Exposure of Neural Crest Cells to Elevated Glucose Leads to Congenital Heart Defects, an Effect That Can Be Prevented by N-Acetylcysteine

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BACKGROUND: Diabetes mellitus during pregnancy increases the risk for congenital heart disease in the offspring. The majority of the cardiovascular malformations occur in the outflow tract and pharyngeal arch arteries, where neural crest cells are essential for normal development. We studied the effects of specific exposure of neural crest cells to elevated glucose on heart development. Antioxidants reduce the damaging effect of glucose on neural crest cells in vitro; therefore, we investigated the effect of supplementing N-acetylcysteine in vivo. **METHODS:** Cardiac neural crest of HH 8–12 chicken embryos was directly exposed by a single injection in the neural tube with 30 mM D-glucose (or 30 mM L-glucose as a control). To examine the effect of a reduction in oxidative stress, we added 2 mM N-acetylcysteine to the injected D-glucose. **RESULTS:** Exposure of neural crest cells to elevated D-glucose–induced congenital heart malformations in 82% of the embryos. In the embryos injected with L-glucose, only 9% developed a heart malformation. As expected, all malformations were located in the outflow tract and pharyngeal arch arteries. The frequency of heart malformations decreased from 82% to 27% when 2 mM N-acetylcysteine was added to the injected D-glucose. **CONCLUSIONS:** These data are the first to confirm that the vulnerability of neural crest cells to elevated glucose induces congenital heart malformations. The fact that N-acetylcysteine limits the teratogenicity of glucose implies that its damaging effect is mediated by an increase of oxidative stress in the neural crest cells. *Birth Defects Research (Part A) 79:231–235, 2007.* © 2006 Wiley-Liss, Inc.

Key words: neural crest cells; congenital heart disease; elevated glucose; oxidative stress; antioxidants; N-acetylcysteine

INTRODUCTION

Diabetes mellitus (DM) during pregnancy is associated with an increased risk for congenital heart disease (CHD) in the offspring of humans and animals (Dunne et al., 2003; Eriksson et al., 2003; Loffredo et al., 2000, 2001; Siman et al., 2000). Women with type I DM have a 2–6 times increased risk of giving birth to a child with CHD (Eriksson et al., 2003; Loffredo et al., 2001). For women with DM type II, a 3–11 times increased risk is observed (Dunne et al., 2003). As the number of women suffering from diabetes during their childbearing age is rapidly increasing, the occurrence of diabetes-induced CHD is a serious and increasing health problem.

Cardiac neural crest cells (NCCs) are essential for cardiovascular development and are postulated to play a prominent role in diabetes-induced CHDs. This idea is rooted in the resemblance seen in the malformations identified in the offspring of diabetic women (Eriksson et al., 2003; Loffredo et al., 2000, 2001), in fetuses of diabetic rats

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(Eriksson et al., 2003; Loffredo et al., 2001; Molin et al., 2004; Siman et al., 2000), and in NCC-ablated chicken embryos (Kirby et al., 1985). In addition, malformations in other organs, in which NCCs are essential for normal development, are frequently identified after pregnancies complicated by diabetes (Digilio et al., 1995; Siman et al., 2000; Wilson et al., 1993).

Similar malformations that are mainly located in the outflow tract and pharyngeal arch artery system are observed in mammalian models with a defect in the cardiac NCCs, such as the *Pax-3* mutated splotch mice (Conway et al., 2000; Epstein et al., 2000; Henderson et al., 1997). PAX-3 is an important NCC transcription factor (Epstein, 1996). Excessive glucose metabolism in mouse embryos (Phelan et al., 1997) inhibits Pax-3 expression, also indicating a role for NCCs in diabetes-induced CHDs.

A direct effect of diabetes on NCCs has been shown in in vitro experiments. Cultured NCCs derived from embryos of diabetic rats show a decrease in proliferation and migration compared to NCCs from control rats. A comparable decrease can be caused in the control NCCs by elevation of the glucose concentration in the culture medium to 50 mM (Suzuki et al., 1996). Antioxidants significantly reduce the damaging effect of elevated glucose on NCC cultures, indicating that the effect of glucose is mediated by an increase in oxidative stress (Suzuki et al., 1996). NCCs are likely to be vulnerable to oxidative stress because they have a lowered antioxidant capacity (Davis et al., 1990).

In this report, we describe the in vivo effect of direct exposure of the NCCs to an elevated glucose concentration by a single injection of 30 mM D-glucose in the neural tubes of chicken embryos at Hamburger–Hamilton stage (HH) 8–12. Exposure of NCCs by neural tube injection has proven to be effective and has resulted in cardiovascular malformations in comparable experiments using homocysteine (Boot et al., 2004). The normal glucose concentration in chicken egg yolk and white is higher than that in human blood samples. In humans, blood glucose values normally vary from 4 mM after fasting to 8 mM directly after a meal. However, comparable to data in the literature (Garcia et al., 1983), the glucose concentration in our fertilized eggs was 12 mM in yolk and 22 mM in white, as measured with a hand-held analyzer. We have chosen to expose NCCs to an elevated level of 30 mM D-glucose, a concentration that is not toxic to NCCs in vitro (Suzuki et al., 1996) and close to the average blood glucose levels identified in the U-rat model we have studied previously (Molin et al., 2004).

Subsequently, the neural tube–injected embryos are allowed to grow until the cardiac post-septation stage, when they were sacrificed and processed to study cardiovascular morphology. Furthermore, we tested if the addition of the antioxidant NAC (N-acetylcysteine) could rescue the embryos from glucose-induced congenital heart malformations.

MATERIALS AND METHODS

Neural Tube Injections

The normal glucose concentration in chicken egg yolk and white is higher than that in human blood samples: 12 mM in yolk and 22 mM in white (measured in >3-μm samples using a hand-held analyzer (Freestyle; Disetronic Medical Systems, Vianen, The Netherlands). We have therefore chosen to expose NCCs to an elevated level of 30 mM D-glucose, which was shown earlier to be non-toxic for rat NCC cultures (Suzuki et al., 1996). It is also close to average blood glucose levels identified in the U-rat model we have studied, in which about one-third of the offspring develop normally despite continuous exposure to intrauterine hyperglycemia (Molin et al., 2004).

We dissolved 30 mM D-glucose (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands; G6125), 30 mM L-glucose (Sigma-Aldrich Chemie; 28,595-1), 30 mM D-glucose with additional 2 mM NAC (Sigma-Aldrich Chemie; A–9165) in 199–medium (Life Technologies, Paisley, United Kingdom; 31153-026), to which 0.25 g/mL of indigo carmine blue (Merck, Haarlem, The Netherlands; 1.04724.0025) was added. Fertilized specific pathogen–free White Leghorn eggs were incubated at 37°C until HH 8–12 (42–44 hr). A small opening was made in the shell of the egg just above the embryo. The glucose solutions were drawn into a glass micropipette mounted on a micromanipulator and then carefully injected using a programmable microinjector (IM–300, Narishige, Tokyo, Japan). The solution was injected into the lumen of the neural tube at somite levels 4–6, filling the neural tube in anterior direction up to the otic placode level (Boot et al., 2003a). After injection, eggs were resealed with Scotch tape and returned to the incubator at 37°C for further development. We studied a total of 28 embryos injected with 30 mM D-glucose, 11 embryos injected with 30 mM L-glucose, and 15 embryos injected with 30 mM D-glucose and 2 mM NAC.

Morphological Analysis of the Heart

The embryos surviving until the post-septation stage (HH 34/35) were dissected and immersion-fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 24 hr. Subsequently, the embryos were dehydrated in graded ethanol, transferred to xylene, embedded in paraffin, and serially sectioned at 5 μm. For immunohistochemistry, alternate sections were rehydrated and stained with hematoxylin-eosin (H-E), antibodies against α–smooth muscle actin (1A4/M851; Sigma-Aldrich Chemie), α/γ-muscle actin (HHF35; DAKO, Heverlee, Belgium), fibronectin (A.0245 DAKO) or fibrillin-2 (JB3, Hybrydomabank, Iowa). Sections to be used for the detection of α–smooth muscle actin were microwave-processed to enhance staining. Sections were incubated overnight at room temperature with a 1:3000 dilution of α–smooth muscle actin, a 1:500 dilution of muscle actin, a 1:2000 dilution for fibronectin, and a 1:2 for fibrillin-2 (in PBS with 0.05% Tween 20 and 1% bovine serum albumin). Subsequently, sections were washed in PBS and incubated for 60 min with a 1:250 diluted peroxidase-conjugated rabbit anti–mouse antibody (DAKO). After washing, sections were incubated with diaminobenzidine tetrahydrochloride (12 mg in trismaleate, pH 7.6) for 10 min, washed, and counterstained with Mayer's hematoxylin. Finally, sections were dehydrated and mounted with Entellan.

Reconstruction

The Amira V3.0 software package (Template Graphics Software, Inc., San Diego, CA) was used to make 3-D reconstructions of 1 normal embryo and 1 with a double-outlet right ventricle (DORV). For the reconstruction we used 5-μm sections every 60 μm with a pixel size of 3.5 × 3.5 μm. Via manual drawing, the cardiovascular lumen

Table 1
Number and Percentage of Congenital Heart Defects (CHDs) after Neural Crest Cell Exposure to D-glucose or L-glucose

	Normal	CHD	Total
L-glucose			
No.	10	1	11
Percent	91%	9%	100%
D-glucose			
No.	1	12[a]	13
Percent	8%	92%	100%

[a]In the D-glucose-injected embryos, a statistically significant increase of heart malformations was observed; $\chi^2_{[1]} = 16.62$; $P < .001$ and all expected values were >5.

was color-labeled and rendered for 3-D visualization. Embryos were processed under comparable conditions described above, allowing for a direct comparison of the reconstructions.

Statistical Analysis

To analyze a possible difference in the number of malformations identified between the embryos injected with either 30 mM D-glucose or 30 mM L-glucose, a Pearson χ^2 analysis was performed with 1 degree of freedom. The same was done for embryos injected with either 30 mM D-glucose or 30 mM D-glucose and 2 mM NAC.

RESULTS

A single exposure of the NCCs to elevated glucose induced a CHD in 92% of the embryos. Only 9% CHD was observed in control embryos injected with 30 mM of the metabolically inactive L-glucose (Table 1). The increase in the number of malformations identified could not be caused by differences in survival because these were comparable, 27% of the D-glucose-injected embryos, 28% of those injected with L-glucose, and 37% in the embryos injected with D-glucose/NAC. The relatively high lethality score is attributed to the experimental set up per se. For the purpose of this study, we analyzed only the later stages HH 34/35, to ensure diagnosis of a full-blown heart malformation.

The addition of the antioxidant NAC led to a two-thirds reduction of the teratogenic effect of elevated glucose, resulting in 27% CHDs compared with the 73% CHDs identified in D-glucose-injected embryos (Table 2).

Table 2
Effect of NAC Addition on Glucose-induced Congenital Heart Defects (CHDs)

	Normal	CHD	Total
D-glucose			
No.	4	11	15
Percent	27%	73%	100%
D-glucose + NAC			
No.	11	4[a]	15
Percent	73%	27%	100%

[a]Addition of NAC to the injected D-glucose resulted in a decrease of CHD from 73% to 27%. This difference was significant; $\chi^2_{[1]} = 6.53$; $P < .01$ and all expected values were >5.

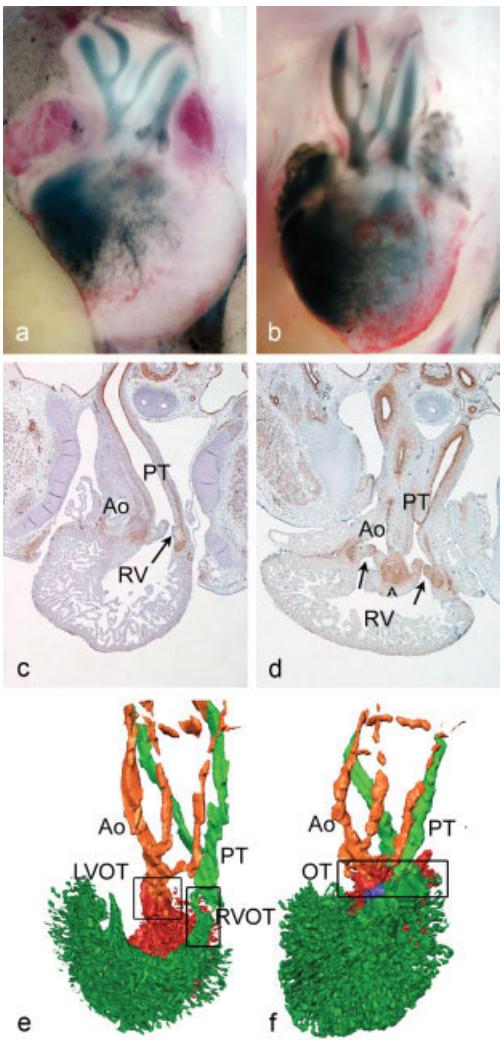

Figure 1. Comparison of a normal heart, in **(a), (c),** and **(e),** to a heart with a double-outlet right ventricle (DORV) with ventricular septal defect (VSD) in **(b), (d),** and **(f).** In (a) and (b), India ink was injected into the right ventricle (RV), illustrating a side-by-side and more parallel positioning of the great arteries in (b). In the normal heart (a), the ink initially went from the RV into the pulmonary trunk (PT), whereas the aorta (Ao) was filled retrograde from the ductus arteriosus after several heartbeats before the heart was photographed. In the heart with a DORV (b), the ink went from the RV into the Ao as well as the PT, indicating the presence of the DORV with obligatory VSD. (c) α–Smooth muscle actin (1A4) immunohistochemical detection on a transverse section through a normal heart. Blood from the RV can only flow to the PT (see arrow), and the Ao is sectioned above the level of the valves/outflow tract cushions. A comparable section of the heart with the DORV shows that the PT and Ao are situated above, and obtain blood from, the RV (see arrows). The presence of smooth muscle actin in the outflow tract septum (see arrowhead) is indicative of the presence of the neural crest cells ($n = 24$). Right-sided view of Amira reconstructions of hearts in (e) and (f). In the heart of the embryo with a DORV (f), there was a marked shortening of the outflow tract (OT) myocardium compared to the normal heart (e). The reconstruction shows the obligatory VSD that belongs to a DORV. Color code: RV and right ventricular outflow tract (RVOT), green; PT, light green; left ventricle and left ventricular outflow tract (LVOT), red; Ao and PAA, brown; VSD, blue.

All types of CHDs in these embryos affected the outflow tract of the heart and/or pharyngeal arch arteries (PAAs). A DORV was the most common malformation identified in 57% of the D-glucose-injected embryos, 9% of the L-glucose-injected embryos, and in 13% of the D-glucose/NAC-injected embryos (Fig. 1b, d, f). In a DORV malformation, both great arteries are positioned side-by-side and arise from the right ventricle. The aortic orifice is not properly wedged in between the right and left artrioventricular orifices, and posterior crossing behind the pulmonary artery has not taken place (Fig. 1a, b). The myocardialization of the outflow tract septum between the right and left ventricular outflow tract has taken place, as is shown by smooth muscle actin staining (Fig. 1c, d). The semilunar valve leaflets have developed normally (Fig. 1c, d). The remaining malformations consisted of an isolated ventricular septal defect (VSD) observed in 25% of the D-glucose-injected embryos and in 13% of the D-glucose/NAC-injected embryos. Malformations of the PAAs (in 21%) were seen only in embryos at the most severe end of the spectrum showing a DORV. Four of these consisted of a fourth PAA malformation resulting in an aortic arch interruption type B. Two embryos had an aberrant subclavian artery and 1 additional case had an absent right ductus arteriosus.

Three-dimensional reconstructions of a normal embryo and an embryo with a DORV were made (Fig. 1e, f), showing the morphological differences in the outflow tract. In the embryo with a DORV, there was a marked shortening of the outflow tract. The aortic orifice was not sufficiently wedged in between the right and left atrioventricular orifices, resulting in a side-by-side position of the great arteries. The reconstruction also shows the obligatory VSD that belongs to a DORV (Fig. 1f).

In the PAAs of some embryos, we observed a striking detachment of stretches of endothelial cells partly floating in the lumen of the vessel. Although the size of these loosened stretches varied, most consisted of 20–50 cells per detached site. Endothelial detachment was identified in 5 of 28 embryos (13%) injected with D-glucose and 2 of 11 embryos (18%) injected with L-glucose, and 1 of 15 embryos (7%) injected with D-glucose/NAC. To see whether extracellular matrix changes had occurred in the wall of the NCC exposed arteries, we studied the expression of fibronectin and fibrillin-2 in these vessel walls. We found no differences between the D- and L-glucose-treated groups. There was no specific preference of endothelial detachment for embryos with or without a detected cardiac malformation.

DISCUSSION

In our study, we have shown a very high incidence of cardiovascular malformations in chicken embryos in which NCCs were exposed to an elevated glucose level. NCCs arise from either side of the neural tube and migrate to the developing heart, where they play a crucial role in the septation and myocardialization of the outflow tract and the remodeling of the PAAs (Bergwerff et al., 1998). If cardiac NCCs are ablated, the resulting malformations, a persistent truncus arteriosus and severe aortic arch anomalies, are more severe than in our experiments (Kirby et al., 1985; Waldo et al., 1996). We observe a milder variation in CHDs and assume that although the NCCs are certainly affected, they remain partly functional. The presence of smooth muscle actin expression in the outflow tract septum also indicates that NCCs have arrived in this structure and initiated myocardialization (Wessels et al., 2004). A last point, which deserves attention, is that PAA anomalies were not seen in all cases in the present study. This suggests a relative normal migration of NCCs and possibly a secondary development of PAA defects on the basis of an abnormal outflow tract development. A similar explanation was provided for the PAA defects in diabetic rat embryos (Molin et al., 2004), in which the role of hemodynamic factors in remodeling has been proposed (Gittenberger-De Groot et al., 2006).

More recently, NCCs were proven to play a role in the addition of cells from the secondary heart field (SHF) to the outflow tract of the heart (Yelbuz et al., 2003). Ablation of NCCs disrupts this process of elongation, resulting in a shorter outflow tract and anomalies such as DORV and tetralogy of Fallot (Waldo et al., 2005; Yelbuz et al., 2002). A DORV with a short outflow tract was identified in 57% of the malformed hearts from the embryos in which NCCs were exposed to elevated glucose. We postulate that the effect of elevated glucose on the NCCs hampers them in the stage of cross-talk with the SHF, thus resulting in the development of DORV with a shortened outflow tract as wells as relative mild VSD. After recovery from the glucose-induced oxidative stress, we believe that the NCCs can still play a role in the septation and muscularization of the septum and remodeling of the PAA.

When we added the antioxidant NAC to the D-glucose solution, the number of cases with CHDs in the injected embryos reduced to 27%, a level not statistically different from the number of malformations identified in the control embryos injected with L-glucose. This suggests that glucose excess causes oxidative stress in our chicken model and that this is the major mechanism for the induction of cardiac malformations. We believe that this is due to the enhanced sensitivity of NCCs to oxidative stress; NCCs have a decreased level of both radical scavenger enzymes superoxide dismutase and catalase (Davis et al., 1990). In two closely related Sprague-Dawley derived rat strains, the U-strain, which is sensitive for development of CHDs, has lower catalase gene expression and enzymatic activity compared to the related H-strain that does not show an increase in CHDs after induced diabetes (Cederberg and Eriksson, 1997). When glucose causes CHDs by increasing oxidative stress, its effects should be diminished by the addition of antioxidants. Supplementation of antioxidants, such as vitamin C/E or folic acid, to pregnant diabetic U-strain rats via the food or water has indeed been proven to decrease the number and severity of CHDs in the offspring (Cederberg et al., 2001; Siman et al., 2000; Siman and Eriksson, 1997; Wentzel et al., 2005). This indicates that the glucose-induced increase in oxidative stress plays an important role in diabetes-induced CHD. Excessive glucose metabolism causes a decrease in the expression of PAX-3 in NCCs of mouse embryos (Loeken et al., 1995). Mutations in this NCC-specific transcription factor cause malformations of the outflow tract of the heart comparable to those found in our NCC-exposed embryos (Epstein et al., 2000). The effect of the excessive glucose metabolism on the expression of PAX-3 can be prevented with the antioxidant vitamin E, again indicating the vulnerability of NCCs to a glucose-induced increase in oxidative stress (Phelan et al., 1997).

We observed the detachment of endothelial cells in a number of embryos of all groups studied. As we could

not find a direct relation with the damaging D-glucose injections or diminished endothelial damage after the D-glucose/NAC injection, we doubt whether this phenomenon was an essential pathological phenomenon. We have given specific attention to this aspect as endothelial detachment was also observed in the homocysteine injected neural tissues (Boot et al., 2003b). In those cases, damage in extracellular matrix composition of the vessel wall was observed in which both fibronectin and fibrillin-2 were decreased. In our glucose-injected embryos, this decrease was not observed which might imply that glucose and homocysteine have their own specific pathways in the induction of vascular pathology.

In conclusion, we have shown in our model that there is a direct influence of glucose on NCCs, which results in CHDs. The malformations observed in our chicken model are comparable to those found in other animal models and, more importantly, in the offspring of diabetic women. The fact that even a single exposure leads to CHDs in the majority of the chick embryos underscores the importance of the NCC in diabetes-induced congenital malformations. It cannot be ruled out that diabetic women with a well-balanced glucose metabolism might suffer an incidental high glucose peak during early pregnancy. It is therefore of importance that the cardiac abnormalities we have detected, really relate to NCC-associated anomalies. The role of oxidative stress in this respect opens up new awareness for therapeutic and preventive strategies.

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