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Adverse effects of high glucose levels on somite and limb development in avian embryos

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ABSTRACT

Gestational diabetes has an adverse impact on fetal musculoskeletal development, but the mechanism involved is still not completely understood. In this study, we investigated the effects of high glucose on the developing somites and their derivate using the chick embryo as a model. We demonstrated that under high glucose, the number of generated somites was reduced and their morphology altered in 2-day old chick embryos. In addition, high glucose repressed the development of the limb buds in 5.5-day old chick embryos. We also demonstrated that high glucose abridged the development of the sclerotome and the cartilage in the developing limb bud. The sonic hedgehog (Shh) gene has been reported to play a crucial role in the development and differentiation of sclerotome. Hence, we examined how Shh expression in the sclerotome was affected under high glucose. We found that high glucose treatment significantly inhibited Shh expression. The high glucose also impaired myotome formation at trunk level – as revealed by immunofluorescent staining with MF20 antibodies. In the neural tube, we established that Wnt3a expression was also significantly repressed. In summary, our study demonstrates that high glucose concentrations impair somite and limb bud development in chick embryos, and suggests that Shh and Wnt genes may play a role in the underlying mechanism.

1. Introduction

Gestational diabetes is defined as glucose intolerance during pregnancy. It is presented as high levels of glucose in the blood during the onset of pregnancy. It has been reported that the incidence of fetal congenital malformations under this condition is 2–5 times higher than in non-diabetic mothers (Ejdesjo et al., 2012). The types of congenital abnormalities encountered include cardiac malformations, central nervous system malformations, macrosomia, phocomelia and spine malformations (Aberg et al., 2001). We noticed that some of these phenotypes are associated with the development and differentiation of the somites in the developing embryo. It has also been suggested that pre-gestational diabetes was a risk factor for the musculoskeletal and central nervous systems (Wahabi et al., 2012). In rat embryos incubated with serum extracted from pregnant women with type I diabetes, there is significant reduction in the number of somites produced by these developing embryos (Ornoy et al., 1995). Furthermore, cellular studies have shown that the teratogenic impact of the diabetic environment was associated with the excess production of reactive oxygen species (ROS) and a reduction in ROS-scavenging enzyme activities (Ejdesjo et al., 2012; Cederberg and Eriksson, 1997; Horton and Sadler, 1983; Zaken et al., 2001; Eriksson and Borg, 1991).

During development, the presomitic mesoderm (PSM) on either side of the neural tube undergoes segmentation to form blocks of paired somites. These somites then differentiate and contribute to the skeletal muscles, cartilage, tendons, endothelial cells and dermis of the embryo. The development of the PSM somites is a rhythmic process that involves a series of developmental steps that include PSM segmentation, epithelialization, somite formation and differentiation (into dermatome, myotome and sclerotome)

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(Nimmagadda et al., 2007). Somitogenesis begins when the embryo completes gastrulation, a process that establishes the embryo's three germ layers (ectoderm, mesoderm and endoderm) (Christ and Ordahl, 1995; Christ et al., 1972). In the gastrula, a variety of progenitor cells for specific tissue types reside within specific domain of the primitive streak. These progenitor cells proliferate and migrate out of the primitive streak to occupy various positions in the PSM. A new somite boundary is formed at the anterior tip of the PSM whenever a new epithelial somite is formed. The newly formed somite begins to mature and differentiate by forming (1) the dermatome which will give rise to the dermis and musculature of the limb, (2) myotome to the musculature of body and (3) sclerotome to the vertebrae and ribs. The neural tube and notochords that face the somites are instrumental in providing the developmental signals that direct the development of the somites (Aulehla and Herrmann, 2004; Maroto et al., 2012).

Somitogenesis is greatly influenced by Shh signaling from the notochord and ventral neural tube. Shh proteins act on the somites by inducing the expression of paired box 1 (Pax1) and Pax9 in the sclerotome (Mise et al., 2008). The epithelium-like dermomyotome expresses Pax3 and Pax7 which specify the formation and differentiation of myotome. It has been reported that Wnt1/3a produced by the neural tube and Wnt8a by the dorsal ectoderm induce Pax3 and Pax7 expression in the dermomyotome (Maroto et al., 2012). Neurotrophin 3 (NTF3) is a member of the neurotrophin family, which has been shown to improve hind-limb functional recovery in animal models (Guo et al., 2007; Johnson et al., 2009). And it expressed by the neural tube induces the dermatome to differentiate into the dermis (Maroto et al., 1997). In this study, we used early chick embryo as an embryonic model to investigate the effects of high-glucose exposure on somite and limb bud development.

2. Materials and methods

2.1. Chick embryos

Fertilized chick eggs were obtained from the Avian Farm of the South China Agriculture University. The eggs were incubated in a humidified incubator (Yiheng Instrument, Shanghai, China) at 38 °C and 70% humidity. After 36 h incubation, the chick embryos were treated with different concentrations of glucose (25, 50 and 100 mM; G8270, Sigma) or mannitol (control). The controls for 25, 50 and 100 mM dose of glucose were 24.7, 49.4 and 98.8 mM dose of mannitol respectively. Briefly, high concentration of glucose and mannitol were directly applied to 1.5-day embryos *in ovo* as previously described (Chen et al., 2013). The treated embryos were then incubated for further 0.5, 2 or 4 days before they were fixed with 4% paraformaldehyde (PFA) for analysis. Therefore, the embryos we harvested eventually were 2, 3.5 or 5.5-day old embryos. The concentration level of blood glucose in the treated embryos was equivalent to levels seen in human diabetes. A prior study showed that hyperglycemia was induced by a single injection of high glucose in early chick embryos. After the high glucose injection, they detected the concentration of blood glucose for 7 days. The blood glucose levels of glucose-injected embryos were as follows: day 1: 15 ± 6.8 mM, day 2: 12.4 ± 2.5 mM, day 3: 12.4 ± 2.0 mM, day 4: 12.3 ± 2.0 mM, day 5: 13.1 ± 3.2 mM, day 6: 12.4 ± 3.3 mM, and day 7: 12.2 ± 3.1 mM. There was no significant changes in the glucose concentration after 7 days of treatment (Larger et al., 2004). The concentration of blood glucose in human diabetes was greater than 11 mM (Sarkisian et al., 2010). So, the blood glucose levels in the observed chick embryos were to be similar to the ones in human diabetics.

2.2. In situ hybridization

In situ hybridization was performed on whole-mount chick embryos using a standard *in situ* hybridization protocol (Henrique et al., 1995). Briefly, digoxigenin-labeled probes were synthesized to detect *Shh* expression (Diez del Corral

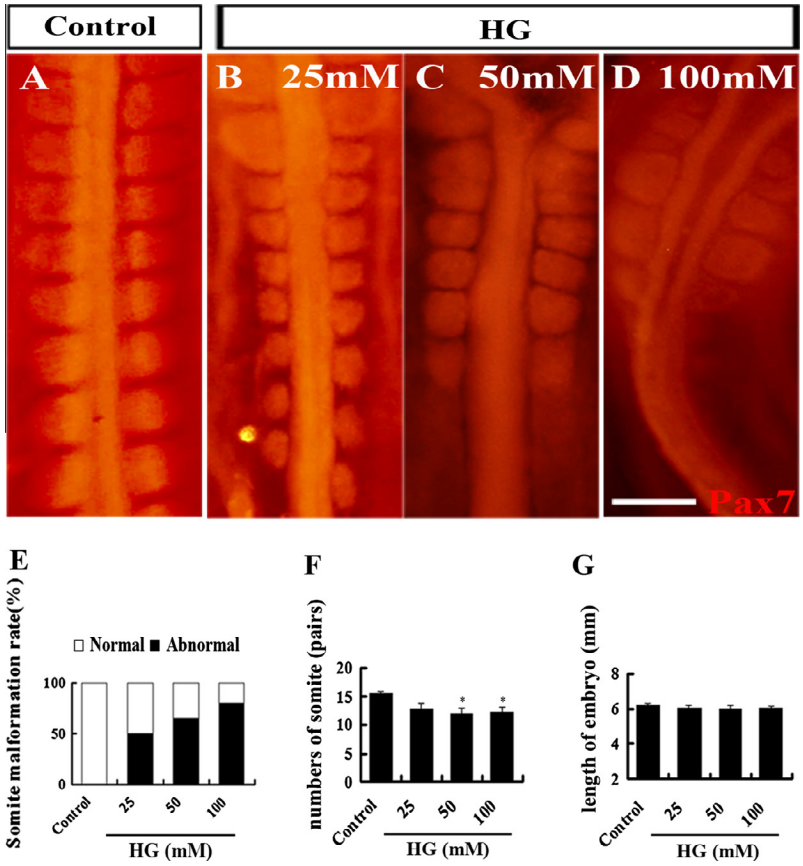

Fig. 1. Effects of high glucose level on somitogenesis in chick embryos. Fertilized eggs were exposed to 0–100 mM glucose and then the embryos were allowed to develop until 2 days. A–D: Representative appearance of somites in 2-day old chick embryos stained with Pax7 following high glucose exposure. The somites were found to be malformed in embryos treated with 25–100 mM glucose. Bar charts showing the incidence rate of somite dysplasia (E), the average numbers of somites formed (F) and the average length of embryos (G). $^{*}p < 0.05$ indicates significant difference between experimental and control embryos. Abbreviations: HG, high glucose. Scale bars = 200 μm in A–D.