Research Paper

Hypertension influences the exponential progression of inflammation and oxidative stress in streptozotocin-induced diabetic kidney

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ABSTRACT

Objective: To investigate the association of hypertension coexisting with diabetes mellitus with oxidative stress and inflammation in the kidneys of streptozotocin (STZ)-induced diabetic rats. **Materials and Methods:** Male Wistar rats were used for the experiments. Blood glucose (BG), urea, blood pressure (BP), and heart rate (HR) were analyzed before and 48 h after STZ injection. Further, these parameters were monitored up to 3 months of diabetes induction. Subsequently, the inflammatory markers (C-reactive protein, tumor necrosis factor-alpha, and nitrate) and oxidative stress markers were estimated after 3 months of diabetes induction in the kidney homogenate. Histological analysis of renal tissue was also carried out. **Results:** Linear elevation of BG, urea, mean arterial pressure (MAP), and HR was observed up to 3 months of diabetes induction. In the same manner, inflammatory and oxidative stress markers were also found to be significantly increased. Notably, the histological analysis revealed the signs of nephropathy such as increased mesangial cell number, thickness of basement membrane, and renal artery. Inflammatory and oxidative stress markers positively correlated with elevated BP and BG, but the correlation was better with BP rather than BG. **Conclusion:** Hypertension has a strong implication in the increased oxidative stress and inflammation of diabetic kidney at the very early stage of diabetes mellitus.

Key words: Diabetes mellitus, diabetic nephropathy, hypertension, streptozotocin and oxidative stress

INTRODUCTION

Hypertension is prone to patients with diabetes mellitus when compared to general population**.** The incidence and prevalence of hypertension among patients with diabetes are well documented in various populations. At the time of diagnosis of type 1 diabetes, blood pressure (BP) levels are usually found to be normal, but hypertension is closely correlated with the diabetic renal disease after its onset.[1] Similarly, during the diagnosis of type 2 diabetes, approximately one-third of the patients have elevated BP,[2] and the prevalence of hypertension

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increases to almost 100% during the manifestation of renal disease.[3] The common signs of diabetic nephropathy (DN) such as increased urinary albumin excretion and decreased glomerular filtration are generally associated with an elevation of BP leading to end-stage renal disease (ESRD).

The coexistence of hypertension in diabetic individuals is a major risk factor for the development of macrovascular and microvascular complications.[4,5] Further, it also fastens the occurrence of microalbuminuria which leads to the progression of DN.[6] Hypertension is induced by the activation of the renin-angiotensin-aldosterone system followed by upregulation of reactive oxygen species and downregulation of nitric oxide (NO). These pathogenic factors accelerate kidney and cardiovascular diseases in patients with diabetes due to local nonhemodynamic effects. Further, an increased levels of NO and various isoforms of NO synthase in the kidney were observed in the early hyperfiltration stages of diabetic kidney disease, but there was a decline in the levels as the disease progressed to overt DN.[7] Chronic kidney disease itself is known to raise oxidative stress and decrease antioxidant defenses.[8,9]

Oxidative stress and inflammation have a strong correlation with the pathogenesis of diabetes and hypertension. It has been well reported that elevation of oxidative stress in diabetes was found to have a significant association with the pathogenesis of DN and its progression to ESRD.[10,11] Consequently, it is believed that inflammation and oxidative stress play a key role in the pathogenesis of DN. The development of inflammation and oxidative stress in different organs, especially in kidney and retina of patients with diabetes has been documented in various studies.[12,13]

Although several studies have reported the role of high BP, oxidative stress and inflammation in the pathogenesis of DN and the progression to ESRD, the exact association or interrelation of blood glucose (BG), BP, and oxidative and inflammatory markers in DN are still unclear. One of the leading causes of ESRD is DN, and yet, the understanding of the role of the DN in the pathogenesis of ESRD is insufficient due to lack of a suitable animal model to study this devastating disease.

Although the streptozotocin (STZ) model is well established and accepted to induce type 1 diabetes and its associated complications, the mechanism of the development of hypertension in diabetes mellitus is still a topic of debate. The dose of STZ and duration of diabetes are also crucial factors in determining the variation in BP. Furthermore, a few studies have reported the contribution of elevated oxidative stress and inflammatory markers in the development and progression of hypertension and diabetes mellitus. However, the change in BP in DN has been neither completely studied nor correlated with metabolic derangement markers.[14] Hence, in this study, we investigated the correlation of BP and BG with inflammatory and oxidative stress markers in the kidneys of STZ-induced diabetes rat model.

MATERIALS AND METHODS

Animals

Healthy male Wistar rats weighing 250–300 g were used for the study. The animals were individually housed and fed a standard commercial diet and water *ad libitum* to adapt to the experimental animal facility for 7 days. The light/dark cycle was 12 h on and 12 h off. All the experiments were executed after the approval of the Institute Animal Ethics Committee (IAEC, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Induction of diabetes mellitus

Animals were divided into two groups; control (n = 8) and diabetic group (n = 8). The diabetic group received STZ in citrate buffer, and the control group received citrate buffer alone. STZ (45 mg/kg body weight [BW]) in 50 mmol/L citric acid buffer (pH 4.5) was used to induce diabetes mellitus by a single i.p. injection to the rats which had been fasted for 16 h. BG, BW, and BP were measured before and 48 h after STZ injection. Further, they were monitored up to 3 months after diabetes induction. BG was estimated using Accu-Chek, Active Glucose Test Strips (Roche Diagnostics India Pvt. Ltd., Mumbai, Maharastra, India), and the BG of >300 mg/dL were considered diabetic.

Biochemical analysis

Blood samples were collected from retro-orbital venous plexus before and after 48 h of STZ injection, and every month up to 3 months of diabetes induction. Urea and creatinine levels were assessed in plasma using fully automated clinical chemistry analyzer (AU400; Mishima Olympus Co., Ltd, Shinjuku, Tokyo, Japan).

Blood pressure measurement

The BP was measured in conscious state in control and diabetes-induced animals. Tail cuff plethysmography (IITC, Woodland Hills, CA, USA) was used to measure the heart rate (HR) and the BP of rats.[15] Briefly, the animals were placed in the rodent restrainer for at least 10 to 15 min before obtaining BP measurements. The BP was recorded before and 2 days after STZ and followed by every 30 days until the end of the study and analyzed using a computerized data acquisition (BPMONWIN software [IITC Life Science Inc. CA, USA]). An average of three consecutive BP measurements was considered final.

Histological analysis

After 3 months of diabetes induction, the animals were sacrificed. The kidney samples were fixed in the 10% neutral buffered formalin for 24 h, dehydrated, and embedded in paraffin. The paraffin-embedded kidney/renal tissues sectioned with the thickness of 5 μm were stained with hematoxylin and eosin.

Sample preparation

After 3 months of diabetes induction, the kidneys removed from the rats were rinsed in ice-cold saline, weighed, finely minced, and homogenized (10% w/v) in ice-cold 0.01M Tris-HCl buffer (pH 7.4) with an all-glass Dounce homogenizer. Aliquots of this homogenate were used for the assay of antioxidant and inflammatory parameters.

Antioxidant parameters

Spectrophotometric estimation of reduced glutathione (GSH) was done by the method of Moron *et al*.[16] Glutathione peroxidase (Gpx) activity was measured by the method of Rotruck *et al*.[17] Malondialdehyde (MDA) levels were estimated using an enzyme-linked immunosorbent assay (ELISA) kit from Cayman. Total antioxidant capacity (TAC) assay activity was estimated according to the method of Benzie *et al*.[18] Free thiol activity was estimated according to the method of Ellman and Lyskol.[19] Protein estimation in each sample was carried out using the method of Lowry *et al*.[20]

Inflammatory parameters

Tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and nitrate levels in kidney were estimated using a commercially available ELISA kit from Diaclone, France, Ray Biotech Inc., and Sigma, respectively as per the manufacturer's instructions.

Data analysis

Continuous data were presented as mean ± standard deviation. Comparison of continuous data between groups was analyzed using paired *t*-test. Continuous data measured at different time points were compared using repeated measures of ANOVA. The association between the parameters was analyzed using Pearson correlation. Fixing the BG values, partial correlation was performed to find out the association between mean arterial pressure (MAP) and markers of inflammation and oxidative stress. Similar analysis was carried between BG and markers of inflammation and oxidative stress fixing the MAP. Data analysis was performed with Statistical Package for Social Sciences version 19.0 for Windows (IBM-SPSS Inc., USA). $P < 0.05$ was considered statistically significant.

RESULTS

Animal glucose and urea levels

The BG significantly elevated in diabetic rats (450 ± 58 mg/dL) as compared to control (111 ± 9 mg/dL) [Figure 1a] after 3 months of diabetes induction. Similarly, serum urea levels significantly increased in diabetes [Figure 1b], but no significant difference was observed in creatinine levels (data not shown).

Blood pressure and heart rate

Systolic BP (SBP), diastolic BP (DBP), and MAP were significantly increased from 125 ± 3 mmHg, 83 ± 2 mmHg, 97 ± 2 mmHg to 160 ± 15 mmHg, 119 ± 10 mmHg, 133 ± 12 mmHg, respectively after 3 months of diabetes induction [Figure 2]. Further, there was a significant change in HR after 3 months of diabetes induction (548 ± 22 bpm) when compared to control (384 ± 13 bpm) [Figure 3].

Histological analysis

The control group [Figure 4a and 4c] showed no changes in kidney tissue morphology, whereas the diabetic group showed a relatively increased number of mesangial cells in association with increase in the thickness of the glomerular basement membrane [Figure 4b] and renal artery [Figure 4d].

Antioxidant parameters

Table 1 represents the GSH, Gpx, MDA, TAC, and free thiol levels in control and diabetic kidney and all the parameters are significantly different between the groups. GSH levels were 1-fold lower in diabetic rats (13.11 ± 0.79 nmol/mg protein; $P < 0.05$) as compared to

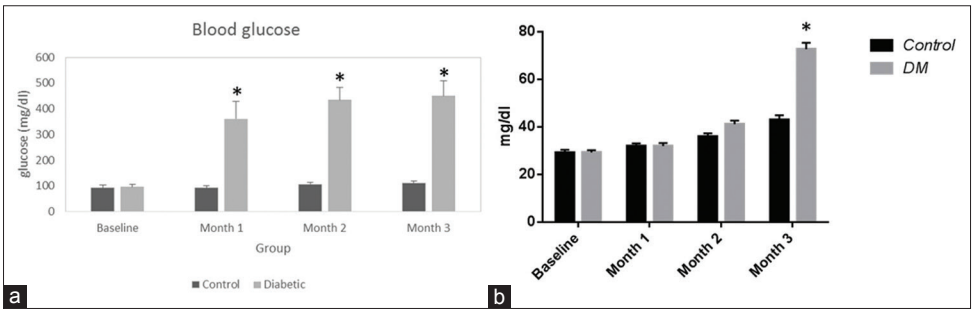

Figure 1: (a) Blood glucose and (b) urea levels in STZ-induced diabetic rats before STZ injection and followed for up to 3 months. Data are expressed as mean ± standard deviation

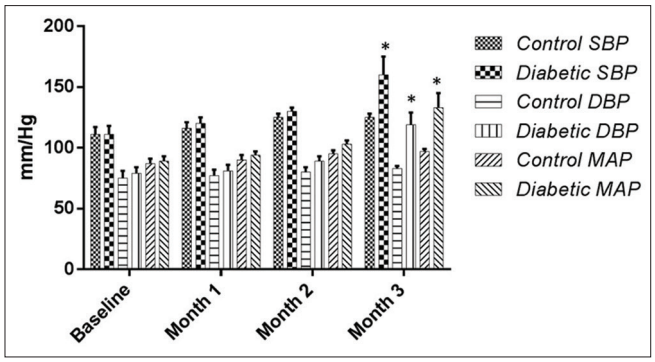

Figure 2: Effect of diabetes mellitus on systolic, diastolic, and mean arterial pressure in STZ-induced diabetic rats before and after STZ injection, and followed for up to 3 months. Data are expressed as mean ± standard deviation

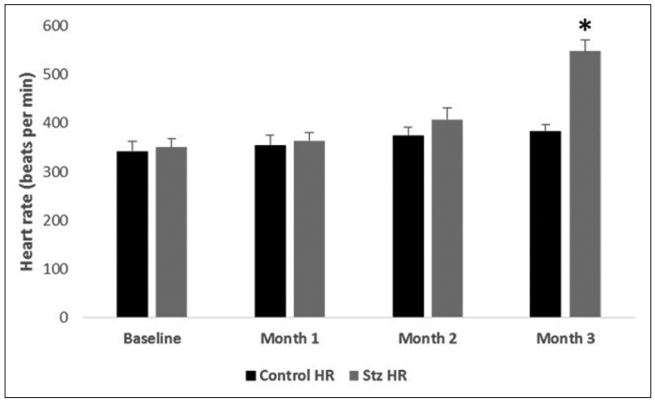

Figure 3: Effect of diabetes mellitus on heart rate in STZ-induced diabetic rats before and after STZ injection and followed for up to 3 months. Data are expressed as mean ± standard deviation

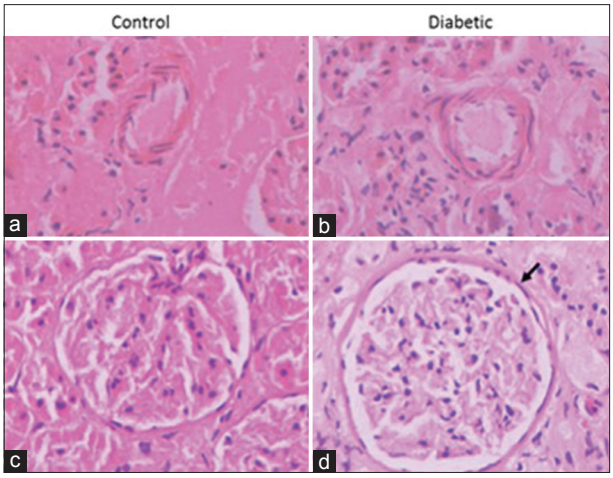

Figure 4: Effect of diabetes mellitus on kidney histopathology of STZ-induced diabetic rats after 3 months of induction (H and E, ×40). Renal artery of control (a) and diabetic (b) rats. Kidney tissue of control (c) and diabetic (d) rats

control rats (26.96 ± 0.75 nmol/mg protein). Gpx activity in diabetic kidney (1.28 ± 0.05 U/mg protein) was significantly increased compared to control (0.79 ± 0.07 U/mg protein). Diabetic kidney (3.45 ± 0.20 µmol/L) showed 1-fold higher MDA levels than control (1.85 ± 0.09 µmol/L). TAC in control (224.86 ± 4.90 µmol/L) was 1.5-fold higher than that in diabetic kidney (147.18 ± 1.81 µmol/L; $P < 0.05$). Similarly, free thiols in control (1.35 ± 0.05 mmol) were significantly increased when compared to diabetic kidney (0.75 ± 0.05 mmol).

Table 1: The oxidative stress and inflammatory markers in kidney homogenate of control and diabetic rats 3 months after diabetes induction

Assay	Control	Diabetic
GSH (nmol/mg protein)	26.96±0.75	13.11±0.79*
Gpx (U/mg protein)	0.79±0.07	1.28±0.05*
MDA (µmol/L)	1.85±0.09	3.45±0.20 *
TAC (µmol/L)	224.86±4.90	147.18±1.81*
Free thiol (mmol)	1.35±0.05	0.75±0.05*
CRP (µg/ml)	434.87±9.17	495.04±12.68*
TNF-α (pg/ml)	153.64±2.74	233.09±3.74*
Nitrate (ng/µl)	140.56±7.00	165.37±2.99*

The values are mean±SD. *n*=6/group. *P<0.05 when compared to control

Inflammatory parameters

TNF-α, CRP, and nitrate levels of diabetic and control kidney are shown in Table 1. TNF-α estimations in diabetic kidney (153.64 ± 2.74 pg/ml) showed more than 0.75-fold higher levels than that in the control kidney (233.09 ± 3.74 pg/ml; $P < 0.05$). The CRP levels in the kidney from STZ-induced rats (434.87 ± 9.17 µg/ml; $P < 0.05$) were significantly increased in comparison with the normal kidney (495.04 ± 12.68 µg/ml). Similarly, nitrate levels were significantly increased in the diabetic kidney (165.37 ± 2.99 ng/µl; $P < 0.05$) when compared to control (140.56 ± 7.00 ng/µl).

Correlation analysis

Both MAP and BG showed a significant positive correlation with HR, CRP, TNF-α, MDA, Gpx, and nitrate and significant negative correlation with TAC and GSH. After fixing for BG, the MAP remained correlated significantly with HR ($r^2 = 0.784$, $P < 0.01$), inflammatory marker, TNF-α ($r^2 = 0.635$, $P < 0.05$), and oxidative stress markers such as MDA ($r^2 = 0.835$, $P < 0.001$), TAC ($r^2 = -0.787$, $P < 0.004$), and GSH ($r^2 = -0.917$, $P < 0.001$). Similarly, BG showed a significant positive correlation with HR, CRP, TNF-α, MDA, Gpx, and nitrate and a significant negative correlation with TAC and GSH. After fixing for MAP, the BG remained significantly correlated only with inflammatory marker, TNF-α ($r^2 = 0.611$, $P < 0.05$).

DISCUSSION

In this study, with single high dose of STZ, which could induce type 1 diabetes, we have shown the correlation of BP and BG with inflammatory markers and oxidative stress over the time course of the development of DN. One month rat is comparable to 3 human years.[21] One day of the animal is approximately

equivalent to 34.8 human days. In our study, diabetes was followed in rats for 9 human years. The STZ induction showed a decrease in BW and an increase in BG up to 438 mg/dL and it is consistent with the previous reports. Further, the serum urea levels were significantly increased after 3 months of diabetes induction, but there is no change in creatinine levels which is in contrast to the previous report where the urea and creatinine levels were found to be higher after 1 month of diabetes induction.[22] These findings confirm the induction of diabetes mellitus in the STZ-induced rats.

Our study demonstrates that SBP is significantly higher in diabetes-induced rats after 3 months when compared to baseline. Significant elevation of BP after 3 weeks of STZ injection have been previously reported in Wistar–Kyoto rats.[23] Similar to our finding, Bunag *et al*. also reported that the elevated BP persisted up to 7 weeks after diabetes induction.[24] In contrary to this report, there are various studies reporting reduced[25] or no change[26] in BP with STZ-induced diabetes. The mechanism behind the contribution of diabetes mellitus in the development of hypertension is poorly understood.

The present study shows that the inflammatory and oxidative stress markers is increased, and the total antioxidant status is reduced after 3 months of diabetes induction. The MAP and BG are well correlated with oxidative stress and inflammation. The positive correlations of MAP and BG with HR, CRP, TNF-α, MDA, Gpx, and nitrate were found to be statistically significant. However, the TAC and GSH showed a significant negative correlation with MAP and BG. Despite tremendous efforts that have been made in the recent years, it remains unclear whether oxidative stress and inflammation lead to the development of hypertension or *vice versa* in STZ-induced diabetes. Several reports showed similar findings, in which the association of elevated oxidative stress with diabetes and hypertension, were corroborated.[27,28]

It is well-known fact that the diabetes mellitus and hypertension individually contribute to the elevation of oxidative stress. However, the degree of individual contribution to oxidative stress in the kidney is unclear. The mechanism behind the association between oxidative stress and the diabetes is a complex interdependency because it may be a consequence of elevated MAP and vascular complications or a primary event.[29,30] The analysis of gene regulatory network has unraveled that oxidative stress is an important key in the molecular mechanism of diabetes and hypertension.[31] A study on the interaction between hypertension and diabetes with inflammation and oxidative stress in spontaneously hypertensive and Wistar–Kyoto rats induced with STZ has provided crucial insights on the mechanism of renal and retinal diseases of diabetes mellitus. It was demonstrated that only hypertensive diabetic rats showed increased oxidative stress markers in kidney after 10 days of diabetes induction but not diabetic rats. These results illustrate that the coexistence of hypertension in diabetes has a strong implication in the renal cortical oxidative stress at the initial stage of diabetes.[32]

There are several similar reports in which the STZ-induced diabetic rat kidney showed an increased albumin permeability and urinary albumin excretion due to the elevation of TNF-α and the oxidative stress markers.[33] These findings demonstrate that TNF-α is a key regulator of oxidative stress in DN. Hence, oxidative stress and TNF-α are inseparable in diabetes.

In addition, the signs of DN such as the increased glomerular volume, mesangial cell number, glomerular basement membrane thickness, and renal artery thickness were observed after 3 months of diabetes induction. The influence of hypertension on vascular diseases showed an instantaneous effect. One of the most important therapeutic interventions to delay the progression of DN is sustained reduction in BP.[34] Several studies have elucidated that the therapeutic intervention for hypertension, irrespective of the agents used, in microalbuminuric and proteinuric Type 1 and 2 diabetic patients showed a positive impact on albuminuria.[35]

In the present study, hypertension rather than diabetes shows a better correlation with inflammatory and oxidative stress markers in kidney. Our results show an increase in oxidative stress-derived inflammation which could be attributed to the pathogenesis and progression of DN at the very early stage of diabetes.[36,37]

CONCLUSION

Hypertension influences the exponential increase in oxidative stress and inflammation in the kidney in the diabetes rat model. The fact that the current treatment strategies for DN include BP control[38] highlights its contribution to the development of DN. However, more detailed understanding of the molecular mechanisms of disease progression is warranted to elucidate the role of BP in DN.

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Conflicts of interest

There are no conflicts of interest.

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