Effects of Maternal Diabetes on Early Embryogenesis

The Role of Insulin and Insulin Therapy

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SUMMARY

Using the system of whole embryo culture the effects of insulin on early mouse embryogenesis were investigated. Three experimental approaches were employed in which (1) serum from diabetic rats receiving insulin therapy was used as culture medium; (2) insulin was added directly to serum collected from diabetic rats for use as culture medium; (3) insulin was added directly to medium (serum) collected from control rats. Mouse embryos were cultured in each of these media for 24 h and then evaluated for growth and malformations. The results demonstrated that insulin therapy was effective in reducing the incidence of malformations and growth retardation produced by serum from diabetic rats receiving no hormone supplement. However, addition of insulin directly to serum collected from diabetic rats was not effective in reducing the rate of abnormalities produced by this medium. Finally, addition of insulin at extremely high concentrations (10,000 μU/ml) produced no abnormal embryos. Thus, it appears that insulin therapy is successful in reducing malformation rates and that the hormone itself is not teratogenic in this system. DIABETES *32*:1070–1074, November 1983.

Although health care of the pregnant diabetic, including strict monitoring and control of blood glucose levels, has improved dramatically, this approach has had little impact on the incidence of congenital malformations occurring among offspring of these mothers.[1-3] In fact, there has been no improvement in the malformation rate among these infants over the past five decades despite decreasing mortality and morbidity figures over this same period. One explanation for the high number of congenital anomalies is that the susceptible period of teratogenesis in the diabetic is during the 4th–7th wk of gestation,[4] a time when most women may not yet realize they are pregnant. Therefore, establishment of strict diabetic control is often not initiated until this period has passed.

It is difficult to determine causes of these high malformation rates among offspring of diabetics. Glucose imbalance, hyperketonemia, abnormal insulin levels, as well as other factors have been implicated as teratogens.[5-8] Due to the interrelationship of these factors, however, animal models have provided controversial results at best. For example, induction of diabetes in laboratory animals results in an alteration of several metabolic parameters including circulating glucose and insulin levels. Likewise, infusion of glucose or insulin alters more than one metabolic parameter making it virtually impossible to independently monitor each altered factor.[9]

In order to circumvent these difficulties, the technique of whole embryo culture has been employed. In this system rat and mouse embryos develop normally and at rates similar to in vivo growth during the organogenic (teratogenic) period.[10,11] Fortuitously, the morphogenetic events that occur in cultured embryos correspond to those observed in human embryos during the 4th–6th wk of gestation, i.e., the sensitive period in diabetics. Cultured embryos develop in an environment where manipulations of blood sugar, ketone bodies, insulin, and other factors can be made independently. Studies using this approach have clearly shown that hyperglycemia is teratogenic to rat[8] and mouse embryos[12] during this early morphogenetic period, although glucose levels required to induce malformations are very high. Other serum factors such as ketone bodies, which have been shown to be teratogenic at levels achieved during severe diabetic crises in humans,[13,14] and somatomedin-inhibiting factor[15] may play a role as teratogens. Furthermore, serum collected from diabetic rats receiving no insulin therapy produces abnormal morphogenesis in mouse embryos at a rate directly related to the severity of the disease.[16] Finally, insulin teratogenicity, although not evaluated in the embryo culture sys-

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:em, has been documented in chick embryos[7] and has been mplied in mice.[17,18]

These types of studies have begun to delineate embryonic stages susceptible to diabetes-induced teratogenesis, the factors responsible for the malformations, and the levels of altered metabolites necessary to produce a congenital defect. As an extension of these studies the relationship between in vivo insulin therapy and the production of malformations was investigated. Also, the direct effects of hyperinsulinemia on mouse embryogenesis were evaluated. These studies were conducted in the whole embryo culture system using three different approaches: (1) serum from diabetic animals receiving insulin therapy was employed as medium for whole embryo culture and effects on embryonic development were recorded; (2) insulin was added directly to medium (serum) collected from diabetic (hypoinsulinemic) animals receiving no insulin therapy to test the effects of hormone replenishment on morphogenesis in diabetic serum; (3) insulin was added directly to medium (serum) collected from control animals to assess the effects of altered insulin levels on embryonic growth and development.

METHODS

Embryo culture. Mice of ICR strain were mated overnight and the presence of a vaginal plug the next morning served as evidence of mating. This day was termed day 1 of pregnancy. On day 9 of gestation, mothers were killed and the embryos were removed, staged, and prepared for whole embryo culture as described previously.[11] Early somite (2–5) embryos were selected and maintained in culture vials containing 1.5 ml of serum from control or diabetic rats. (Since embryonic growth and development is not affected by strain, age, or sex differences,[19,20] rats were selected by availability only. However, 200-g animals were employed for induction of diabetes because of the ability to maintain more uniform doses of streptozotocin with a resultant consistency of hyperglycemia.) Vials were gassed with 5% O_2, 5% CO_2, and 90% N_2 at 0 and approximately 10 h, placed on a rotator wheel (30 rpm), and maintained at 37°C for 24 h. At termination, each embryo was assessed for malformations and then processed for total protein content using the Lowry method.[21] Morphologic assessments included monitoring neural tube closure, brain vessicle formation, and facial development (formation of the mandible and maxilla). Embryos were categorized as malformed if they demonstrated one or more of these abnormalities regardless of severity. Embryonic circulation and heartbeat as well as visceral yolk sac blood flow were also monitored as a means of determining viability. Embryos having no heartbeat or circulation were classified as failure to thrive (FTT).

Effects of insulin therapy. Diabetes was induced in 200-g male and female Sprague Dawley rats by a single i.p. injection of 75 mg/kg streptozotocin in 0.4 ml of 0.1 M Na citrate buffer, pH 4.5. Three days after streptozotocin treatment, insulin therapy was initiated. In group I, 5 IU of insulin (NPH, U-100 Lilly, Indianapolis, Indiana) was administered subcutaneously on each of the first 2 days. Thereafter, insulin concentrations were adjusted daily based on serum glucose levels [blood was drawn from the tail, collected in a capillary tube, and assayed for glucose on a Beckman Glucose Analyzer (Beckman Instruments, Fullerton, California)]. Treatments were continued for 10 days and every effort was made to maintain strict control of blood glucose levels. In group II, a single subcutaneous injection of insulin was made on the afternoon of each of the next 5 days according to the following dosage: 5, 4, 4, 3, 3 IU. On the morning of the last day of treatment, rats from both groups were ether-anesthetized and blood was drawn from the abdominal aorta. Serum from the animals was then prepared for culture medium as described by Steele and New.[22] Samples were immediately centrifuged (3000 × *g*, 5 min), fibrin clots were squeezed, and the bloods recentrifuged (3000 × *g*, 5 min). Serum was decanted, heat inactivated (59°C, 30 min), and stored frozen at 0°C. Streptomycin sulfate (50 μg/ml) was also added. Samples for use as culture medium were assayed for glucose concentrations and then employed in culture.

Effects of direct insulin addition. As a correlate to insulin therapy studies, a separate group of 200–220-g male Sprague Dawley rats was administered 75 mg/kg streptozotocin i.p. followed by no insulin therapy. Ten days after this injection, these animals were killed and serum collected for use as culture medium as described above. All samples from this group were pooled and, prior to culture, 50–300 μU/ml of insulin was added directly to the serum.

As a means of assessing the teratogenicity of insulin on mammalian embryos, hormone (Na salt of bovine insulin) concentrations ranging from 10 (500 μU/ml) to 250 (10,000 μU/ml) times physiologic levels were added to serum collected from control rats. This serum was then used as medium for maintaining embryos in culture. All embryos were scored for malformations and analyzed for total protein.

A χ^2 analysis was used to analyze the frequency of malformations. The Fisher's exact test was employed to evaluate the significance of the three subgroups receiving insulin therapy. Protein data were subjected to an analysis of variance comparison with the alpha set at 0.005 in order to control the experiment wise error rate.

RESULTS

For the first 24 h of culture in serum collected from control rats, early somite mouse embryos undergo normal growth and development compared with their in vivo counterparts. At the start of culture conceptuses are in the initial stages of organogenesis such that cranial and caudal neural folds are open and no heartbeat or circulation exists. During the next 24 h the neural tube closes (with exception of the posterior neuropore, which normally stays open at this time), otic and optic vessicles appear, somite numbers increase (from 2–5 to 19–20), forelimb bud development is initiated, and a heartbeat with embryonic and visceral yolk sac circulation is established. Total protein content of each embryo also increases from approximately 15 μg at the start of culture to 70–90 μg at 24 h.

Rats treated with 75 mg/kg streptozotocin (i.p.) developed a severe hyperglycemia with glucose levels of 6.11 (SD ± 0.5) mg/ml compared with 1.5 (SD ± 0.2) mg/ml in controls (concentrations recorded at the time of culture). Daily monitoring of serum glucose levels and subsequent adjustment of insulin dosages failed to provide strict control of the diabetic state. Glucose levels fluctuated widely in vir-

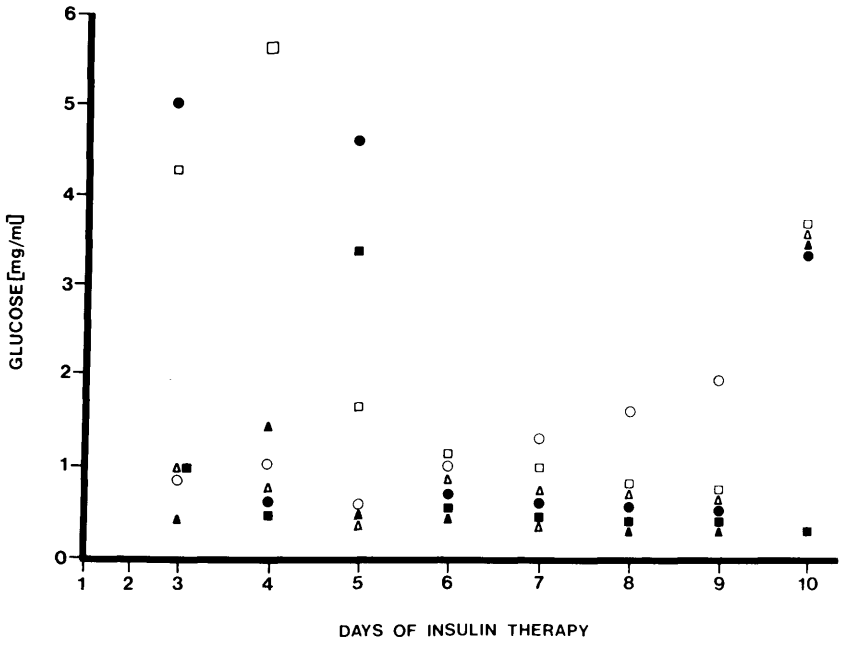

FIGURE 1. Graph of fluctuating glucose levels in diabetic rats (group I) given insulin therapy over a 10-day period. Glucose concentrations are randomly distributed over the first 5 days, then stabilize at or below normal levels, and finally rise dramatically by day 10. (Each symbol represents serum samples from the same animal analyzed at different days.)

tually every animal regardless of the concentration of insulin administered (Figure 1). In most cases glucose levels were erratic during the first 5 days of treatment, stabilized for 1–2 days at or near control levels, then became hypoglycemic, and finally became erratic again. It was therefore decided to employ only a 5-day fixed pattern of insulin dosages (group II) and to kill animals on the morning of day 6.

Insulin therapy in group II animals produced a range of glucose from 0.8 to 6.79 mg/ml. Based on glucose concentrations routinely employed in whole embryo culture,[8,12] these samples were classified as normoglycemic (1.0–2.0 mg/ml), hypoglycemic (<1.0 mg/ml), and hyperglycemic (>2.0 mg/ml) (Table 1). Effects of these samples on embryonic development varied depending on the group being tested. Embryos cultured in normoglycemic samples showed no adverse effect on total protein although 21.7% were malformed. The defects consisted primarily of open neural folds (exencephaly), which in two embryos were only slightly open and may have represented a delay in this morphogenetic process. In the other three a severe separation of the cranial folds existed.

Serum from hypoglycemic animals produced only a slight reduction in total embryonic protein, but induced a high malformation rate consisting of exencephaly, facial hypoplasia, and abnormal rotation. In eight samples from this group there was sufficient serum to culture paired embryos in each sample. Therefore, one embryo for each sample was cultured in the serum as it was collected, while a similar staged embryo was cultured in the same serum, but with the addition of sufficient glucose to bring the final concentration to control levels (1.5 mg/ml). In each case of paired embryos, those cultured in serum containing no additional glucose were all malformed (exencephaly), whereas their counterparts with additional glucose showed a reduced incidence of abnormalities. Total proteins were also increased in samples with additional glucose.

Serum from hyperglycemic animals produced a trend toward decreased growth, but defects in this group were not severe. In one there was a narrowing of the craniofacial region, whereas the others exhibited microcephaly. The two microcephalics were cultured in serum containing the highest glucose levels (6.52 and 6.79 mg/ml).

Addition of insulin directly to serum collected from severely diabetic rats receiving no insulin therapy failed to reverse the high incidence of malformations and low protein values demonstrated previously with this serum. Thus, only 2 of 23 embryos escaped serious deformity and all showed significant protein reductions from controls. Malformations in affected embryos were severe, with inhibition of cranial and caudal neural tube closure, anopthalmia, and hypoplasia of the craniofacial area.

Insulin itself was not teratogenic even at the highest doses tested (10,000 μU/ml). However, embryos grown in the pres-

TABLE 1
Effects of insulin on malformation rates and growth (total protein) in early somite mouse embryos grown in whole embryo culture

Treatment	% Malformed	Protein (μg ± SD)
Control serum	0 (N = 26)	77.0 ± 18.2
Diabetics + insulin therapy		
Normoglycemic serum (1–2.0 mg/ml)	21.7 (N = 23)*	96.4 ± 33.4
Hyperglycemic serum (>2.0 mg/ml)	25.0 (N = 8)†	55.6 ± 9.1
Hypoglycemic serum (<1.0 mg/ml)	83.3 (N = 12)‡	62.8 ± 28.6
Hypoglycemic serum + glucose	37.5 (N = 8)§	88.4 ± 44.2
Diabetics, no insulin therapy		
− direct insulin addition	68.6 (N = 51)‡	42.2 ± 13.0‡
+ direct insulin addition	100 (N = 26)‡	32.4 ± 15.1‡
Control serum + insulin		
750–5000 μU/ml	0 (N = 9)	89.1 ± 18.7
10,000 μU/ml	0 (N = 8)	64.6 ± 9.4

All P values compared with controls.
*P < 0.02; †P < 0.05; ‡P < 0.001; §P < 0.01.

ence of the highest concentrations showed a trend toward decreased growth as evidenced by their lower protein content compared with controls.

DISCUSSION

The present investigation represents an extension of previous efforts in which serum from streptozotocin-induced diabetic animals receiving no insulin therapy was shown to be teratogenic.[16] Glucose levels in serum from these animals ranged from 3.5 to 6.11 mg/ml depending on the dose of streptozotocin. Teratogenicity of the serum was dependent on the severity of the disease and both malformation rates and growth were most severely altered in serum from animals with the highest glucose levels.[16] In the present study, insulin therapy was shown to reduce the malformation rates among the severely diabetic samples and to restore normal growth among severely diabetic animals that were brought under strict control, i.e., normoglycemic. Lack of control of the diabetic state, including hyper- and hypoglycemia continued to produce a high rate of malformations (exencephaly) and also adversely affected total growth in both groups.

Factors responsible for the production of malformations and effects on growth are not clear. For example, even normoglycemic samples resulted in a significant percentage of abnormalities (21.7 versus 0% in controls), which would indicate that something other than glucose is responsible for the defects. One explanation may be that these animals do not remain normoglycemic throughout the 5 days of insulin therapy. Similarly treated animals in which glucose and insulin levels were carefully monitored displayed glucose levels that fluctuated drastically during one or more of the days of therapy. Consequently, other serum factors, such as amino acids, ketone bodies, and free fatty acids, may still be altered and play a role in production of the defects. A similar explanation can be provided for the results with hyperglycemic serum from insulin-treated animals. Malformation rates in this group are lower than in untreated diabetics, but higher than those in normoglycemic group. Furthermore, protein values are lower than controls or embryos cultured in serum from control animals to which excess glucose (0.5 mg/ml) was added.[12] Again these data suggest that factors other than hyperglycemia alone must be operating to affect development and/or growth.

Results with hypoglycemic serum suggest that low glucose levels are also teratogenic in vitro. However, this effect may represent an artifact induced by the system, since embryos continuously draw from a small pool of glucose (1.5 ml of medium) metabolizing as much as 0.5–0.75 mg/ml in a 24-h period (unpublished results). Therefore, if a serum sample starts with low glucose levels, the embryo may exhaust its major fuel supply by the end of culture. Thus, the system does not mimic the in vivo situation as it exists in the diabetic mother where the ratio of serum, i.e., glucose pool, to embryo is much greater than in culture. Additional experiments whereby glucose levels are strictly maintained at constant levels in culture will be necessary to determine the effects of hypoglycemia on the embryo. Interestingly, the addition of glucose to hypoglycemic samples restores total growth, but fails to eliminate the malformations. Again this result suggests that factors other than glucose may be responsible for the altered morphogenesis.

With regard to which factors might adversely affect embryonic development in the diabetic state, it does not appear that insulin itself plays a direct role. In the present study addition of insulin directly to serum collected from untreated severely diabetic animals (blood glucose = 6.11 mg/ml) failed to reduce the malformation rate or the adverse effects of this serum on growth. If anything, the results suggest that insulin may potentiate the effects of this serum on growth and morphogenesis. However, insulin by itself has no teratogenic effects on cultured embryos even at levels 250 times higher than physiologic concentrations. Only at the highest dose was there a slight decrease in growth and even at this concentration no malformations were observed. Thus, the mammalian conceptus appears not to be as sensitive to insulin as the avian embryo.[7] The absence of insulin-induced teratogenesis in culture also suggests that malformations observed after insulin administration to the pregnant dam[18] may be due to the metabolic sequelae such a treatment produces and are not a result of direct effects of the hormone.

Other factors present in serum from diabetics that may have an effect on embryogenesis include ketone bodies (B-hydroxybutyrate and acetoacetate), amino acids, and free fatty acids. Indeed, one recent report indicates that the ketone body B-hydroxybutyrate has teratogenic potential and produces neural tube defects in mouse embryos exposed in vitro.[13,14] Since serum from untreated diabetic rats is known to have elevated levels of this compound[23] it is reasonable to suggest that it may play a role in the high incidence of defects produced by this serum. Little is known about the teratogenic effects of amino acids (branched chain) and free fatty acids, although both are elevated in the uncontrolled diabetic (ref. 23, unpublished results).

Results from the present study are consistent with those obtained by other investigators using in vivo and in vitro models to show that insulin therapy reduces the risk of congenital defects in the offspring from diabetics.[15,24–26] Malformation rates are slightly higher in this study than reported by others, but in most cases in vivo malformation rates were based on examination of term fetuses and did not include resorptions and dead conceptuses. In the present investigation embryos were examined early in gestation during the period of organogenesis. Thus, embryos that may not have survived to term or those that had only a minor defect were included in the results. Either or both of these factors would therefore add to the higher rate of malformations. Furthermore, the severity of diabetes, i.e., glucose levels, in the other studies was much lower than those observed in the present investigation.

Although later gestational stages were not tested, the study clearly indicates that early somite embryos can be affected by altered serum factors characteristic of the uncontrolled diabetic state. This stage represents an early period of development that would occur during the 4th–5th wk of human gestation. It is also the period of greatest susceptibility for the production of neural tube defects such as spina bifida. Interestingly, the preponderance of abnormalities observed in this study were neural tube closure anomalies although most occurred in the cranial region.

In summary, these results support the contention that insulin therapy and strict control of the diabetic state reduce

the risk of occurrence of congenital anomalies in offspring of the diabetic. Furthermore, early periods of gestation are particularly sensitive. It is therefore imperative that diabetic mothers seek the earliest care possible, preferably before fertilization.

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REFERENCES

[1] Soler, N. B., Walsh, C. H., and Malins, J. M.: Congenital malformations in infants of diabetic mothers. Q. J. Med. 1976; 178:303–13.

[2] Molsted-Pederson, L.: Pregnancy and diabetes: a survey. Acta Endocrinol. 1980; 94:13–19.

[3] Gabbe, S. G.: Congenital malformations in infants of diabetic mothers. Obstet. Gynecol. Sur. 1977; 32:125–32.

[4] Mills, J. L., Baker, L., and Goldman, A. S.: Malformations in infants of diabetic mothers occur before the seventh gestational week: implications for treatment. Diabetes 1979; 28:292–93.

[5] Zwilling, E.: Micromelia as a direct effect of insulin—evidence from in vitro and in vivo experiments. J. Morphol. 1959; 194:159–79.

[6] Watanabe, G., and Ingalls, T. H.: Congenital malformations in the offspring of alloxan-diabetic mice. Diabetes 1963; 12:66–72.

[7] Landauer, W.: Is insulin a teratogen? Teratology 1972; 5:129–36.

[8] Cockroft, D. L., and Coppola, P. T.: Teratogenic effects of excess glucose on head-fold rat embryos in culture. Teratology 1977; 16:141–46.

[9] Van Lancker, J. L.: Molecular and Cellular Mechanisms in Disease. New York, Springer-Verlag, 1976:423–57.

[10] New, D. A. T.: Whole embryo culture and the study of mammalian embryos during organogenesis. Biol. Rev. 1978; 53:81–122.

[11] Sadler, T. W.: Culture of early somite mouse embryos during organogenesis. J. Embryol. Exp. Morphol. 1979; 49:17–25.

[12] Sadler, T. W.: Effects of maternal diabetes on early embryogenesis: II. Hyperglycemia-induced exencephaly. Teratology 1980; 21:349–56.

[13] Horton, W. E., Jr., and Sadler, T. W.: Effects of maternal diabetes on early embryogenesis: alterations in morphogenesis produced by the ketone body, B-hydroxybutyrate. Diabetes 1983; 32:610–16.

[14] Sadler, T. W., Horton, W. E., and Warner, C. W.: Whole embryo culture: a screening technique for teratogens? Terat. Carcin. Mutagen. 1982; 2:243–53.

[15] Cockroft, D. L., Freinkel, N., Phillips, L. S., and Shambaugh, G. E.: Metabolic factors affecting organogenesis in diabetic pregnancy. Clin. Res. 1981; 29:577A.

[16] Sadler, T. W.: Effects of maternal diabetes on early embryogenesis: I. The teratogenic potential of diabetic serum. Teratology 1980; 21:339–47.

[17] Smithberg, M., and Runner, M.: Teratogenic effects of hypoglycemic treatments in inbred strains of mice. Am. J. Anat. 1963; 113:479–89.

[18] Cole, W. A., and Trasler, D. G.: Gene-teratogen interaction in insulin-induced mouse exencephaly. Teratology 1980; 22:125–39.

[19] New, D. A. T.: Development of rat embryos cultured in blood sera. J. Reprod. Fertil. 1966; 12:509–24.

[20] New, D. A. T.: Development of explanted rat embryos in circulating medium. J. Embryol. Exp. Morphol. 1967; 17:513–25.

[21] Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193:265–75.

[22] Steele, C. E., and New, D. A. T.: Serum variants causing the formation of double hearts and other abnormalities in explanted rat embryos. J. Embryol. Exp. Morphol. 1974; 31:707–19.

[23] Topping, D. L., and Targ, M. E.: Time course of changes in blood glucose and ketone bodies, plasma lipids and liver fatty acid composition in streptozotocin-diabetic male rats. Horm. Res. 1975; 6:129–37.

[24] Baker, L., Egler, J. M., Klein, S. H., and Goldman, A. S.: Meticulous control of diabetes during organogenesis prevents congenital lumbrosacral defects in rats. Diabetes 1981; 30:955–59.

[25] Eriksson, U., Dahlstrom, E., Larsson, K. S., and Hellerstrom, C.: increased incidence of congenital malformations in the offspring of diabetic rats and their prevention by maternal insulin therapy. Diabetes 1982; 31:1–6.

[26] Horii, K., Watanabe, G., and Ingalls, T. H.: Experimental diabetes in pregnant mice. Prevention of congenital malformations in offspring by insulin. Diabetes 1966; 15:194–204.