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Effects of Impaired Maternal Insulin Secretion on Preimplantation Embryo Development in ICR Mice

P. REHÁK, J. MIHÁLIK, J. VESELÁ, Š. ČIKOŠ, V. BARAN, J. KOPPEL

Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic

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Summary

To investigate the significance of impaired insulin secretion on preimplantation embryo development, outbred ICR female mice received an injection of a single dose of streptozotocin 200 mg.kg^{-1} 14–17 days before fertilization. Oocytes were collected 24–26 h after hCG injection. Morphological evaluation revealed a lower percentage of oocytes with second polar bodies from streptozotocin-treated females in comparison with controls. Furthermore, in this group the incidence of degenerated embryos significantly increased after 120 h *in vitro* cultivation. Insulin (5 U per 100 g b.w.) administered twice daily to streptozotocin-treated mice significantly improved the Embryonic development. Morphological analysis of oocyte maturation in streptozotocin-treated mice showed no significant differences in comparison with control mice. It could be concluded that marked changes in preimplantation embryo development were detected in outbred ICR mice after streptozotocin administration and this process was partly reversible by insulin treatment. Furthermore, it was shown that the process of fertilization was negatively influenced and that during *in vitro* cultivation the delayed effects of impaired insulin secretion resulted in an increase of embryo degeneration at the time following the third mitotic cleavage.

Key words
Preimplantation embryo – Development – Diabetes – Insulin – Streptozotocin

Introduction

Functional studies in several species have emphasized the importance of the insulin family of growth factors (insulin, insulin-like growth factors I and II) in preimplantation Embryonic development. In the maternal reproductive tract, insulin and insulin-like growth factors are present and the preimplantation embryo may synthesize endogenous insulin-like growth factors. During early Embryonic development, the expression of insulin and insulin-like growth factors and their specific receptors is regulated temporally and spatially. These findings suggest that insulin and insulin-like growth factors could influence the preimplantation embryo in a para- and autocrine fashion (reviewed by Schultz and Heyner 1993).

Impaired maternal insulin secretion induced in rodents by chemically induced diabetes (streptozotocin, alloxan) influenced preimplantation embryo development (Diamond *et al.* 1989, Beebe and Kaye 1991, De Hertogh *et al.* 1992). We have recently observed that the subdiabetic state in inbred BALB/c mice induced by streptozotocin (65 mg.kg^{-1}) resulted in impaired preimplantation embryo development at the 8-cell stage and almost one half of the morphologically normal 2-cell embryos isolated from subdiabetic females was incapable of development up to the 8-cell Embryonic stage even in a non-diabetic maternal environment (Veselá *et al.* 1993, 1994). These observations emphasize the important role of insulin during preimplantation development and the possibility that impaired maternal insulin secretion has negative effects on early embryo development.

The aim of our study was to further investigate the significance of impaired insulin secretion induced by the streptozotocin treatment on preimplantation embryo development in outbred ICR mice.

Methods

Animals

The study was conducted on mice of the outbred ICR strain, 4–5 weeks old. Mice were given free access to food (ST-1 diet, TOP DOVO, Dobrá Voda, Slovak Republic) and water. Animals were maintained in a 12 h light/12 h dark cycle (light on at 06.00 h).

Streptozotocin treatment

Streptozotocin (STZ, Sigma, Germany) was dissolved in a sterile sodium citrate buffer (0.1 $mol.l^{-1}$, pH 4.5) and injected i.p. into females within 5 min after preparation. Mice received an injection of a single dose of STZ 200 $mg.kg^{-1}$ body weight, 14–17 days before fertilization. Control animals were administered a citrate buffer.

Ultralente insulin (Pur-Insulin-Superdep Spofa, Prague) was injected twice daily s.c. in a dose of 5 U per 100 g starting 9 days after STZ treatment. Control animals and STZ mice without insulin treatment were treated twice daily with NaCl 154 $mmol.l^{-1}$

Blood glucose determinations

Blood glucose determinations were made by glucose-oxidase method (Oxochromglucose, Lachema, Brno, Czech Republic). Blood samples (tail vein) were obtained from animals in each group (fasted overnight) ten days after STZ administration.

Fertilization

Mice were superovulated by s.c. injection of 5 IU pregnant mare serum gonadotropin followed 46 h later by i.p. 5 IU human chorionic gonadotropin. Females were mated with males of the same strain overnight. Mating was confirmed by identification of a vaginal plug.

Oocyte collection and culture in vitro

Females were killed by cervical dislocation 24–26 h after hCG injection (13.00–15.00 h). To eliminate experimental bias, at least two independent series were performed in each group and the results were pooled. Oocytes were obtained from both fallopian tubes using a dissecting microscope (Technival, Poland) by flushing the oviduct with KSOM medium (Erbach *et al.* 1994). The oocytes were observed under a differential interference contrast microscope (Jenamed, Variant, Germany) and assessed for their morphological aspects according to the following criteria: a) degenerated – oocytes with signs of degeneration, b) unfertilized – oocytes without second polar bodies, c) normal – oocytes with second polar bodies.

The normal oocytes with second polar bodies were cultured *in vitro* for 120 h in 40 μl droplets of the KSOM medium in 5 % CO_2 in air at 37 °C. Each droplet contained oocytes isolated from one mouse. During cultivation *in vitro*, embryos were examined under the differential interference contrast microscope and assessed according to the morphological criteria.

Oocyte collection and maturation in vitro

Fully grown oocytes were collected from ICR mice (18–20 days after STZ injection) killed by cervical dislocation 46–48 h after injection of 5 IU PMSG. Ovaries were removed and placed into an E199 medium (USOL, Prague, Czech Republic) containing 10 % (v/v) foetal bovine serum (Veterinary University, Brno, Czech Republic) supplemented with HEPES and calcium lactate (Sigma, Germany). Antral follicles were ruptured with a sterile needle and oocytes surrounded by cumulus cells were collected and incubated *in vitro* for 15 h in 50 μl droplets of the E199 medium in 5 % CO_2 in air at 37 °C. Each droplet contained oocytes isolated from one mouse. The oocytes were fixed and stained with 2 % orcein and examined under the differential interference contrast microscope (Jenamed, Variant, Germany).

Statistical analysis

Results are given as means ± S.D. The chi-square (χ^2) test was used to detect differences in the distribution of preimplantation embryos. Statistical analysis of the oocyte number and glycaemia was done by Student's t-test.

Results

Morphology of oocytes recovered 24–26 h after hCG injection from control and streptozotocin-treated mice as well as after in vitro culture (120 h)

Oocytes were collected 24–26 h after hCG injection from control and streptozotocin-treated (STZ 200 $mg.kg^{-1}$) mice. The number of oocytes per mouse did not differ significantly ($p>0.05$) between the STZ and control groups. We observed significantly higher basal glycaemia in females treated with streptozotocin compared with control animals ($p<0.05$, $p<0.01$). Morphological evaluation of oocytes from STZ mice without insulin treatment revealed a lower percentage of normal oocytes with second polar bodies in comparison with the controls ($p<0.001$). Insulin administration twice daily resulted in a significant difference between the STZ groups ($p<0.001$) and the proportion of oocytes with second polar bodies was comparable to control levels ($p<0.05$). Furthermore, oocytes with second polar bodies from insulin-treated STZ mice after *in vitro* 120 h cultivation (Day 6) developed no significant changes ($p>0.05$) in the distribution pattern compared with the controls. However, morphological analysis of embryos from STZ mice without insulin treatment after 120 h *in vitro* cultivation (Day 6) has shown an increased incidence

($p<0.05$) of degenerated embryos (32 %) in comparison with the controls (19 %). The proportion of degenerated embryos in this group (compared to the controls and insulin-treated STZ mice) increased between Day 3 and Day 5. Insulin administration twice daily resulted in a significant difference ($p<0.001$) between the STZ groups (Tables 1 and 2, Fig. 1).

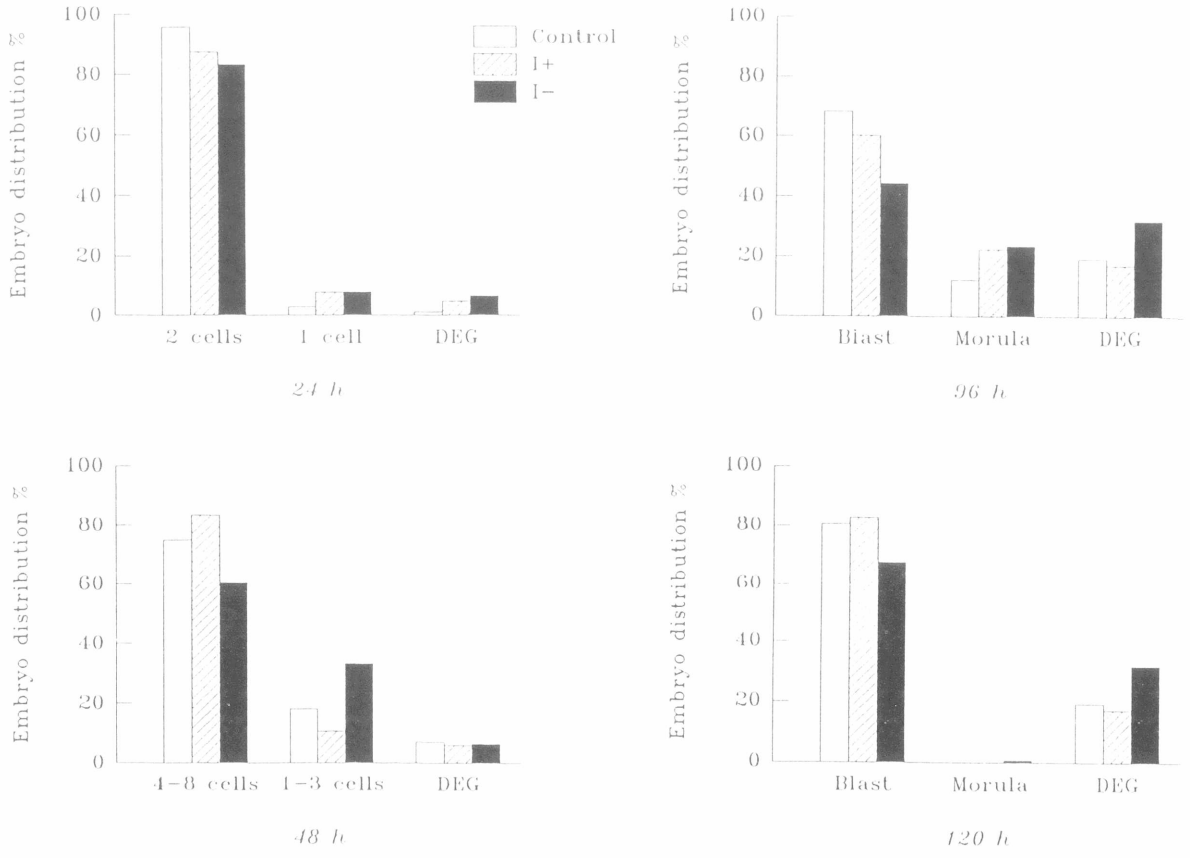

Fig. 1
Morphology of oocytes recovered 24–26 h after hCG injection from control and streptozotocin-treated mice after culture in vitro. Control – open squares, streptozotocin: insulin-treated – hatched squares (I+), insulin non-treated – full squares (I−), DEG – degenerated embryos.

Table 1
Number of oocytes 24–26 h after hCG injection in streptozotocin-treated (STZ 200 $mg.kg^{-1}$) and control mice

Parameter	Controls	STZ + insulin	STZ − insulin
Number of mice	10	13	11
Blood glucose ($mmol.l^{-1}$)	3.1±0.5	5.1±2.8^{+}	7.6±4.9^{++}
Total number of oocytes	239	245	283
Oocytes/mouse	23.9±8.8	18.9±8.3	25.7±18.7
% with 2nd polar body	71.5	69	55.1
% without 2nd polar body	22.6	18.4	37.1
% degenerated	5.9	12.6	7.8
χ^2 test			
STZ versus Controls		$p<0.05$	$p<0.001$
Insulin+ versus Insulin−		<0.001	

Significantly different from controls - $^{+}p<0.05$, $^{++}p<0.01$

Morphology of oocytes recovered from control and streptozotocin-treated mice as well as after in vitro culture (15 h)

In the second experiment, oocytes were collected 46–48 h after PMSG from streptozotocin-treated (STZ 200 mg.kg^{-1}) mice and control mice. Morphological evaluation of oocytes from STZ mice with and without insulin treatment revealed no apparent changes in the number of oocyte-cumulus complexes in comparison with the controls. There were no significant changes between the experimental groups and controls in the proportion of MII oocytes after 15 h of *in vitro* culture (Table 3).

Table 2
Results of *in vitro* culture of oocytes with second polar bodies from streptozotocin-treated and control mice

Parameter	Controls	STZ + insulin	STZ − insulin
Number of oocytes	171	169	156
		48 h in vitro	
% 4–8 cell	74.9	83.4	60.3
% 1–3 cell	18.1	10.7	33.3
% degenerated	7.0	5.9	6.4
χ^2 test			
STZ versus Controls		p>0.05	p<0.01
Insulin + versus Insulin −		p<0.001	
		120 h in vitro	
% blastocysts	80.7	82.8	67.4
% morulae	0	0	0.6
% degenerated	19.3	17.2	32
χ^2 test			
STZ versus Controls		p>0.05	p<0.05
Insulin + versus Insulin−		p<0.001	

Table 3
Morphology of oocytes recovered from control and streptozotocin-treated mice and after *in vitro* culture (15 h)

Parameter	Controls	STZ + insulin	STZ − insulin
		At recovery	
Number of collected oocytes	21	33	22
% GV oocyte	90.5	100	100
% degenerated	9.5	0	0
χ^2 test			
STZ versus Controls		p>0.05	p>0.05
		15 h in vitro	
Number of cultured oocytes	83	56	79
% MII	96.4	96.4	97.5
% degenerated	3.6	3.6	2.5
χ^2 test			
STZ versus Controls		p>0.05	p>0.05

Discussion

Our results indicate the delayed effects of impaired insulin secretion as the proportion of degenerated embryos increased between 48 h (Day 3) and 96 h (Day 5) of culture *in vitro* from day 1 after hCG injection. *In vivo* insulin therapy (twice daily) appeared to reverse this delayed developmental alteration as there were little differences in the distribution pattern of embryo stages between controls and streptozotocin-treated animals receiving insulin injections. These results confirm our previous findings and show the importance of maternal insulin secretion for the process of fertilization and very early preimplantation development.

Diamond et al. (1989) reported delayed oocyte maturation and a reduced number of 2-cell embryos in diabetic mice (inbred strain $B_6C_3F_1$, 330 $mg.kg^{-1}$ STZ, or 300 $mg.kg^{-1}$ alloxan) and their slower development *in vitro*. Nevertheless, Beebe and Kaye (1990), showed that STZ (190 $mg.kg^{-1}$) induced diabetes in mice (outbred strain Quackenbush) but did not influence the proportion of 2-cell embryos, which developed into blastocysts after 48 h cultivation *in vitro*. The differences were explained by the use of different strains of mice or by the administration of various STZ doses, but they did not correlate with the differences in the genetic background between outbred and inbred strain (Beebe and Kaye 1990). Wolf *et al.* (1984) demonstrated that the genetic background influenced significantly the susceptibility of different inbred strains of mice to streptozotocin-induced diabetes.

Recently, it has been shown in a detailed study that protein synthesis in preimplantation mouse 8-cell embryos was influenced by insulin and IGFs, and further, each growth factor affected specific proteins (Shi *et al.* 1994). It is known that maternal insulin and IGF-I are present in the maternal reproductive tract. Studies at the mRNA level have confirmed the temporal expression pattern of receptors for insulin and IGF-I in late 8-cell murine embryos (reviewed by Schultz and Heyner 1993). A direct insulin influence on the preimplantation embryo remains questionable as Diamond *et al.* (1991) observed that insulin addition to the culture media had no detectable growth-promoting effects on 2-cell embryos *in vitro*.

Our findings show that impaired maternal insulin secretion during the pre- and earliest post-conception stage has detrimental effects on embryo development which persist despite their removal from the maternal diabetic milieu and culture *in vitro*. Insulin replacement therapy appeared to reverse these negative effects, as we observed a higher percentage of fertilized embryos and better embryo development during *in vitro* culture. It is probable that the main impact of maternal impaired insulin secretion is on fertilization and preimplantation embryo development since we did not detect any significant changes in oocyte maturation in streptozotocin-treated females.

It could be concluded that, in outbred ICR mice, marked changes in preimplantation embryo development were detected after streptozotocin administration and this process was partly reversible by insulin treatment. Streptozotocin treatment of ICR mice also apparently lowered the proportion of fertilized oocytes. During *in vitro* cultivation, delayed effects of impaired insulin secretion increased the incidence of morphologically degenerated embryos after the third mitotic cleavage.

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Reprint Requests

Dr. J. Koppel, Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4, 040 01 Košice, Slovak Republic.