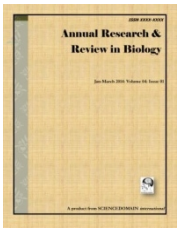

Annual Research & Review in Biology
4(1): 71-78, 2014

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Fetal Myocardial Reduction in Hyperglycemic Mouse Pregnancy is Associated with Dys-regulated Expression of the Anti-apoptotic Gene Bcl-2 in the Developing Heart. Preliminary Results

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JCG and PN designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MRP and SDH managed the analyses of the study and reviewed the manuscript. All authors read and approved the final manuscript.

Research Article

Received 31st May 2013
Accepted 25th August 2013
Published 4th October 2013

ABSTRACT

Aims: We previously detected significant late-gestation ventricular myocardial reduction in fetal mouse hearts from hyperglycemic dams. Flow cytometric analysis of the myocardial cells showed an enhanced rate of apoptosis, suggesting dysregulated cell death as a mechanism associated with the myocardial defect. The present study therefore examined expression of the anti-apoptotic gene Bcl-2 in fetal myocardium from hyperglycemic mouse

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dams on days 14 and 17 of gestation.
Methodology: The hyperglycemia was induced in female Rockefeller (inbred CD1) mice, 6 to 8 weeks old, by pre-breeding streptozotocin (STZ) injection.
Results: Expression of Bcl-2 in the fetal myocardium from the diabetic dams was decreased by 53% and 51% at GD14 and 17, respectively.
Conclusion: These results suggest maternal hyperglycemia may damage the developing myocardium by altering expression of gene pathways that regulate cell death.

Keywords: Diabetes mellitus; apoptosis; Bcl2; heart defects.

1. INTRODUCTION

Pregnancy may be complicated by maternal diabetes mellitus under three different scenarios: type 1 diabetes (insulin dependent diabetes) where the mother produces vastly inadequate insulin; type 2 diabetes (insulin resistant diabetes) which is the most common form of diabetes (90 – 95 % of all diagnosed cases) and in which insulin levels may be moderately reduced to normal; and gestational diabetes mellitus (GDM) which is also insulin resistant, probably a variety of type 2 diabetes, and in which the mother develops hyperglycemia in late gestation. For type 1 and type 2 diabetes, the embryo experiences hyperglycemia from the first stages of development. With GDM, the pregnancy is affected by hyperglycemia only during advanced gestation, when the fetus is rapidly growing [1,2]. Under any of the scenarios above, the embryo/fetus may suffer harmful effects of hyperglycemia.

Cardiovascular complication is one of the most common causes of morbidity and mortality in adult diabetic humans [3]. Maternal hyperglycemia is also an inducer of birth defects that include a high incidence of cardiovascular malformations, however little is known about mechanisms underlying the heart damage. Our laboratory detected ventricular chamber dilation and myocardial reduction in late gestation fetal mouse hearts, collected from dams in which pre-breeding diabetes was induced by streptozocin (STZ) [4]. Subsequent flow cytometric studies showed a significant increase in early apoptotic myocardial cells and in late apoptotic/necrotic cells in the late gestation fetus, compared to fetuses from non-diabetic control mice [5]. Mice in the present experiments were identically treated with vehicle or STZ, and expression of the anti-apoptotic gene Bcl-2 was evaluated in fetal myocardium on GD 14 and 17.

2. MATERIALS AND METHODS

Female Rockefeller mice, an inbred CD-1 strain, 6 to 8 weeks of age were obtained from Immunology Institute, Austral University of Chile, Valdivia, Chile. Experiments were performed in the Pharmacology and Morphophysiology Institute of Austral University of Chile. All experiments were reviewed and approved by the Bio-ethic committee of Austral University of Chile prior to initiation. Mice were housed 5 per cage for a 1-week acclimation period. Mice were fed a standard rodent diet and tap water was provided ad libitum. Mice were maintained under controlled conditions of temperature (22ºC), humidity (40-60 %) and lighting (12/12 hour light/dark cycle). For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated as day 0 of gestation (GD 0).

2.1 Study Design

Mice were divided prior to breeding into two treatment groups in a randomized complete block design. Dosing occurred as previously described [5] and as shown in Fig. 1.

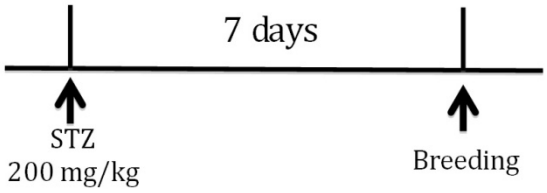

Fig. 1. Timeline for treatments

Blood glucose (BG) levels in tail vein blood were determined every 3-5 days post-STZ administration using an Accuchek active blood glucose glucometer (Roche Applied Sciences, Indianapolis, IN). Females displaying BG levels ≥ 250 mg/dl were considered hyperglycemic (females with lower levels of hyperglycemia became normal after some days).

2.2 Fetal Body Weights and Lengths

Pregnant dams were euthanized on GD 14 and GD 17 by cervical dislocation. Fetuses were collected by cesarean section, dried, and weighed individually. Fetal length was determined using NIH software Image J, designed for morphometric analysis [4]. Fig. 2 shows a fetus imaged for measuring.

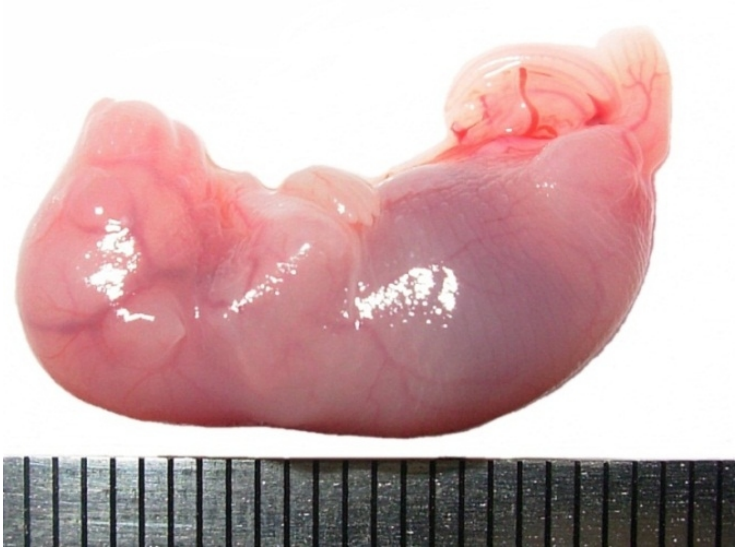

Fig. 2. Determination of fetal length by image J in a 17 day-old control fetus. Scale lines: 1 mm between lines

2.3 Determination of the Expression of the Gene Bcl2

Fetal hearts were collected by micro-dissection under sterile conditions using a Stereomicroscope. The left and right auricles, as well as the great vessels were removed, after which ventricles were pooled by dam (i.e., the pregnant dam was maintained as the statistical unit). The pooled ventricles were placed in saline sterile solution (1 – 2 min), and then transferred to RNA later and stored to – 20ºC for later analysis. Samples were analyzed by RT-PCR for quantification of gene Bcl2, as follows. The RNeasy fibrous tissue mini kit was used to extract RNA (Qiagen,

Valencia, CA). Hearts were removed from RNA later and weighed to achieve 20–30 mg of tissue, which equated to 6–7 hearts per litter. Tissue was placed in a collection tube and 400 uL of B-mercaptoethanol 1 RLT buffer were added. Hearts were immediately homogenized for 1 min in the collection tube using a tissue disruptor (Qiagen).

RNase free water and 10 ul of proteinase K were added to the mix to facilitate disruption of the heart tissue. Samples were then incubated with DNase I for 15 min at room temperature for RNA extraction. RNA quality and quantity were read using a Biophotometer (Eppendorf, Westbury, NY), after which the script cDNA synthesis kit from Bio-Rad (Hercules, CA) was used with 1 ug of RNA from each sample. Primers were designed using software Beacon designer and sequences submitted to Invitrogen (Carlsbad, CA) for elaboration. Two primers were used: CCT TGG CGT GTC TCT CTG and TCC TGT GAT TCT CCC TTC. Samples were analyzed by real time polymerase chain reaction (RT-PCR) for quantification of Bcl-2 using the SYBRgreen supermix from Bio-Rad (Hercules, CA).

2.4 Statistical Analysis

JMP software from the SAS family was used to run a T test, to detect differences between groups ($p < .05$).

3. RESULTS AND DISCUSSION

Twenty-three mice were dosed with STZ and became significantly hyperglycemic. These mice were bred overnight as described, producing 6 pregnant females (26%). This approximate pregnancy rate is normal for mice showing moderate to severe hyperglycemia [4] (Table 1). Fetuses from diabetic dams were smaller by length and weight at GD 14, but not at GD 17. Fetal number per litter was also not different between groups (Table 2). Fetal myocardial Bcl-2 expression was reduced to 53% of control at GD 14 and to 51% of control at GD 17 (Figs. 3-4). Externally-visible birth defects (e.g., excencephaly, other neural tube defects, craniofacial malformation and tail alterations) were increased in the diabetic pregnancies, again as previously seen (Fig. 5).

Table 1. Maternal parameters and pregnancy outcome

	Control	STZ
Number of females bred	6	23
BG at breeding	112±18.5	375.6±88*
% pregnant	100	26.1*
% fetuses with external defects	0	21.7*
Number of litters with defects	0	6*

*BG: Blood Glucose Levels. *significant difference between groups, p < 0.05. Data is presented as Mean ± SD.*

Table 2. Fetal parameters at GD 14 and 17

	GD 14		GD 17	
	Control	STZ	Control	STZ
Number of females	3	3	3	3
Fetuses/litter	10.3±0.6	9.0±1.7	10.3±2.9	6.6±0.6
Fetal weight (g)	0.3±0.01	0.2±0.05*	1.1±0.1	0.9±0.3
Fetal length (mm)	13.2±0.4	12.3±0.4	20.4±1.0	20.7±2.8
External defects	0 (0%)	6 (22%)*	0 (0%)	4 (21%)*
Litters with defects	0 (3%)	3 (100%)*	0 (0%)	3 (100%)*

**Significant difference between groups, p < 0.05. Data is presented as Mean ± SD.*

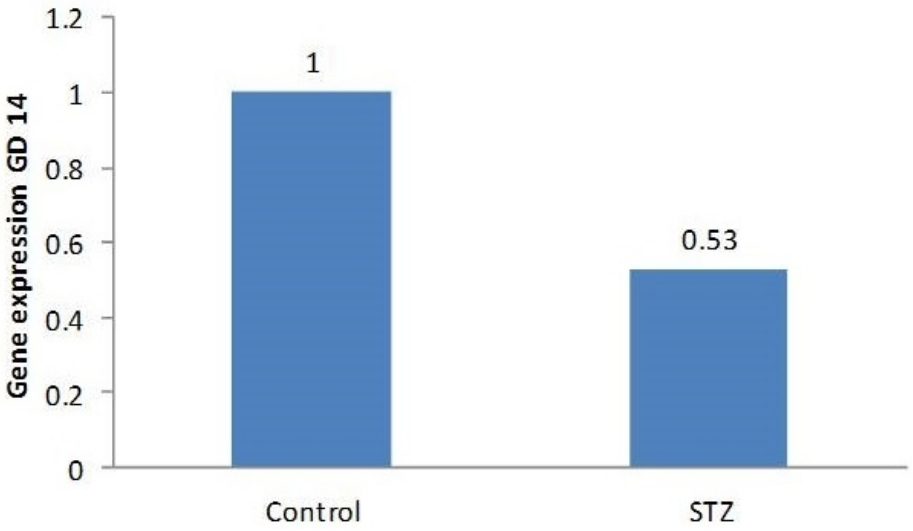

Fig. 3. Bcl2 myocardial gene expression levels determined by ΔΔCt, at GD 14

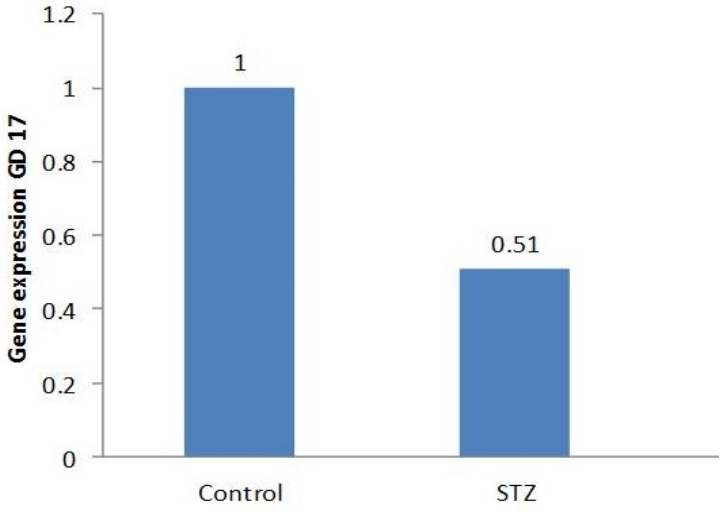

Fig. 4. Bcl2 myocardial gene expression levels determined by ΔΔCt, at GD 17

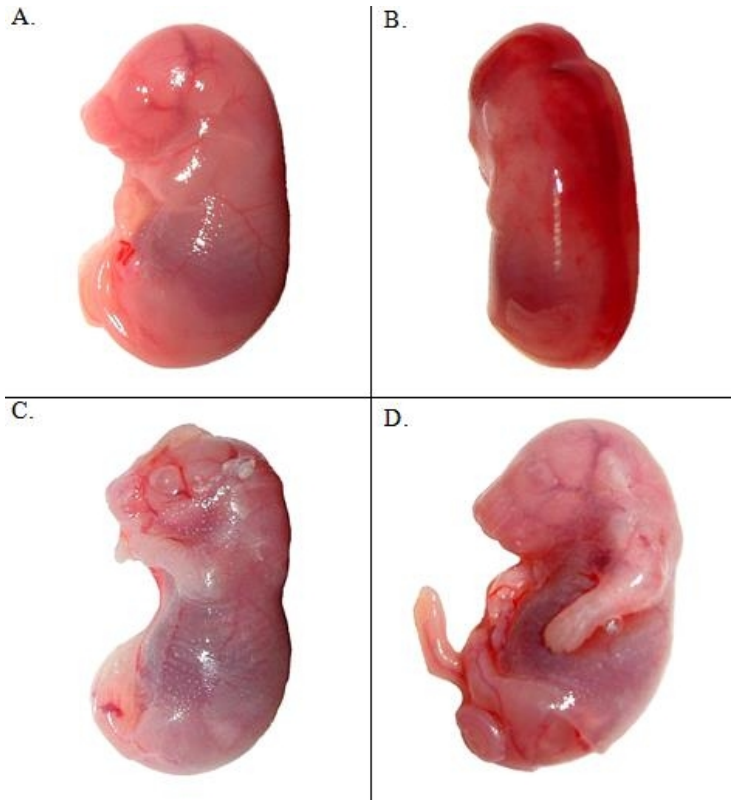

Fig. 5. External birth defects. A: Control. B: Excencephaly and spina bífida. C: Facial malformation. D: Tail alteration

Available data in the literature are limited but support the idea that hyperglycemia may increase adult heart myocardial apoptosis. Fiordaliso et al. [6] reported activation of p53 and p53-regulated pro-apoptotic genes, as well as increased cell death in murine myocardial cells. In humans, Frustaci et al. [7] detected increased apoptosis and necrosis in myocytes, endothelial cells and fibroblasts of adult hearts of diabetic and diabetic/hypertensive patients. Previous flow cytometric results in our laboratory supported a hypothesis for hyperglycemia-related dysregulated apoptosis in the fetal mouse heart [5]. The present fetal hearts from hyperglycemic dams displayed down-regulation of the anti-apoptotic gene Bcl-2 at both GD 14 and 17. Such down-regulation of Bcl-2 is consistent with the observed elevated myocardial apoptosis, however is also different from related previous observations. Schaffer et al. [8] exposed neonatal Wistar rat cardyomyocites for 3 days to 25 mM/L hyperglycemic media, and detected significantly increased Bcl2 gene expression. In those studies, two pro-apoptotic factors, Bax and Bad remained unaltered. Ricci et al. (2008) [9] similarly found that myocytes from newborn and adult rats over-expressed Bcl2 and Akt factor under chronic exposure to a highly hyperglycemic media. In a previous report in our lab, we detected up-regulation of the gene Bcl-2 in the fetal heart at GD17 under maternal diabetes. In that report, an outbred CD1 strain was used [5]. The expression of the gene Bcl-2 may suffer variations during the diabetic pregnancy.

4. CONCLUSION

The finding of down-regulated Bcl-2 in the present mouse model, where myocardial damage and increased apoptotic cells have been documented, suggests the need for additional experiments that analyze a broader panel of apoptotic regulatory genes in fetal myocardial cells in the diabetic pregnancy. Candidate genes include Bax, Bad, Akt, caspase 3, caspase 9 and p53 and ideally should be extended to also examine earlier windows in fetal heart development.

ACKNOWLEDGEMENTS

We gratefully thank the Center for Science and Global Sustainability (Virginia Tech - UACh) for financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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