Synchronization of the factors critical for diabetic teratogenesis: An in vitro model

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OBJECTIVE: Our goal was to determine the relationship between critical factors and conditions such as gestational age and exposure time to elevated glucose levels in diabetic embryopathy.

STUDY DESIGN: A postimplantation rat embryo culture was used as a model for investigation. The effect of various factors on embryonic development was studied. Experiments were conducted with increasing glucose concentrations (150 to 905 mg/dl, n = 186), at various gestational ages (10 to 12 days, n = 169), and for varying durations of exposure (30 to 180 minutes, n = 169). Gross morphologic characteristics of the yolk sac and embryo were assessed.

RESULTS: Embryopathy was induced by hyperglycemia in a dose-related fashion: a 20% rate at two times control glucose concentration, almost a 50% rate at four times control, and approximately a 100% abnormality rate at more than six times control. A critical window in gestational age, days 10 to 11, and a minimum exposure time to hyperglycemia of 2 hours were necessary to induce teratogenesis.

CONCLUSIONS: Diabetic teratogenesis occurs in a dose-related fashion and requires a minimum exposure time and critical gestational age. Only synchronization of these critical conditions induces embryonic maldevelopment. Furthermore, nonsynchronized aberrant conditions may result in apparently normal embryonic development. (AM J OBSTET GYNECOL 1996;174:1284-8.)

Key words: Diabetic embryopathy, rat embryo culture

Despite improved management of diabetic pregnancies, congenital anomalies are still a major cause of perinatal morbidity and mortality among the offspring of diabetic mothers. The frequency of major congenital anomalies among infants of diabetic mothers has been estimated at 6% to 10%, which represents a twofold to fivefold increase over the nondiabetic population.1-3

Although many etiologic factors have been proposed regarding the mechanism of diabetes-related malformations, our knowledge of the exact cause is still limited and unclear. However, at present, altered metabolic control associated with hyperglycemia during organogenesis is considered a teratogen.1-5

Embryonic malformations similar to those described in humans have been reported in animals in which a diabetic state has been induced chemically by streptozocin.6-8 Although clinical and experimental studies suggest that perturbations in metabolic control may be causative in diabetic malformations, embryopathy does not always occur under hyperglycemic conditions.

The purpose of this study was to determine critical conditions associated with diabetic embryopathy. We used an in vitro postimplantation rodent conceptus culture model to investigate the teratogenic effects of different levels of hyperglycemia at various gestational ages with varying durations of exposure to hyperglycemia.

Material and methods

Sprague-Dawley rats (CD strain, Charles River, Mass.) were used for the study. Rats were mated overnight and vaginal smears were examined the next morning. If spermatozoa were present in the vagina, pregnancy day 0 was determined. Rat embryos were removed from the uterus on pregnancy day 10. Culture day 12 is equivalent to conception day 11.5. The conceptuses were explanted, and cultures were performed as previously described.9-11 The embryos were maintained in polypropylene tubes in a roller incubator at 38°C. Each tube contained 5 to 6 embryos in 5 milliliters of culture medium consisting of 80% (vol/vol) rat serum and 20% (vol/vol) 0.9% (wt/vol) NaCl. Serum was collected from adult male rats, heat inactivated, and treated with penicillin and streptomycin as described previously.7-8 The embryonic chorion and parietal yolk sac were removed, exposing a neurula stage embryo and visceral yolk sac. The conceptuses were transferred to petri dishes containing 0.9% (wt/vol) NaCl and viewed under the dissecting microscope for morphology.

Three sets of studies were performed under the conditions described here.
Control conceptuses. Ninety-six rat conceptuses were cultured for 48 hours in heat-inactivated male rat serum medium only (mean glucose concentration 150 mg/dl).

Increasing doses of hyperglycemic medium. One hundred eighty-six rat conceptuses were cultured in glucose concentrations of 300, 600 and 950 mg/ml, representing two, four, and six times the normal glucose concentration (mean 150 mg/dl) for 48 hours.

Varying gestational ages and duration of exposure. One hundred sixty-eight conceptuses were exposed to a hyperglycemic medium (950 mg/dl) at different gestational ages (days 10 through 12) and for varying durations (0.5 hour, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 6 hours, 12 hours, and 24 hours) to assess the critical susceptible gestational age and the minimum exposure time required to induce anomalies. The osmolality of the control and hyperglycemic sera was measured at the start and end of the culture period.

Conceptuses were washed with normal saline solution, and gross morphologic features of the yolk sac and embryo were assessed. The embryos were categorized as morphologically normal or showing evidence of frank malformations with a systematic checklist approach and two independent evaluators. Normal embryos exhibited an entirely normal body flexure and closure of both anterior and posterior neural pores. Results were dichotomized into “malformed” and “normal” for statistical analysis. The significance of differences between the groups was evaluated by $\chi^2$ analysis.

Results

Malformations were induced in a dose-related fashion (Fig. 1). At a glucose concentration of 300 mg/dl about 20% of the embryos demonstrated malformations; at a concentration of 600 mg/dl almost 50% of the conceptuses were malformed; and at a concentration of 950 mg/dl a malformation rate of approximately 100% was observed. The malformation rate in the control group was 3.5%. These differences were statistically significant ($p < 0.05$).

Exposure of conceptuses to serum containing 950 mg/dl of D-glucose resulted in a significant reduction in conceptus (embryo and yolk sac) size. In addition, embryos demonstrated gross anomalies such as open neural tube defects, microcephaly, dorsal tail flexion, heart defects, and pericardial effusion (Fig. 2).

A minimum exposure to hyperglycemia of 2 hours was required to induce malformations. If exposure to the 100% teratogenic dose of hyperglycemia (950 mg/dl) was initiated on day 10, the malformations were primarily defects of the neural tube; on day 11 they were principally cardiac defects; and beyond day 11.5 no defects were induced (Fig. 3). Exposure to a 100% teratogenic dose for 0.5, 1.0, or 1.5 hours did not result in an increased malformation rate as compared with controls. However, exposures of 2.0, 2.5, and 3.0 hours or more resulted in a 100% malformation rate (Fig. 4).

Comment

Although clinical and experimental studies suggest that perturbations in metabolic control may be causative in diabetic teratogenesis, embryopathy does not always occur even under the aforementioned conditions. In an attempt to determine critical factors and conditions for diabetic teratogenesis, we used the postimplantation rat embryo culture as a model for investigation. Malformations were induced in a dose-related fashion—a 20% rate at two times control glucose concentration, a 50% rate at
Fig. 2. Exposure of conceptus to serum media containing 950 mg/dl d-glucose. A, Rat embryo with neural tube defect outlined by arrows. B, Rat embryo with heart defect (1), pericardial effusion (2), neural tube defect (3), and dorsal tail flexion (4).

four times control, and a 100% rate at six times control. A 100% malformation rate, however, has never been reported in humans, even with extremely high (15 SD above the mean) glycosylated hemoglobin levels. This difference may be attributed to the fact that higher glucose levels can be achieved in vitro or, perhaps, that other coexisting conditions are necessary to induce these malformations.

Various human studies have also suggested a relationship between metabolic control and malformations. Elevated first-trimester glycosylated hemoglobin levels are associated with an increased occurrence of birth defects. Measurement of hemoglobin A₁c provides a retrospective index of glycemic control over the previous 4 to 8 weeks, and therefore first-trimester levels are believed to reflect the degree of metabolic control during organogenesis. It is within the first 5 or 6 weeks of the human pregnancy (equivalent to days 10 through 12 in rats) that morphogenesis and organogenesis occur. Greene et al. examined the relationship between the level of hemoglobin A₁c in the first trimester and major malformations and spontaneous abortions in 303 insulin-requiring diabetic gravid women. They found the risk of major malformations was 3.0% with hemoglobin A₁c ≤6 SDs above the mean and 40% for levels >15 SDs above the mean. Although they found the risk for adverse pregnancy outcomes was markedly elevated for women in poor metabolic control, there was a wide range of glycemia over which the risks were not substantially elevated. Indeed, we have demonstrated that a critical window (days 10 and 11) of exposure to the presence of hyperglycemia is needed to induce these malformations. Moreover, a minimum exposure time is also needed to induce malformations. Exposure times of <2 hours did not result in malformations. Conversely, exposure times >2 hours did result in malformation of conceptuses when the potent teratogenic concentrations of hyperglycemia were used.

Therefore our data appear to support the clinical observation that a number of synchronized conditions and events are necessary to induce congenital malformations. Significant elevations in glycosylated hemoglobin levels generally represent prolonged periods of significant hyperglycemia, whereas more modest elevations most likely reflect shorter and more transient periods of hyperglycemia. Therefore one can assume that the glucose level, the duration of hyperglycemia, and the embryonic gestational age are all critical in inducing embryopathy. In fact, the clinical data of various investigators have demonstrated that most major malformations are associated with significant elevations in first-trimester glycosylated hemoglobin levels, representing a critical threshold for the induction of malformations.

There are also reports indicating that several intermediary metabolites, such as lactate, pyruvate, ketone bodies, and branch chain amino acids, show increased serum concentrations in poorly controlled diabetes. The complimentary assumption that glucose may not be the sole teratogenic factor involved in diabetic embryopathy has also gained experimental support. The synergistic and additive effects of fuel interactions have been examined in numerous studies. Exposure to combinations of β-hydroxybutyrate, hyperglycemia, and somatomedin inhibitors were shown by Sadler et al. to have a synergistic effect in causing malformations in rat embryo cultures. Therefore one can speculate that a possible synergistic effect may also exist in humans between glucose and other fuel metabolites and may increase the likelihood of malformations.

In addition to the biochemical factors involved in the etiology of diabetic embryopathy, genetic susceptibility may also play a role. Eriksson demonstrated the inter-
play between genetic background and alterations in fuel metabolism in the induction of diabetes-associated malformations. Using a rat model he compared the outcome of diabetic pregnancy in two substrains of Sprague-Dawley rats. One strain had a low incidence of skeletal malformations (H) in its offspring and the other strain (U) had a high incidence. When the strains were mated to form hybrids and then exposed to specific teratogens (glucose and β-hydroxybutyrate) and the offspring were of the mixed H/U type, there was an increase in skeletal malformation rates. These findings suggest that a mixed genetic-environmental cause is responsible for diabetic embryopathy.

In conclusion, in this study we have demonstrated that diabetic teratogenesis requires the synchronization of a number of environmental and developmental conditions or factors. Conversely, apparently normal embryonic development may occur even under severe aberrant conditions if these events are nonsynchronized.

REFERENCES