic non-ischemic group (DNI). Group IV: diabetic rats underwent left renal ischemia, served as diabetic ischemic group (DI).

Collection of blood samples

Blood samples were collected (24 h after ischemia) from the ophthalmic venous plexus of animals under halothane anesthesia. Sera were separated from clotted blood after 20 - 30 min by centrifugation at 4000 rpm for 15 min then divided into aliquots and kept at - 20°C for further assay of fructosamine, creatinine, and TNF- , while, serum glucose was determined immediately.

Harvesting of kidney specimen

Kidney specimens were harvested 24 h after ischemia. Animals were anaesthetized with high dose of sodium thiopental intraperitoneal. Then, abdomen was rapidly opened and left kidney was removed rapidly and cut into two equal halves by a scalpel. One half of the kidney was rapidly placed in 10% neutral buffered formalin for immunostaining and H and E staining and the other half was rapidly frozen in liquid nitrogen, and stored at -80°C for reverse transcriptase (RT)- polymerase chain reactions (RT-PCR) assessments.

Determination of serum glucose, fructosamine, creatinine, and TNF-

Serum glucose was determined by using diagnostic kits (Spinreact, Spain), following the manufacturer instructions. Serum fructosamine was estimated by using commercial kits (Spinreact, Spain), following the manufacturer instructions. Serum creatinine was determined by a determined using auto analyzer apparatus (CX7, Beckman, USA). Serum TNF- was determined by ELISA using rat TNF- kits (Ray Biotech, USA) according to the manufacturer's instructions.

Table 1. Serum glucose, fructosamine and creatinine in different groups.

	Normal control	Diabetic non-ischemic	Normal ischemic	Diabetic ischemic
S. Glucose (mg/dl)	98.1 ± 11.29	495 ± 42.16*	103.6 ± 2.30˘	484.6 ± 46.94#
S. Fructosamine (mol/L)	103.89 ± 10.46	319 ± 24.75*	107.14 ± 11.23˘	315.4 ± 39.59#
S. Creatinine (mg/dl)	0.32 ± 0.067	0.64 ± 0.084*	1.21 ± 0.035˘	2.56 ± 0.13 #

Determination of apoptosis by DNA fragmentation

Genomic DNA was extracted and purified from tissues by wizard genomic DNA purification kits (Promega, Madison, WI, USA). Ten micrograms of DNA were loaded into 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. DNA electrophoresis was carried out at 80V for 1 - 2 h. DNA ladders, an indicator of tissue apoptotic nucleosomal DNA fragmentation, were visualized under ultraviolet light and photographed for permanent records.

Detection of acute tubular necrosis (ATN) by histological examination

The kidney specimens were processed for paraffin blocks and sections of 3 m thickness were made and stained with haemtoxylin and eosin and examined blindly by an experienced renal pathologist under light microscope. The severity of lesions of the tubular damage was graded according to Jablonski et al. (1983) scale.

Immunohistochemical examination

The selected paraffin blocks for immunohistochemical staining were sectioned (4 m thickness) and stained with Bcl -2 monoclonal antibody, using the avidin-biotin peroxidase method (Chaudhary et al., 1997).

Detection of TGF- , NF- B, iNOS and survivin gene expression by PCR in kidney tissues

RNA extraction

Total RNA was extracted from tissue homogenate by using total RNA isolation system (Promega, Madison, WI, USA) according to manufacturer's recommendation. To determine the concentration and purity of RNA, measure the absorbance at 280 nm in a spectrophotometer.

Reverse transcriptase (RT)-PCR

Synthesis of cDNA: The extracted RNA was reverse transcribed into cDNA using RT- PCR kit (Stratagene, USA) . Three μl of random primers were added to the 10 μl of RNA which was denaturized for 5 minutes at 65°C in the thermal cycler. Primers sequences of studied genes are TGF-beta, forward

5'AGCGTGGTCGCGGCCGAGGT-3'; NF-kappaB forward
5'TACCATGCTGTTTGGTTCA-3',reverse
5'TCAAGCTACCAATGACTTTC-3'; iNOS, forward
5'GAATTATACACGGAAGGGCCAA -3', reverse
5'AAATGAACCACCCGACTGAAG-3'; survivin forward
5'GAATTATACACGGAAGGGCCAA -3', reverse
5'GAATTATACACGGAAGGGCCAA -3', beta-actin forward

5'TCACCCTGAAGTACCCCATGGAG-3',reverse 5'TTGGCTTGGGGGTTCAGGGGG-3'. The RNA primer mixture was cooled to 4°C. 19 μl of cDNA master mixture (containing 5 μl first strand buffer, 2 μl of dNTPs (10 mmol/L), 1 μl units of MMLV-reverse transcriptase (50 U/ul), 1 μl of RNase inhibitor (40 U/μl)and 10 μl of DEPC-treated water) was added to the 31 μl RNA-primer mixture resulting in 50 μl of cDNA. The last mixture was incubated in the thermal cycler one hour at 37°C followed by inactivation of enzymes at 95°C for 10 min, and finally cooled at 4°C.

PCR: The PCR was performed by adding 45 1 μl PCR mix (containing 5 μl 10 x PCR buffer, 2 μl of dNTPs (10 mmol/L), 1 μl of the 2 primers (50 pmol for each), 1 μl of Taq polymerase and 37 μl of DEPC-treated water) to about 5 l of cDNA. The PCR cycling conditions were 95°C for 1 min for denaturation followed by 55°C for 1 min and 72°C for 2 min; for 35 cycles with final extension at 72°C for 12 min.

Gel electrophoresis and semi-quantitative determination of PCR products: 10 l of PCR product was analyzed on 2% agarose gel with ethidium bromide staining and the product was visualized on ultraviolet transilluminator, then gel documentation was performed. PCR products were semi-quantified by gel documentation system (BioDocAnalyze).

Statistical analysis

Statistical analysis was done by using SPSS computer program (version 10). One-way analysis of variance (ANOVA) was done to study test of significance within treated groups, within Scheffe s posthoc test. Pearson's correlation co-efficient was used to study correlation between variables. Significance was considered when P values were less than 0.05.

RESULTS

Serum glucose, fructosamine and creatinine

Results of serum glucose, fructosamine and creatinine are mentioned in Table 1. It shows that diabetic non-ischemic rats showed significant rise in serum glucose levels by 404% when compared with normal control and normal ischemic rats (P < 0.001). However, there is no significant difference in serum glucose between diabetic non-ischemic and diabetic ischemic rats. As regard, serum fructosamine, it exhibited a significant increase in diabetic non-ischemic rats by 207% when compared with normal control and normal ischemic rats (P < 0.001).Also, there was no significant difference in serum fructosamine between diabetic non-ischemic and diabetic ischemic rats. Serum creatinine showed significant increment in diabetic non-ischemic rats (by 100%) when compared
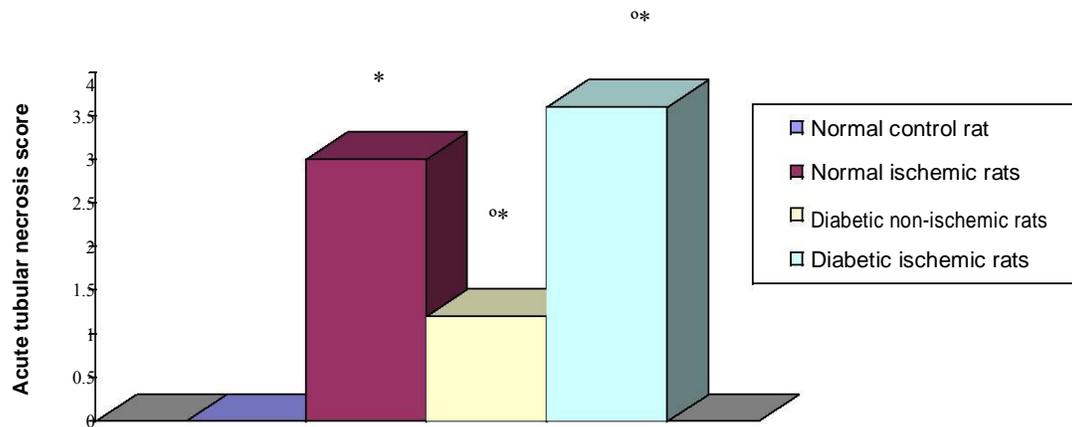
Figure 1. Acute tubular necrosis score in different groups. Data are expressed as means.* Significant vs. normal non ischemic rats. ºSignificant vs. normal ischemic rats.
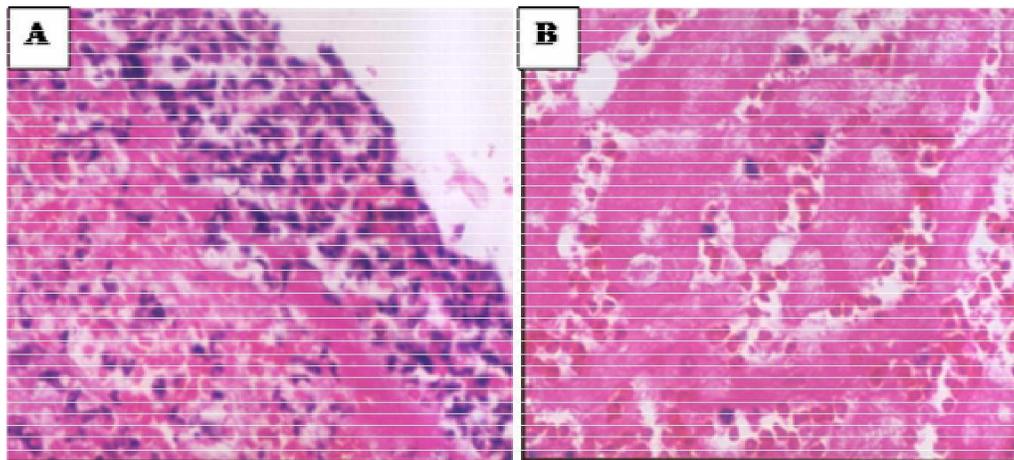

Figure 2. kidney section shows leucocytic infiltrations mostly neutrophils (A) and severe coagulative necrosis of the renal tubules and intertubular hemorrhages (B) (H and E) 1200 (diabetic ischemic group).

with normal control rat (P < 0.05). Also, serum creatinine was significantly increased in normal ischemic rats by 100% when compared with diabetic non-ischemic rat (P < 0.05). Diabetic ischemic rats showed significant increase in serum creatinine when compared with normal ischemic rats by 111.57% (P < 0.001).

Acute tubular necrosis score

There was a significant increase in the renal tubular necrosis scores in ischemic non-diabetic, and diabetic ischemic rats when compared with normal rats and diabetic non-ischemic rats (p < 0.034) (Figure 1). Kidney sections from ischemic diabetic rats show massive coagulative necrosis, and inflammatory infiltrate mostly

neutrophils (Figure 2).

Expression of proinflammatory cytokines TNF-alpha, TGF-beta, and NF$_k$B and iNOS

Table 2 shows significant rise in serum TNF - levels in diabetic non- ischemic rats by 93.81% and in normal ischemic rats by 51% when compared with normal control rats (P < 0.01). Also, there was a significant increase in serum TNF- level was observed in diabetic ischemic animals in comparison with diabetic non-ischemic one by 23.03% (P < 0.05). As regard, TGF - 1 in kidney tissues was significantly elevated in diabetic non-ischemic rats by 242.2% and normal ischemic rats by 151% when compared with the normal control rats (P< 0.001). Also, it

Table 2. Levels of serum TNF- and expression of TGF- 1, NF- B, iNOS, and survivin in renal tissues in different groups.

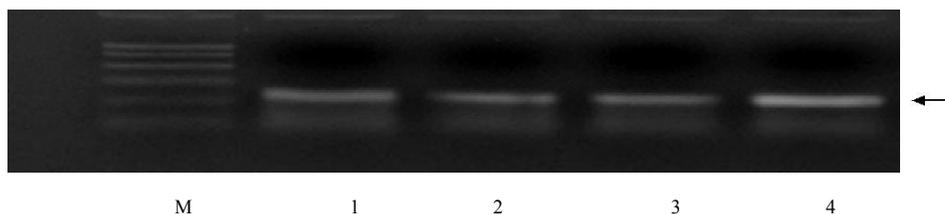|	Normal control (n=15)	Diabetic non-ischemic (n=15)	Normal ischemic (n=15)	Diabetic ischemic (n=15)
TNF- (pg/ml)	28.62 ± 1.73	55.47 ± 3.82	43.13 ± 2.33	68.25 ± 5.55 #
TGF- 1 (µg/ml)	1.09 ± 0.16	3.73 ± 0.46	2.74 ± 0.23	5.74 ± 0.31 #
NF- B (µg/ml)	2.58 ± 0.24	6.36 ± 0.32	7.89 ± 0.13	12.12 ± 0.21 #
iNOS (µg/ml)	1.73 ± 0.37	4.24 ± 0.28	4.99 ± 0.34	7.28 ± 0.24 #
Survivin (µg/ml)	15.03 ± 0.71	11.52 ± 0.12	19.24 ± 0.07	10.15 ± 0.15 #

Figure 3. mRNA gene expression levels of renal TGF- 1 and control gene (- actin) in all rats (n = 15/group). Lane M: 100 bp DNA marker, Lane 1, 2, 3, and 4 are representative for normal non ischemic rats, diabetic ischemic rats, diabetic non-ischemic rats, and ischemic non-diabetic rats, respectively.
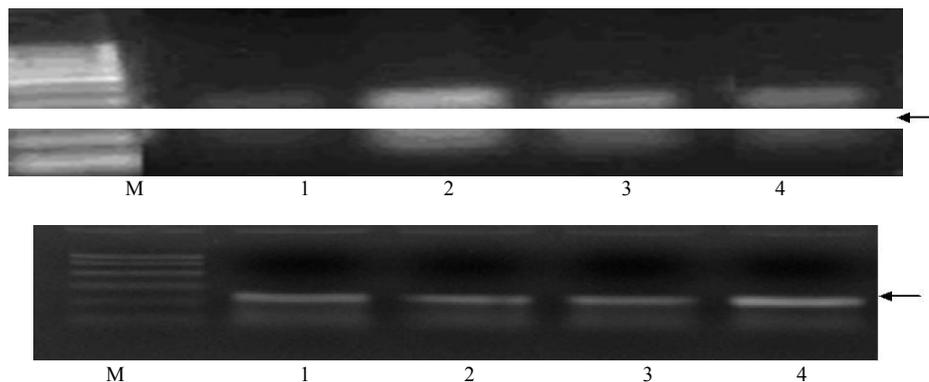

Figure 4. mRNA gene expression levels of renal NF- B and control gene (- actin) in all rats (n = 15/group). Lane M: 100 bp DNA marker, Lane 1, 2, 3, and 4 are representative for normal non ischemic rats, diabetic ischemic rats, diabetic non-ischemic rats, and ischemic non-diabetic rats, respectively.

was found that renal tissue content of TGF- 1 was significantly increased in diabetic ischemic group in comparison with diabetic non-ischemic one by 27.07% (P < 0.01) (Figure 3) . As regard, NF- B, there was a significant elevation in NF- B content in renal tissue in diabetic non-ischemic rats by 146.51% and in normal ischemic rats by 205.81% when compared with normal control group (P < 0.001). Also, there was a significant rise in NF- B content in renal tissue in diabetic ischemic rats in comparison with diabetic non -ischemic group by 99.56% (P < 0.01) (Figure 4). Also, from Table 2, it can be noticed that, compared to the normal control group,

the diabetic non-ischemic and normal ischemic rats showed significant increase in iNOS contents in renal tissue by 145.08 and 188.23% respectively (P < 0.001). Also, there was a significant rise in iNOS contents in renal tissue in diabetic ischemic group in comparison with the diabetic non-ischemic group by 71.69% (Figure 5).

Apoptosis and apoptotic markers (Bcl-2 and survivin)

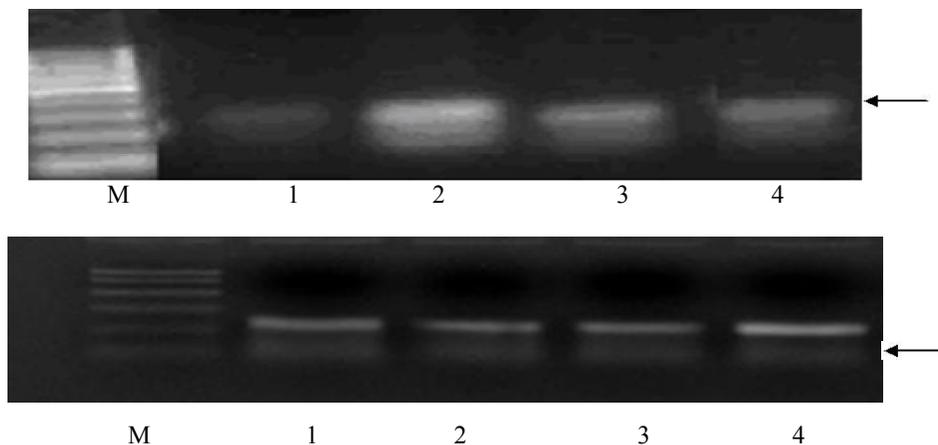The non quantitative analysis of DNA fragmentation revealed typical laddering of fragmented DNA in

Figure 5. mRNA gene expression levels of renal iNOS and control gene (- actin) in all rats (n = 15/group). Lane M: 100 bp DNA marker, Lane 1, 2, 3, and 4 are representative for normal non ischemic rats, diabetic ischemic rats, diabetic non-ischemic rats, and ischemic non-diabetic rats, respectively.
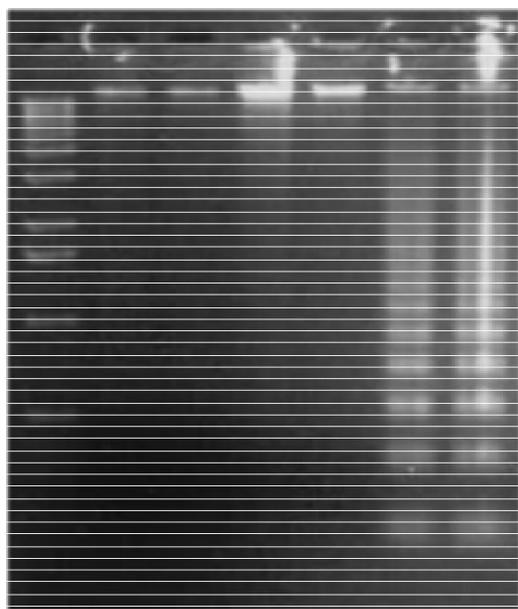

Figure 6. Genomic DNA agarose gel electrophoresis showing a non quantitative analysis of DNA fragmentation which is characteristic of apoptosis. Lane 1 contains commercial 100 bp DNA markers (arrow indicates 200 bp). DNA fragmentation analysis revealed typical laddering of fragmentated DNA in the normal ischemic rat and diabetic ischemic rats respectively (lane 6 and 7). No laddering pattern in normal rats and diabetic ischemic rats (lane 2, 3, 4, 5) 24 hours after ischaemia.

specimens from non diabetic-ischemic and ischemic – diabetic rats indicating cell apoptosis (Figure 6). Table 2 shows a significant decrease in renal tissue content of survivin in diabetic non-ischemic rats (by 23.35%) and

significant increase in normal ischemic rats (by 28.03%)when compared with the normal control ones (P < 0.05). Meanwhile, survivin content of diabetic ischemic group was significantly decreased in comparison with the corresponding value of diabetic non-ischemic group by 11.89% (P < 0.05) (Figure 7). As regard immunohistochemistry of kidney sections for Bcl-2, the various sections showed different scales of positivity. A significant positive staining was noticed in the diabetic non ischemic, normal ischemic and diabetic ischemic, while negative staining was observed in the normal non-ischemic group (Figure 8).

Correlations between serum glucose and various measured parameters

There were significant positive correlations between serum glucose and serum fructosamine (r = 0.98, P < 0.01, Figure 9a), serum creatinine (r = 0.832, P < 0.01, Figure 9b) and serum TNF- (r = 0.873, P < 0.01, Figure 9c). Also, our data showed significant positive correlations between blood glucose and renal tissue contents of TGF -B (r = 0.881, P < 0.01, Figure 9d), NF-B (r = 0.891, P < 0.01, Figure 9e) and iNOS (r = 0.89, P < 0.01, Figure 9f). On the other hand, a significant negative correlation was observed between blood glucose and renal tissue content of survivin (r = -0.833, P < 0.01, Figure 9g).

Correlations of serum TNF- to various measured parameters in kidney tissues

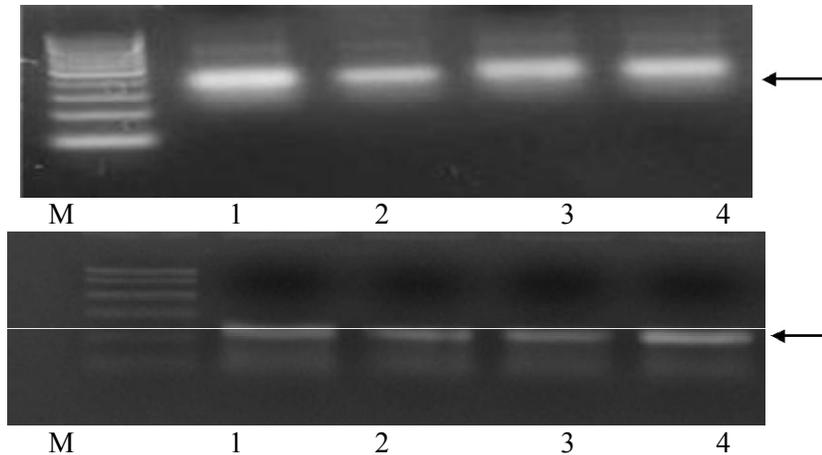There were significant positive correlation between TNF-

Figure 7. mRNA gene expression levels of renal survivin and control gene (- actin) in all rats (n = 15/group). Lane M: 100 bp DNA marker, Lane 1, 2, 3, and 4 are representative for normal non ischemic rats, diabetic ischemic rats, diabetic non-ischemic rats, and ischemic non-diabetic rats, respectively.
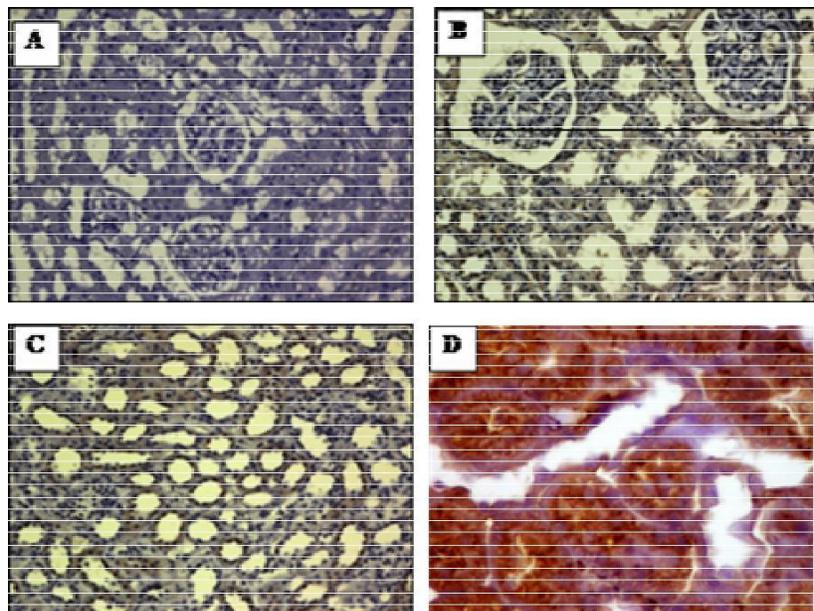

Figure 8. Immunohistochemistry for Bcl-2 stained with immunoperoxidase of kidney sections. Positively stained cells were identified by brown cytoplasmic coloration (*arrows* indicate positively stained cells). A: Negative staining for Bcl-2 in normal control group, B and C: Moderate positivity for Bcl-2 in diabetic non-ischemic and non-diabetic ischemic groups, respectively ×200. D: Marked positivity for BCL-2 in diabetic ischemic group × 400.

and renal tissue contents of TGF-B (r = 0.937, P < 0.01, Figure 10a), NF- B (r = 0.937, P < 0.01, Figure 10b) and iNOS (r = 0.89, P < 0.01, Figure 10c). However, a significant negative correlation was observed between serum TNF- and renal tissue content of survivin (r = - 0.854, P < 0.01, Figure 10d).

DISCUSSION

Diabetes mellitus (DM) especially type II causes organ dysfunctioning and increases the sensitivity of organs to damages. In the present study, we tested the hypothesis that DM and hyperglycaemia render the kidney more
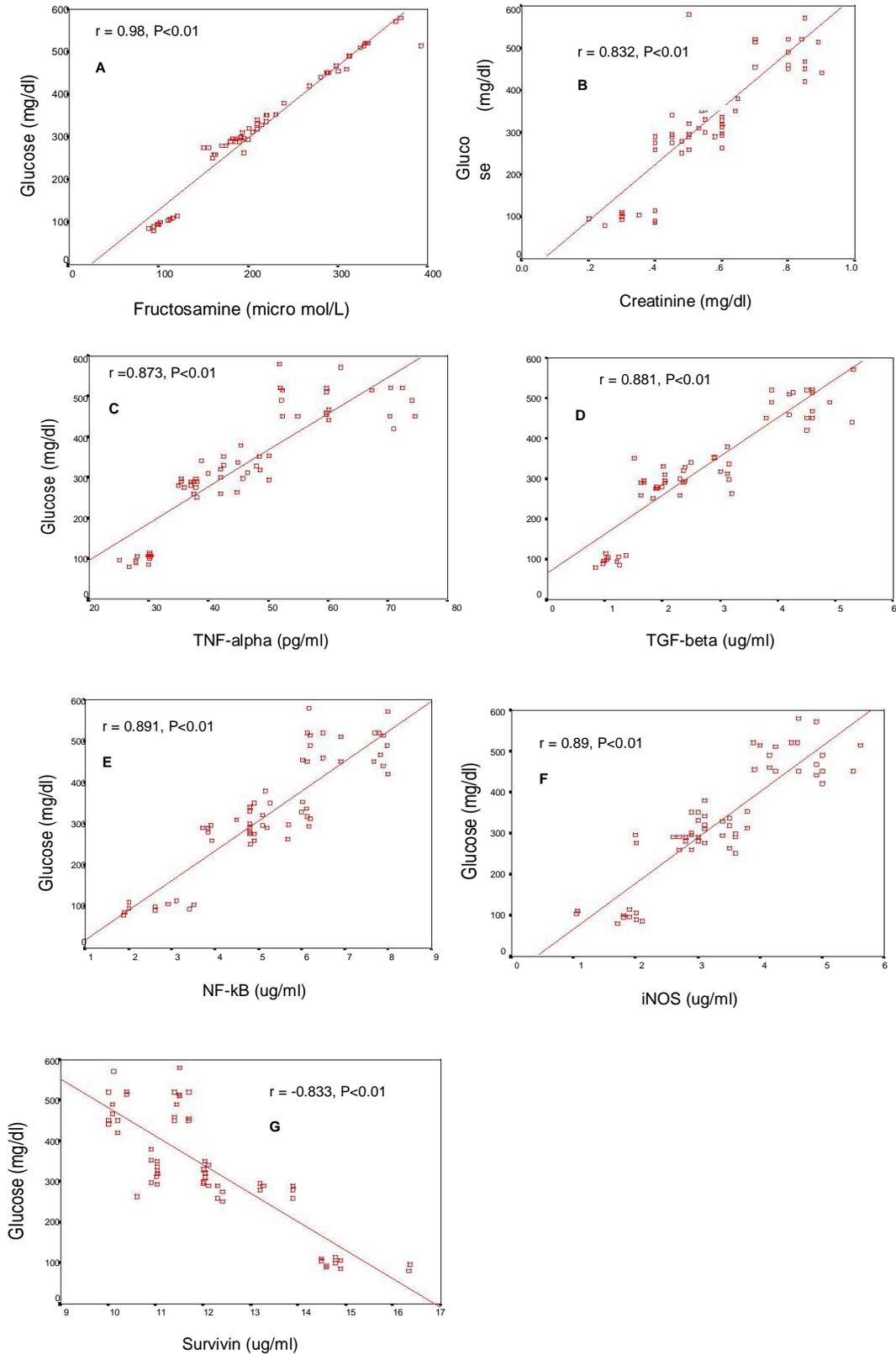
Figure 9. Correlation between serum glucose and different measured parameters. With serum fructosamine (a), serum creatinine (b); serum TNF- (c), TGF- content in kidney (d), NF- B content in kidney (e), iNOS content in kidney (f), and survivin content in kidney (g).
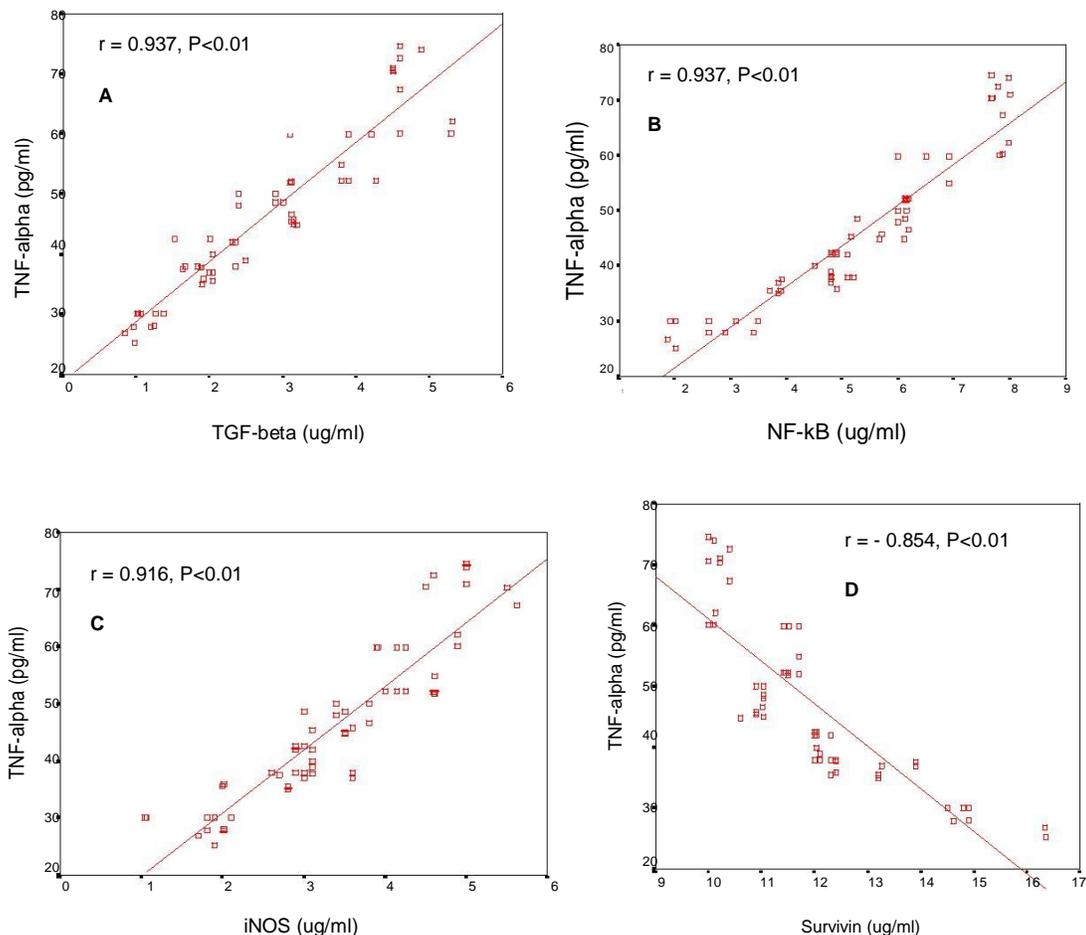
Figure 10. Correlation of serum TNF- with kidney contents of TGF- (a), NF- B (b), iNOS (c), and survivin (d).

more susceptible to ischemic injury and examined the effect of left renal ischemia for 45 min with right nephrectomy on renal functions, histological damage, proinflammatory cytokines TNF- , TGF- 1, and NF- B, as well as on the expression of iNOS and apoptotic markers Bcl-2 andsurvivin in a rodent model of STZ-induced type II DM. Diabetic rats in the present study showed significant increase in the serum blood glucose, creatinine, fructosamine, and TNF- , and increased expression of TGF- 1, NF- B, iNOS, and Bcl-2 with decreased expression of survivin. Expression of these markers became significantly marked in diabetic rats after the episode of ischemia. At the same time, there is a positive correlation between blood glucose and these parameters except survivin which has a negative correlation with blood glucose. These findings suggest the possible role of proinflammatory cytokines; TNF - , TGF- 1, and NF- B, iNOS, and apoptotic cell death in pathogenesis of ischemic injury in diabetic kidney and the involvement of hyperglycemia in pathogenesis of increased susceptibility of diabetic kidney to ischemic injury.

As mentioned before in literature, hyperglycaemia is most probably a contributing factor in the development of ischaemic ARF. The present study showed significant increase in the blood glucose diabetic rat, with no further increase in blood glucose by renal I/R injury. These results are consistent with those reported in many studies (Qian et al., 2008; Akbarzadeh et al., 2007; Abu-Abeeleh et al., 2009). Also, the diabetic rats showed significant elevation in serum creatinine level in diabetic rats in comparison with non diabetic rats. These data are in agreement with several studies which reported that, in experimentally diabetic rats there is a significant increase in serum creatinine levels when compared with normoglycemic ones and this is considered as an indicator of deteriorated renal function (Umrani and Goyal, 2003; Chen et al., 2005; Kuhad and Chopra, 2009). In addition, our results confirmed the findings of Yousef et al., (2005) who found that combination of renal ischemia with DM raised serum creatinine more than did DM alone suggesting significant impairment of glomerular function.

In consistence with renal function impairment,

histopathological examination revealed significant increase in ATN score in diabetic ischemic rat. These findings are associated with marked leucocytic infiltration, which suggest increased inflammatory state in diabetic ischemic kidney. So, the present study was established to provide new insights on the factors involved in the exaggerated inflammatory response to I/R injury in diabetes. The first one is TNF - . The present study showed significant rise in serum TNF- level in diabetic non- ischemic, normal ischemic, and diabetic ischemic rats, when compared with normal control rats, but the marked rise was found in diabetic ischemic rats. These findings are in consistent with many studies that demonstrated significant rise in serum TNF - level in renal I/R injury (Tu tepe et al., 2007; Sener et al., 2006), as well as in diabetic rats (Kuhad and Chopra, 2009; Satoh et al., 2003). The enhanced TNF- production ischemic diabetic rat may result from constitutive overproduction by adipose tissue in type II diabetes, the effects of hyperglycemia and AGEs (Liu et al., 2006), and increased generation of ROS in renal I/R injury and DM (Bonventre and Zuk, 2004). Hyperglycemia induced oxidative stress, along with AGEs and products of lipid peroxidation, possibly serves as activators of transcription factors, leading to induction of gene expression of pro-inflammatory cytokines and release of many inflammatory cytokines as TNF - and IL-6 through NF- B (Yamagishi et al., 2007). Also, the oxidative stress in ischemia alone has been shown to activate NF- B and the activation of this transcription factor may play a role in the sequence of I/R induced TNF- production (Bonventre and Zuk, 2004; Meldrum et al., 2002). TNF-can induce renal injury via induction of apoptosis and necrotic cell death (Boyle et al., 2003), alterations of intraglomerular blood flow and glomerular filtration rate as a result of the hemodynamic imbalance between vasoconstrictive and vasodilatory mediators (Baud et al., 1998), alterations of endothelial permeability, as well as, alterations of the barrier function of the glomerular capillary wall leading to enhanced albumin permeability (Koike et al., 2007) . At the molecular level TNF-augments the release of many inflammatory factors from renal mesangial cells (Furuichi et al., 2006; Yeboah et al., 2008) and this may confirm our results in which we found that there is a significant positive correlation between serum TNF- and renal tissue content of TGF- , NF- B and iNOS.

TGF- 1 is another cytokine that stimulates extracellular matrix (ECM) synthesis, cell clustering, tubulogenesis, and apoptosis in many cell types including renal cells (Schuster and Krieglstein, 2002). The present study demonstrated increased expression of TGF- in renal biopsies ischemic non diabetic, diabetic non-ischemic and diabetic ischemia with marked expression in diabetic ischemic rats. These findings are in agreement with other studies which reported that increased expression of TGF- 1 in several animal models of diabetes (Kuhad and

Chopra, 2009; Pantsulaia, 2006) and in animal models of renal I/R injury (Spurgeon et al., 2005) . Increased expression of TGF- in diabetes may occur in response to many mediators such as high glucose concentration; AGEs; oxidative stress and activation of protein kinase C (Ziyadeh, 2004) and in I/R injury occur as a direct consequence of hypoxia, angiotensin II signaling and loss of ECM integrity (Docherty et al., 2002). In STZ induced diabetic mice, neutralization of TGF- by anti- TGF-antibodies attenuated renal hypertrophy and enhanced ECM gene expression, suggesting a causal association of TGF- activity and kidney disease in diabetes (Ziyadeh, 2004).

NF- B is an inducible transcription factor that is sequestered in the cytoplasm in inactive form and when activated, it translocates from cytoplasm to nucleus, where it binds to B DNA promoter sites and transactivates a number of downstream proinflammatory genes, including IL-1, IL-2, NOS, intercellular adhesion molecule, vascular cell adhesion molecule, endothelial selectin and TNF - (Barnes and Karin, 1997). The current experiment reported that NF - B expression was significantly increased in diabetic nonischemic and normal ischemic rats when compared to normal control one. Moreover, the current study elucidated a significant elevation in NF- B level in renal tissue in diabetic ischemic rats when compared with diabetic non ischemic and normal ischemic. Previous studies reported activation of NF- B in the kidney of STZ diabetic rats (Wang et al., 2007). Donnahoo et al. (2000) documented that renal ischemia, with or without reperfusion, activates NF- B and increases TNF- mRNA content and protein bioactivity in the kidney.

NF- B can be activated by oxidative stress which plays a critical role in diabetes as well as I/R in induction of renal injury (Kim et al., 2009). The common point of the interaction between ROS on the NF-kB activation pathway is unknown. The most likely scenario is that ROS, from either DM or renal I/R, promote the activation pathway by activating I B kinase leading to phosphorylation of critical serine residues in IkB, resulting in the liberation of NF- B (Chen et al., 2009).

The next point in the present study was to investigate the involvement of NO system in development of renal I/R injury in DM. NO is synthesized by 2 isoforms of NOS; inducible NOS which produces large amounts of NO that can be toxic and pro-inflammatory, whereas, constitutively expressed isoforms produce only low to modest amounts of NO (Staels and Fruchart, 2005). The present study reported that iNOS expression was significantly increased in diabetic nonischemic and normal ischemic rats when compared to normal control one. Moreover, there was a significant elevation in iNOS level in renal tissue in diabetic ischemic rats when compared with diabetic non ischemic. These findings suggest the involvement of iNOS in ischemic injury in diabetic kidney. This result coupe with many authors who

documented that iNOS is activated in the kidney of rats, soon after the induction of diabetes, thus suggesting its involvement in the increased production of NO observed immediately after the onset of diabetes (Umrani and Goyal, 2003; Chen et al., 2005). In diabetic kidney, AGEs were detected in the mesangial area in glomeruli, which were associated with a paralleled increased of iNOS production (Chang et al., 2004).

On the other hand, many investigators are contradictory to the current results (Veelken et al., 2000; Schwartz et al., 2001) . They found that iNOS and endothelial NOS mRNA levels measured by RT-PCR in diabetic rats were not changed as compared with levels in controls. Therefore, renal NO generation, at least *via* endothelial NOS and iNOS, is not the primary cause of glomerular hyperfiltration in diabetes (Schwartz et al., 2001). Veelken et al. (2000) demonstrated that in glomeruli isolated from diabetic and control rats, neither iNOS mRNA nor protein expression was detected.

The last point in this study was to examine apoptosis and the expression of apoptotic markers survivin and Bcl-2 in diabetic ischemic injury. The present study demonstrated a typical laddering of fragmented DNA kidney specimens obtained from ischemic non-diabetic and ischemic-diabetic rats indicating apoptotic cell death. To our knowledge there are no previous studies discussing the role of survivin in renal I/R. Our data showed that there was a significant decrease in renal tissue content of survivin in diabetic non-ischemic rats and significant increase in normal ischemic rats when compared with the normal control ones. Meanwhile, survivin content of diabetic ischemic group was significantly decreased in comparison with the corresponding value of diabetic non-ischemic group. Lechler et al. (2007) reported that focusing on the kidney, survivin is strongly expressed in proximal tubule, particularly at the apical membrane, which can be verified in rat, mouse, and human kidneys. Mechanisms by which survivin suppresses apoptosis are still under considerable debate (Johnson and Howerth, 2004).

Previous studies reported that renal I/R is associated with up-regulation of two anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-2 X ligand) in the damaged renal tubule (Oberbauer et al., 2001). Bcl-2 augmentation protects renal tubular epithelial cells from I/R, and subsequent interstitial injury by inhibiting tubular apoptosis (Suzuki et al., 2008; Isaka et al., 2009). The results present studies are in agreement with these previous reports. Contradictory to our study, Yamamoto et al. (2004) suggested that the proapoptotic mechanisms of hypoxia in renal epithelial cells largely depend on a significant decrease in Bcl-2.

It is concluded that, renal ischemia for 45 min caused enhanced renal injury in type II DM. Proinflammatory cytokines TNF- , TGF- , and NF- B and iNOS as well as Bcl-2 and survivin may contribute to the enhanced renal ischemic injury in type II DM. Also, hyperglycaemia may be involved in hypersensitivity of kidney to ischemic injury in DM.

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