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Subdiabetogenic Streptozocin Treatment Impairs Preimplantation Development of Mouse Embryos

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Summary

To estimate the significance of insulin in the regulation of preimplantation embryo growth, female mice received a single subdiabetogenic dose of streptozocin (65 mg/kg intraperitoneally) 8–11 days or 14–17 days before fertilization. Mean glycaemia levels and the number of embryos per mouse did not differ significantly between the streptozocin-treated and control groups. Morphological analysis of preimplantation embryos collected on day 3 of pregnancy revealed significant changes in the distribution pattern of preimplantation embryo stages recovered from streptozocin-treated females. Continuous insulin treatment of streptozocin-treated mice improved the impaired development of preimplantation embryos only in short-lasting experiments. After a long subdiabetic period (14–17 days) the incidence of degenerated embryos was increased in both streptozocin-treated groups. It can be concluded that the subdiabetic state in female mice impairs preimplantation embryo development which could partly be prevented by insulin treatment.

Key words

Subdiabetic state – Pregnancy – Preimplantation embryo – Development – Insulin

Introduction

The mechanisms underlying cellular proliferation and differentiation of the early mammalian embryo are of fundamental interest. Although the development of the early mammalian embryo is controlled primarily by the embryonic genome, it is also regulated by hormones and growth factors and by a continuing supply of energy. Because insulin is an important growth factor for many rapidly proliferating cells, it appeared of interest to investigate its influence on the development of early preimplantation embryos.

Experimental evidence supports insulin involvement in the early development. Although it is clear that insulin receptors are expressed during early stages of mammalian development (Rosenblum *et al.* 1986), the functional role of insulin still remains to be clarified. The appearance of insulin receptors on compacting 8-cell embryos coincides with the *in vitro* stimulatory effects of insulin on protein synthesis at embryo compaction associated with a switch in energy dependence from lactate and pyruvate to glucose (Harvey and Kaye 1988, 1991). Chemically induced diabetes affects embryonal development during the preimplantation period in rodents (Diamond *et al.* 1989, Beebe and Kaye 1990, 1991, De Hertogh *et al.* 1992). Recently, Vercheval *et al.* (1990) reported that embryo morphology and nuclear counts of blastocysts in spontaneously ovulating rats after subdiabetogenic streptozocin treatment were affected similarly to those of the fully diabetic group on day 5 of pregnancy. Physiological levels of insulin stimulated DNA, RNA and protein synthesis in preimplantation mouse embryos cultured *in vitro* with significant effects observed at the morula stage of development. On the contrary, neither an insulin-like growth factor-I (IGF-I) nor IGF-II had any appreciable effects under the same experimental conditions (Rao *et al.* 1990). These observations provide considerable support for the assumption that insulin plays a key role during preimplantation development and that the absence of insulin *in vivo* possibly affects embryonal development.

The aim of our study was to evaluate the developmental stage of preimplantation embryos recovered from mice treated with a subdiabetogenic

dose of streptozocin after spontaneous ovulation and mating.

Materials and Methods

Animals

The study was conducted on female mice of the inbred BALB/c strain (VELAZ, Prague, Czechoslovakia), 6–8 weeks old. Mice had free access to food (Purina-like, DOS 2 b diet, VELAZ, Prague, Czechoslovakia) and water. Animals were maintained under a 12 h light-dark cycle. Females with confirmed normal 4–5 days oestral cycle were used exclusively.

Induction of the subdiabetic state

Streptozocin (STZ, Serva, Germany) was dissolved in a sterile sodium citrate buffer (0.1 mol/l, pH 4.5) and administered i.p. to female mice within 5 min after preparation. Animals received a single subdiabetogenic dose of STZ (65 mg/kg body weight) either 8–11 days (group S [short]) or 14–17 days (group L [long]) before fertilization. In preliminary experiments STZ induced changes of glucose tolerance in a dose of 65 mg/kg without changes of basal glycaemia in female mice (data not shown).

Ultralente insulin (Pur-Insulin-Superdep Spofa, Prague, Czechoslovakia) was injected once daily s.c. in a dose of 2–3 IU/100 g starting 4 days (group S) or 6 days (group L) after STZ treatment. Group S was insulin-treated for 4 days, group L for 8 days. Subsequently, the animals were divided into two subgroups. In one subgroup, insulin treatment was continued throughout the remainder of the experiment (I+), the second subgroup was treated with NaCl 154 mmol/l only (I–). Each experimental subgroup consisted of at least 14 mice. Control animals (n=28) received an injection of the citrate buffer and afterwards NaCl (154 mmol/l) every day. Blood glucose was determined by the glucose-oxidase method before the animals were killed.

Embryo collection

Spontaneous ovulation was used to preclude effects of exogenous hormones used for superovulation. Females were kept with males of the same strain overnight. If a vaginal plug was found on the morning of the next day, this was considered as the first day of pregnancy. After four negative matings, animals were excluded from the experiments.

To assess the *in vivo* development of preimplantation embryos, the animals were killed by cervical dislocation on day 3 between 0700 h and 0800h. Embryos were obtained from both fallopian tubes using a dissecting microscope (Technival, Poland) by flushing the oviduct with HAM's F-10 media (Sigma, USA) containing 10 % (v/v) bovine foetal serum. The embryos were inspected under a differential interference contrast microscope (Jenamed, Variant, Germany) and their morphological characteristics were classified according to the following criteria:

a) degenerated embryos, including unfertilized oocytes and zygotes which could not be morphologically differentiated,
b) abnormal 1, 2, 3-cell embryos,
c) normal 4, 5, 6, 7 or 8-cell embryos.

Statistical analysis

Results are given as means ± S.D. Statistical comparisons between the groups were carried out by two different methods. The chi-square (χ^2) test was used to detect differences in the distribution of preimplantation embryos. Statistical analysis of blood glucose concentrations and embryo number was performed by analysis of variance.

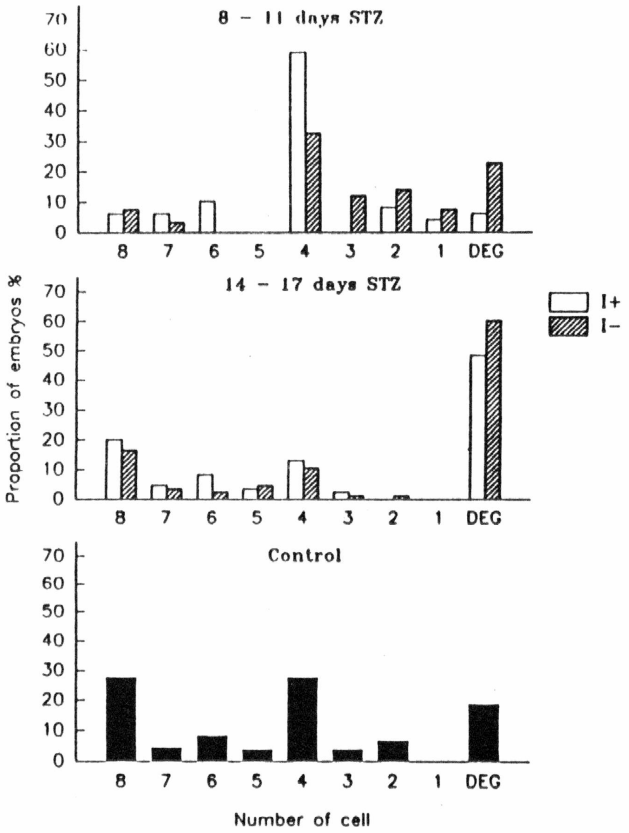

Fig. 1
The distribution of the number of cells/embryo (day 3) recovered from insulin-treated (I+) and untreated (I–) subdiabetic and control mice.

Results

The morphological analysis of preimplantation embryos collected on day 3 of pregnancy are shown in Table 1. The subdiabetic state in mice did not induce significant changes in basal glycaemia. The number of embryos in the control and STZ-treated groups did not differ significantly. However, the distribution pattern of preimplantation embryo stages (Fig. 1) was different in the STZ and control groups.

STZ administration 8–11 days before fertilization (group S)

Subdiabetic mice without insulin treatment had a lower proportion of 4-8 cell embryos albeit compensated by a higher percentage of 1-3 cell embryos ($p<0.001$). Insulin administration resulted in a highly significant difference between the STZ groups ($p<0.001$) and returned the impaired development of preimplantation embryos after STZ to control levels.

STZ administration 14–17 days before fertilization (group L)

The overall distribution of cell/embryo was significantly different in the STZ-treated groups when compared to the control group ($p<0.001$). After the long subdiabetic period, insulin treatment did not influence the embryo distribution in the experimental group significantly. In both STZ-treated groups the incidence of degenerated embryos was markedly increased. There were 6 mice in the I+ group ($n=11$) and 7 mice in the I− group ($n=11$) which produced only degenerated embryos.

Table 1
Morphological stages of embryos recovered from day 3 pregnant control and subdiabetic mice (mean ± S.D.)

		STZ 8–11 days before fertilization		STZ 14–17 days before fertilization	
	Control mice	I+	I−	I+	I−
Number of mice	17	7	10	11	11
Glycaemia (mmol/l)	3.73±0.49	4.20±0.20	3.84±0.57	3.90±0.79	3.75±0.74
Total no. of embryos	135	49	92	85	85
Embryos/mice	7.9±2.2	7.0±3.9	9.2±2.7	7.7±1.4	7.7±2.0
% of 4 – 8 cells	71.1	81.6	43.5	49.4	37.6
% of 1 – 3 cells	10.4	12.2	33.7	2.4	2.4
% of degenerated	18.5	6.1	22.8	48.2	60.0
χ^2 test					
STZ vs control		$p>0.05$	$p<0.001$	$p<0.001$	$p<0.001$
I+ vs I−		$p<0.001$		$p>0.05$	

Discussion

The present study has confirmed that the preimplantation stage of embryo development is highly sensitive to a single subdiabetogenic dose (65 mg/kg) of streptozocin. We have demonstrated that the subdiabetic state in mice without apparent changes in glycaemia induces significant changes in the distribution pattern of preimplantation embryo stages recovered on day 3 of pregnancy.

Uncontrolled spontaneous diabetes mellitus (diabetic NOD mice) *per se* was shown to retard mouse embryo development 72 h after HCG (Moley *et al.* 1991). Diamond *et al.* (1989) reported a reduced number of 2-cell embryos in diabetic mice (330 mg/kg STZ or 300 mg/kg alloxan) and their slower development *in vitro*. Nevertheless, this observation is partly at variance with the data reported by Beebe and Kaye (1990), showing that STZ-induced diabetes (190–240 mg/kg) in mice did not influence the proportion of 2-cell embryos which developed into blastocysts *in vitro*. However, these authors showed that the rate of protein synthesis of blastocysts collected on day 4 from diabetic mice (190 mg/kg STZ) was significantly lower in comparison to the controls and that insulin treatment led to recovery (Beebe and Kaye 1991). In the above mentioned studies (Diamond *et al.* 1989, Beebe and Kaye 1991,

Moley *et al.* 1991), gonadotropins were used to induce superovulation thus bypassing, to a certain extent, normal hypothalamic-pituitary regulation of folliculogenesis, which could be impaired in streptozocin-induced diabetes (Vomachka and Johnson 1982). Mice with spontaneous cycles were used in the present study and a comparison of the number of embryos showed no significant difference between control and STZ-treated groups. It can therefore be concluded that endogenous gonadotropin levels were adequate for ensuring normal folliculogenesis, at least as far as the number of embryos is concerned.

Daily doses of insulin administered to STZ-treated mice normalized the impaired development of embryos in our study only in the short STZ-mating interval. Similarly, insulin replacement therapy (once daily) only partly prevented the diabetes-induced impairment of rat embryo preimplantation development (De Hertogh *et al.* 1992). More frequent insulin treatment of subdiabetic mice in the long STZ-mating experiment might be more effective than insulin once a day, similarly as was shown in diabetic female mice (Diamond *et al.* 1989, Beebe and Kaye 1991).

Since direct insulin effects were mainly observed in embryos after compaction, it is probable that the subdiabetic state influenced embryo development through an alteration of maternal metabolism. It was shown that elevation of glucose levels (220 mg/dl) *per se* directly retarded the development from the 2-cell stage to the blastocyst cultured *in vitro* (Diamond *et al.* 1991). Since subdiabetogenic streptozocin treatment altered the glucose tolerance of mice without detectable changes of basal glycaemia (data not shown), it is possible that greater variations of blood glucose levels connected with absorption of nutrients exerted a negative influence on preimplantation embryo development.

It is interesting to note the evident difference in the proportion of degenerated embryos. Whereas in control mice and after short STZ-mating interval the rate of degeneration was less than 22.8 %, there was a marked increase to 48–60 % after the long STZ-mating interval. Our findings suggest that 14-17 days of the subdiabetic state, despite insulin treatment, permanently impairs embryonal development. The discrepancy of these findings with those of Diamond *et al.* (1989) and Beebe and Kaye (1990) who reported no apparent increase in the number of degenerated embryos, may be due to the fact that they used a shorter STZ-mating interval, i.e. 6 days.

Our morphological analysis of the developmental period of cleavage represented only a static examination of the dynamic process which reflects only a part of the complex regulation. Nevertheless, it has been shown that the subdiabetic state of female mice does impair preimplantation embryo development, but it is difficult to determine whether this is due to the direct action of insulin or to changes in maternal metabolism. It appears that strict metabolic and hormonal control during the earliest phases after fertilization is required to ensure normal development of the embryo. More studies are needed to elucidate the explicit role of insulin during the early stages of mammalian development.

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