

# High Glucose Level Induces Cardiovascular Dysplasia During Early Embryo Development

## Authors

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## ABSTRACT

The incidence of gestational diabetes mellitus (GDM) has increased dramatically amongst multiethnic population. However, how gestational diabetes mellitus damages the developing embryo is still unknown. In this study, we used yolk sac membrane (YSM) model to investigate angiogenesis in the developing chick embryo. We determined that in the presence of high glucose, it retarded the growth and extension of the embryonic vascular plexus and it also reduced the density of the vasculature in yolk sac membrane model. Using the same strategy, we used the chorioallantoic membrane (CAM) as a model to investigate the influence of high glucose on the vasculature. We established that high glucose inhibited development of the blood vessel plexus and the blood vessels formed had a narrower diameter than control vessels. Concurrent with the abnormal angiogenesis, we also examined how it impacted cardiogenesis. We determined the myocardium in the right ventricle and left atrium were significantly thicker than the control and also there was a reduction in glycogen content in cardiomyocytes. The high glucose also induced excess reactive oxygen species (ROS) production in the cardiomyocytes. We postulated that it was the excess reactive oxygen species that damaged the cardiomyocytes resulting in cardiac hyperplasia.

## Introduction

GDM is defined as carbohydrate intolerance diagnosed during pregnancies [1]. It occurs ubiquitously among many ethnic groups and

increases the risk of the woman developing diabetes post-partum [2, 3]. Moreover, GDM is characterized by increased insulin resistance and the failure of the  $\beta$ -cells to compensate for the deficiency. These disorders have an adverse effect on maternal health, as well as short- and long-term complications in the offspring, as revealed in a number of epidemiological studies [4–6]. Undoubtedly,

\* contribute to the work equally

the altered glucose metabolism in pregnant mothers has negative impact on their offspring [7–12]. Pedersen and Freinkel established the theory of fuel-mediated teratogenesis, in which the intrauterine environment is influenced by excessive maternal fuel and in turn adversely affects fetus development [13, 14]. Caesarean section and pre-mature delivery are considered consequence of glucose intolerance during pregnancy.

The vascular system is composed of a complex network of branching and tubular structures of different dimensions which support the growth of the embryo. It is also the first system to emerge during embryo development. It is irregularly distributed in the various tissues according to the requirement of blood supply. Vasculogenesis have been analyzed by counting and measuring the blood vessels. There is no doubt that factors that causes angiodyplasia will result in embryonic dysplasia and death of the embryos. It has been proposed that vascular diseases could be predicated from measurement of the dimension of the blood vessels and density of the vasculature [15].

The chick embryonic yolk sac is an extra-embryonic structure that is involved providing nutrition to the developing embryo and it is first site where blood vessels and angioblasts develop. During development, the chorionic membrane and allantois fuses together to form the CAM. The YSM model is an adequate model for studying blood vessel formation [15] but the CAM model also possesses many advantages because it is highly vascular, highly reproducible, simplistic and low-cost. In addition, as the CAM assay is a closed system, it makes small molecules, peptides and drugs under study more stable [16]. Therefore, we utilize the advantages of both YSM and CAM models to investigate the effects of high glucose on angiogenesis.

ROS are produced by aerobic cells and includes  $O_2^-$ ,  $H_2O_2$  and  $HO_2$ . Oxidative stress is regarded as being an imbalance between the production of ROS and the antioxidant defense mechanism and which may damage cellular proteins, lipids and DNA [17]. During pregnancy, the placenta is highly vascular and abundant in mitochondria making it a prime target for ROS induced oxidative stress. Hence, it is reasonable to assume the sensitivity of this tissue will impact on the developing embryo - especially during the early stages of organogenesis when the antioxidant defense is still immature. It has been reported that oxidative stress induced by excess ROS may severely damage the embryo to produce different types of embryonic malformation [18]. The pathological mechanism involved may entail ROS inducing apoptosis and abbreviating gene expression, leading to abortions, embryonic malformation or fetal intrauterine growth retardation [19, 20].

To date, there few studies exploring the relationship of cardiovascular disease (CVD) in the offspring of GDM mothers. We hypothesized that the intrauterine environment of women with GDM promotes CVD etiology and the adverse effects of high glucose is mediated via excess ROS production. In our previous study (unpublished data), we have established that high glucose exposure would increase ROS production in embryonic tissues. Therefore, we investigated the harmful effects of high levels of glucose/ROS on angiogenesis and cardiogenesis.

## Materials and Methods

### Reagents

Silicon rings (external diameter: 10mm, internal diameter: 9 mm) were obtained from the caps of culture bottle. Glucose, Formamide, Deionised formamide, mannitol, Eosin and Haematoxylin were purchased from Sigma-Aldrich Company. Fertilized Leghorn eggs were purchased from the Avian Farm of the South China Agriculture University (Guangzhou, China).

### Chick embryo yolk sac membrane (YSM) model

Fertilized chicken eggs were incubated in an incubator (Yiheng Instruments, Shanghai, China) at 38 °C and 70 % humidity for 2.5 days. The eggshell was cracked open with the egg placed into a sterilized glass dish. It was covered with a crystal dish and incubated for 1 h to allow the embryos adapt to their new environment. The eggs containing the embryos were selected for mannitol (control) or high glucose (25 mM, 50 mM and 100 mM) treatment. The embryos were orientated at the center of the eggs and then silicon rings were placed in over the leading edge of YSM as shown in ► **Fig. 1**. The ring was marked with black ink to indicate the starting position of the YSM within the ring. Into each ring was added 50 µl of mannitol or 25 mM - 100 mM of glucose. The extent of angiogenesis was determined and photographed after 12, 24, 36 and 48 h incubation.

### Chick embryo chorioallantoic membrane (CAM) model

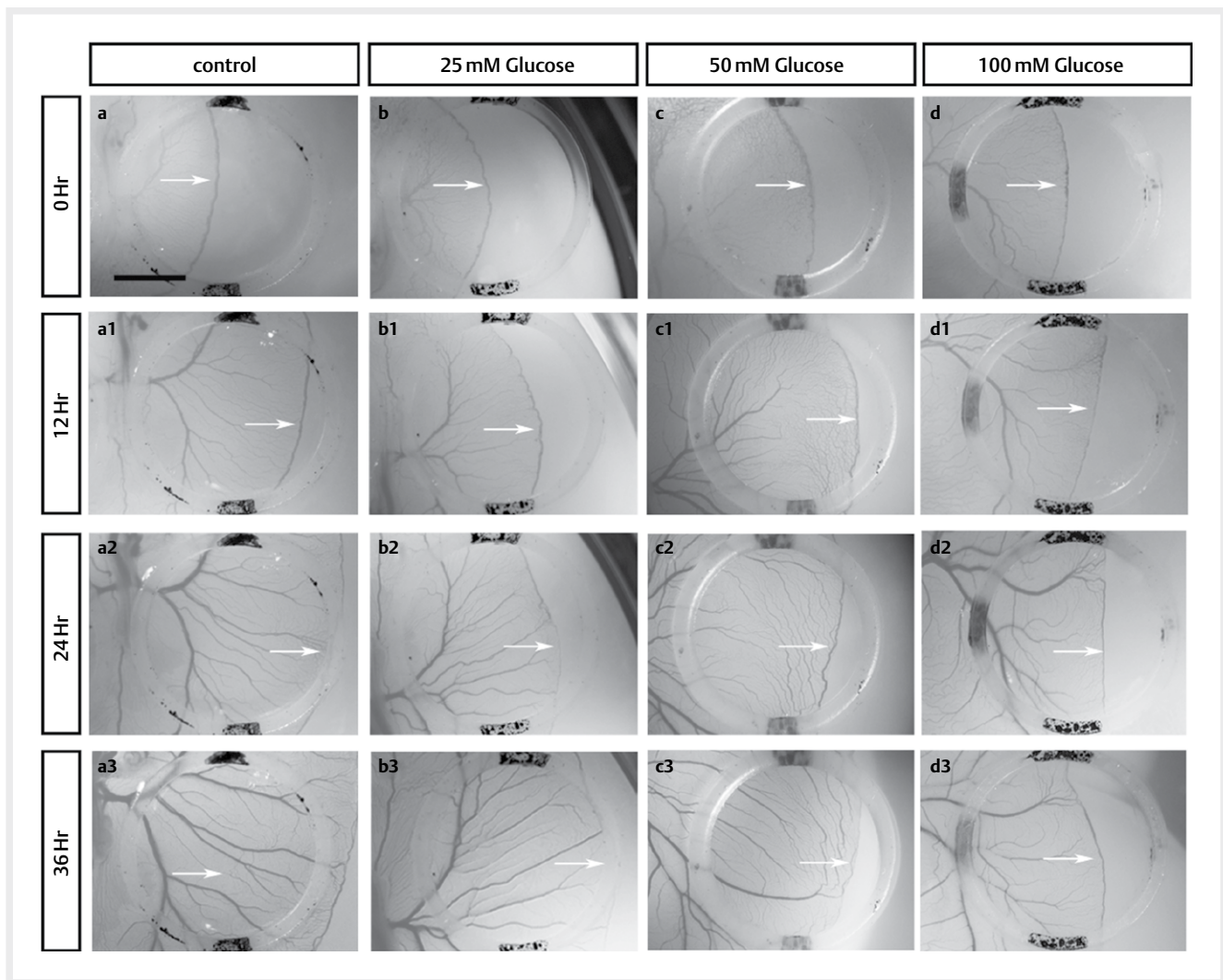
Fertilized eggs were incubated for 3 days and divided randomly into four groups. The eggs were injected with either 50 ml of mannitol (control) or 50 ml glucose solutions (0mM, 25 mM, 50 mM and 100 mM). When the embryos reached the desired stage (1.5, 4 and 9 days after treatment), the egg were opened and place on a culture with the CAM intact. The embryos and CAM were photographed using a stereomicroscope (Olympus MVX10, Japan) as previously described [20].

### Evaluating embryonic development

The morphology and developmental stage of chick embryos produced was established according to Hamburger and Hamilton (HH) staging scheme [21]. The embryos were first determined whether to be live or dead by the presence of heart beat. The embryo malformation rate was then established by determining the ratio of number of abnormal embryos versus total number of embryos. For each embryo, the YSM was also photographed using a stereomicroscope [20].

### Periodic Acid Schiff (PAS) staining of the heart

The embryos were embedded in wax, sectioned at 5 µm, dewaxed and hydrated. The sections were treated with 1 % periodic acid for 10 min, washed in distil water and then incubated in Schiff solution for 10–20 min. The sections were next treated with sulphurous acid (2X) and wash thoroughly in distil water for 5–10 min. Finally, the sections were stained with Celestine Blue dye (3 min), Hematoxylin (3 min), washed in distil water (5–10 min) and stained with 0.5 % orange G (30 s).



► **Fig. 1** High glucose inhibits extension of the vascular plexus in YSM. Chick embryo YSM was treated with **a** mannitol (control), **b** 25 mM HG, **c** 50 mM HG and **d** 100 mM HG introduced into silicon rings. **a–d** are representative appearance of the growing vascular plexus in the YSM, 12 h **a1–d1**, 24 h **a2–d2** and 36 h **a3–d3** after mannitol or glucose treatment. The position of leading edge of vascular plexus at start of experiment was marked on the silicon ring with black ink. The white arrows indicate the extent of the vascular plexus growth and extension in the YSM. The presence of HG inhibited vascular plexus development in a dose-dependent manner. Scale bar = 4 mm. Abbreviations: HG, high glucose; Hr, hours.

### MTT and ROS assay for cardiomyocyte

Cell viability was assessed using MTT assay. Cell viability was evaluated by the ratio of the absorbance value of high glucose-treated cells relative to the control. The final results were determined from analyzing three independent experiments [20]. we used an ROS assay kit to measure the ROS generated by the cardiomyocytes. The reaction was measured using a fluorescent spectrophotometer (Thermo Scientific).

### Statistics

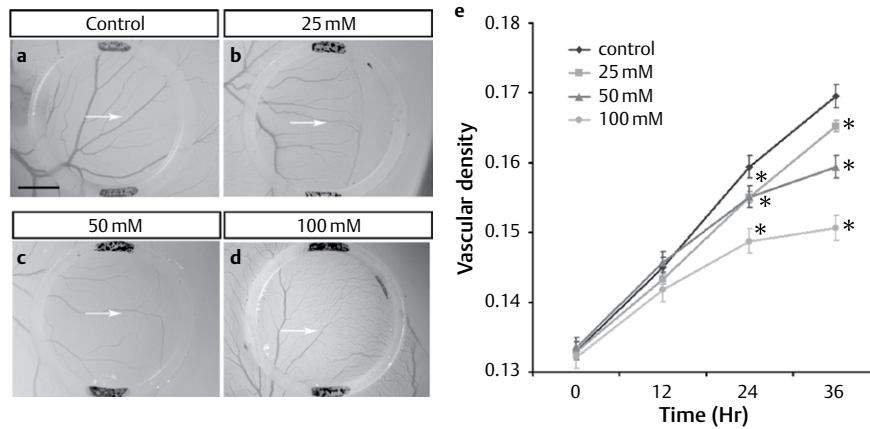
Using Image-Pro Plus, the density of the vasculature in both CAM and YSM before and after exposure to glucose was analyzed. Data analyses and construction of statistical charts were performed using a Graphpad Prism 5 software package (Graphpad Software, CA, USA). The results were presented as mean  $\pm$  SE. All data were analyzed using ANOVA, which was employed to test the difference

between control and experimental groups.  $p < 0.05$  was considered to be significantly different [20].

## Results

### Effects of high glucose on the developing vascular plexus of chick embryos

The effects of high glucose on angiogenesis were investigated using the chick yolk sac membrane (YSM) of chick embryos as a model. The model allows the development of the vascular plexus to be easily observed and followed in the presence of glucose. Silicone rings were placed on the leading edges of the vascular plexus, to limit the area exposed to high glucose when it was added to the rings (► **Fig. 1a**). Mannitol was used as a control to normalize the effect of changes in osmolarity due to high glucose. In the control group,



► **Fig. 2** High glucose reduces the density of the vascular plexus in YSM. Chick embryo YSM was exposed to **a** mannitol (control), **b** 25 mM HG, **c** 50 mM HG and **d** 100 mM HG introduced into silicon rings for 24 h. The vascular plexus were photographed and the densities of vascular plexus were analyzed using an Image-Pro Plus 6.0 programme. The results were statistically analyzed and presented as mean  $\pm$  SE. Statistical significance was determined using one-way analysis of variance (ANOVA). \*  $P < 0.05$  was considered as significantly different. Scale bar = 4 mm. Abbreviations: HG, high glucose; Hr, hour.

the leading edges of vascular plexus expanded with the growth of the embryo, and reached 2/3 rd of the way into the silicone ring after 12-h incubation (► **Fig. 1a1**). The vascular plexus completely invaded the ring after 24-h incubation (► **Fig. 1a2**) and passed beyond the ring after 36-h (► **Fig. 1a3**). In contrast, the growth of the vascular plexus was visibly retarded in the presence of 25 mM (► **Fig. 1b**), 50 mM (► **Fig. 1c**) and 100 mM (► **Fig. 1d**) glucose at all corresponding stages compared with the control. The criteria used to assess vascular plexus development are described in Supplementary data 1. The high glucose not only slowed growth of vascular plexus but also creased the leading edges of the blood vessels (► **Fig. 1c2–d3**). Furthermore, we analyzed the density of the vascular plexus in the presence of 0–100 mM glucose (► **Fig. 2a–d**). We established that high glucose significantly reduced the density of the vascular plexus in a dose dependant manner (► **Fig. 2e**). In addition, the blood vessels were narrower and provided even thinner branches in ► **Fig. 2**.

### High glucose disrupts angiogenesis in CAM of chick embryo

The effects of high glucose on angiogenesis in CAM were investigated. In the absence of glucose, the blood vessels emanating from the embryos appeared relatively large which gradually branch into smaller versions and then end up being capillaries (► **Fig. 3a**). We treated the embryos with 3 different concentration of glucose and determined that high glucose abridged angiogenesis (► **Fig. 3b–d**).

The density and diameter of blood vessels in CAM were carefully examined following the high glucose treatment. We found that the vascular density was significantly reduced following glucose treatment (► **Fig. 3e**). The diameters of blood vessels were measured by taking the largest main branch of the CAM in each experimental group that have been sectioned transversely (dotted lines in ► **Fig. 3a–d**). Both low (► **Fig. 3a'–d'**) and high magnifications

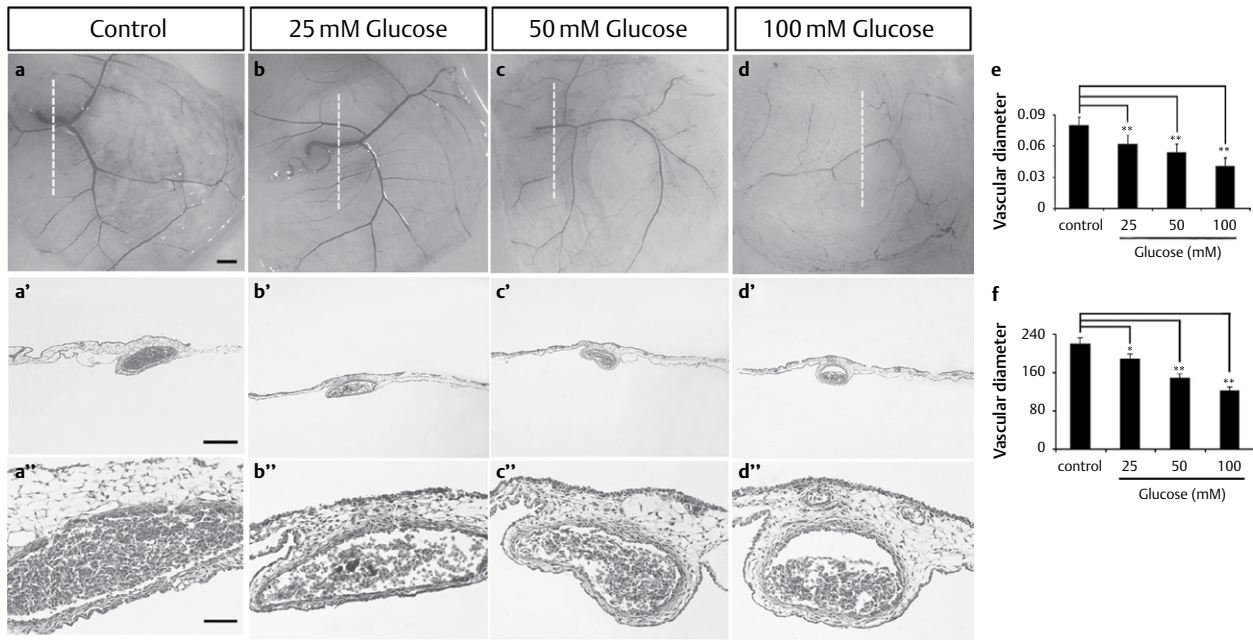
(Fig. A''–D'') of these blood vessels showed that the diameter of blood vessels narrowed dramatically with increase in glucose levels (► **Fig. 3f**). These results imply that exposure to high glucose impaired angiogenesis during early chick embryo development.

### Influence of high glucose on cardiogenesis

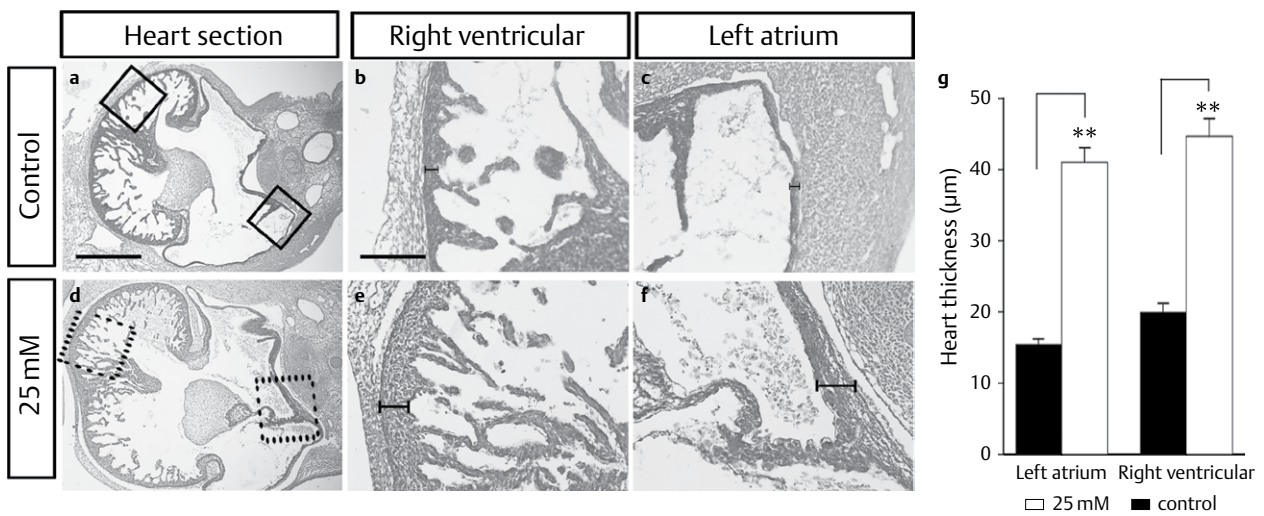
The heart and vasculature are interlinked during development, hence we examined the effect of high glucose exposure on cardiogenesis (► **Fig. 4**). PAS staining revealed that the hearts of control and glucose-treated embryos appeared grossly similar. However, transverse section of these hearts revealed that the right ventricle and left atrium in embryos treated with 25 mM glucose were thicker (► **Fig. 4d–f**) than corresponding sites in control embryos (► **Fig. 4a–c**). Measurement and comparison of the thickness of the heart walls confirmed that the right ventricle and left atrium of high glucose-treated heart were significantly thicker than the control (► **Fig. 4g**). We observed that PAS staining was weaker in the high glucose-treated heart than the control heart (Figs. A–F). This indicates that high glucose exposure reduces the glycogen content of the developing heart. This might be part of the reason why the right ventricular and left atrial walls of high glucose-treated hearts were thicker, through some compensatory mechanism.

### High glucose reduces cardiomyocyte viability by inducing excess ROS generation

The effects of high glucose exposure on cardiomyocytes were investigated. Cardiomyocyte primary cultures were produced from hearts were harvested from HH12 chick embryos. Immunofluorescent staining using MF20 antibody (cardiomyocyte marker) revealed that most of the cells in our primary culture were MF20 positive (► **Fig. 5a**). The cultures were treated with 0–100 mM glucose (► **Fig. 5b–d**). Compared with the control (► **Fig. 5b**), the presence of high glucose inhibited the growth of cardiomyocytes (► **Fig. 5c and d**). We calculated the number of cardiomyocytes in the different

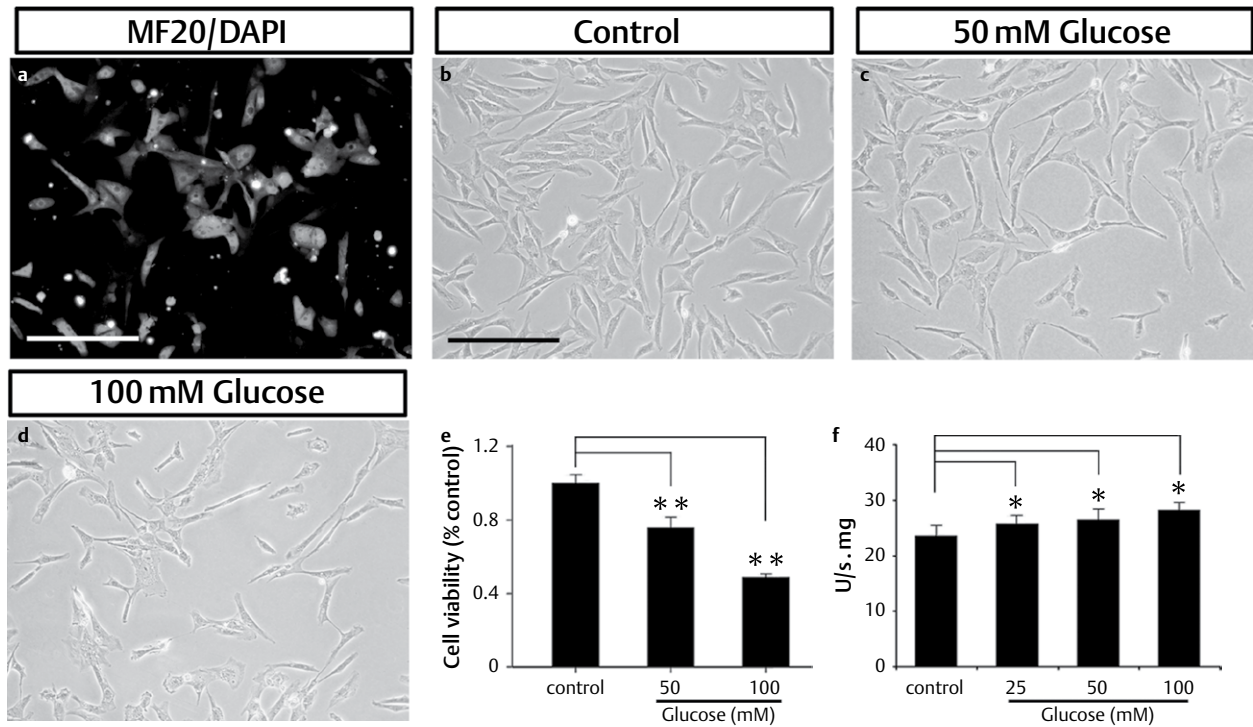


► **Fig. 3** High glucose retards growth of the vascular plexus in CAM. The photography of growth of vascular plexus in CAM was performed following either mannitol as control or HG administration as indicated three times at day 1.5, 4 and 8. **a** representative appearance of blood vessels in CAM treated with physiological concentration of glucose (control). **b–d** representative appearance of blood vessels in CAM treated with 25 mM HG **b**, 50 mM HG **c** and 100 mM HG **d** respectively. **a'–d'** transverse sections of the main artery in CAM were performed at the sites indicated by the dotted lines in **a–d**. **a''–d''** high magnification of blood vessels in **a'–d'**. The densities of the vascular plexus **e** and diameters of blood vessels **f** in control and HG-treated CAM were photographed and analyzed using an Image-Pro Plus 6.0 Programme. The results revealed that HG exposure significantly reduced the caliber and density of blood vessels. \*  $P < 0.05$ , \*\*  $p \leq 0.01$  are significantly different. Scale bar = 1 mm in **a–d**, 500  $\mu\text{m}$  in **a'–d'** and 100  $\mu\text{m}$  in **a''–d''**.



► **Fig. 4** High glucose increases the thickness of the myocardium. PAS staining was performed on sections of embryonic hearts following mannitol **a–c** or 25 mM HG **d–f** for 4 days. **b, c** are framed areas in **a** showing the right ventricle **b** and left atrium **c** of control embryos. **e, f** are framed areas in **d** showing the right ventricle **e** and left atrium **f** of 25 mM HG treated embryos. **g** The thickness of the myocardium of mannitol and HG-treated embryos were compared. It revealed that HG significantly increased the thickness of the left atrium and right ventricle. \*\*  $p \leq 0.01$  significantly different. Scale bar = 400  $\mu\text{m}$  in **a** and **d**, 100  $\mu\text{m}$  in **b, c** and **e, f**.

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► **Fig. 5** High glucose reduces cell viability and increases accumulation of ROS. Primary cardiomyocyte cultures were established from day 9 chick embryo hearts. **a** immunofluorescent staining with MF20 antibody confirmed that most of the cells in the culture were cardiomyocytes. **b–d** the cardiomyocytes were treated with mannitol **b**, 50 mM HG **c** and 100 mM HG **d**. **e** MTT assay revealed HG significantly reduced cardiomyocyte viability. **f** HG also significantly enhanced ROS production cultured cardiomyocyte. The data are presented as mean  $\pm$  SE. Statistical significance was determined using one-way analysis of variance and \*  $P < 0.05$ , \*\*  $p \leq 0.01$ . Scale bar = 200  $\mu$ m in **a–d**.

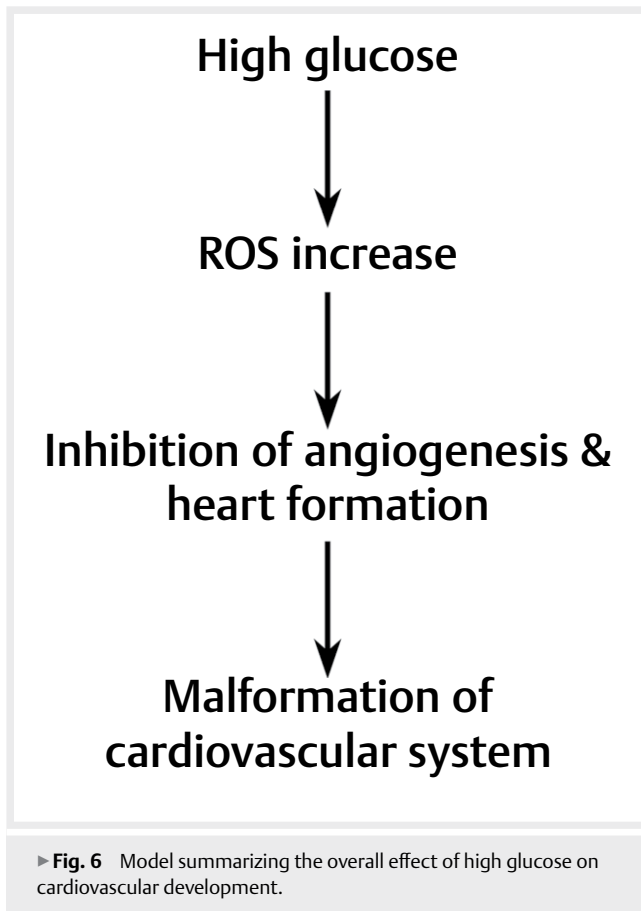
cultures and established that cell viability were significantly reduced in the presence of high glucose, in a dose-dependent manner (\*\*  $p < 0.01$ ). In addition, we determined that ROS increased in the cardiomyocytes following high glucose treatment (► **Fig. 5f**), suggesting that it might be the excess ROS that were reducing the cardiomyocytes' viability (► **Fig. 6**).

## Discussion

It has been reported that the coronary collateral vessels (CCV) develop poorly in patients with diabetes mellitus (DM) compared with individuals without DM. Hence, it was speculated that DM is an important factor in the etiology of abnormal CCV development [22]. Ning et al. [23] demonstrated that high glucose caused a decrease in endothelial cell proliferation. It also increased endothelial collagen IV expression, apoptosis and mitochondrial fragmentation. Venkatesan et al. [24] reported that enhanced cytokine and adhesion molecule expression was characteristic of high glucose-induced endothelial dysfunction, in which the CIKS gene played a crucial role. Despite numerous studies have been conducted on the relationship of DM and CCV [25], there is still lack of information on the relationship between high blood glucose induced by gestational DM and angiogenesis in the developing embryos. In this context, we employed the chick embryo model to investigate the effects of high glucose on cardiovascular development. We used YSM

and CAM assays to examine how high glucose influenced angiogenesis. Each of these models had their own advantage and disadvantage in respect to evaluating angiogenesis. From the view point of development, YSM forms earlier than CAM so combining both assays provided a more comprehensive picture on how high glucose affects angiogenesis. YSM is derived from extra-embryonic structure, in which blood islands form and develop into the beginning of blood vessels and hemocytoblast during development [26]. Using YSM assay, it has been demonstrated that magnetic fields changed the dynamic behavior of vascular tissues and bilaterally effected the growth of blood vessels [15]. Therefore, YSM model was chosen in this study since vasculogenesis and angiogenesis occur there in chronological order. Using the YSM model, we provided experimental evidence that high glucose reduced vascular plexus extension and density of blood vessels in the vascular plexus in a dose dependent manner. High glucose exposure had an adverse influence on embryonic vasculogenesis and early angiogenesis.

CAM has been extensively used as an in vivo model for studying tumor angiogenesis and evaluation of anti-angiogenesis/cancer drugs [27, 28]. Therefore, we used CAM as an angiogenesis model and established that high glucose impaired angiogenesis, as also seen in YSM. The impairments included reduction in the diameter and density of blood vessels. We also investigated the effect of high glucose in the cardiogenesis. We speculated that exposure to high glucose might damage cardiomyocytes by causing these cells to



overproduce and accumulate excess ROS [29]. Hence, we examined the morphology and histology of the heart exposed to high glucose. We found the right ventricle & left atrium were significantly thicker in high glucose treated embryos than control embryos. In addition, the glycogen content of high glucose treated heart was significantly reduced. Our findings also suggest that the cardiac walls had undergone compensatory hyperplasia due to direct high glucose-induced cardiomyocyte damage and indirectly through vascular dysplasia. This correlates with what occurs clinically that chronic hypertension results in compensatory hyperplasia of the myocardium.

We have also established in our primary cardiomyocyte culture that high glucose was accompanied by an increase in ROS. It has been ROS accumulation in cells activates signaling pathways associated with trauma and the resultant positive feedback amplify the oxidative injury [30]. ROS are intermediate metabolic products formed by the homolysis of simple substance or organic compound carrying atoms and radicals with un-paired electrons. ROS are highly reactive and causes nuclear acid, protein, carbohydrate and lipid to degenerate. Moreover, ROS can also alter the chemical structure of enzymes causing them lose their of enzyme activity. All the deleterious effects of ROS on the cellular structure and function causes the destruction of cells and tissue [31]. ROS is generated when the cells are stimulated by external factors such as high oxygen tension, radiation, anti-cancer agent, anti-bacterium agent, insecticide, aesthetics, smoke and air pollutant. ROS can be efficiently

neutralized in cells by the intrinsic antioxidant systems where free radical scavenger clears the cells of ROS or block ROS induced oxidation reaction. However, if excess ROS was produced by p66Shc and MAO in heart mitochondria, cardiomyocyte death and tissue damage would occur eventually leading to heart failure.

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## Conflict of interest

No conflict of interest has been declared by the authors.

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