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In vitro development of rat embryos obtained from diabetic mothers

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Abstract. Rat embryos of 9.5 or 10 days of gestation were removed from control or streptozotocin-diabetic mothers and cultured in normal rat serum (180 mg% glucose) or in diabetic serum (600 mg% glucose). The development of control embryos in normal serum was adequate. Embryos from normal mothers cultured in diabetic serum showed signs of developmental retardation. The development of embryos obtained from diabetic mothers was severely impaired, regardless of the gestational age or the culture medium. These results suggest that a diabetic maternal milieu produces irreversible effects in the embryo very early in gestation.
Key words. Diabetes; malformations; embryo culture.

Pregnancy in human diabetics has been associated with an increased risk of congenital malformations. This problem has been extensively studied during the last two decades both from the clinical and experimental points of view.

Congenital malformations, resorptions, and developmental retardations have been reported as common results of experiments carried out on pregnant laboratory animals made diabetic with Streptozotocin (Stz)[1-3]. Genetic predisposition[4] and maternal/embryonic nutrition[5,6] were found to be major factors in producing diabetic embryopathies in rats.

In vitro studies have been used to show that some spedific components of the diabetic status, like ketone bodies or somatomedin inhibitors, can be implicated in diabetic embryopathies. This suggests a multifactorial basis for these pathologic conditions[7,8].

Clinical studies showed that when a strict metabolic control of the mother was initiated very early in pregnancy[9] or before conception[10,11] the risk of fetal malformations diminished.

In pregnant rats, the strict control of diabetes by administering insulin reduces the embryolethality and the incidence of malformations[1,12]. Eriksson et al.[13], using intermittant insulin treatment of diabetic rats during pregnancy, obtained the highest rates of resorptions and malformations when the interruption of maternal treatment occurred on days 2–7 post coitum.

These results highlight the relevance of embryonic age as a factor for the induction of diabetic embryopathies, and suggest that the highest susceptibility is at a very early stage of development, probably before the major organogenetic events begin.

We carried out this experiment in order to test whether maternal diabetic milieu may cause irreversible alterations in very early stages of embryogenesis, and to investigate the ability of embryos exposed to a diabetic environment to recover when cultured in normal serum.

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Materials and methods

Female CD:Crl albino rats (Charles River, Calco, Italy) weighing 150–175 g were maintained in an air-conditioned room (20 ± 2 °C, 60% humidity) with a light/dark cycle of 12 h each. Food (Italiana Mangimi) and water were allowed ad libitum. After one week of acclimatization, one group of animals was given i.v. 50 mg/kg of Stz dissolved in citrate buffer, pH 4.8. One week after Stz treatment, blood glucose levels were determined (Destrostix Kit, Ames) and rats with blood concentrations below 400% were revived from the trial. Treated and untreated females were caged overnight with males of the same strain. The day on which spermatozoa were found in vaginal smear was called day 0 of pregnancy.

Embryos were dissected from the uterus in the early afternoon of day 9 (9.5 day-old embryos, 1–3 somite stage) or in the morning of day 10 (10 day-old embryos, 8–10 somite stage). The culture medium consisted of 100% rat serum prepared from blood collected from normal or diabetic animals, immediately centrifuged, heat inactivated, pooled, and stored at − 80 °C. Serum glucose levels were measured before storage (GOD-PAP Peridochrom Glucose, Boehringer Mannheim). The medium was supplemented with penicillin (100 I.U./ml) and streptomycin (100 µg/ml).

For each stage we prepared the following groups: control embryos in normal or diabetic serum; embryos from diabetic mothers in normal or diabetic serum. Embryos of 9.5 days were cultured for 48 h while embryos of 10 days were cultured for 30 h. Culture bottles containing 5 ml of serum and 4–5 embryos were equilibrated with gas mixtures of 5% O_2, 5% CO_2 and 90% N (9.5-day embryos), or 10% O_2, 5% CO_2 and 85% N

Table 1. Effects of normal and diabetic sera on growth of 9.5- and 10-day-old embryos explanted from normal or diabetic mothers.

Embryo	Serum	Somite number	Yolk sac diameter (mm) (M ± SD)	Crown-rump length (mm) (M ± SD)	Head length (mm) (M ± SD)	Total score (M ± SD)	Embryo protein (M ± SD)	Yolk sac protein (M ± SD)
Embryos explanted at day 9.5								
Control N = 10	normal	24.40 ± 0.84	4.35 ± 0.30	3.71 ± 0.31	1.73 ± 0.09	40.10 ± 0.57	202.08 ± 26.53 N = 7	147.16 ± 21.11 N = 7
Control N = 9	diabetic	22.89 ± 1.05	4.03 ± 0.19[a] N = 10	3.38 ± 0.18[a]	1.59 ± 0.12[a]	38.67 ± 0.87[a]	177.15 ± 19.40 N = 8	126.66 ± 20.58 N = 8
Diabetic N = 15	normal	21.80 ± 1.52[a]	4.03 ± 0.32[a]	3.53 ± 0.18	1.62 ± 0.10	37.93 ± 1.10[a]	182.46 ± 34.17 N = 13	124.55 ± 26.11 N = 13
Diabetic N = 10	diabetic	21.70 ± 1.89[a]	3.67 ± 0.17[abc] N = 13	3.25 ± 0.23[ac]	1.51 ± 0.12[a]	38.40 ± 0.70[a]	166.24 ± 30.34	119.70 ± 18.58
Embryos explanted at day 10								
Control N = 14	normal	25.07 ± 0.83	4.45 ± 0.15 N = 13	4.02 ± 0.23	1.95 ± 0.14	41.14 ± 0.66	252.32 ± 22.47 N = 9	155.41 ± 16.79 N = 9
Control N = 14	diabetic	27.21 ± 0.70[a]	4.45 ± 0.23 N = 13	3.88 ± 0.23	1.93 ± 0.21	40.50 ± 0.76	212.13 ± 34.21 N = 13	121.64 ± 17.04[a] N = 13
Diabetic N = 14	normal	22.78 ± 2.39[ab]	3.90 ± 0.45[ab] N = 20	3.52 ± 0.45[ab]	1.56 ± 0.27[ab]	38.67 ± 2.40[ab]	173.39 ± 54.84[a] N = 15	107.67 ± 30.40[a] N = 15
Diabetic N = 10	diabetic	23.00 ± 1.41[ab]	3.60 ± 0.26[abc] N = 14	3.35 ± 0.15[ab]	1.66 ± 0.09[ab]	38.8 ± 1.13[ab]	162.87 ± 35.88[ab] N = 11	102.26 ± 17.57[a] N = 11

[a]Significantly different from control embryo in normal serum.
[b]Significantly different from control embryo in diabetic serum.
[c]Significantly different from diabetic embryo in normal serum.

Table 2. Effects of normal and diabetic sera on development of 9.5- and 10-day-old embryos explanted from normal or diabetic mothers: external anomalies.

Embryo serum	*Embryos explanted at day 9.5*				*Embryos explanted at day 10*			
	control normal N = 10	control diabetic N = 10	diabetic normal N = 15	diabetic diabetic N = 12	control normal N = 14	control diabetic N = 14	diabetic normal N = 19	diabetic diabetic N = 13
Abnormal embryos %	-	10.00[a]	33.33[a,b]	41.67[a,b,d]	-	7.14	47.37[a,b]	30.76[a,b]
Plurimalformed	-	-	13.13	-	-	-	-	7.69
Ventrally convex	-	10.00	-	16.67	-	-	-	15.38
Dextrocardia	-	-	20.00	-	-	-	-	-
Heart malrotation	-	-	13.13	8.33	-	-	5.25	-
Irregular somites	-	-	-	-	-	-	5.25	7.69
Microcephaly	-	-	-	-	-	-	21.05	-
Situs inversus	-	-	-	8.33	-	-	-	-
Hypoplastic forebrain	-	-	20.00	25.00	-	7.14	21.05	-
Delayed development:								
Total embryos %	-	50.00[a]	66.66[a,b]	66.66[a,b]	-	21.42[a]	42.10[a,b,c]	23.07[a]
Posterior neuropore	-	30.00	40.00	50.00	-	21.42	36.83	15.38
Optic vescicle	-	-	20.00	16.67	-	-	15.77	7.69
Otic vescicle	-	50.00	33.13	33.34	-	-	47.36	15.38

[a]Significantly different from control embryo in normal serum.
[b]Significantly different from control embryo in diabetic serum.
[c]Significantly different from diabetic embryo in diabetic serum.
[d]Significantly different from diabetic embryo in normal serum.

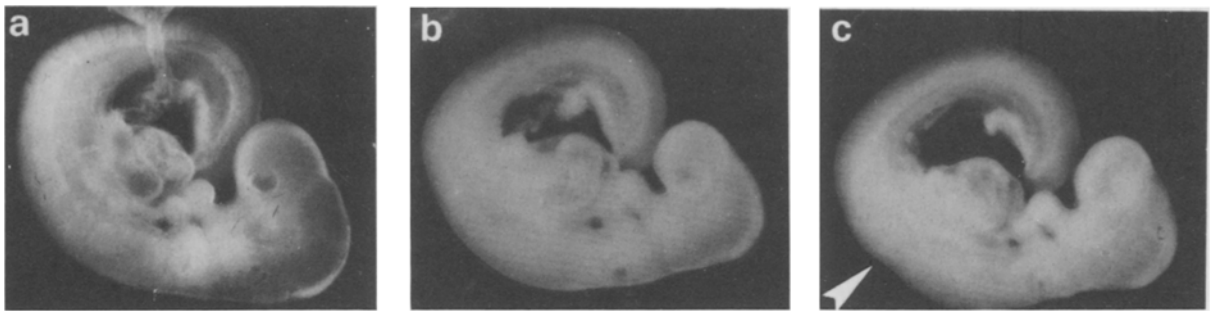

Figure 1. External morphology of rat embryos cultured in vitro:
a) embryo explanted from normal mother and cultured in normal serum;
b) embryo explanted from a diabetic mother and cultured in diabetic serum. Note the hypoplasia of forebrain and the delayed closure of the optic vescicle;
c) embryo explanted from a diabetic mother and cultured in diabetic serum; showing hypolasia of forebrain and irregular somites (arrow).

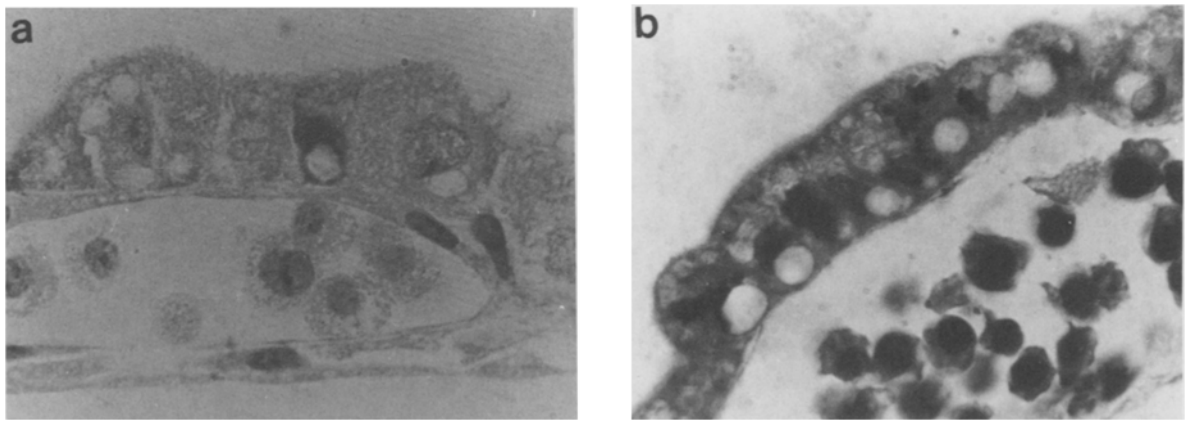

Figure 2. Visceral yolk sac of a control rat embryo cultured in normal serum (*a*) and of a rat embryo explanted from a diabetic mother and cultured in diabetic serum (*b*). The endodermal cells of control contain few spread vacuoles; in contrast the endodermal cells of yolk sac in *b*) contain numerous vacuoles.

(10-day embryos). During the last 24 h of culture, the bottles were re-equilibrated twice with a gas mixture containing 20% O_2 (ref. 14). The bottles were inserted in a roller apparatus (30 rpm), and incubated at 38 °C.

At the end of the culture period, embryos were examined under a dissecting microscope. Development was assessed by a morphological score as described by Brown and Fabro[15]. Yolk sac diameter, crown-rump length, and head length were measured, and embryonic protein was determined according to Bradford[16]. Some embryos were fixed in 4% formaldehyde, embedded in JB4 resine, and examined histologically.

Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons, and the chi-square-test. The level of significance was set at $p < 0.05$.

Results

The glucose levels were found to be 180 mg% in control serum and 660 mg% in the pooled diabetic serum.

The development of control embryos in control sera was adequate although the embryos explanted on day 10 developed better than those explanted on day 9.5 (tables 1 and 2). Embryos of 9.5 days from normal mothers cultured in diabetic serum showed some degree of developmental retardation revealed by the significantly reduced value of several parameters like crown–rump length, yolk sac diameter, head length, and total score (table 1). Delay in closure of posterior neuropore and otocyst was also observed in a few embryos. Only one embryo in this group was malformed (failure in turning, table 2). The development of embryos from diabetic mothers cultured in normal serum was comparable to that of control embryos in diabetic serum. However, in this group 5/15 embryos were severely malformed: hypoplastic prosencephalon, heart malformations, and severe general malformations (table 2). When embryos from diabetic mothers were cultured in diabetic serum, only a slight worsening was observed in general development in comparison to the previous group (table 1). In these conditions there was also a

high rate of malformed embryos: two failed to turn, three had hypoplastic prosencephalon, and one had a complete situs inversus (table 2).

The development of 10-day-old embryos in control serum mimics that of in vivo embryos of the same age. The development of control embryos cultured in diabetic serum was also quite good and differed from that of the previous group only in the lower protein content per embryo (table 1). Only one embryo was malformed in this group. The development of embryos from diabetic mothers resulted in severe impairment, both when cultured in normal serum and when cultured in diabetic serum. All developmental parameters were significantly reduced and a high percentage of embryos showed severe malformations or morphological developmental delays (tables 1,2; fig.1).

It is interesting that a common result found in embryos from diabetic mothers cultured in diabetic serum was a dramatic reduction in their yolk sac diameter (table 1). Furthermore, the yolk sacs were more opaque than normal. Histological examination revealed many vacuoles in the endodermal cells of these sacs, whereas few or no vacuoles were seen in visceral endoderm of control yolk sacs (fig. 2)

Discussion

The postimplantation whole embryo culture has been used for studying several aspects of embryotoxicity and also to clarify some problems connected with diabetic embryopathies. In general, normal embryos have been cultured in normal serum with glucose added in order to obtain very high and quite unphysiological concentrations of glucose. The results of these studies indicate that glucose concentrations of about 50 mM (900 mg%) are necessary to obtain clear dismorphogenic effects[17,18]. In our experiment we cultured both control embryos and embryos from diabetic mothers in normal or diabetic serum. The results clearly indicate that, independent of the culture medium, the embryos obtained from diabetic mothers are unable to develop normally. This means that the maternal milieu has irreversible effects in the embryo very early in gestation. Otherwise, diabetic serum has no effect on 10-day-old embryos (8–10 somites), but is able to induce some alterations in younger embryos (9.5 days). These data suggest that rat embryos are sensitive to diabetes-induced alterations until the stage of about 4–8 somites. During these early stages of development a number of important events are going on: gastrulation, neural induction, lateral mesoderm assembly, embryo symmetry formation etc. Moreover, in this period the genes controlling the major events of development are activated. It is difficult at the moment to establish what kinds of alterations have been induced, at what level, and by what mechanisms. What is clearly shown is that these alterations compromise the subsequent development of the embryo.

These results strongly support the necessity of the monitoring of diabetic women as early as possible in gestation.

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