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Preimplantation Embryo Development in ICR Mice after Streptozotocin Treatment

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Summary

To investigate the significance of impaired insulin secretion on preimplantation embryo development, outbred ICR female mice received a single injection of streptozotocin 130 mg (low) and 160 mg (subdiabetic) kg^{-1}, 14–17 days before fertilization. Preimplantation embryos were collected on day 3 of pregnancy, four to eight-cell embryos were cultured *in vitro* 48 h (day 5) and their cell number was estimated. After spontaneous ovulation, the significantly different distribution pattern in comparison with the controls was detected only in preimplantation embryos isolated from subdiabetic (160 $mg.kg^{-1}$ streptozotocin) mice. Furthermore, the incidence of degenerated embryos was significantly increased after 48 h *in vitro* cultivation. The analysis of cell number distribution in embryos after cultivation *in vitro* indicated a significant delay in cell proliferation in both experimental groups (130 and 160 $mg.kg^{-1}$ streptozotocin) in comparison with control mice. After superovulation, the only significant difference was found in the distribution pattern of embryos isolated on day 3 of pregnancy from subdiabetic (160 $mg.kg^{-1}$ streptozotocin) mice. No significant differences were found after embryo cultivation *in vitro*. It could be concluded that, in outbred ICR mice, lower streptozotocin treatment (130 $mg.kg^{-1}$) influenced only cell distribution of *in vitro* cultured embryos after spontaneous ovulation. In ICR mice, marked changes in preimplantation embryo development were detected only after subdiabetic (160 $mg.kg^{-1}$) streptozotocin treatment. During *in vitro* cultivation delayed effects of impaired insulin secretion resulted in an increase of embryo degeneration at the time after the third mitotic cleavage. Our results indicate that the effects of impaired maternal insulin secretion on preimplantation embryo development in mice are marked and consistent after spontaneous ovulation. Superovulation apparently disguises subtle changes in preimplantation embryo development after low and subdiabetic streptozotocin treatment.

Key words
Preimplantation embryo – Streptozotocin – Superovulation – Insulin – ICR mice

Introduction

Growth factors are known to play a significant role during preimplantation embryonic development. Numerous studies have emphasized the importance of the insulin family of growth factors (insulin, insulin-like growth factors I and II) in embryonic development. During preimplantation embryonic development, the expression of insulin and insulin-like growth factors and their specific receptors is regulated temporarily and spatially. Insulin and insulin-like growth factors could influence the preimplantation embryo in a para- and autocrine fashion (reviewed by Schultz and Heyner 1993).

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It has been shown that rodents with chemically induced diabetes (streptozotocin, alloxan) are a suitable model for studying the influence of impaired maternal insulin secretion on 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, even from the two-cell stage (Veselá *et al.* 1993, 1994). Furthermore we have demonstrated that insulin changes in subdiabetic BALB/c mice had a deleterious influence on oocyte fertilization (Veselá *et al.* 1995).

The aim of this study was to estimate the effects of impaired insulin secretion induced by streptozotocin treatment on preimplantation embryo development in ICR mice after spontaneous ovulation and superovulation.

Methods

Animals

The study was conducted on female mice of the outbred ICR strain, 4–5 weeks old. Mice were given free access to food (ST-1 diet, TOP DOVO, Dobrá Voda, Slovakia) and water. Animals were maintained in a 12 h light/12 h dark cycle (light on at 06:00 h). Only females with a confirmed normal 4–5 days cycle were used.

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. 5 min after preparation. Mice received a single dose of STZ – 130 and 160 $mg.kg^{-1}$ body weight, 14–17 days before fertilization. Control animals were given a citrate buffer solution.

Blood glucose determinations

Blood glucose determinations were made by the glucose-oxidase method (Oxochrom-glucose, Lachema, Brno, Czech Republic). Ten days after STZ administration, animals in each group (fasted overnight) received an i.p. injection of 10 % glucose solution in a dose 1 $g.kg^{-1}$ b.w. Blood samples (tail vein) were obtained immediately before glucose administration and 30 min after.

Fertilization

Spontaneous ovulation: females were kept with males of the same strain overnight. If a vaginal plug was found in the following morning, this was considered day 1 of the pregnancy. After five negative matings, animals were excluded from the experiments.

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

Embryo collection and culture in vitro

Females were killed by cervical dislocation on day 3 of pregnancy (07:00–09:00 h, 66–68 h after hCG administration). To eliminate experimental bias at least two independent series were taken in each group and the results were pooled. Embryos were obtained from both fallopian tubes and uterus using a dissecting microscope (Technival, Poland) by flushing the oviduct with E199 medium (USOL, Prague, Czech Republic). The embryos were observed by differential interference contrast microscope (Jenamed, Variant, Germany) and assessed for their morphological properties according to the following criteria: a) degenerated – embryos with signs of degeneration, 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.

The normal embryos (the 4 to 8-cell stage) were cultured *in vitro* 48 h in 40 μl droplets of E199 medium supplemented with GPBoS (growth proteins from bovine serum, USOL, Prague, Czech Republic) in 5 % CO_2 in air at 37 °C. After 48 h cultivation *in vitro* the embryos were examined under a differential interference contrast microscope (Jenamed, Variant, Germany) and assessed according to the following morphological criteria: a) degenerated embryos, b) morulae, c) blastocysts. Blastocysts were placed in 0.9 % sodium citrate solution for 15 min. A microdrop of this solution was placed on a grease-free slide for fixation (methanol and acetic acid in a ratio 3:1). After air drying, the preparations were stained with 4',6-diamidino-2-phenylindole (Sigma, Germany). After mounting, the cell number was counted using UV light epifluorescence (Jenalumar a/d contrast; Carl Zeiss Jena, 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 blood glucose concentrations and embryo number was done by Student's t-test.

Results

Morphology of embryos recovered from pregnant (day 3) control and STZ-treated mice and embryos cultured in vitro 48 h – spontaneous ovulation

The basal glycaemia and glucose tolerance were unchanged after 130 $mg.kg^{-1}$ streptozotocin. We observed significantly altered glucose tolerance in females treated with 160 $mg.kg^{-1}$ streptozotocin compared with control animals ($p<0.001$). The number

of embryos per mouse did not differ significantly ($p>0.05$) between streptozotocin-treated and control groups (Table 1).

Preimplantation embryos were collected on day 3 of pregnancy and four to eight-cell embryos were cultured *in vitro* for 48 h (day 5). Morphological analysis of embryos from mice treated with 130 mg.kg^{-1} STZ on day 3 and after 48 h cultivation did not reveal any significant changes ($p>0.05$) in the distribution pattern compared with the controls. Preimplantation embryos isolated from subdiabetic mice (160 mg.kg^{-1} STZ) had a significantly different distribution pattern in comparison with the controls ($p<0.025$). Furthermore, the incidence of degenerated embryos significantly ($p<0.025$) increased in this group after 48 h *in vitro* cultivation (Tables 1 and 2).

Analysis of cell number distribution in embryos after cultivation *in vitro* indicated a significant ($p<0.025$, $p<0.01$) delay in cell proliferation in both experimental groups (130 and 160 mg.kg^{-1} streptozotocin) in comparison with control mice (Table 3).

Table 1. Morphological stages of embryos collected from day 3 (spontaneous ovulation) pregnant streptozotocin (130 and 160 mg.kg^{-1}) treated mice and control mice (mean ± S.D.)

	130 mg STZ	Control	160 mg STZ	Control
Number of mice	19	12	16	20
Glycaemia[a] time 0	3.3±0.5	3.0±0.4	2.6±0.7	2.7±0.4
after 30 min	7.5±1.8	6.4±1.2	7.3±1.5^{+++}	5.3±1.1
Total no. of embryos	169	101	175	242
Embryos/mice	8.9±2.3	8.4±4.1	10.9±3.3	12.1±3.4
% of 4–8 cell	85.2	90.1	90.3	96.3
% of 1–3 cell	14.2	9.9	9.1	2.9
% of degenerated	0.6	0	0.6	0.8
χ^2 test: streptozotocin versus control	$p>0.05$		$p<0.025$	

[a]Blood glucose (mmol.l^{-1}) time 0 (basal value) and 30 min after i.p. glucose 1 g.kg^{-1}. Streptozotocin versus control significantly different (t test): $^{+++}p<0.001$

Table 2. Distribution pattern of embryos collected from streptozotocin (130 and 160 mg.kg−1) treated mice and control-mice (spontaneous ovulation) cultured *in vitro* for 48 h

	130 mg STZ	Control	160 mg STZ	Control
No. of cultured 4–8 cell embryos	142	72	151	228
% blastocysts	97.9	97.2	92.0	93.9
% morula	1.4	0	2.0	4.8
% degenerated	0.7	2.8	6.0	1.3
χ^2 test streptozotocin versus control	$p>0.05$		$p<0.025$	

Table 3. Distribution of cell number of embryos collected from streptozotocin (130 and 160 mg.kg^{-1}) treated mice and control mice (spontaneous ovulation) cultured *in vitro* for 48 h

	130 mg STZ	Control	160 mg STZ	Control
No. of stained blastocysts	124	62	46	103
% 33–48 cells	16.1	17.7	43.5	22.3
% 49–64 cells	32.3	27.4	30.4	23.3
% 65–96 cells	46.8	35.5	26.1	46.6
% 97–128 cells	4.8	19.4	0	7.8
χ^2 test: streptozotocin versus control	$p<0.025$		$p<0.01$	

Morphology of embryos recovered from pregnant (day 3) control and STZ-treated mice and embryos cultured in vitro (48 h) – superovulation

The basal glycaemia and glucose tolerance were unchanged after 130 mg.kg^{-1} streptozotocin ($p>0.05$). We observed significantly altered basal glycaemia and glucose tolerance in females treated with 160 mg.kg^{-1} streptozotocin compared with control animals ($p<0.01$). The number of embryos per mouse did not differ significantly ($p>0.05$) between streptozotocin-treated and control groups (Table 4).

Table 4. Morphological stages of embryos collected from day 3 pregnant (superovulation) streptozotocin (130 and 160 mg.kg^{-1}) treated mice and control mice (mean ± S.D.)

	130 mg STZ	Control	160 mg STZ	Control
Number of mice	9	8	11	13
Glycaemia at time 0	2.9±0.6	3.0±0.4	4.1±0.5++	3.1±0.5
after 30 min	6.1±2.3	6.2±1.2	8.9±1.6++	5.2±0.8
Total no. of embryos	174	183	178	179
Embryos/mice	19.3±6.3	22.9±11.8	16.2±6.3	13.8±4.5
% of 4–8 cell	78.2	79.8	72.5	83.3
% of 1–3 cell	12.7	9.8	11.2	11.2
% of degenerated	9.2	10.4	16.3	5.6
χ^2 test: streptozotocin versus control	$p>0.05$		$p<0.01$	

[a]Blood glucose (mmol.l^{-1}) time 0 (basal value) and 30 min after i.p. glucose 1 g.kg^{-1}. streptozotocin versus control significantly different (t test): ++ $p<0.01$.

Table 5. Distribution pattern of embryos collected from streptozotocin (130 and 160 mg.kg^{-1}) treated mice and control mice (superovulation) cultured *in vitro* for 48 h

	130 mg STZ	Control	160 mg STZ	Control
No. of cultured 4–8 cell embryos	113	130	127	149
% blastocysts	97.3	98.5	97.6	98.7
% morula	0	0	0.8	0
% degenerated	2.7	1.5	1.6	1.3
χ^2 test: streptozotocin versus control	$p>0.05$		$p>0.05$	

Table 6. Distribution of cell number of embryos collected from streptozotocin (130 and 160 mg.kg^{-1}) treated mice and control mice (superovulation) cultured *in vitro* for 48 h

	130 mg STZ	Control	160 mg STZ	Control
No. of stained blastocysts	90	98	90	104
% 33–48 cells	14.4	22.4	25.6	32.7
% 49–64 cells	32.2	33.7	41.1	35.6
% 65–96 cells	46.7	38.8	30	30.7
% 97–128 cells	6.7	5.1	3.3	1.0
χ^2 test: streptozotocin versus control	$p>0.05$		$p>0.05$	

Preimplantation embryos were collected on day 3 of pregnancy and four to eight-cell embryos were cultured *in vitro* for 48 h (day 5). Morphological analysis of embryos from mice treated with 130 mg.kg^{-1} STZ on day 3 exhibited no significant changes ($p>0.05$) in the distribution pattern compared with controls. Preimplantation embryos isolated from subdiabetic mice (160 mg.kg^{-1} STZ) had a significantly different distribution pattern in comparison with the controls ($p<0.01$). No significant changes ($p>0.05$) were detected in either of the experimental groups after *in vitro* 48 h cultivation (Tables 4 and 5).

Analysis of the cell number distribution in embryos after cultivation *in vitro* revealed no significant changes in cell proliferation in both experimental groups (130 and 160 mg.kg^{-1} streptozotocin) in comparison with control mice (Table 6).

Discussion

Our results indicate that the effects of the impaired insulin secretion on preimplantation embryo development depend on streptozotocin dose, on the fertilization procedure and the analytical method employed. We have shown that ICR mice after 130 mg.kg^{-1} STZ were without apparent changes in glycaemia and only after spontaneous ovulation and embryo cultivation *in vitro* a delay in cell proliferation of blastocysts was observed in the experimental group. In both experimental groups (spontaneous ovulation and superovulation) changes were observed in preimplantation embryo development only in the subdiabetic state (after 160 mg.kg^{-1} STZ). In this case, the higher number of significant changes was also detectable after spontaneous ovulation. Furthermore, the present results have shown that morphologically normal four to eight-cell embryos recovered from subdiabetic females on day 3 of pregnancy and cultured *in vitro* have a significantly greater ratio of degeneration at the time of recovery (48 h later) in comparison with the control group. It could be considered that a considerable number of embryos was damaged on day 3 of pregnancy, but this was not detectable using light microscopy. During *in vitro* cultivation, delayed effects of impaired insulin secretion on embryo development appeared. Similarly, it was shown that embryos recovered from diabetic rats on day 5 of pregnancy and incubated *in vitro* for 72 h exhibited a marked impairment in growth, a higher rate of degeneration and they contained fewer cells than control embryos (Pampfer *et al.* 1994). Very recently, we have detected marked changes in preimplantation embryo development in ICR mice treated with 200 mg.kg^{-1} STZ and this process was partly reversible by insulin treatment (Rehák *et al.* 1996). Furthermore, it was shown that the process of fertilization was negatively influenced and that delayed effects of impaired insulin secretion during *in vitro* cultivation resulted in an increase of embryo degeneration at the time after third mitotic cleavage.

Our results indicate the possibility that ovarian stimulation resulting from the gonadotropin treatment might mask some subtle effects of impaired maternal insulin secretion (subdiabetic state) on preimplantation embryo development. However, studies dealing with the effects of diabetes on preimplantation embryo development (Diamond *et al.* 1989, Beebe and Kaye 1991, Moley *et al.* 1991, 1994, 1996) used superovulation. In a recent study (Rehák *et al.* 1996,) we were also forced to use superovulation as the rate of fertilization in diabetic ICR mice (200 mg.kg^{-1} STZ) was very low after spontaneous ovulation. It could be hypothesized that superovulation could influence preimplantation embryo development after a mild STZ dose by elevation of IGF-I levels decreased in diabetes (Edwards *et al.* 1996, Katagiri *et al.* 1996), or simply by the fact that the increased embryo loss after superovulation is sufficient to mask subtle effects due to the subdiabetic state.

Studies on animal diabetes models indicated a correlation between the degree of metabolic control and preimplantation embryo development (Diamond *et al.* 1989, Vercheval *et al.* 1990, Moley *et al.* 1994, 1996). We detected only subtle changes in ICR mice (the delay in blastocyst cell proliferation) during preimplantation development using the low dose of STZ (130 mg.kg^{-1}) whereas impaired preimplantation embryo development in ICR mice emerged only after the subdiabetic dose of STZ (160 mg.kg^{-1}). Similarly, when we used subdiabetic BALB/c mice (Veselá *et al.* 1993, 1994) we observed only marginal changes in glucose levels, which were unlikely to affect directly preimplantation embryos.

Our findings show that slightly impaired maternal insulin secretion during the pre- and earliest post-conception stage has detrimental effects on embryo development which continues despite their removal from maternal diabetic milieu and culture *in vitro*. Our results open a promising approach to studies of the insulin role in preimplantation embryo development. We have partly analyzed two strains of laboratory mice (outbred ICR – Rehák *et al.* 1996 and inbred BALB/c – Veselá *et al.* 1993, 1994, 1995) for their early embryo development after STZ treatment. The detailed analysis of their hormonal profile (insulin, IGF) and metabolic characterization (ketoacids etc.) can serve as a key to the difference in their reaction to STZ treatment that could be related to the observed impaired embryo development.

It can be concluded that lower streptozotocin treatment (130 mg.kg^{-1}) in outbred ICR mice influences only cell distribution of *in vitro* cultured embryos after spontaneous ovulation. In ICR mice marked changes in preimplantation embryo development were detected only after subdiabetic (160 mg.kg^{-1}) streptozotocin treatment. During *in vitro*

cultivation delayed effects of impaired insulin secretion increased embryo degeneration at the time after third mitotic cleavage. Our results indicate that the effects of impaired maternal insulin secretion on preimplantation embryo development in mice after spontaneous ovulation are clear and consistent. Superovulation apparently disguises some subtle changes in preimplantation embryo development after low and subdiabetic streptozotocin treatment.

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