

Role of Apoptosis and Oxidative Stress in High Glucose-Induced Endothelial Dysfunction in Isolated Aortic Rings

Eman M. Awad^{1*}, Al-Shaimaa F. Ahmed¹, Mahmoud El-Daly¹, Nashwa F. G. El-Tahawy², AlShimaa Wagdy³, Ashraf Taye⁴

¹Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, Minia, Egypt.

²Department of Histology and Cell Biology, Faculty of Medicine, Minia University, Minia, Egypt.

³Department of Pathology, Faculty of Medicine, Minia University, Minia, Egypt.

⁴Department of Pharmacology and Toxicology, Faculty of Pharmacy, South Valley University, Qena, Egypt.

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Abstract

Diabetes and its associated hyperglycemia represent a major health problem. One of the common complications of diabetes is endothelial dysfunction which leads to several cardiovascular diseases. Studying the effect of hyperglycemia and possible treatment strategies necessitates the induction of diabetes *in vivo* that requires a long time for endothelial dysfunction to develop. Recently, *in vitro* models of hyperglycemia have been introduced in which vascular tissues are incubated in media containing a high concentration of glucose which ultimately requires much less time for endothelial dysfunction development. In this study, we examined the effect of incubation of isolated aortic rings in a high glucose medium on their relaxation and contractile responses as well as the histopathological changes of aortic tissues. We also examined the involvement of oxidative stress and apoptosis in hyperglycemia-induced endothelial dysfunction. The results of our study showed that incubation of aortic rings with high glucose media resulted in a significant reduction of relaxation response of precontracted aortic rings along with an enhanced contractile response to epinephrine. This was associated with obvious histopathological alterations of aortic tissues. Aortic rings incubated in high glucose also exhibited increased oxidative stress and a reduction of the anti-apoptotic marker, Bcl2. In conclusion, *in vitro*, high glucose can be used as a model for studying endothelial dysfunction and its possible mechanisms involving oxidative stress and apoptotic pathway.

Keywords

high-glucose, Ach induced relaxation, SNP, Bcl2

1. Introduction

Diabetes mellitus (DM) is one of the most prevalent metabolic disorders, which is a worldwide health problem [1]. Hyperglycemia, a hallmark of DM, contributes to endothelial dysfunction that precedes the microvascular and macrovascular complications characteristic of DM [2]. The definition of endothelial dysfunction as a disrupted balance between vasorelaxation and vasoconstriction mechanisms is well-accepted [3]. The initiating signal of this imbalance is increased hyperglycemia-induced reactive oxygen species (ROS) production, nitric oxide (NO) depletion, and subsequent impairment of endothelium-dependent vasorelaxation [4]. Nitric oxide plays a pivotal role in the regulation of vascular homeostasis and cell-cell communication [5]. Under the hyperglycemic condition, increased superoxide anion ($O_2^{\cdot-}$) output reduces the bioavailability of NO [6]. Intracellular $O_2^{\cdot-}$ molecule is relatively short-lived because of its neutralization by superoxide dismutase (SOD). However, under hyperglycemia, oxidative stress is predominant via the increased production of ROS and/or the reduced endogenous antioxidant capacity [7]. High glucose induces superoxide anion production via several pathways including; mitochondrial electron transport chain, xanthine oxidase, and the NADPH oxidase systems [8-11]. In

addition, increased cellular accumulation of glucose uncouples the endothelial nitric oxide synthase (eNOS), which contributes

to superoxide anion rather than nitric oxide production [11-13]. Taken together, the hyperglycemia-augmented production of $O_2^{\cdot-}$ which exceeds the capacity of endogenous SOD.

Studying *in vivo* animal models of DM, which demonstrate persistent prolonged hyperglycemia resulted in a better understanding of diabetes-induced endothelial dysfunction and opportunities for possible protection [14, 15]. Although these models mimic human DM and DM-induced endothelial dysfunction to variable extents, they have several limitations such as time consumption and animal mortality, not to mention the metabolic complexity of each model. Introducing simple *in vitro* models facilitated the study of endothelial dysfunction pathophysiology under the influence of high glucose [9], high fructose [16], advanced glycation end products (AGEs) [17, 18], and other insults, either alone or in combination. Besides saving time and animal lives, these *in vitro* models have the potential to clarify the effect of different insults on vascular function both in cell- and vessel-based systems, avoiding the interference by endogenous feedback and repair mechanisms prominent *in vivo*. Based on the well-established association between DM and hyperglycemia with the development of vascular diseases [3, 4, 8], we hypothesized that incubation of rat aortic vascular rings

* Correspondence: Eman M. Awad

Tel.: (+2)01094473394; Fax: +2086-236-90-75.

Email Address: eman.mftah@mu.edu.eg

under hyperglycemia-like conditions will alter the vascular function and precipitate endothelial dysfunction. This study aimed to test the role of oxidative stress and antiapoptotic signaling in the effect of *in vitro* high glucose on vascular reactivity of isolated aortic rings.

2. Methods

2.1. Animals

Adult female Wistar rats weighing 180-200 g were obtained from the animal house facility of the Faculty of Pharmacy, Nahda University at Beni-Suef (NUB), Egypt. The animals were kept under constant environmental conditions with free access to water and standard food until the day of the experiment. All experiments were reviewed and approved by the Commission on Ethics of Scientific Research, Faculty of Pharmacy, Minia University (Code number of the project: ES05/2020).

2.2. Experimental design

Since the aim of this study was to investigate the effect of high glucose on vascular reactivity, we established an *in vitro* model of hyperglycemia-like conditions by incubating the aortic rings in physiological buffers containing low glucose (10 mM; LG), high glucose (40 mM; HG), or high mannitol (30 mM of mannitol + 10 mM glucose). We used high mannitol as a control to exclude the effect of osmolarity of the high glucose solution.

Aortic ring preparation

Rats were anesthetized by an i.p. injection of pentobarbital sodium (80 mg/kg) as previously described [19], the thoracic aorta was exposed by a midline incision through the abdomen and thoracic cage and carefully dissected free of the surrounding tissues in oxygenated ice-cold Krebs-Henseleit solution as described in other studies [9, 20].

2.3. Assessment of *in vitro* vascular reactivity

The thoracic aorta was cut into segments of 4-5 mm in length. Each ring was mounted between two stainless steel hooks (with enough care to avoid mechanical damage of the tissue) in an organ bath containing 10 ml Krebs-Henseleit solution continually bubbled with carbogen. The tissues equilibrated under 1.5 g tension for 45 min. The vascular reactivity was monitored via a computer-based data acquisition system (Powerlab® system and LabChart® 7.0 software, AD Instruments). The tissues were challenged with 80 mM KCl solution every 15 min until a stable response was obtained. Tissue segments precontracted with 10^{-6} M PE that showed more than 65% relaxation to 10^{-6} M ACh were considered endothelium-intact and were used in this study. Each tissue was then incubated in LG, HG, or high mannitol for 3 h after which the contractile and relaxation responses were assessed.

The contractile responses to cumulative additions of phenylephrine (PE; 10^{-9} to 1.5×10^{-5} M) calculated as a percentage of the maximal effect of 80 mM KCl were used to establish PE response curves. On the other hand, the relaxation responses of 10^{-6} M PE-contracted rings to acetylcholine (10^{-9} to 10^{-5} M) were used for establishing the concentration-effect curves of ACh as endothelium-dependent relaxations. The same protocol was repeated using sodium nitroprusside (SNP; 10^{-10} - 10^{-4} M) to test the endothelium-independent vasodilatation responses.

2.4. Assay of aortic superoxide dismutase, catalase, and total nitrite/nitrate

Tissue rings exposed to different glucose conditions for 3 h were frozen in liquid nitrogen then finely minced and homogenized (10% w/v) in 20 μ M Tris-buffer, pH 7.4 in a glass tissue grinder (Corning KONTES Tissue Homogenizer PYREX® with glass Pestles) in presence of liquid nitrogen to facilitate tissue grinding as aortic tissues are elastic. The supernatant was recovered after centrifugation for 10 min (15,000 rpm, 4°C) and used for the assessment of biochemical parameters. The activity of SOD was determined by using pyrogallol (autoxidized substance in presence of superoxide anion) according to a previously described procedure [21] with slight modifications. Detection of catalase activity in the supernatant was carried out using a commercial kit (Biodiagnostic, Egypt). The total nitrite/nitrate content was measured colorimetrically [22].

2.5. Histological analysis and immunohistochemical staining of aortic tissues

After incubation for 3 h under different experimental conditions, the tissues were fixed in 5% formal saline for 24 h, then dehydrated in ascending alcohol series and embedded in paraffin as per the standard protocol [23]. Thin sections (~5 μ m thickness) were stained with hematoxylin and eosin (H&E) for observation of morphological changes. The morphometric measurements were done for each aorta (n=6) [24]. The scores for endothelial lining integrity were 1 for completely confluent endothelium, 2 for partially confluent endothelium, 3 for loosely netted endothelium, 4 for islands of the endothelium, and 5 when no endothelium was detected. The adventitia thickness (AT) was scored from 1 to 5 based on the measurement assessed at 10x magnification. Besides, the sections were scored for histopathological changes (40x magnification) such as degeneration of smooth muscle layer and mononuclear cellular infiltration. Also, other sections were immune-stained with Bcl2 (Rabbit polyclonal antibody, Catalog No. A0208, ABclonal technology, Woburn, Massachusetts, USA) according to the manufacturer's directions then and visualized with a suitable chromogen such as diaminobenzidine (DAB) that produce a stable dark brown end product [25], then the tissue will be counterstained with a nuclear stain as hematoxylin [26]. The score for immunoreactivity of tissues to Bcl2 marker was assessed using image J program to evaluate the density of DAB stain in each section of aortic segments on each slide.

2.6. Statistical analysis

Results are presented as mean \pm SEM and were analyzed for statistically significant differences at $p < 0.05$ using unpaired Student's t-test for differences between two groups. GraphPad Prism® software was used for statistical calculations (Version 7.00 Windows, GraphPad Software, San Diego California USA). Concentration-effect curves were fitted using a built-in Dose-Response function. The difference between responses to a given concentration was analyzed using unpaired t-tests followed by the Holm-Šidák multiple comparisons test.

3. Results

3.1. Effect of high glucose on the contractility of the isolated rat aorta

Data in Figure 1 show the change in contractility of isolated rat aortic rings induced by incubation in high glucose (40 mM) for 3 h under experimental *in vitro* conditions. We observed unaltered responses of the segments exposed to high glucose upon addition of a maximal concentration (80 mM) of KCl in comparison to low glucose (Figure 1A). When we treated the tissues with

increasing concentrations of PE (10^{-9} to 1.5×10^{-5} M), robust concentration-effect curves were obtained. The data in Figure 1B illustrate that the contractile responses of HG-incubated tissues were significantly higher than those incubated in low glucose starting from 100 nM PE up until the maximal concentration (1.5×10^{-5} M). Besides, incubation of the thoracic aortic rings in 40 mM glucose significantly ($p < 0.05$) enhanced the sensitivity of the tissue to PE as the EC₅₀ value was 387.2 nM (95% CI: 329.5 to 455.1) compared with 684.8 nM (95% CI: 534.8 to 878.8) in the LG-incubated ones.

Moreover, the maximum contraction observed in these HG-incubated tissues was significantly elevated ($p < 0.05$) at 114.0 ± 1.849 % versus 93.75 ± 2.586 % in the LG-treated tissues. Tissues incubated in high mannitol showed contractile responses like those in low glucose (not shown).

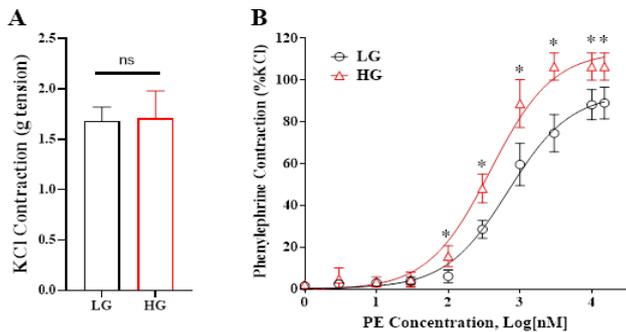


Figure 1: Effect of high glucose on vascular contractility. Thoracic aorta rings were equilibrated for 45 min in 10 mM glucose Krebs-Hensleit buffer under 1.5 g tension, then challenged with 80 mM KCl until a stable contraction was obtained. The integrity of the endothelium was confirmed by addition of 1 μ M ACh to PE (1 μ M)-precontracted rings, and tissues that showed $>65\%$ relaxation were used in the experiments. Tissue segments were incubated in either low (LG) or high glucose (HG) physiological buffers. The contractile effect to 80 mM KCl did not differ after 3 h of LG or HG incubation (A). The cumulative concentration effect curves of phenylephrine (PE)-induced contractions are shown (B). * Denotes significant differences ($p < 0.05$) between the means of LG- and HG-incubated tissues at the specified concentrations (10^{-7} - 1.5×10^{-5} M PE). Data represent the mean \pm SEM of at least 4 independent experiments. Unpaired t tests for each concentration point in the concentration-effect curves were used for comparison between means followed by Holm-Sidak multiple comparisons test.

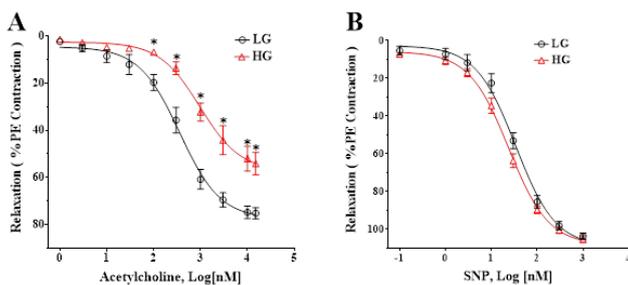


Figure 2: Effect of high glucose on the relaxation of isolated rat aorta. The vascular rings were treated as described in the methods section. Following the contraction studies as in Figure 1, the rings were washed and allowed 15 minutes of resting before being constricted with phenylephrine (10^{-6} M). After reaching a plateau contractile response, tissues were exposed to cumulative increasing concentrations of acetylcholine in the range of 10^{-9} - 1.5×10^{-5} M (A) or sodium nitroprusside (SNP) in the range 10^{-10} - 10^{-3} M (B). * Denotes significant differences at $p < 0.05$ between the means of LG- and HG-incubated tissues in the concentration range of 10^{-7} - 1.5×10^{-5} M acetylcholine. Data represent the mean \pm SEM of at least 4 independent experiments. Unpaired t tests for each concentration point in the concentration-effect curves were used for comparison between means followed by Holm-Sidak multiple comparisons test.

3.2. Effect of high glucose on the relaxation responses of the isolated rat aorta.

To test the effect of HG on endothelium-dependent and endothelium-independent relaxations, the phenylephrine-precontracted rat thoracic aortic rings were challenged with

increasing cumulative concentrations of acetylcholine or sodium nitroprusside, respectively. Incubation of the aortic segments in high glucose (40 mM) buffer for 3 h resulted in a significant ($p < 0.05$) reduction in acetylcholine-induced relaxations when the total concentration of acetylcholine reached 100 nM or higher in comparison with the aortic segments incubated with low glucose. Importantly, vessels incubated in HG showed a significant ($p < 0.05$) reduction (26%) of the maximal relaxation responses and increased (2.68-fold) EC₅₀ value of acetylcholine. No significant difference was observed in SNP-induced relaxation. Incubation of aortic segments with the high mannitol buffer containing 10 mM glucose plus 30 mM mannitol showed relaxation responses comparable to those produced by tissue incubated in the low glucose experiments (data not shown).

3.3 Effect of incubation with high glucose on the histopathological appearance of aortic tissue

As shown in Figure 3, LG and HM groups showed normal endothelial cell lining with normal smooth muscle cells of tunica media-oriented horizontal to the aortic lumen. Also, they exhibited a normal amount of elastic collagenous elements and connective tissues in the tunica adventitia with no change in tunica adventitia thickness. On the other hand, HG showed disrupted integrity of endothelial lining with multifocal degeneration of smooth muscles with a significant ($p < 0.05$) increase in elastic collagenous elements. Besides, there was a remarkable inflammatory cell infiltration with profound edema showed by a significant ($p < 0.05$) increased adventitial thickness in the tunica adventitia.

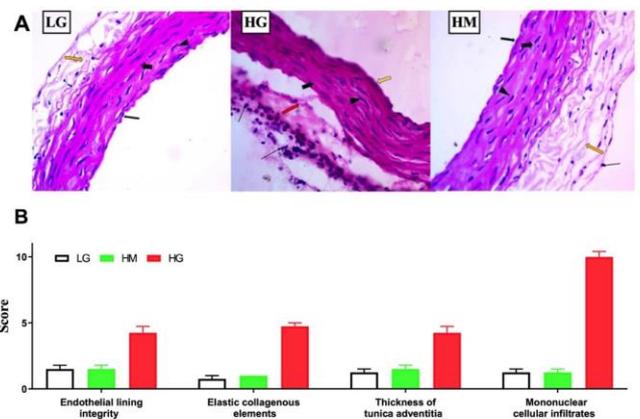


Figure 3: Effect of high glucose on the histopathological appearance of aortic tissue. (A) Representative photomicrographs showing H&E stained aortic tissue from different groups (B) Bar charts showing semiquantitative scores for H&E stained aortic sections.

Thin arrow: endothelial cells lining on the tunica intima, **arrowhead:** smooth muscle cells of the tunica media oriented horizontal to the arterial lumen; **thick arrow:** elastic tissue of the tunica media, **yellow arrow:** amount of collagen fibers and connective tissue in the tunica adventitia. LG: low glucose, HG: high glucose; HM: high mannitol.

3.4 Effect of incubation with high-glucose on apoptosis of aortic tissue

To study the effect of high glucose on apoptosis, we measured Bcl2 (an anti-apoptotic marker) immunoreactivity. Figure 4 shows that aortic rings incubated in high glucose media resulted in a significantly ($p < 0.05$) lower immunoreactive area to Bcl2 antibody compared to both LG and HM. This reflects an attenuated anti-apoptotic pathway and thus increases apoptosis.

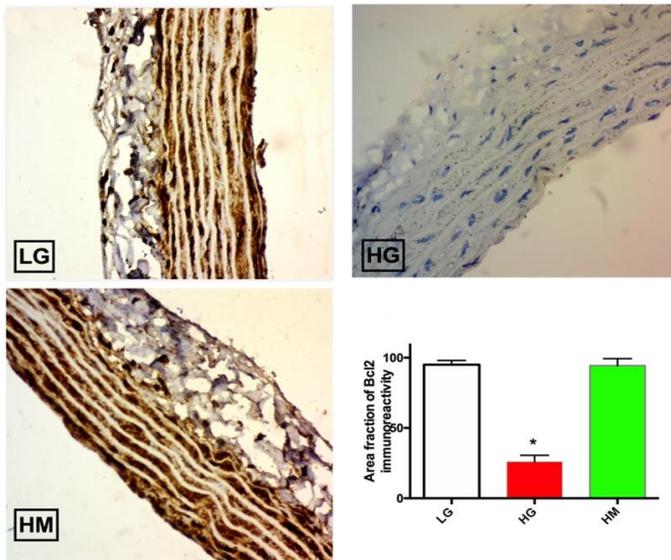


Figure 4: Effect of high glucose on the Bcl2 immunoreactivity of aortic tissue. Representative photomicrographs showing Bcl2 immunoreactivity of aortic tissues incubated in low glucose (LG), high glucose (HG), or high mannitol (HM). The bar charts show the semiquantitative scores for Bcl2 immunoreactivity in the corresponding groups. Analysis was done using one-way ANOVA followed by Tukey-Kramer post hoc test. * Denotes significant difference from LG group.

3.5. Effect of incubation with high-glucose on oxidative stress parameters of aortic tissue

As shown in Table (1), aortic rings incubated in high glucose media resulted in a significant ($p < 0.05$) increase in SOD and catalase activity. Meanwhile, it caused a significant ($p < 0.05$) increase in total nitrite /nitrate concentration. Noteworthy, incubation with low glucose and mannitol did not cause a significant change in these parameters.

Table 1: Effect of high glucose on SOD, catalase, and total nitrite/nitrate levels in aortic tissues.

Group	SOD	Catalase	Total nitrite/nitrate
HG	643 ± 102 *	22.4 ± 1.9*	10.1 ± 1.6*
LG	388 ± 30	6.8 ± 0.2	1.7 ± 0.1
HM	249 ± 34	2.2 ± 0.2	2.6 ± 0.4

Data represent the mean ± SEM of 6 aortic rings from each group. Data analysis was carried out by one-way ANOVA followed by Tukey-Kramer post hoc test. * Denotes significant difference from LG. LG: low glucose, HG: high glucose; HM: high mannitol.

Discussion

Hyperglycemia is the most prominent metabolic derangement in diabetic patients and is critical to the development of all diabetes-associated complications [27]. The pathogenesis of these diabetes complications, notably the cardiovascular ones, is highly dependent on glycemic control [28-30]. In this study, we aimed to investigate the effect of short-term high glucose exposure on vascular function. We used a simple *in vitro* model to mimic the

hyperglycemia encountered *in vivo* in diabetic patients in an organ bath system. We also explored the possible mechanisms mediating high glucose induced vascular dysfunction. We studied the effect of high glucose on vascular contractile as well as relaxant response in the isolated rat aorta. Our results showed that the incubation of thoracic aortic rings with high glucose induced endothelial dysfunction through an observed attenuation of the vasorelaxant response to Ach. The effect of high glucose on the response to Ach has been previously reported in different models of diabetes. For example, in a model of type I diabetes using STZ, 8 weeks for hyperglycemia resulted in an impaired endothelial-dependent relaxation showing a maximum relaxation response to Ach of 64% compared to 87% in normal rats [31]. Also, in a model of genetic type-2 diabetes, 30 days of elevated blood glucose level resulted in an impaired endothelial-dependent relaxation in female isolated thoracic aortic rings [32]. Moreover, in a mouse model of diabetes-induced by injection of an insulin receptor antagonist, impaired relaxation response to Ach in the thoracic and abdominal aorta was reported 1 month after induction using MRI imaging technique [33]. The mechanism by which hyperglycemia induces ED can be explained through its ability to activate protein kinase C by increasing diacylglycerol leading to decreased eNOS expression, increased ET-1 expression, NF- κ B activation, increased transforming growth factor- β expression, and NADPH oxidase activation, which all contribute to ED [34].

In addition to the reduced relaxation response to Ach, we observed an augmented contractile response to phenylephrine (PE) which is consistent with previous findings in chronic diabetic experimental models [31, 35] as well as in human diabetic patients [36]. This enhanced contractility can be explained by the work of Hien and colleges who found that incubation of vascular smooth muscle cells in high glucose media enhances the expression of contractile proteins [37].

The impaired relaxation and increased contraction can be both linked to the oxidative stress status associated with hyperglycemia which we observed in our study as an increased level of aortic total nitrate and a compensatory increase in the antioxidant defense enzymes (SOD and catalase). This link between hyperglycemia and increased oxidative stress has been previously reported [35, 38, 39]. Various studies have established that increased ROS production may be accompanied by a compensatory increase in antioxidant enzymes but not much enough to overcome this oxidative burst in hyperglycemia-induced endothelial dysfunction. This phenomenon was previously reported in many studies involving *in vivo* models of diabetes and examining the effect of uncontrolled hyperglycemia on different organs [39-42].

Incubation with high glucose for three hours resulted in profound histopathological changes evident in inflammation of the adventitia and reduction of endothelial lining integrity which resembles those observed in chronic endothelial dysfunction models [43] and explains the predisposing factor of endothelial dysfunction and vascular complication in chronic metabolic disorders-induced vascular inflammation [44].

Enhanced apoptotic signals in response to hyperglycemia are widely described in different models of endothelial dysfunction [45-48]. A study by Tawfik et al. demonstrated that incubation of endothelial cells in high glucose or ROS-containing media resulted in activated apoptotic signals that were attributed to Janus kinase 2 activations [45]. Also, enhanced ROS production, especially nitrosative stress has been linked to the stimulation of apoptotic pathways in various pathological conditions [49]. These studies emphasize the role of oxidative stress in apoptosis

consistent with the finding of our study where we report that high glucose-mediated a robust reduction of Bcl2 expression along with an increase in total nitrate level in thoracic aortic tissue. The anti-apoptotic Bcl2 protein is one of the important markers controlling cell fate. Activation of PKC α which is reported to be increased in response to hyperglycemia [50], has been previously reported to induce apoptosis [51]. Persistent hyperglycemia caused a reduction in Bcl2 expression and an increase in the apoptotic protein caspase-3 in a study inducing hyperglycemia by continuous iv glucose infusion [48]. Oxidative stress, the imbalance between antioxidant capacity and ROS production; and enhanced cellular apoptosis are key factors in the initiation of inflammation which predisposes to various metabolic disorders such as obesity-induced hypertension [52]. In conclusion, our study confirms the suitability of the high glucose-incubated aortic rings in studying vascular dysfunction. We showed evidence of dysfunction as impaired relaxation response and elevated contractile response. Histopathological examination of aortic tissue exposed to high glucose for three hours emphasized the functional data showing distorted structure and signs of inflammation. In our model of endothelial dysfunction, we suggested that increased oxidative stress and apoptosis are the underlying mechanisms of impaired vascular dysfunction in response to increased glucose levels. From our study, we can conclude that this in vitro model closely resembles other in vivo models which often require a long period to develop vascular dysfunction. This model will provide a tool to study different pathways and treatment options which is less time-consuming and more economic.

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