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Embryonic growth impaired by maternal hypoglycemia during early organogenesis in normal and diabetic rats

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Abstract. The effect of maternal hypoglycemia on early organogenesis was studied in normal and diabetic rats. Female Wistar rats were made diabetic by an intravenous injection of streptozotocin (45 mg/kg) 2–3 weeks before conception. On day 9.5 or 10.5 of embryo development, both control and diabetic dams received saline or Actrapid human insulin (400 mU/rat) intraperitoneally after 19-h starvation. The fasting plasma glucose levels in diabetic dams decreased from approximately 23 to 8 mM. Hypoglycemia as low as 3.5 mM was maintained for 60 min in insulin-treated mother rats. Pregnancy was terminated on day 11.6 of embryo development. A significant growth retardation was found in diabetic embryos as compared with normal embryos. Maternal hypoglycemia lowered the DNA content in normal but not diabetic embryos, while the teratogenic effect of maternal hypoglycemia was not pronounced in either normal or diabetic embryos. These data may suggest that maternal hypoglycemia in vivo in early pregnancy influences the embryogenesis but not teratogenesis of rat embryos.

Key words: Hypoglycemia – Maternal diabetes – Rat embryo – Early organogenesis

Introduction

Accumulating evidence indicates that poorly controlled maternal diabetes during pre-pregnancy and the initial stage of pregnancy is associated with an increased risk of congenital anomalies in the infant [1, 2]. Mills et al. [3] found that most anomalies occurred before the 4th to 7th week of gestation. Therefore, meticulous control of blood glucose before and during the first 6–8 weeks of gestation should be maintained [4, 5]. A tight control of blood glucose with insulin, however, may be associated with frequent hypoglycemic attacks. It has been noted that long-term diabetic patients are frequently unaware of these hypoglycemic episodes [6, 7], especially if treated with human insulin [8–10]. We previously reported that maternal hypoglycemia during early organogenesis resulted in an increased incidence of skeletal anomalies in rat offspring [11]. In addition, anomalies were markedly enhanced in diabetic offspring. In the present investigation, we further studied the short-term effect of maternal hypoglycemia on embryogenesis in nondiabetic and diabetic rat mothers.

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Methods

Female Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), aged from 13 to 17 weeks, were used throughout the experiments. They were housed at 24 °C under 12-h light-dark cycles (lights on 08:00–20:00 h). Tap water and laboratory chow pellets (60% carbohydrate, 13% fat and 27% protein; Japan Clea, Tokyo, Japan) were given ad lib. Rats were made diabetic by a single intravenous injection of streptozotocin (STZ, 45 mg/kg bw; Sigma, St. Louis, Mo., USA) dissolved in citrate buffer (0.01 M, pH 4.5) 2–3 weeks before mating. Control rats received only citrate buffer. One week later, the mean blood glucose concentrations of the rats treated with STZ had increased to over 28 mM.

Mating was accomplished by overnight housing of female rats with male rats and confirmed by the presence of sperm in a vaginal smear the following morning. Midnight on the night of mating was considered the start of day 0 of embryogenesis.

Between 12:00 and 13:00 h on the 10th or 11th day of gestation (day 9.5 or 10.5 of embryo development, respectively), crystalline human insulin (Actrapid; Novo, Copenhagen) at a concentration of 400 mU/ml was injected intraperitoneally (i.p.) into 19-h starved, nondiabetic, gravid (referred to as N-I9 and N-I10) rats, respectively, and diabetic, gravid (referred to as D-I9 and D-I10) rats, respectively. The corresponding control groups of nondiabetic and diabetic rats received 1 ml of saline (referred to as N-S9, N-S10, D-S9, and D-S10 rats, respectively). Insulin for injection was diluted in a sterile solution of 0.9% NaCl containing 0.01 ml autologous plasma per milliliter. After a plasma sample was obtained from the tail vein at 90 min, hypoglycemic animals were immediately given dextrose (1 or 5% wt/vol in water) as drinking water to raise the blood sugar to preinjection levels, so that the influence of maternal hypoglycemia was limited to the rather short period of 60 min.

At 14:00 h on the12th day of gestation (day 11.6 of embryo development), the rats were killed by cervical dislocation. The embryos and associated membranes were freed from surrounding de-

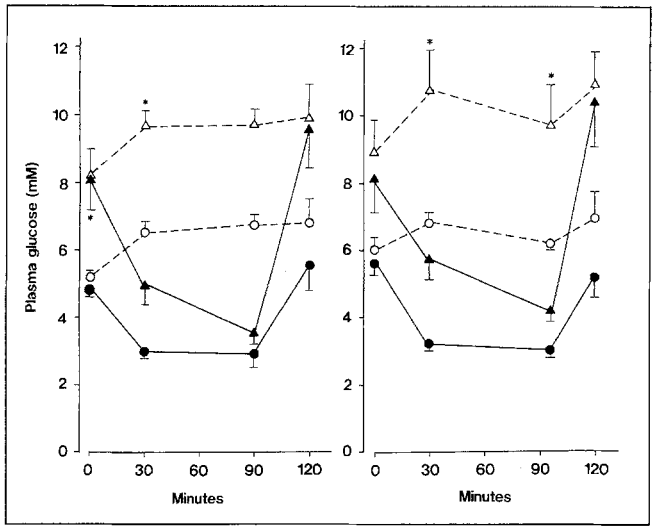

Fig. 1. Plasma glucose concentration after i.p. injection of either saline (*dotted lines*) or insulin (*solid line*) in nondiabetic (*circles*) and diabetic (*triangles*) dams on day 9.5 (*left panel*) and 10.5 (*right panel*) of embryo development. Ninety minutes after injection of saline or insulin, 5% glucose solution was given per os to hypoglycemic animals of the day 9.5 group, and D-I10 dams were given 1% glucose solution to avoid marked hyperglycemia. *$P< 0.05$ vs corresponding nondiabetic rats

cidua and transferred to a petri dish containing saline. Embryos and investing membranes were teased apart with fine jewelers' forceps through a stereomicroscope. These procedures were performed within 15 min. The total number of somites and the crown-rump length were determined for each embryo, without the examiner knowing the maternal treatment group. Embryos were then individually inspected to determine whether the morphology of the brain spheres, neural tube, heart, optic and otic vesicles, limb buds, visceral arches, and axial curvature conformed to that expected at day 11.6 of development. Embryos not conforming to normal in any of the above structures were considered dysmorphic. After visual inspection, individual embryos were placed in 0.5 N NaOH for subsequent determination of total protein [12] and DNA content [13]. These embryos were observed by scanning electron microscopy after they were fixed in phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde. After 3–4 h of fixation, they were washed with phosphate buffer. These specimens were postfixed with 1% OsO_4 and stained with 2% tannic acid followed by 1% OsO_4. After dehydration, they were then mounted on aluminum stubs, coated with gold, and observed under a Hitachi S-450 scanning electron microscope at 20 kV.

Data are presented as means ± SE. Statistical significance was determined by one-way analysis of variance and Scheffe's multiple-comparison test. Differences were considered significant if values of $P< 0.05$ were obtained.

Results

Table 1 shows the body weight and plasma glucose levels in the normal and diabetic rat mothers killed on day 11.6 of embryo development. The body weight increased between day 0 and day 12 of pregnancy, but the weight gain was smaller in the diabetic mothers than in the nondiabetic mothers (24.3 ± 0.8 vs 35.7 ± 0.4 g, $P< 0.005$). There was a significant difference in body weight between nondiabetic and diabetic mothers of both day 9.5 and 10.5 groups. Plasma glucose levels were 28.1 ± 0.7 mM one week after STZ injection, and 21–23 mM in diabetic mothers on day 11.6 of gestation. Maternal plasma glucose levels were fourfold higher in diabetics than in nondiabetics. There was no significant difference in plasma glucose levels between the day 9.5 and day 10.5 groups.

Table 1. Body weight and plasma glucose levels in normal and diabetic rat mothers killed on day 11.6 of embryo development (mean ± SEM)

Maternal group	Litter (*n*)	Body weight (g)	Plasma glucose (mM)
Day 9.5			
Nondiabetic, treated with saline	12	270.8 ± 4.6	5.5 ± 0.3
Nondiabetic, treated with insulin	14	277.7 ± 3.4	5.7 ± 0.3
Diabetic, treated with saline	10	250.0 ± 3.7*	22.8 ± 2.4**
Diabetic, treated with insulin	10	249.2 ± 4.0**	21.0 ± 1.4**
Day 10.5			
Nondiabetic, treated with saline	11	271.9 ± 2.4	4.6 ± 0.3
Nondiabetic, treated with insulin	11	272.5 ± 4.3	5.4 ± 0.2
Diabetic, treated with saline	8	238.2 ± 3.1**	23.8 ± 2.6**
Diabetic, treated with insulin	7	250.3 ± 5.5	21.0 ± 1.8**

On postconceptional day 9.5 or 10.5, both control and diabetic dams received saline or Actrapid human insulin (400 mU/rat, i.p.). They were killed on day 11.6 of embryo development
* $P< 0.05$, ** $P< 0.005$ vs corresponding nondiabetic dams. There were no significant differences in the corresponding body weight or plasma glucose levels between the day 9.5 and day 10.5 groups

Plasma glucose levels during hypoglycemia

Plasma glucose levels during the period of 120 min after i.p. injection of saline or insulin on day 9.5 or 10.5 of embryo development are shown in Fig. 1. In the day 9.5 group, after saline injection the mean plasma glucose levels were not changed during the 120-min period in D-S9 dams. After insulin injection, the mean plasma glucose level of the N-I9 dams decreased from 4.8 to 2.9 mM within 30 min, remained at the same level at 90 min, and then increased at 120 min after drinking 5% glucose solution ad lib. There was a profound reduction in fasting plasma glucose level in diabetic rats after 19-h fasting, since the plasma glucose level in fed diabetic dams was 21–23 mM. After insulin injection, the mean plasma glucose level in D-I9 dams declined from 8.1 to 4.9 mM within 30 min, dropped further to 3.5 mM at 90 min and then increased to 9.5 mM at 120 min. There was no significant difference in plasma glucose level after insulin injection between N-I9 and D-I9 animals. Similar patterns were noted in N-S10 and D-S10 animals. After insulin injection, the mean plasma glucose

levels in N-I10 dams decreased from 5.6 to 3.2 mM within 30 min, fell further to 3.0 mM at 90 min, and then increased to 5.0 mM at 120 min. The hypoglycemic effect of insulin was slightly but not significantly less in D-I10 dams than in D-I9 dams. There was no significant difference in plasma glucose levels immediately before and 30 min after insulin injection (8.1 ± 1.0 vs 5.7 ± 0.6 mM, NS). Ninety minutes after insulin injection, the plasma glucose levels were significantly ($P<0.005$) lower than the preinjection levels. However, there was no significant difference in plasma glucose levels at 30 and 90 min between N-I10 and D-I10 dams. Plasma glucose levels were returned to the basal level at 120 min by the oral administration of 1% and 5% dextrose solution at 90 min after insulin injection in D-I10 dams and N-I10 dams, respectively.

Table 2. Effect of maternal insulin-induced hypoglycemia on embryogenesis (mean ± SEM)

Maternal group	Litter (*n*)	Implantations (*n*)	Resorptions (*n*)
Day 9.5			
Nondiabetic, treated with saline	12	12.5 ± 0.5	0.2 ± 0.2
Nondiabetic, treated with insulin	14	13.3 ± 0.7	0.2 ± 0.1
Diabetic, treated with saline	10	11.4 ± 0.5	0.5 ± 0.2
Diabetic, treated with insulin	10	11.9 ± 0.5	0.5 ± 0.3
Day 10.5			
Nondiabetic, treated with saline	11	13.3 ± 0.6	0.9 ± 0.3
Nondiabetic, treated with insulin	11	13.0 ± 0.6	0.9 ± 0.4
Diabetic, treated with saline	8	10.5 ± 0.5	0.8 ± 0.2
Diabetic, treated with insulin	11	11.1 ± 0.7	0.4 ± 0.2

Implantation and resorption

On day 11.6 of gestation, hysterectomy was carried out. Somewhat fewer implantations were obtained in D-S9 dams than in N-S9 dams (11.4 ± 0.5 vs 12.5 ± 0.5, NS) (Table 2). The number of implantations was not affected by maternal hypoglycemia in N-I9 and D-I9 dams. There was no difference in the incidence of resorption (early death) among the groups.

Analysis of embryos

There was no difference in somite numbers, crown-rump length, and total protein levels as indices of embryo development between the embryos of N-I9 dams and those of N-S9 dams (Table 3). Only DNA levels were significantly lower in embryos of N-I9 dams than in those of N-S9 dams. Embryo development was impaired when the mother rats had severe diabetes. The crown-rump length of embryos from D-S9 dams was significantly less than that of N-S9 dams. Insulin-induced hypoglycemia did not significantly enhance growth retardation in embryos from D-I9 dams.

Growth retardation was also found in embryos of N-I10 dams, accompanied by 9.3% and 17.0% decreases in total protein and total DNA compared with those of N-S10 dams, although only the difference in DNA levels was statistically significant. Growth retardation observed in embryos from D-S10 dams was greater than that in N-S10 dams. Maternal hypoglycemia on day 10.5 of embryo development slightly but not significantly reduced the growth of diabetic embryos.

Embryonic abnormalities (retardation and malformations) in 11.6-day rat embryos from nondiabetic and diabetic mothers are shown in Table 4 and Fig. 2. Dysmorphogenic changes such as microencephaly and abnormal somites were found in only two out of 150 embryos from N-S9 rats. Insulin-induced hypoglycemia did not enhance dysmorphogenesis in nondiabetic rats from the day 9.5 and day 10.5 groups. In the embryos from N-I10 dams, open posterior neuropores, abnormal somites, and abnormal

Table 3. Somite number, crown-rump length, total protein, and total DNA of 11.6-day rat embryos from nondiabetic and diabetic mothers (mean ± SEM)

Maternal group	Embryos (*n*)	Somites (*n*)	Crown-rump (mm)	Protein (μg)	DNA (μg)
Day 9.5					
Nondiabetic, treated with saline	150	26.7 ± 0.1	4.03 ± 0.03	446 ± 5	40.4 ± 0.9
Nondiabetic, treated with insulin	186	26.3 ± 0.1	4.00 ± 0.03	430 ± 7	36.1 ± 0.7***
Diabetic, treated with saline	113	26.0 ± 0.1	3.72 ± 0.03*	420 ± 7	28.3 ± 0.6
Diabetic, treated with insulin	119	25.3 ± 0.2**	3.61 ± 0.04	410 ± 9	26.2 ± 0.8**
Day 10.5					
Nondiabetic, treated with saline	146	26.8 ± 0.1	3.96 ± 0.02	454 ± 5	38.9 ± 0.9
Nondiabetic, treated with insulin	143	26.2 ± 0.2	3.87 ± 0.05	412 ± 13	32.3 ± 0.7***
Diabetic, treated with saline	84	25.8 ± 0.2*	3.62 ± 0.04**	406 ± 9	26.7 ± 0.5**
Diabetic, treated with insulin	78	25.0 ± 0.2*	3.53 ± 0.04**	389 ± 15	25.0 ± 1.1

* $P< 0.05$, ** $P< 0.005$ vs corresponding nondiabetic rats; *** $P< 0.005$ vs the saline group

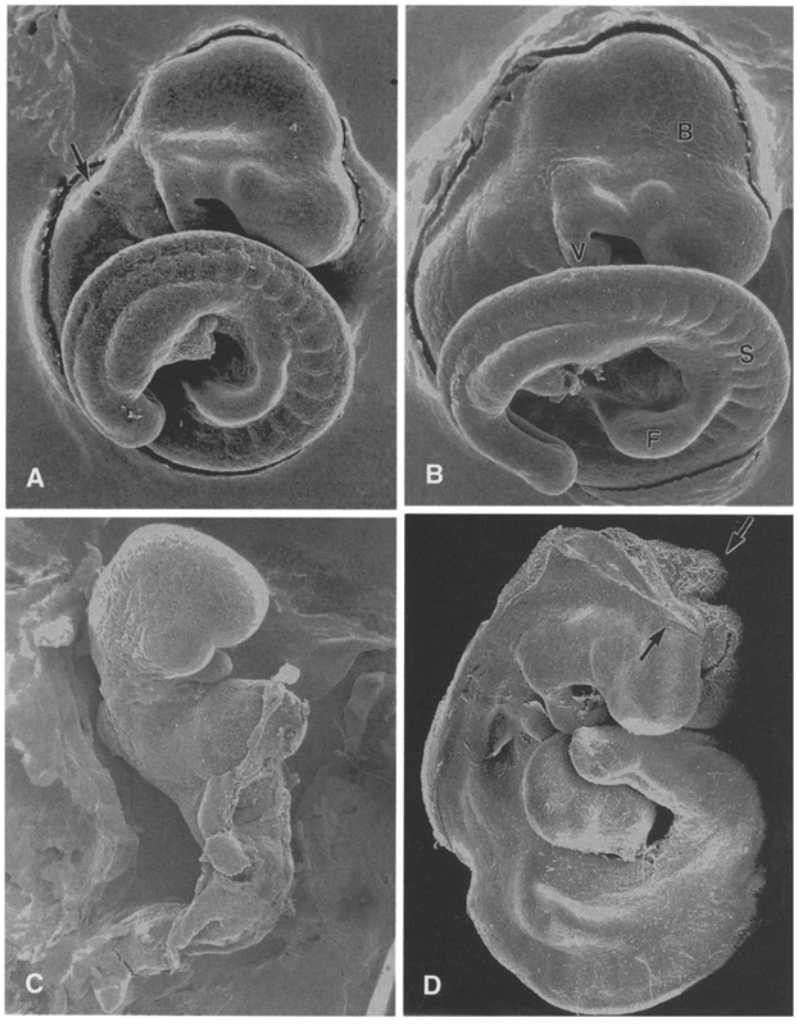

Fig. 2 A–D. Scanning electron micrographs of 11.6-day embryos from nondiabetic and diabetic mother rats which had received either saline or 400 mU of insulin on day 9.5 or 10.5 of embryo development (A,C, D, × 45; B, × 56). **A** Nondiabetic rat embryo from an N-S9 dam. The embryo is normal, showing rotation, well-developed brain vesicles, and closed neuropores. *B*, brain; *V*, visceral arches; *F*, forelimb bud; *S*, somites. **B** Nondiabetic rat embryo from an N-I9 dam. The otic vesicle (*arrow*) is not yet closed, and somite formation is delayed. **C** Diabetic rat embryo from a D-S10 dam. Note the open anterior neuropore (*arrow*) and marked growth retardation. **D** Diabetic embryo from a D-I9 dam. There is abnormal axial rotation, and the heart and head regions are less developed than in the normal control

Table 4. Effect of maternal insulin-induced hypoglycemia on the development of 11.5-day rat embryos

	Embryos	Brain		Neuropore			Abnormal axial rotation
	(*n*)	Micro-encephaly *n* (%)	Exen-cephaly *n* (%)	Open anterior *n* (%)	Open posterior *n* (%)	Abnormal somite *n* (%)	*n* (%)
Day 9.5							
Nondiabetic, treated with saline	150	1 (0.6)				1 (0.6)	
Nondiabetic, treated with insulin	183			1 (0.5)			1 (0.5)
Diabetic, treated with saline	113			1 (0.9)			
Diabetic, treated with insulin	119	1 (0.8)		1 (0.8)	2 (1.7)		1 (0.8)
Day 10.5							
Nondiabetic, treated with saline	146						
Nondiabetic, treated with insulin	143				3 (2.1)	1 (0.7)	1 (0.7)
Diabetic, treated with saline	84	1 (1.2)	1 (1.2)	1 (1.2)	7 (8.3)*	1 (1.2)	2 (2.4)
Diabetic, treated with insulin	78				1 (1.3)	5 (6.4)*	

* $P < 0.05$ vs corresponding embryos from the day 9.5 group

axial rotation (Fig. 2) were found. These anomalies were combined in two embryos. Maternal diabetes itself did not cause any morphological anomalies in embryos from the day 9.5 group. On the other hand, 13 out of 84 diabetic embryos from the day 10.5 group were abnormal, and 8.3% of the embryos showed an open posterior neuropore that suggests growth retardation. Although maternal hypoglycemia did not enhance dysmorphogenesis in D-I10 dams, 5 out of 78 embryos had abnormal somites.

Discussion

When neurulation begins (day 9.3–9.5), the energy source for the normal development of rat embryos largely depends on uninterrupted anaerobic glycolysis [14, 15]. Rodent embryos utilize glucose at a very high rate to produce lactate. Once the yolk sac is developed and circulation begins (day 10.3–10.5), anaerobic glycolysis declines over a period of 2–3 days [16], and almost all glucose is metabolized via the Krebs cycle. Therefore, interference with glycolysis by ambient mannose [17, 18] or hypoglycemia [19, 20] disrupts early organogenesis in the rodent embryos.

We previously demonstrated that maternal insulin-induced hypoglycemia during early organogenesis (day 9.5 or 10.5 of gestation) induces skeletal malformations in rat offspring [11]. In addition, moderate hyperglycemia in the rat mothers amplifies the hypoglycemic effect. In the present study, we focused on the short-term effect of transitory hypoglycemia in pregnant rats at two points in early organogenesis in vivo. We also addressed whether maternal diabetes could modify the embryotoxic effect of insulin-induced hypoglycemia on days 9.5 and 10.5 of gestation.

Buchanan et al. [20] demonstrated that brief maternal hypoglycemia during early organogenesis (days 9.5 or 9.75 of embryo development) impairs normal embryo development in the rat. They also found no embryotoxic effect of maternal hypoglycemia during late neurulation (day 10.6 of embryo development) [21]. The magnitude and period of hypoglycemia in the present study were the same as or a little greater than the results reported by those authors. The results agree well, because maternal hypoglycemia did not significantly reduce the number of somites, crown-rump length or protein level of embryos from N-I10 dams. Their gravid rats fasted for 12 h and ours for 19 h. Thus, the fasting plasma glucose levels in N-I10 dams were to some extent lower than their data [21]. The prolonged fasting period may be one of the reasons for the decrease in total DNA in the embryos from N-I10 dams. In fact, the number of somites of 11.5-day embryos in that study was greater than in the present study (29.7 ± 0.2 vs 26.8 ± 0.1).

Rat fetuses of diabetic dams show retarded development during the preimplantation period [22]. In the viable embryos from diabetic rats on gestational day 11.5, crown-rump length, somite numbers, and protein and DNA content were significantly diminished [23]. Various aspects of the disturbed metabolic state in the diabetic mother have been suggested as possible teratogenic effects of hyperglycemia [24, 25], hyperketonemia [26], and somatomedin inhibitors [27, 28]. Since maternal diabetes promotes growth retardation [23], prolonged starvation and maternal hypoglycemia promoted the greater growth retardation observed in the embryos from D-I10 dams. In addition, the increase in counterregulatory hormones induced by maternal hypoglycemia might worsen embryogenesis further. Whether the pronounced growth retardation in the diabetic embryos from hypoglycemic mothers is a consequence of the overall disturbed metabolic status remains to be clarified.

Dysmorphogenic changes in the embryo were most frequently observed in D-S10 but not D-I10 dams. In support of our present observations, clinical studies [29, 30] have shown a lack of teratogenic effect of maternal hypoglycemia. In contrast, rat embryos showed an increased frequency of dysmorphogenic lesions in in vitro embryo culture when they were briefly exposed to hypoglycemic (2.2–2.5 mM) serum during culture in hyperglycemic medium supplemented with subteratogenic concentrations of glucose (33.3 mM) [31]. To explain the difference in effect of maternal hypoglycemia in vivo on the mechanisms between embryogenesis and teratogenesis, further studies are required.

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