Reproductive Toxicology 46 (2014) 129–136

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Hyperglycemia induces embryopathy, even in the absence of systemic maternal diabetes: An *in vivo* test of the fuel mediated teratogenesis hypothesis

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ARTICLE INFO

Article history:
Received 29 April 2013
Received in revised form 31 March 2014
Accepted 31 March 2014
Available online 8 April 2014

Keywords:
Embryopathy
Teratogenesis
Hyperglycemia
Diabetes
Fuel-mediated teratogenesis
Animal model

ABSTRACT

Embryonic exposure to excess circulating fuels is proposed to underlie diabetic embryopathy. To isolate the effects of hyperglycemia from the many systemic anomalies of diabetes, we infused 4 mg/min glucose into the left uterine artery of non-diabetic pregnant rats on gestation days (GD) 7–9. Right-sided embryos and dams exhibited no glucose elevation. Embryos were assessed on GD13, comparing the left *versus* right uterine horns. Hyperglycemic exposure increased rates of embryopathy, resorptions, and worsened embryopathy severity. By contrast, saline infusion did not affect any of these parameters. To assess for possible embryopathy susceptibility bias between uterine horns, separate dams were given retinoic acid (25 mg/kg, a mildly embryopathic dose) systemically on GD7.5. The resultant embryopathy rates were equivalent between uterine horns. We conclude that hyperglycemia, even in the absence of systemic maternal diabetes, is sufficient to produce *in vivo* embryopathy during organogenesis.

1. Introduction

Diabetes mellitus exposes the embryo to a myriad of maternal insults including hyperglycemia, altered lipid levels, and inflammation. The effects of diabetes during pregnancy include devastating consequences to the developing embryo. Seven to twenty percent of women with pregestational diabetes experience adverse pregnancy outcomes, including spontaneous abortions, stillbirths, neonatal death and congenital malformations [1–3]. With improved care of diabetes before and during pregnancy and of infants born to diabetic mothers, perinatal mortality has declined. However congenital malformations have emerged as the leading cause of death in this population of infants [4,5]. Infants born to mothers with pregestational diabetes are eight times more likely to have major malformations than those born to mothers without diabetes [6]. Associated anomalies include brain and neural tube defects, caudal regression syndrome, skeletal dysplasia, congenital heart defects, gastrointestinal and genitourinary tract anomalies [5,7]. Multiple birth defects are also more common [7]. Significant progress has been made understanding the fetal-sided molecular mechanisms of diabetes-induced teratogenesis [8], which include oxidative stress [9,10], reduced Pax3 expression [11], and PKC activation [12].

The fuel mediated teratogenesis hypothesis states that maternal-diabetes induced embryopathy is caused by exposure of the embryo to excess circulating maternal energy fuels [13]. These exposures include excesses of glucose, ketones, fatty acids, triglycerides and variably glycerol [14,15]. Because improved glycemic control is associated with a lower incidence of pregnancy loss and congenital malformations [5,16–18], glucose is generally implicated as the major teratogen. *In vitro*, embryos cultured in defined media have supported this hypothesis for excesses of glucose [19,20] and/or ketones [21–23]. *In vivo*, glucose administration at hyperglycemia-inducing doses is sufficient to induce embryopathy [24]. Because systemic hyperglycemia induced by glucose administration produces a myriad of other acute systemic aberrations including altered circulating fuels, systemic oxidative stress, and inflammation [25–30], it is uncertain whether the consequences are related to localized effects of hyperglycemia on the embryo and/or downstream systemic maternal effects of hyperglycemia. It thus could be the case that localized hyperglycemia is insufficient to induce embryopathy *in vivo*, lacking required synergy with other perturbations of diabetes and systemic hyperglycemia [23].

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http://dx.doi.org/10.1016/j.reprotox.2014.03.013
0890-6238/

The objective of this study was to determine whether hyperglycemia local to the developing embryo is sufficient to induce embryopathy *in vivo*, even in the absence of maternal diabetes. To meet this objective, we utilized a recently developed rat model that exposes select embryos to isolated hyperglycemia without maternal systemic hyperglycemia [31]. Our approach takes advantage of the bicornate nature of the rat uterus whereby each horn has a distinct blood supply. Glucose is infused directly into the left uterine artery circulation, producing hyperglycemia in the blood supply of that uterine horn alone, while the mother and contralateral uterine horn experience no significant elevation of blood glucose [31]. Here, we report use of this approach to test the fuel mediated teratogenesis hypothesis *in vivo*, finding that hyperglycemia in the left uterine artery during organogenesis is sufficient to induce embryopathy even in the absence of maternal diabetes.

2. Materials and methods

2.1. Animals and breeding

All procedures were performed within the regulations of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Iowa Institutional Animal Care and Use Committee. Hsd:Sprague-Dawley SD rats (Harlan Laboratories, Inc., Indianapolis, IN) were housed in a temperature controlled, 12 h light-dark cycled animal care facility with free access to water and conventional diet. Rats were bred and checked daily. Gestational day (GD) 0 was defined as the morning of a positive vaginal swab for the presence of spermatozoa.

2.2. Catheter placement

On GD1, a vascular catheter was inserted into the left femoral artery and secured in place just proximal to the left uterine blood supply as previously described [31]. Catheterization was performed at this point in gestation to allow stabilization of uterine blood flow prior to implantation, which typically occurs on GD 4 or 5. The catheter was kept patent utilizing an indwelling solution of 0.3 ml of 500 U/ml heparin in autoclaved glycerol. The distal end of the catheter was tunneled subcutaneously to the interscapular region for subsequent access.

2.3. Glucose infusion

On GD7, the distal end of the catheter was isolated from the interscapular region under brief isoflurane-oxygen anesthesia and connected to a single channel swivel (Instech Laboratories Inc., Plymouth Meeting, PA), affording the animal free movement about the cage. Glucose (20% dextrose in normal saline containing 5 U/ml heparin) was infused at 4 mg/min (20 μl/min) for 48 h on GD7-9 (Fig. 1A). Glucose infusion at this rate produces marked diabetic-level hyperglycemia in the uterine artery while there is no significant increase in maternal systemic or right uterine horn blood glucose [31]. Non-fasting, whole blood glucose concentrations were measured by tail nick sampling with a One Touch Ultra meter (LifeScan Inc., Milpitas, CA) at GD1, 7, 9 and 13. As a control for the effects of the catheter presence and infusion, some mothers underwent an identical protocol infusing normal saline containing 5 U/ml heparin rather than glucose on GD7-9.

2.4. Retinoic acid

To assess for possible lateralizing embryopathy predilections, we utilized a mildly embryopathic dose of the teratogen retinoic acid (RA) administered systemically [32]. On GD 7.5, select dams received 25 mg/kg of RA intraperitoneally. RA (Sigma–Aldrich Inc., St. Louis) was prepared immediately before administration in a light protected manner by dissolution in a minimal amount of dimethyl-sulfoxide followed by suspension in medium chain triglyceride or peanut oil to a concentration of 2 mg/ml. A portion of the retinoic acid treated dams also underwent left uterine artery catheter placement on GD1 with or without infusion of saline containing 5 U/ml heparin on GD7-9 to assure that catheter infusion, alongside a mild systemic teratogen, did not increase embryopathy on one side *versus* the other.

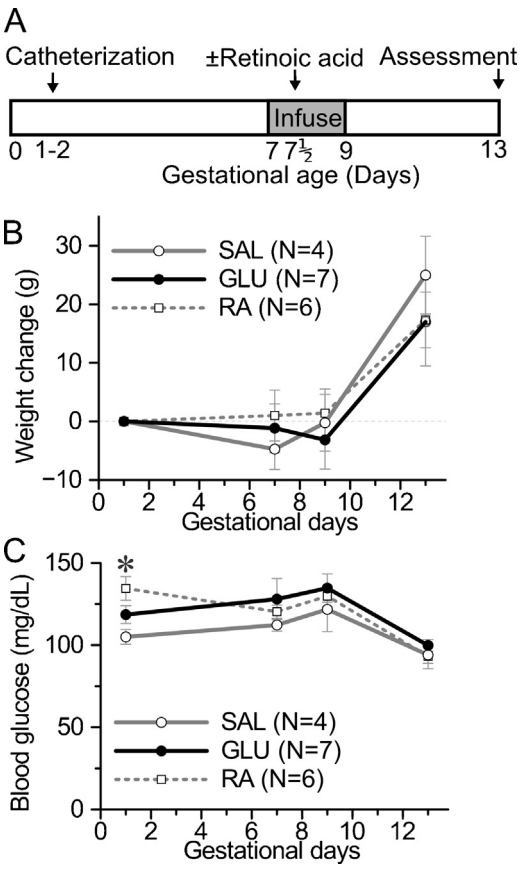

Fig. 1. Experimental design and maternal characteristics by experimental group. (A) Timeline: a catheter infusing into the left uterine artery was placed on gestational day 1. (B and C) There were three experimental groups of dams: glucose (GLU, black line, filled circle), saline (SAL, gray line, open circle), and retinoic acid (RA, dotted line, open square). GLU and SAL dams received glucose (4 mg/min) or normal saline, respectively, infused *via* the catheter on gestational days 7–9. Retinoic acid (25 mg/kg) was administered to RA dams intraperitoneally on gestational day 7.5. A portion of RA dams also received a catheter with or without saline infusion. Embryos were assessed on gestational day 13 (GLU and RA) or 14 (SAL). (B) Maternal weight change from baseline (gestational day 1) by experimental group. (C) Maternal systemic blood glucose by experimental group. The day 9 blood glucose was measured prior to discontinuing infusions. *$p < 0.05$ for difference between RA and SAL mothers by ANOVA and Tukey's HSD post-doc analysis. Note that at day 1 the three groups had not yet received differing treatments. There were no other statistical differences between groups.

2.5. Outcome measures

Embryos were accessed by terminal cesarean section on GD13 (glucose and RA dams) or GD14 (saline dams). Rats completely lacking any viable embryos were not considered. The physical location of the embryos was recorded, being from the left or the right uterine horn and numbered consecutively by proximity to the cervix. Embryos were collected, within the amniotic-sac when intact, and placed in 10% neutral buffered formalin. Embryos were carefully