6-shogaol protects against diabetic nephropathy and cardiomyopathy via modulation of oxidative stress/NF-κB pathway

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Abstract: Diabetes dramatically increases the risk of numerous heart and kidney troubles. Diabetic nephropathy (DN) and cardiomyopathy (DC) are major causes of death. The pathophysiology of DN/DC includes inflammatory and oxidative stress mechanisms. NF-κB is one of the transcription factor that mediates signal transduction processes. Nowadays, it is suggested that inhibition of NF-κB activation could delay the development of DN and DC. 6-shogaol was reported to modulate NF-κB besides its anti-oxidant and anti-inflammatory activities. Therefore, it is worth testing it against diabetic complications. Rats were divided to 4 groups: Normal control (NC), 6-shogaol (6S), diabetic control (DC), diabetic rats treated with 6-shogaol (DC+6S). BGL, BUN, serum creatinine, total urine protein, creatine kinase (CK), LDH, NO, TNF-α, NF-κB were determined in serum. Heart and kidney tissues were isolated for GSH, MDA, SOD measurement and histopathology. NF-κB was estimated in kidney tissues using immunohistopathology and western blot techniques. Results showed that diabetic rats left untreated for 16 weeks showed kidney injury as evidenced from elevated BUN, serum creatinine, urine protein, TNF-α and NF-κB. Heart tissue damage was evidence from elevated CK, LDH. Diabetic rats simultaneously treated with 6-shogaol showed a protective effect on both kidney and heart as evidenced biochemically and histopathologically. Therefore, using 6-shogaol may be of value in protection against diabetic complications in kidney and heart of rats.

Keywords: Diabetic cardiomyopathy, diabetic nephropathy, BUN, NF- κB, CK.

INTRODUCTION

Diabetic nephropathy (DN) and diabetic cardiomyopathy (DC) are major causes of high death rats. Diabetic nephropathy is found in 80% of type 1 diabetics and in 25-40% of the people diagnosed with type 2 diabetes (Lim, 2014). Combination of cardiomyopathy with diabetic nephropathy in the same patient make a poor prognosis. There is increasing incidence of diabetes and DN, warning a medical disaster in dialysis units which results in a greater consumption of economic resources (Jin *et al.*, 2015).

Concomitant presence of retinopathy and proteinuria are major risk factors for progression of DN (Alwakeel *et al*., 2015). Moreover, severe dehydration, lipid disorders, hyperglycemia and uncontrolled systolic blood pressure (>130 mm Hg) are major contributing factors for progress to nephropathy (Alwakeel *et al.,* 2015). The mechanisms of the pathophysiology of DN include inflammatory and oxidative stress mechanisms. NF-κB is a major factor that mediates signal transduction processes. The inhibition of NF-κB activation could delay the development of DN. On the other hand, there is a direct relationship between activation of NF-κB in kidney biopsies and severity of proteinuria (Schmid *et al*., 20060. Based on the above data, any compound which can inhibit NF-κB may be of value in protection against DN.

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Uncorrected obesity is directly linked to higher incidence of diabetes which my progress to diabetic cardiomyopathy. The increase in cardiac output due to excessive lipid metabolism ultimately leads to left ventricular dilation, hypertrophy and eventually heart failure (Lorenzo-Almorós *et al.,* 2017). Several reports, describing different factors including oxidative stress (Mahmoud, 2017), NF-κB (Frati *et al.,* 2017) and insulin resistance (Jia *et al*., 2017) which are implicated in diabetes-associated cardiac functional defects. Therefore, further investigation into the molecular pathological mechanisms of DN/heart damage and identifying new therapies against DN/heart damage are crucial for postponing its further development.

6-shogaol, a natural compound from Zingiber officinale has many pharmacologic actions, it is used for treatment of cancer (Wu *et al.,* 2018), reduction of oxidative stress (Si *et al*., 2018), treatment of inflammatory diseases (Zhang *et al*., 2017) and antihypertensive effects (Wang *et al*., 2013). Pan *et al*., (2008) proved that 6-shogaol abrogated the stimulation of COX-2 and NOS in murine cells. They suggested that 6-shogaol may inhibit NF-κB and consequently reduced iNOS and COX-2 gene expression in macrophages. The antioxidant and anti-inflammatory characteristics of 6-shogaol may be due to unsaturated ketone moiety (Dugasani *et al.*, 2010). Therefore, it is worth testing 6-shogaol against the progression of diabetic nephropathy and cardiomyopathy

as it possesses antioxidants and anti-inflammatory and can decrease the expression of NF-κB.

MATERIALS AND METHODS

Drugs and chemicals

6-shogaol and streptozotocin were obtained from Sigma-Aldrich Inc. (Saint Louis, MO, USA).

Animals

Adult male Wistar albino rats weighing 160-180 g were used in this study. Control and treated animals were fed standard diet and water *ad-libitum*. All other groups were given for four weeks a diet with high content of fat (HFD) to establish the insulin resistant model. The internationally accepted ethical guidelines of animal care were followed in these experiments (Health Research Extension Act of 1985).

Experimental protocol

A total of 80 rats are randomly divided into 4 groups (N = 20), as follow:

Group I: (NC): Normal control group. Rats are given standard animal pellet and water ad labitum. and received a single *ip* injection of vehicle (0.1 M citrate buffer, pH 4.4) only.
Group II: 6-Shogaol (6S): Normal control group treated with 6S for 16 weeks
Group III: (DC): Diabetic control rats without any treatment for 16 weeks to develop complications on kidney and heart.
Group IV: (DC+6S): Diabetic rats treated with protective drug (6S) for 16 weeks.
The rats were anaesthetized with pentobarbital sodium and sacrificed four months after protective drug administration. Blood samples and heart/kidney tissues were collected for biochemical, histopathological and immunohistochemical analyses.

Induction of diabetes

To establish the insulin resistant model, the animals were fed high fat diet (HFD) for 4 weeks. After 4 weeks, rats fed with HFD are intraperitoneally injected with a single small dose of streptozotocin at the dose of 30 mg/kg dissolved in 100 mM citrate buffer pH 4.5. Control rats were fed with normal chow (4% calories from fat) for 4 weeks and intraperitoneally injected with an equivalent volume of citrate buffer. Blood glucose levels were measured 72 h after STZ injection using hand-held glucometer. Body weights are recorded every week. Rats which will have blood sugar values ≥16.7mmol/L will be used for this study (Yan *et al.,* 2017). FBG was determined every month throughout the experimental time to confirm the diabetic state.

Progression of Type-2 diabetic rats to diabetic nephropathy (DN)

After treatment the animals were left for 16 weeks to show renal histological lesions similar to DN patients. This duration was sufficient to increase blood sugar level and cause frequent urination with weight loss (Cruzado *et al.,* 2004).

Progression of Type-2 diabetic rats to diabetic cardiomyopathy (DC)

With 16 weeks of high-fat, high-carbohydrate feeding, diabetic rats will develop myocardial hypertrophy and dysfunction with metabolic changes (Zhang *et al.,* 2014).

Biochemical analysis

Determination of blood urea nitrogen (BUN), serum creatinine and total protein contents in urine

Blood urea nitrogen level was estimated colorimetrically using urea enzymatic kit (Bio-Assay Systems, CA, USA). Serum creatinine level was evaluated colorimetrically using creatinine kit (Bio-Assay Systems, CA, USA). For total urinary protein: protein concentrations were measured using rat urinary protein assay kit (Chondrex, USA).

Determination of lactate dehydrogenase (LDH) activity and creatine kinase (CK)

Both LDH and serum CK activities were measured by commercially available LDH kit and CK kit respectively (Linear Chemicals, S.L., Spain).

Determination of reduced glutathione (GSH), malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity in kidney /heart tissues

GSH level was determined in the homogenates using commercially available GSH kit (Linear Chemicals, S.L., Spain). MDA was measured in rat tissues homogenates using commercially available MDA kit (Linear Chemicals, S.L., Spain). The activity of renal SOD was assessed in tissue homogenate using commercially available SOD kit (Linear Chemicals, S.L., Spain).

Determination of NF-κB activity, TNF-α, and NO levels in serum

NF-κB was measured in the serum using rat NF-κB ELISA kit according to manufacturer instructions (MyBioSource, Inc. San Diego, USA). TNF-α content was measured in serum using rat TNF-α ELISA kit, Biosource, Belgium, according to the manufacturer's protocol. Total serum nitric oxide levels were estimated using a colorimetric assay kit that measures total nitrate and nitrite as an index of total nitric oxide produced, and hence NOS enzyme activity indirectly by commercially available NO assay kit (Bio-Assay Systems, CA, USA).

NF-κB protein expression

Western blot technique was used to estimate NF-κB protein expression according to the manufacturer

instructions using mouse monoclonal anti-NF-κB p65 or mouse monoclonal anti-actin (Santa Cruz Biotechnology, Inc.). AIDA Image Analyzer software was uses to quantify the scanned intensities of protein bands.

Detection of NF-κB in renal tissues by immunohistochemical technique

The samples were prepared to quantify nuclear factor kappa beta (NF-κB) protein expression using a primary antibody mouse monoclonal anti-NF-κB. (MyBioSource Comp, California, USA). Then linked with biotinylated goat ani-mouse lgG antibody (Dako, LASB Universal). After exposing slides to 3,3-Diaminobenzidine (DAB) solution to produce colored reaction. Cells stained for NF-κB were calculated as percentage/filed.

Histopathological evaluations: Hematoxylin and Eosin stains (H&E)

3µm thick sections from kidney/heart were deparaffinized and hydrated in ethyl alcohol and distilled water. The sections were stained using Hematoxylin and Eosin (H&E) stain then examined under the microscope.

STATISTICAL ANALYSIS

Results were expressed as the means ± SEM. Evaluation of data was achieved using GraphPad Prism version 6.00. Statistical significant difference was determined by ANOVA followed by Tukey's multiple comparison test.

RESULTS

Body weight of diabetic and normal rats throughout the experimental time

Table 1 showed that body weight (g) increased gradually in normal control and drug treated group. In diabetic rats the body weight started to decrease after 8 weeks and became obvious after 12 weeks. Treatment of diabetic rats with 6-shogaol arrested body weight reduction (table 1).

Fasting blood glucose level (FBG) levels during experimental time

Estimation of blood glucose levels were done in diabetic and control normal groups at the end of experiment. FBG for each individual rat was not decreased below 200 mg/kg but increased gradually with time till the end of the experiment. Also administration of 6-shogoal showed anti-hyperglycemic effects as shown in (table 2).

Kidney function tests

Untreated diabetic rats showed significant elevation of BUN (mg/dl) by 400% (fig. 1a), serum creatinine levels by 169% (fig. 1b) and protein in urine by 400% (fig. 1c) when compared with normal control rats. Furthermore, giving 6-shogaol to diabetic rats decreased the elevated BUN, serum creatinine and urine protein levels by 64%, 36% and 65% respectively as compared to diabetic group (fig. 1a, b and c). BUN, serum creatinine and urine protein levels were at normal levels in control normal rats and 6-shogoal groups (fig. 1a, b and c).

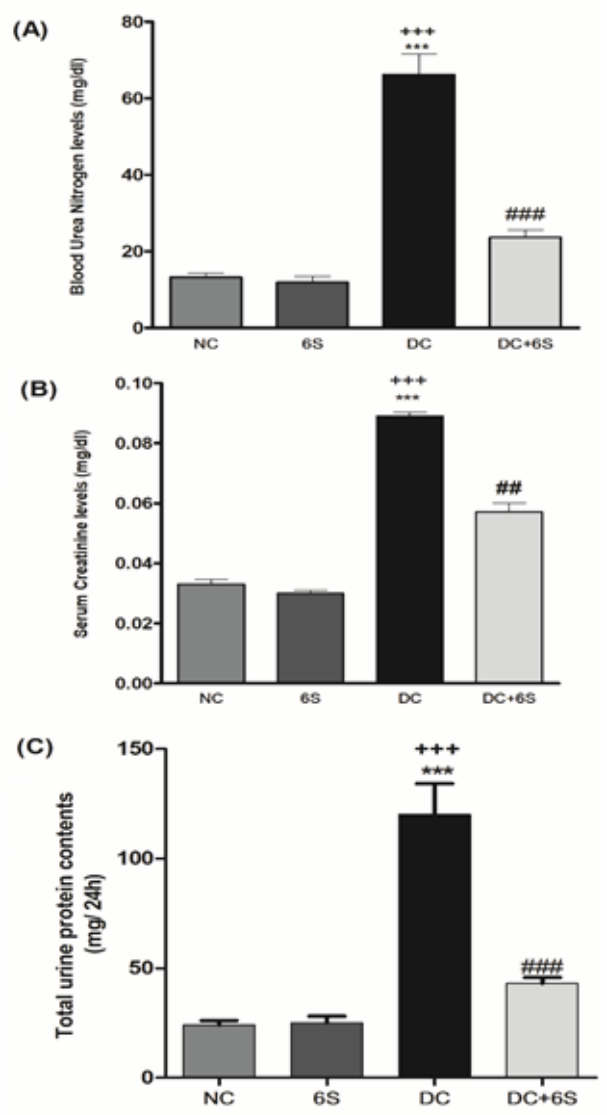

Fig. 1: BUN, serum creatinine and total urine protein contents in normal control (NC), 6-Shogaol (6S), diabetic control (DC), and diabetic rats treated with 6-Shogaol (DC+6S). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from NC group. ###p<0.001: Statistically significant difference from DC group. +++p<0.001: Statistically significant difference from 6S group.

Cardiac enzymes: LDH and CK levels in serum

CK and LDH were enhanced by 445% and 450 % in diabetic control group after 16 weeks compared to control level. Concomitant treatment of diabetic rats with 6-shogaol resulted in a marked reduction in CK (68%) and LDH (73%) levels compared to diabetic group (fig. 2a and b).

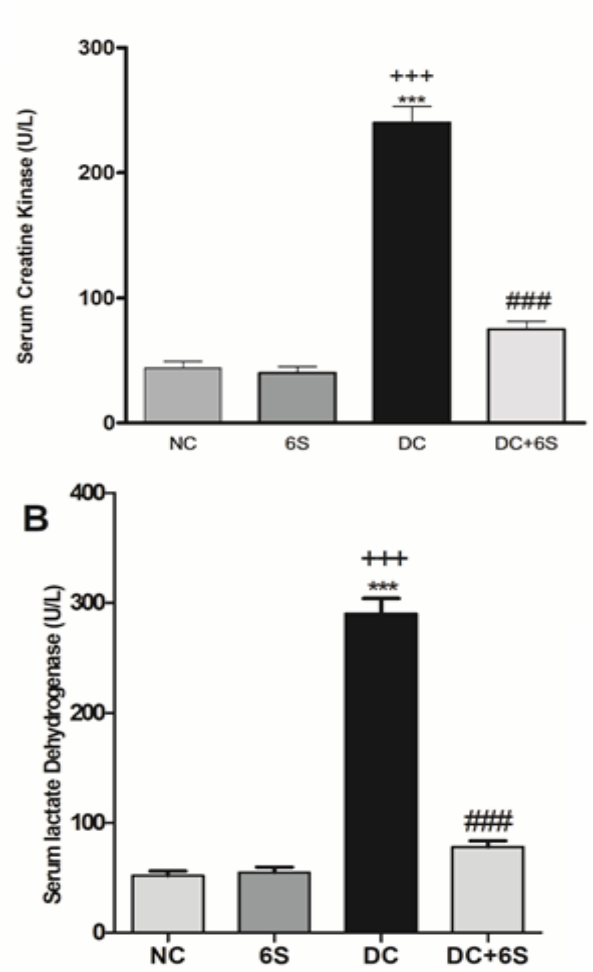

Fig. 2: Serum creatine kinase (CK) and lactate dehydrogenase (LDH) in normal control (NC), 6-Shogaol (6S), diabetic control (DC), and diabetic rats treated with 6-Shogaol (DC+6S). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from NC group. ###p<0.001: Statistically significant difference from DC group. +++p<0.001: Statistically significant difference from 6S group.

Effects of 6-shogaol on oxidative stress biomarkers in kidney/heart tissues

In this study, tissues from kidney/heart of diabetic rats showed a decrease in GSH levels (nmol/g tissue) by 46/42% compared with control group respectively. 6-shogoal attenuated GSH decline and bring back its level to normal through rising GSH levels by 71/52% as compared with diabetic control group [fig. 3a]. MDA also was elevated by 480/350% in kidney/heart tissues of diabetic rats as compared with control group. 6-shogaol returned MDA level to a normal level by reducing its level by 41/42% (fig. 3b). SOD activity was diminished in tissues of obese diabetic rats by 100/52% in kidney/heart tissues as for control group. However, the declined SOD activity was enhanced by 82/66% after 6-shogoal administration (fig. 3c).

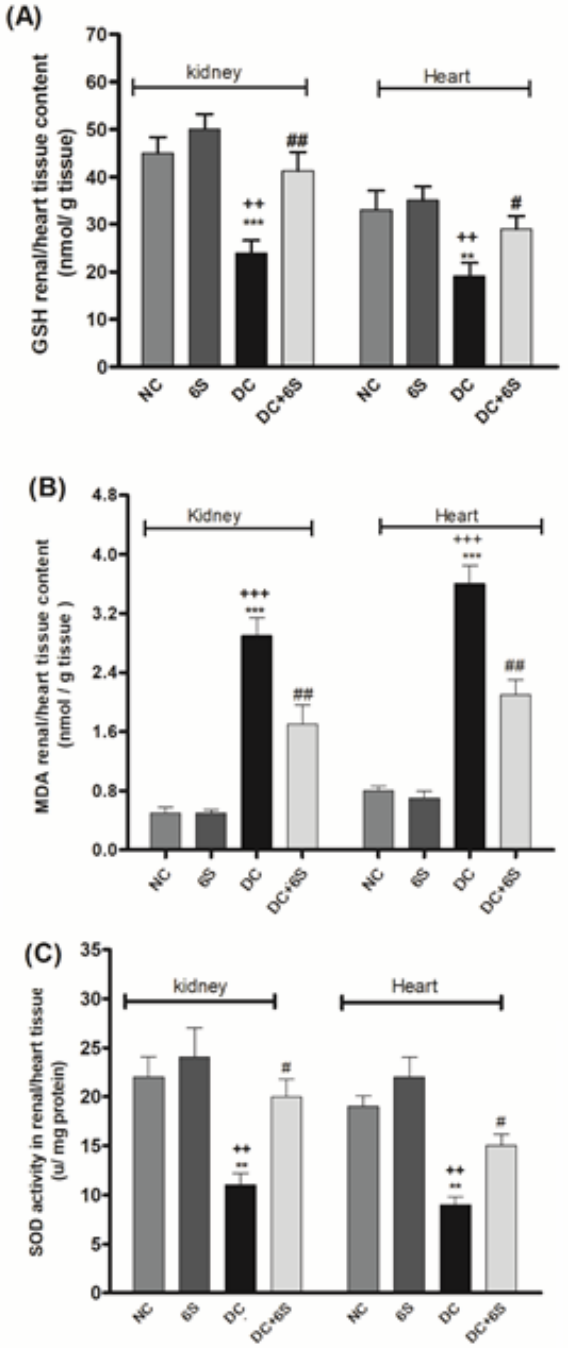

Fig. 3: GSH, MDA and SOD in kidney and heart tissues of normal control (NC), 6-Shogaol (6S), diabetic control (DC), and diabetic rats treated with 6-Shogaol (DC+6S). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001, **p<0.01: Statistically significant difference from NC group. ##p<0.001, #p<0.05: Statistically significant difference from DC group. +++p<0.001, ++p<0.01: Statistically significant difference from 6S group.

Effects of 6-shogoal on NF-κB, TNF-α and NO in serum

The serum levels of NF-κB, TNF-alpha and NO were increased by 322%, 433% and 374% in diabetic groups as compared with control group. Administration of 6-shogaol attenuated the activity by 40%, 33% and 57% when compared with diabetic one (fig. 4a, b and c).

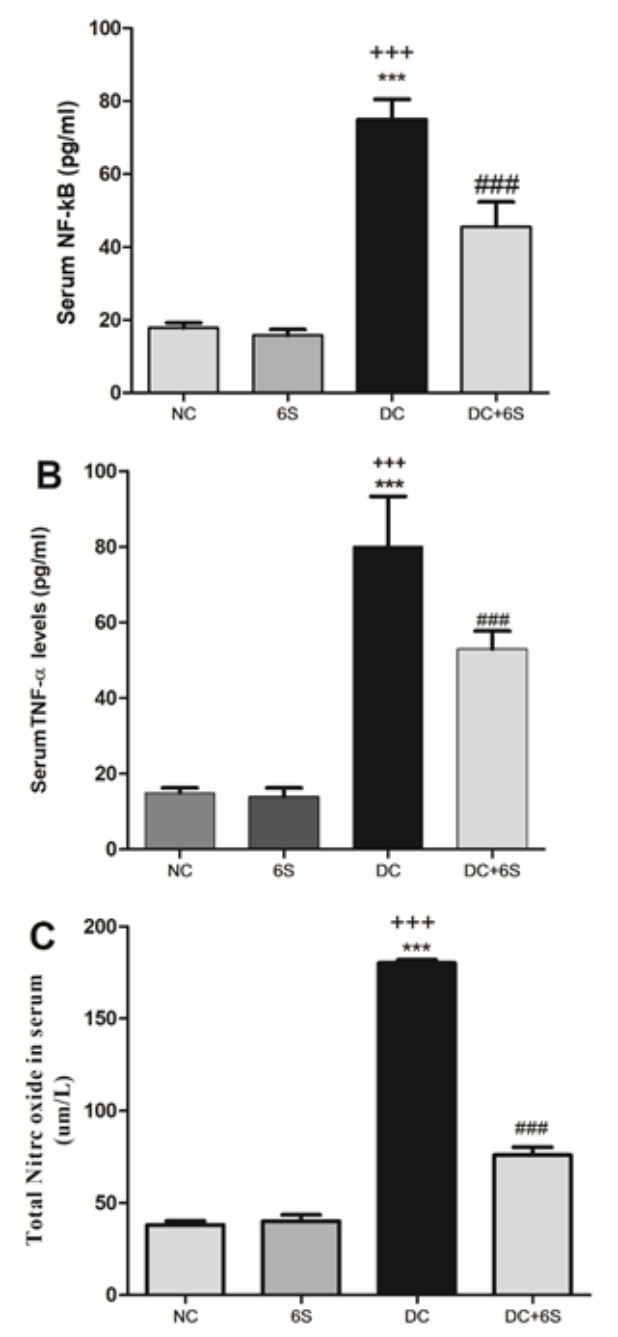

Fig. 4: Serum NF-kB, TNF-alpha and total nitric oxide (NO) in normal control (NC), 6-Shogaol (6S), diabetic control (DC), and diabetic rats treated with 6-Shogaol (DC+6S). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from NC group. ###p<0.001: Statistically significant difference from DC group. +++p<0.001: Statistically significant difference from 6S group.

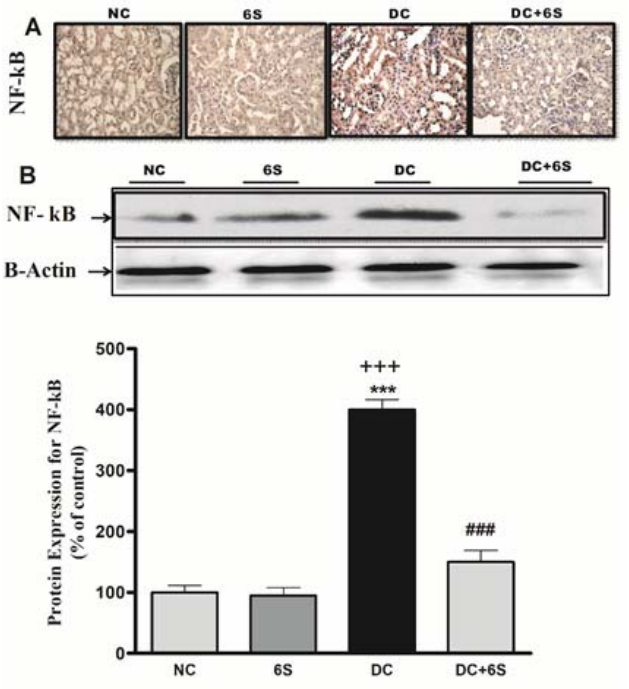

Fig. 5: NF-kB protein expression using immunohisto-chemically analysis (A) and western blot analysis (B) in normal control (NC), 6-Shogaol (6S), diabetic control (DC), and diabetic rats treated with 6-Shogaol (DC+6S). The significant difference between two groups was determined by ANOVA followed by Tukey's multiple comparison test. ***p<0.001: Statistically significant difference from NC group. ###p<0.001: Statistically significant difference from DC group. +++p<0.001: Statistically significant difference from 6S group.

Immunohistochemical detection of NF-κB in renal tissues

Weak immune staining of NF-κB appeared in sections of control and 6-shogaol groups and covered 15% of glomeruli/tubules field (fig. 5a). Severe activity of NF-κB that covered about 85% of kidney tubules field was obvious in diabetic rat's renal tissues (fig. 5a). Diabetic rats kidney samples administered 6-shogaol showed mild activity of NF-κB (about 25% of the field in tubules and masengel cells) (fig. 5a).

Expression NF-κB p65 using western blot technique

The band of diabetic kidney tissues revealed overexpression of NF-κB p65 protein as compared to normal control group (fig. 5b). However, administration of 6-shogaol to diabetic rats reduced NF-κB p65 expression by 62% in comparison to the diabetic (fig. 5b).

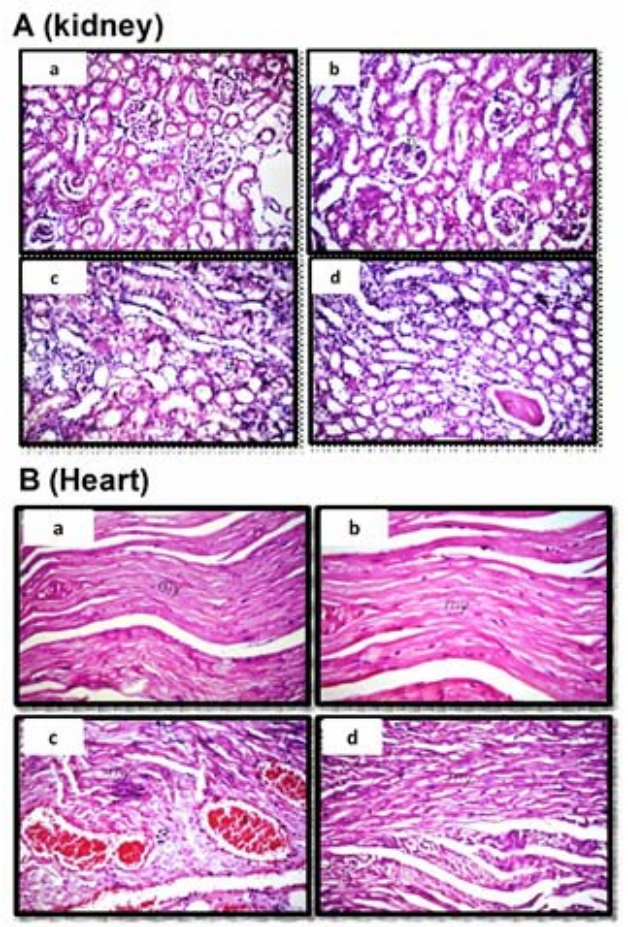

Fig. 6: Histopathological analysis of kidney and heart tissues in normal control (a), 6-Shogaol (b), diabetic control (c), and diabetic rats treated with 6-Shogaol (d). For kidney tissues: Sections from control and diabetic groups showed normal histological structure of the glomeruli and tubules at the cortex (photo, a and b). In renal sections from untreated diabetic rats, focal inflammatory cells infiltration was detected in between the degenerated tubules at the cortex (photo c), as well as in the corticomedullary portion (photo c). Homogenous eosinophilic casts were detected in the tubular lumen of the medullary portion (photo c). Photographs from treated diabetic group revealed normal glomeruli with absence of glomerular atrophy or hypertrophy with mild focal inflammatory cells infiltration in between the tubules (photo d). In addition, shogoal reduced the mononuclear leucocytes inflammatory cells infiltration (photo d). For Heart tissues: group kept as control or treated with 6-shogoal there was no histopathological alteration and the normal histological structure of the myocardial muscle bundles were recorded (photo, a and b). in diabetic rats there was severe congestion was noticed in the myocardial blood vessels (photo c), associated with focal haemorrhage in between the myocardial bundles (photo c). in treated diabetic rats there was no histopathological alteration as recorded (photo d).

Histopathological studies

Kidney: Control and diabetic samples showed no histopathological alteration and the normal histological structure of the glomeruli and tubules at the cortex were recorded in fig. 6a and b). In renal sections from untreated diabetic rats, the photos showed focal inflammatory cells infiltration was detected in between the degenerated tubules at the cortex, as well as in the corticomedullary portion. Homogenous eosinophilic casts were detected in the tubular lumen of the medullary portion (fig. 6c). Photographs from treated diabetic rats showed normal glomeruli with mild focal inflammatory cells infiltration in between the tubules. In addition, shogoal decreased inflammatory cells infiltration (fig. 6d).

Heart: Group kept as control or treated with 6-shogoal there was no histopathological alteration and the normal histological structure of the myocardial muscle bundles were recorded (fig. 6a and b). In diabetic rats' severe congestion was noticed in the myocardial blood vessels (fig. 6c), associated with focal hemorrhage in between the myocardial bundles (fig. 6c). In treated diabetic group there was no histopathological alteration as recorded in (fig. 6d).

DISCUSSION

The higher number of type 2 diabetics is directly linked to higher incidence of diabetic complications. Findings suggest that obesity and diabetes mellitus are closely interconnected with the worldwide increase of obesity (Wahlqvist *et al.,* 2015). The growing number of patients with complications such as nephropathy, cardiomyopathy and neuropathy increase significantly the medical costs (Jose *et al*., 2017). Therefore, it is a global interest to discover new therapeutic drugs to diminish or prevent these disabling conditions.

To construct DN model in type-2 diabetic rats it should be based on the functional and structural lesion of human DN as well as metabolic abnormalities (Al-Awar *et al*., 2016). Feeding the rats with HFD may induce obesity and insulin resistance in outbred rats. Therefore, described non-genetic rat models of DM2 are produced by small single dose of STZ in combination with high-fat diet (Srinivasan *et al*., 2005). In this study, groups that have fed HFD and single low dose of STZ produced type 2 diabetes as indicated from elevated blood glucose levels throughout the experimental time. Body weight loss is observed in diabetes as a sign of disease progression (Kodikonda and Naik, 2017). Our results were matched with this observation and the animals showed significant diabetes-associated weight loss when compared with control animals which gain weight.

Table 1: Body weight of diabetic and normal rats throughout the experimental time

Groups	Week 0 BW (g)	Week 4 BW (g)	Week 8 BW (g)	Week12 BW (g)	Week16 BW (g)
NC	160±4.1	200*±3.8	245*±2.3	275**±2.2	310**±2.5
6S	164±2.1	210±*3.4	250**±2.7	284**±2	315**±2.1
DC	166±2.1	240*±2.1	300**±2.4	270**±2.1	250*±2.9
DC+6S	165±2.6	250*±2.7	320**±2.1	300**±2.2	280**±2.3

Table 2: Fasting blood glucose level (FBG) levels during experimental time

Groups	Week 0 BGL (mg/dl)	Week 4 BGL (mg/dl)	Week 8 BGL (mg/dl)	Week12 BGL (mg/dl)	Week16 BGL (mg/dl)
NC	118±2.4	122±2.5	125±2.6	123±2.3	127±2.1
6S	122±2.3	118±2	115±2.4	117±1.9	119±1.8
DC	245±3.1	270*±3	290*±2.9	310**±2.8	350**±2.7
DC+6S	250±2.9	210*±2.5	189*±3	176**±2.5	162**±2.7

*p<0.05: Statistically significant from week zero
**p<0.01: Statistically significant from week zero

Previous studies showed that untreated obese diabetic rats for 16 weeks is enough to develop kidney and heart damage (Cruzado *et al.,* 2004). Our study demonstrated that STZ-induced diabetes in obese rats resulted in kidney damage as evidenced biochemically through increased BUN and serum creatinine as well as increase in proteinuria. Histopathological studies showed that, kidney tissues of untreated DC rats showed severe lesions such as focal inflammatory cells infiltration and degenerated tubules. Homogenous eosinophilic casts were detected in the tubular lumen. Moreover, the elevated LDH and CK in untreated diabetic rats also revealed cardiomyopathy after 16 week of diabetes induction (Hou *et al.,* 2016). Examination of the heart tissues by pathologist showed severe congestion and hemorrhage in between the myocardial bundles.

Growing evidence indicates that oxidative stress is abnormal state that connected with the development of diabetic tissue injury (Pradeep and Srinivasan, 2017). In our study we observed enhanced formation of ROS and decreased activities of key antioxidant enzymes. Continuous elevation of blood glucose level and elevated FFA may contribute to enhanced mitochondrial formation of free radicals and subsequently to ROS (Newsholme *et al.,* 2007). When the neutralizing response is absent, the system becomes overwhelmed resulting in stimulation of signaling pathways which are sensitive to stress, including nuclear factor NF-κB (Patel and Santani, 2009). In our model, there is elevated levels of serum NF-κB and over-expression in renal tissues by western blot and immuno-histochemical analysis.

Many literatures suggested that pro-inflammatory gene expression is regulated by NF-κB. Our results showed elevated levels of TNFα levels which can be explained on the bases that cardiac/renal cells are sensitive for pro-inflammatory cytokines (e.g. TNFα). It was reported that activation of NF-κB was matched with an increase of TNFα in some diabetic cardiomyopathy models (Umapathy *et al*., 2017). Elevated nitric oxide levels are attributed to endothelial NO synthase which generates superoxide anion in addition to NO. The over-produced nitric oxide may lead to peroxynitrite formation after reaction with superoxide radical, which is a harmful ROS resulting in more powerful tissue damage (Pacher *et al*., 2005).

The main result of stimulation of signaling pathway which sensitive to stress is the overproduction of NO that which is responsible for alteration in glomerular filtration and microalbuminuria that characterize early diabetic nephropathy (Boels *et al.,* 2017). Many studies reported overexpression of iNOS in liver of diabetic rats and consequently elevated NO level was found in liver at early stage of diabetes (Dias *et al.,* 2005).

Interestingly, some pharmacologically active compounds of ginger were reported to have antioxidant, anti-inflammatory and inhibition of NF-κB activities (Ha *et al*., 2012). 6-shogaol, present in dried ginger, has superior biological activity and higher stability compared to its counterpart in fresh ginger extract, (Chen *et al*., 2014). The present study demonstrated that 6-shogoal reduced the elevated blood sugar level. Consistent with this observation, some reports found a hypoglycemic effect of 6-shogoal in streptozotocin-diabetic rats which is attributed to the ability of shogaol to stimulate glucose utilization (Wei *et al.,* 2017). Also it was found that 6-paradol the main metabolite of 6-shogaol decreased blood glucose and cholesterol levels in HFD mice (Wei *et al*., 2017).

Administration of 6-shogoal to diabetic rats significantly abrogated diabetic complication in kidney and heart as evidenced biochemically and histologically. Most of kidney and heart function enzymes (BUN, creatinine, CK, LDH) were ameliorated and major histopathological lesions are abolished in groups pretreated with 6-shogoal. This effect is attributed to its antioxidant and anti-

inflammatory properties as well as its anti-hyperglycemic effects (Na *et al*., 2014). The presence of alpha, beta-unsaturated ketone moiety in 6-shogaol is responsible for its potent antioxidant and anti-inflammatory activities. In this study, 6-shogoal treatment attenuated oxidative injury in the kidneys/hearts of diabetic rats, indicating the antioxidant effect of 6-shogoal (Mashhadi *et al*., 2013).

Some studies explained the anti-inflammatory activity of 6-shogaol on the bases of inhibiting glial cell activation and inflammatory cytokine production (Moon *et al.,* 2014). This the case in this study, we noticed that 6-shogaol reduced ROS generation, reduced elevated NO levels, and attenuated inflammatory cytokines (TNF-alpha) production.

CONCLUSION

6-shogaol showed protective activity against diabetic tissues injury due to its anti-hyperglycemic effects and anti-oxidative and anti-inflammatory effects. Collectively, the finding of this study indicates that 6-shogaol is a promising candidate for the reducing the kidney/heart damage caused by diabetes.

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