Cardioprotective effect of 6-shogaol against hyperglycemia-induced toxicity in H9c2 cardiomyocytes via suppressing of NF-κB pathway

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Abstract: Diabetic cardiomyopathy (DC) is a serious complication of diabetes. Apoptosis, inflammatory and ROS production are among the factors that are involved in the progression of diabetic cardiomyopathy. 6-shogaol is reported to inhibit apoptosis and reduce inflammatory and ROS production. This study aimed to study the effect of 6-shogaol (6S) on the progression of diabetic cardiomyopathy in vitro. To develop DC model, H9c2 cell line was exposed to high glucose (HG) level (33 M glucose) for 24 h and used as a model for diabetic cardiomyopathy. Another set of H9c2 cell lines were 1 h pretreated with different conc. of 6-shogaol (5-20 µM). Cell viability, apoptosis, ROS production, IL-6, TNF-alpha and NF-kB were estimated in these cell lines treated with HG level or pretreated with 6-shgoal before HG. Exposing cardiomyocytes H9c2 cells to HG produced dramatic changes in cell biology and chemistry. There is a significant reduction in cell viability and enhancement in cell apoptosis as compared with control. In addition, ROS production, IL-6, TNF-α levels were increased in H9c2 line treated with HG. Also, there is overexpression of NF-κB in cells treated with HG levels alone. On the other hand, pretreatment of cardiomyocytes H9c2 cells with 6-shogaol (5-20µM) significantly improved cell viability and reduced apoptosis, in addition, 6S at a dose of 10 µM abrogated the deleterious effects of HG on oxidative stress and inflammatory parameters via modulation of NF-κB pathway. Therefore, 6S has a potential protective effect against hyperglycemia-induced DC *in vitro*.

Keywords: Cardiotoxicity, H9c2, TNF-alpha, IL-6, high glucose.

INTRODUCTION

Cardiovascular diseases are the major complication of diabetes. Mortality due to cardiovascular problems is always associated with hyperglycemia, in the diabetic population (Giles, 2003). Various metabolic perturbations are implicated in mitochondrial disruptions and structure alterations which eventually leading to myocardial dysfunction. Many studies reported that oxidative stress is directly associated with cardiac lesions noticed in a diabetic heart (Mayyas and Alzoubi, 2018).

The NF-κB is an important factor of cellular exposure to oxidants. NF-κB plays an essential role in the production of oxidants and inflammatory mediators (Kumar and Singh, 2019). NF-κB is crucial for oxidative reactions as heart tissues deprived from NF-κB showed less ROS synthesis when exposed to harmful stimuli (Morgan and Liu, 2011). Natural products now are the main source of phytochemicals from food substances that could prevent oxidative injury by down-regulating NF-κB expression (Ye *et al*., 2018).

6-shogaol attenuates inflammation, cell proliferation via modulation of NF-κB expression. Therefore, it is used in treatment of ulcerative colitis (Hassan and Hassan, 2018). Also, 6-Shogaol reduces progression of experimental endometriosis *in vivo* and *in vitro* (Wang *et al*., 2018). In one study the researcher found that 6-shogaol has a wound healing activity in mice (Bakht *et al*., 2014). In another study it reduced oxidative and inflammatory reactions in mice with middle cerebral artery occlusion (Na *et al*., 2016). Overall, 6-shogaol could be a promising therapeutic treatment to ameliorate diabetes and the development of diabetic cardiomyopathy.

MATERIALS AND METHODS

Drugs and chemicals

6-shogaol, MTT, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Sigma (St Louis, MO, USA). All other chemicals are of analytical grade.

Cell culture and treatment

An H9c2 cell line, was purchased through Sigma Aldrich, UK. These cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C under 95% O2 and 5% CO2. Cells were nearly 80% confluent. To mimic the increased glucose level in diabetes, the H9c2 cells were cultured in 33.3 mM glucose or high glucose (HG) or 5.5 mM glucose (normal glucose), the latter of which was used as a control for indicated time. Cells treated with different concentrations of 6S (5-20 µM) for 1 h before addition of 33 *m*M or 5.5 mM for 24h. This dose range was selected based on previous study of (Ling *et al*., 2010).

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Cell viability assay

MTT Assay principle

This colorimetric assay uses reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Formazan crystals are then dissolved using a solubilizing solution and absorbance is measured at 500-600 nm using a plate-reader. The darker the solution, the greater the number of viable, metabolically active cells. Briefly, 50 µL of MTT reagent was added to each well. The formed MMT crystals were dissolved in isopropanol and the purple colored product measured at 570 nm.

Caspase-3 activation assay (Apoptosis assay)

Caspase-3 is an enzyme that plays a key role in mediating apoptosis. Caspase-3 activity was measured using caspase-3 assay kit (GenScript, USA) according to the manufacturer's protocol. Briefly, cells were exposed to HG in the presence or absence of 6-shogaol, then adding caspase reagent and incubation. The level of fluorescence was measured with excitation/emission at 499/521 nm

ROS production

ROS production in cell homogenate was measured using lucigenin (5 µM)-chemiluminescence (BMG Labtech GmbH, Germany). The specificity of the assay was confirmed by adding superoxide dismutase or tiron.

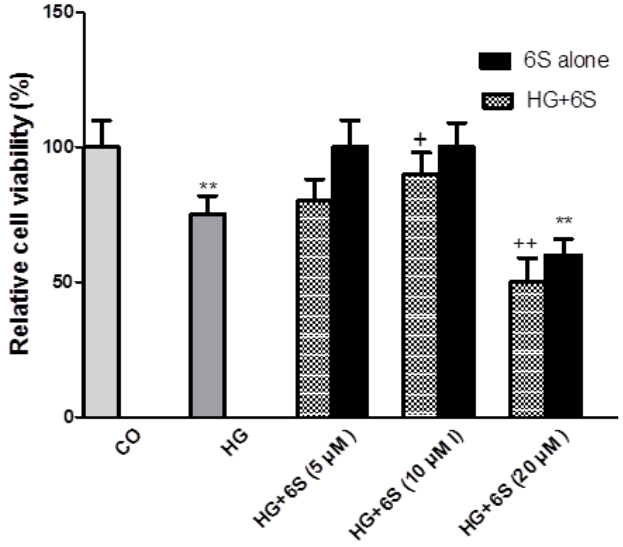

Fig. 1: Effect of different concentrations of 6-shogaol (5-20 µM) alone or in combination with high glucose (33 µM) on cell viability of H9c2 cardiomyocyte cells. Data reflect the mean ± SEM of three independent experiments. $^{**}p<0.01$ indicate a significant difference compared with the control (CO) group. $^{+}p< 0.05$ and $^{++}p<0.01$ indicate a significant difference compared with the HG group.

Estimation of IL-6 and TNF-α in H9c2 cell line

The levels of Il-6 and TNF-alpha were measured in H9c2 cell line. The cell is treated with HG in the presence or absence of 6-shogaol. Il-6 and TNF-alpha were measured using ELISA kit (Glory Bioscience, USA) according to the manufacturer's instructions. All the experiments were performed in triplicate.

Detection of NF-κB protein expression

Western blot technique was applied to detect the NF-κB protein bands using mouse monoclonal anti-NF-κB p65 (Santa Cruz Biotechnology, Inc.), following manufacturer protocol. AIDA Image Analyzer software was uses to quantify the scanned intensities of protein bands.

STATISTICAL ANALYSIS

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

RESULTS

Cell viability

Exposure of cells to high glucose markedly decreased cell viability, but this effect was reduced by 1 hr pretreatment with 10 µM of 6-shogaol but 5 µM has no effect (fig. 1). The effects of 10 µM was more pronounced than 5 µM. 5 µM and 10 µM of 6-shogaol itself have no effects on cell viability. The dose of 20 µM of 6-shogaol alone caused a severe decrease in cell viability (fig. 1).

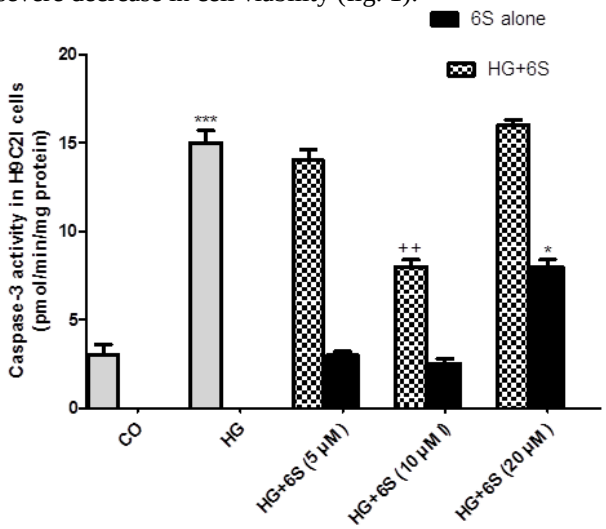

Fig. 2: Effect of different concentrations of 6-shogaol (5-20 µM) alone or in combination with high glucose (33 µM) on caspase-3 activation in H9c2 cardiomyocyte cells. Data reflect the mean ± SEM of three independent experiments. $^{*}p<0.05$ and $^{***}p<0.001$ indicate a significant difference compared with the control (CO) group. $^{++}p<0.01$ indicate a significant difference compared with the HG group.

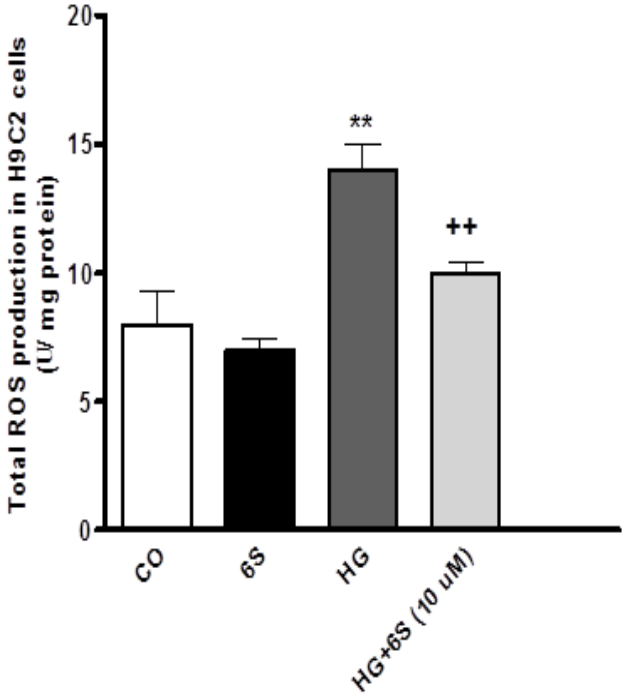

Fig. 3: Effect of 6-shogaol alone or in combination with high glucose (33 µM) on ROS production using chemiluminescence method in H9c2 cardiomyocyte cells. Data reflect the mean ± SEM of three independent experiments. $^{**}p<0.01$ indicate a significant difference compared with the control (CO) group. $^{++}$ $p<0.01$ indicate a significant difference compared with the HG group.

Caspase-3 activation assay (Apoptosis assay)

In cells treated with different concentrations (5-20 µM) of 6-shogaol alone the level of caspase-3 did not change from control in 5 and 10 µM. However, 20 µM of shogaol increased apoptosis compared to control (fig. 2). HG exposure enhance the formation of cleaved form of caspase-3, which was inhibited by 10 µM of 6-shogaol pretreatment not by 5 µM of 6-shogaol (fig. 2). Pretreatment with 20 µM of 6-shogaol leads to increase in apoptosis. Therefore, 10 µM of shogaol was selected for further experiments as it is superior to 5 µM and nontoxic for cells as in case of 20 µM.

Lucigenin-derived chemiluminescence (CL) assay for detection of cellular reactive oxygen species (ROS)

To investigate if exposures to HG can trigger ROS production in H9c2 cells. Lucigenin-derived chemiluminescence (CL) assay was performed to detect the presence of superoxide radicals after incubation with HG. The results suggest that HG, at concentration 33 µM, induces significant superoxide production in H9c2 cells (fig. 3). 1 hr pretreatment with 6-shogaol at dose of 10 µM significantly attenuated these delirious effects and inhibited ROS production (fig. 3).

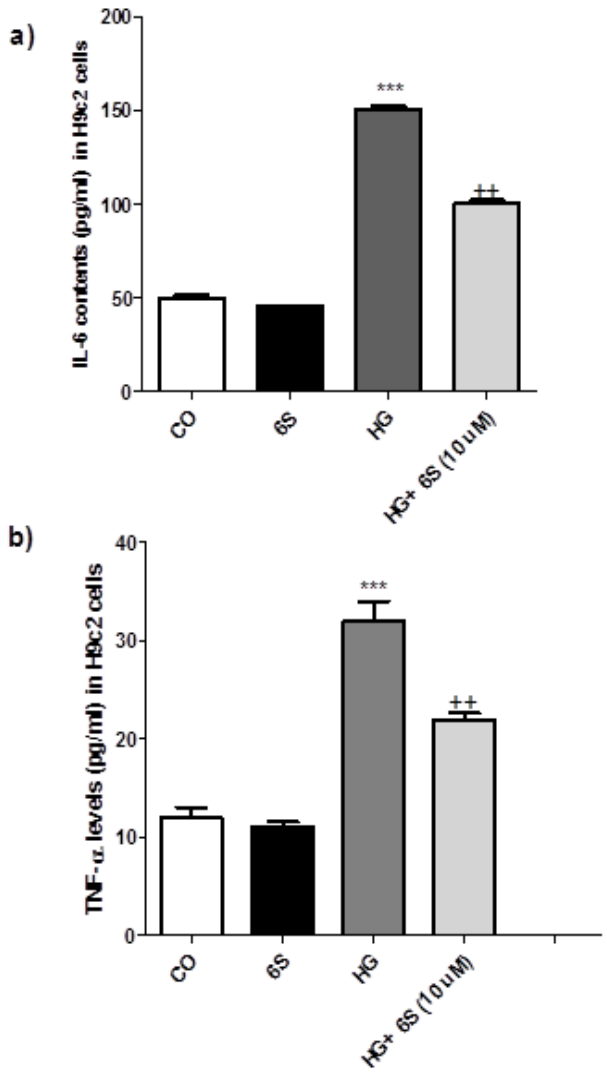

Fig. 4: Effect of 6-shogaol alone or in combination with high glucose (33 µM) on inflammatory mediators (a) IL-6 and (b) TNF-α in H9c2 cardio myocyte cells. Data reflect the mean ± SEM of three independent experiments. $^{***}p<0.001$ indicate a significant difference compared with the control group. $^{++}p<0.01$ indicate a significant difference compared with the HG group.

Measurement of IL-6 and TNF-α in H9c2 cell line

Incubation of the cells with HG for the specified time significantly increased the produced level of inflammatory cytokines (IL-6 and TNF-α) (fig 4 a and b). However, pretreatment with 6-shogaol 10 µM markedly reduced this increase in both cytokines (fig. 4 a and b).

Detection of NF-κB protein expression using western blot technique

The band from H9C2 cells treated with HG revealed overexpression of NF-κB p65 protein as compared to normal control group (fig. 5). However, pretreatment with of 6-shogaol at dose of 10 µM reduced NF-κB p65 expression by 62% in comparison to the HG (fig. 5).

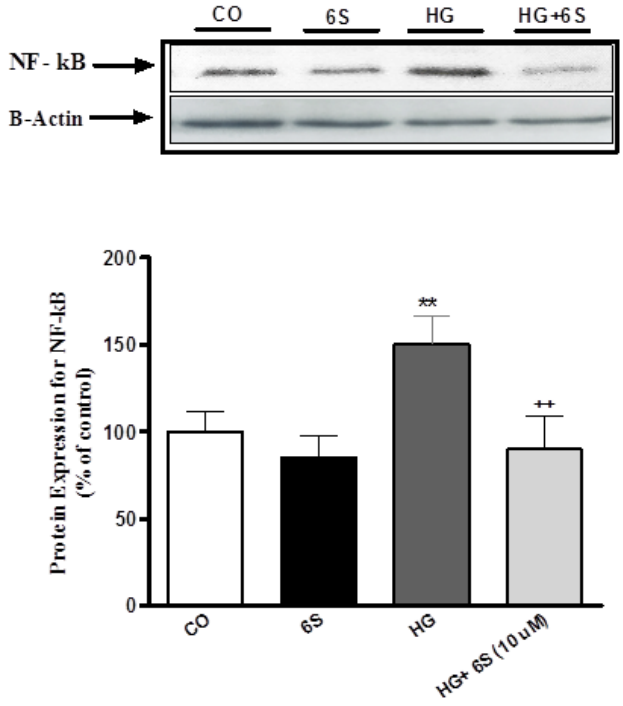

Fig. 5: Effect of 6-shogaol alone or in combination with high glucose (33 µM) on NF-κB protein expression using western blot method in H9c2 cardiomyocyte cells. Data reflect the mean ± SEM of three independent experiments. $^{**}p<0.01$ indicate a significant difference compared with the control group. $^{++}p<0.01$ indicate a significant difference compared with HG group.

DISCUSSION

The cause of heart failure usually seen with diabetic cardiomyopathy is due to hypertrophy as a result of overload on cardiac muscle in diabetic patients**.** Many factors are implicated in the molecular mechanism of diabetic cardiomyopathy including ROS, inflammatory mediator and NF-κB overproduction (Fuentes-Antrás *et al*., 2014). Therefore, further investigation into the molecular pathological mechanisms of diabetic cardiomyopathy and identifying new therapies are crucial for postponing its further development.

The H9c2 cell line is derived from cardiac origin and is now widely used in cardiac research. It is used to mimic the diabetic cardiomyopathy in human or animals (Wu *et al*., 2018). The main advantages of this cell line are it is an animal-free alternative, used directly to test the effect of HG/6S on the myocardium without the influence of any variables that would be introduced when using an animal model (Wu *et al*., 2018). Also, the presence of multiple enzymes in H9c2 cells like that found in the rat heart make this cell line a good *in vitro* tool to examine drug effects on heart.

To determine the safety of 6-shogaol on cell viability H9c2 cells were exposed to different concentration of shogaol (5-20 µM) alone or in combination with HG (33 mM glucose). Then examine its effect on cell viability and programmed cell death. The results of this study revealed that both 5 and 10 µM of shogaol have no effect on cell viability or apoptosis. However, 20 µM caused a deleterious effect on both parameters, i.e. decreased cell viability and increased cell death. This result indicates that both doses are safe to the cells and that 20 µM l is toxic to the cells. This cytotoxic effect of higher dose of 6-shogaol may result from the excessive enhancement of NF-kB activity that led to suppression of platelet-derived growth factor receptor (PDGFR) as previously reported in H9C2 cells **(**Hoesel and Schmid, 2013**)**. By examining the protective effect of pretreatment with the 3 doses against the deleterious effect of HG on H9c2 cells viability and apoptosis. we found that only 10 µM has a protective effect and that 5 µM did not show any significant protective effect from HG group. And 20 µM is cytotoxic. Therefore, 10 µM was selected for further experiments.

Reactive oxygen species production plays a key role in high glucose induced cardiac damage. Pretreatment with 10 µM of 6-shogaol has been shown to reduce HG-induced ROS generation and oxidative stress in H9c2 cells. In line with the result of Mashhadi *et al*. (2013) who reports that 6-shogaol reduced ROS production in H9C2 cells.

Several recent studies suggested that overproduction of ROS stimulates inflammatory response. This one of the potential mechanisms by which oxidative stress links multiple risk factors to disease (Mittal *et al*., 2014). We found here that hyperglycemia-induced cardiac cell damage is associated with enhanced inflammatory response as evidenced by the significant increase in proinflammatory cytokines (TNF-*α* and IL-6) (Dludla *et al*., 2017). 6-shogaol at a dose of 10 µM mitigated these increases in both cytokines (Han *et al*., 2017).

An interesting article revealed that administration of exogenous ROS caused activation of NF-kB in H9C2 cells (Morgan and Liu, 2011). In line with the result of this study we found that hyperglycemia caused cardiac damage and induced cardiac ROS overproduction which was accompanied by NF-kB over-expression, which was inhibited by 6-shogaol administration.

CONCLUSION

In conclusion, the finding of this study showed a cardioprotective effect of 6-shogaol in hyperglycemia-induced damage in H9c2 cardiomyocytes. Consistent with our previous report (Al Malki *et al*., 2018), that was performed *in vivo* and clearly proved that 6-shogaol

markedly attenuated diabetic cardiomyopathy in rats, this study revealed the 6-shogaol also attenuated HG-triggered cardio toxicity *in vitro*. This cardio protective effect is attributed to attenuating oxidative and inflammatory pathways as well as suppression of NF-κB pathway. Therefore, 6-shogoal is a promising candidate for prevention of diabetic complication especially that associated with heart and kidney.

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