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The Influence of Insulin on the *in vitro* Development of Mouse and Bovine Embryos

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Summary

To further investigate the role of insulin during preimplantation embryo development, we compared the effects of insulin on the development of mouse and bovine preimplantation embryos and on cell proliferation during culture *in vitro* in simplex media. The influence of insulin on the development of mouse zygotes was determined during cultivation in mSOF medium, alone or supplemented with glucose. Similarly, the effects of insulin on the bovine preimplantation embryo development were studied in mSOF medium. The addition of insulin into mSOF medium enhanced significantly the number of cells per mouse blastocyst. Moreover, when mSOF medium was supplemented with insulin and 0.2 $mmol.l^{-1}$ glucose, the percentage of hatched blastocysts and the mean cell number of mouse blastocysts were significantly higher. Insulin had no significant effect on the development of bovine embryos, produced by *in vitro* fertilization of *in vitro* matured oocytes. Neither the rates of developing embryos nor the mean number of cells in blastocysts were different in comparison with control embryos. Our results suggest that the *in vitro* development of mouse embryos could be enhanced by the addition of insulin to the culture medium and is further improved by the addition of glucose. In contrast to this our results indicate that insulin has no detectable beneficial effect on the preimplantation development of bovine embryos in mSOF medium.

Key words
Preimplantation embryo • Mouse • Bovine • Insulin • Development

Introduction

Active substances from the oviduct environment, namely stimulation and regulation macromolecules, which control processes of fertilization and preimplantation embryo development, are not included in defined simplex media under *in vitro* conditions. Embryos during *in vitro* cultures (IVC) often undergo a species-specific block in development and a reduction in viability. Cocultures with somatic cells, with "conditioned" media or after addition of sera to complex media, are used for the stimulation of embryo development during *in vitro* culture. However, complex culture media containing serum and/or used with cocultures may contain infectious agents (Stringfellow and Wrathall 1995). The culture media or culture conditions need to be further modified to support a higher percentage of blastocyst development *in vitro* (Eckert and Niemann 1996, Grupen *et al.* 1997, Tsuzuki *et al.* 1998, Tsai *et al.* 1999). The development of embryo to the blastocyst stage is regulated at least partially by autocrine and paracrine mechanisms, as several growth factors are

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synthesized by the preimplantation embryo itself. Growth factors, such as intercellular signals during development, may act directly by regulating the proliferation and/or differentiation of competent target cells, or as indirect signals by inducing the expression of a cascade of other genes, and this in turn affects specific developmental events. Insulin and insulin-like growth factors IGF-I, IGF-II (for review see Kane *et al.* 1997) play an important role in regulations of preimplantation embryo cell proliferation and differentiation. In our experiments we have shown the importance of maternal insulin secretion for ensuring normal preimplantation embryo development (Veselá *et al.* 1993, 1994, 1995, Rehák *et al.* 1996, Mihalik *et al.* 1998).

The aim of this study was to estimate the effects of insulin addition to culture media on the development of mouse and bovine preimplantation embryos and on cell proliferation during culture *in vitro* in simplex media.

Methods

Mouse embryos

The study was performed 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, Slovakia) and water. Animals were maintained in a 12 h light/12 h dark cycle (light on at 06:00 h). Females were superovulated by s.c. injection of 5 IU pregnant mare serum gonadotropin followed by i.p. 5 IU human chorionic gonadotropin 46 h later. Females were mated with males of the same strain overnight. Mating was confirmed by identification of a vaginal plug. Females were killed by cervical dislocation on day 2 of pregnancy (15:00 – 16:00 h, 50-51 h after hCG administration). Embryos were obtained from both fallopian tubes using a dissecting microscope (Technival, Poland) by flushing the oviduct with mSOF medium. Two- to four-cell embryos were randomly allocated into control and experimental groups. To eliminate experimental bias, at least four independent series were done in each group and the results were pooled.

Embryos were cultured in a chemically defined mSOF medium with BSA, supplemented with a solution of essential and non-essential amino acids (Takahashi and First, 1992). In the first experiment, 170 $\mu mol.l^{-1}$ insulin (bovine, Sigma, Germany) was added to the culture media. In the second experiment (control and experimental groups), we used mSOF media with 0.2 $mmol.l^{-1}$ glucose. The media for the experimental group were enriched with 170 $\mu mol.l^{-1}$ insulin.

The embryos were examined after 72 h of culture in 5% CO_2 in humidified air at 39 °C. Embryos were fixed and the percentage of blastocysts, morulae and degenerated embryos and their total cell number were recorded.

Bovine embryos

Standard techniques for maturation and fertilization of oocytes and embryo development *in vitro* were employed. Briefly, bovine ovaries were recovered from slaughtered animals at the local abattoir and transported to the laboratory at 30-32 °C in physiological saline. Oocytes were collected by aspiration of 2-7 mm

Table 1. Distribution pattern of mouse embryos and cell number in blastocysts cultured in mSOF media with insulin.

	mSOF+insulin		mSOF
No. of cultured embryos (%)	221 (100%)		233 (100%)
No. of hatching blastocysts (%)	72 (32.6%)		57 (24.4%)
No. of other blastocysts (%)	113 (51.1%)		123 (52.8%)
No. of morulae (%)	27 (12.2%)		30 (12.9%)
No. of degenerated (%)	9 (4.1%)		23 (9.9%)
χ^2 test insulin versus control		p>0.05	
Cell number in blastocysts	63.45±19.67		56.47±12.44
t-test insulin versus control		p<0.001	

follicles. Only oocytes with a complete dense cumulus and a dark cytoplasm were selected for *in vitro* maturation (IVM). 25-30 cumulus-oocyte complexes were transferred into 500 µl drops of maturation medium under mineral oil and incubated at 39 °C in a humidified incubator, under 5% CO_2 in air for 24 h. The medium used for IVM was Medium 199 10x with Earle's salts (Sigma, Germany), supplemented with 15 % cow serum (Vajta *et al.* 1996). After maturation, the oocytes were placed into a TALP medium containing 6 mg/ml fatty acid free bovine serum albumin and 10 $\mu g.ml^{-1}$ heparin. The oocytes were inseminated *in vitro* (IVF) with frozen semen of one bull (Slovakian pinzgau breed) of the proven fertility in the preliminary experiments at a concentration 1.5 x 10^6 $spermatozoa.ml^{-1}$ (Day 0). Motile spermatozoa were obtained by the swim-up procedure (Parrish *et al.* 1986). After about 20 h of coincubation (Day 1), presumptive zygotes were transferred into the drop of culture mSOF medium without removal of cumulus cells (Takahashi and First 1992).

On Day 2 the embryos were stripped of cumulus cells by careful pipetting. The percentage of cleaved embryos (with 2 cells at least) represented 75.4 % and those with 4-8 cells 58.7 %, respectively. Embryos with 4-8 cells were then randomly subjected to one of two treatments: mSOF + BSA (control group) or mSOF + BSA + 170 $\mu mol.l^{-1}$ of insulin. Embryos in both groups were examined at 168 h of culture in 5 % CO_2 in humidified air at 39 °C. Embryos were fixed on Day 8 and the percentage of blastocysts, morulae and degenerated embryos and their total cell number were counted.

Embryo morphological analysis and cell counting

The embryos were observed under a differential interference contrast microscope (Jenamed, Variant, Germany) and assessed for their morphological characteristics according to the following criteria: a) degenerated embryos, b) morulae, c) blastocysts. Blastocyst cell numbers were counted using the same procedure as was described previously (Pursel *et al.* 1985) with a slight modification. The fluorescent DNA-specific dye 4',6–diamidino–2–phenylindole (DAPI) (Sigma, Germany) was used for nucleus staining instead of Hoechst 3342. Briefly, the embryos were transferred onto a glass slide in a minimum volume of the medium into 5-10 µl drops of working solution and incubated for 5 min at room temperature. The excess of working solution was removed and embryos were embedded between a slide and coverslip with mowiol. Fresh working solution was prepared daily by combining 0.75 ml of 80 $mmol.l^{-1}$ sodium citrate dihydrate, 0.25 ml ethanol and 1 µl DAPI water stock solution (1 $mg.ml^{-1}$). Cell number counting was performed 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 the total cell number in blastocysts was done by Student's t-test. $P<0.05$ was considered as significant

Results

Insulin effect on morphology of mouse embryos after 72 h culture in vitro - mSOF

The morphological analysis of insulin-treated embryos after 72 h cultivation in mSOF revealed no significant changes ($p>0.05$) in the distribution pattern compared with controls (Table 1). The number of cells

Table 2. Distribution of cell number of mouse blastocysts (DAPI staining) cultured in mSOF media with insulin.

	mSOF+insulin	mSOF
No. of stained blastocysts	177	171
% 33-48 cells	24.3	31
% 49-64 cells	32.2	40.4
% 65-96 cells	37.9	28.6
% 97-128 cells	4.5	0
% >128 cells	1.1	0
χ^2 test insulin versus control	$p<0.01$	

per blastocyst increased significantly ($p<0.001$) in the experimental group (Table 1). Analysis of cell number distribution in blastocysts after *in vitro* cultivation indicated a significantly increased ($p<0.01$) cell proliferation in the experimental group in comparison with the control group (Table 2).

Insulin effect on morphology of mouse embryos after 72 h culture in vitro – mSOF with glucose

Morphological analysis of insulin-treated embryos after 72 h cultivation in mSOF with glucose revealed significant changes ($p<0.005$) in the distribution pattern compared with the controls, mainly due to an increase in the proportion of hatching blastocysts (Table 3, Fig. 1). Furthermore, the cell number in blastocysts increased significantly ($p<0.001$) in the experimental group (Table 3). The analysis of cell number distribution in embryos after cultivation *in vitro* revealed significantly increased cell proliferation in the experimental group ($p<0.025$) in comparison with the control group (Table 4).

Table 3. Distribution pattern of mouse embryos and cell number in blastocysts cultured in mSOF media with insulin and glucose.

	mSOF+insulin+glucose		**mSOF+glucose**
No. of cultured embryos (%)	135 (100%)		130 (100%)
No. of hatching blastocysts (%)	95 (70.4%)		68 (52.3%)
No. of other blastocysts (%)	23 (17.%)		50 (38.5%)
No. of morulae (%)	2 (1.5%)		2 (1.5%)
No. of degenerated (%)	15 (11.1%)		10 (7.7%)
χ^2 test insulin versus control		p<0.005	
Cell number in blastocysts	88.59±23.2		77.64±20.1
t-test insulin versus control		p<0.001	

Table 4. Distribution of cell number of mouse blastocysts (DAPI staining) cultured in mSOF media with insulin and glucose.

	mSOF+insulin+glucose		**mSOF +glucose**
No. of stained blastocysts	111		115
% 33-48 cells	4.6		5.2
% 49-64 cells	10.8		16.5
% 65-96 cells	48.6		61.7
% 97-128 cells	32.4		15.7
% >128 cells	3.6		0.9
χ^2 test insulin versus control		p<0.025	

Insulin effect on morphology of bovine embryos after 168h culture in vitro – mSOF

Morphological analysis of insulin treated bovine embryos after 168 h cultivation in mSOF revealed no significant changes in the distribution pattern compared with controls as well as no significant changes in cell number (Table 5, Fig. 1). The analysis of cell number distribution in blastocysts after *in vitro* cultivation indicated no significant changes of cell proliferation in experimental group in comparison with the control group (Table 6).

Table 5. Distribution pattern of bovine embryos and cell number in blastocysts cultured in mSOF media with insulin.

	mSOF+insulin		mSOF
No. of cultured embryos (%)	150 (100%)		148 (100%)
No. of hatching blastocysts (%)	10 (6.7%)		8 (5.4%)
No. of other blastocysts (%)	48 (32%)		44 (29.7%)
No. of morulae (%)	21 (14%)		12 (8.1%)
No. of degenerated (%)	71 (47.3%)		84 (56.8%)
χ^2 test insulin versus control		p>0.05	
Cell number in blastocysts	76.1±28.31		72.84±26.26
t-test insulin versus control		p>0.05	

Table 6. Distribution of cell number of bovine blastocysts (DAPI staining) cultured in mSOF media with insulin

	mSOF+insulin		mSOF
No. of stained blastocysts	58		52
% 33-48 cells	12.1		25
% 49-64 cells	27.6		11.5
% 65-96 cells	37.9		40.4
% 97-128 cells	13.8		19.2
% >128 cells	8.6		3.9
χ^2 test insulin versus control		p>0.05	

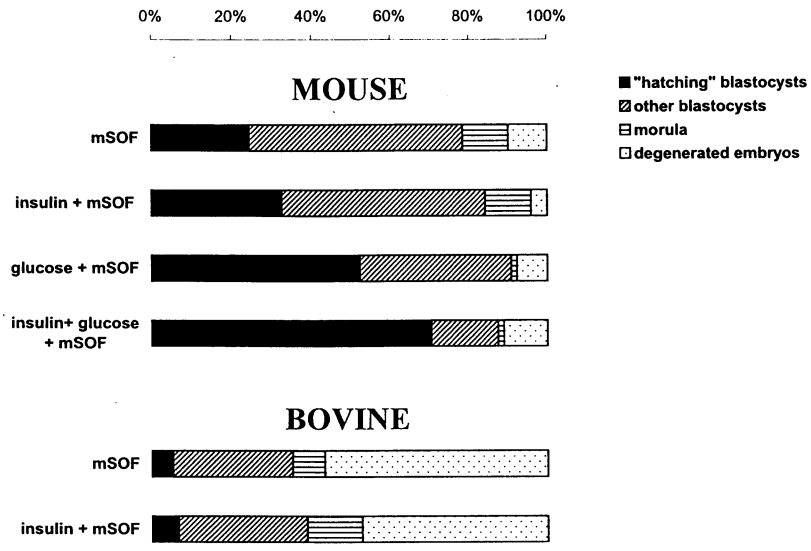

Fig. 1. *Morphological analysis of mouse and bovine embryos at the end of in vitro culture. Results are given as the percentage of total embryos. Embryos were cultured in media mSOF or mSOF with glucose (control groups) and in the same media in the presence of insulin (experimental groups).*

Discussion

Our results demonstrated that the addition of insulin into the mSOF medium significantly improved the development of mouse blastocysts as indicated by the increase in their cell number. Moreover, when mSOF was supplemented with insulin and 0.2 mmol.l^{-1} of glucose, the percentage of hatched blastocyst and the mean cell number in mouse blastocysts were significantly higher. In contrast to this, insulin had no significantly positive

effect on the development of bovine embryos, produced by *in vitro* fertilization of *in vitro* matured oocytes. Neither the rates of developing embryos nor the mean cell number in blastocysts were significantly different in comparison with the controls. However, bovine embryos cultured *in vitro* in the presence of insulin had a slightly greater number of cells per blastocysts and more embryos reached the blastocyst stage than in the control group. However, these differences were not significant.

In our experiment, we have chosen the identical culture conditions using the same simplex media for two different species, mouse and bovine oocytes, to allow a direct comparison of insulin effect on the *in vitro* preimplantation embryo development. Usually, IVM/IVF/IVC of bovine oocytes and embryos is performed at 39 °C, respecting thus the rectal temperature in cattle (Grøndahl *et al.* 1996). Although the temperature 37 °C is widely used by many laboratories for *in vitro* culture of mouse embryos, Salahuddin *et al.* (1995) observed no apparent impairment of the developmental capacity of mouse embryos cultured at 38.7 °C. Our preliminary results had shown that mouse embryos were blocked at the two-cell stage in the mSOF medium (data not shown). Therefore we had to use in our experiments *in vitro* cultures of mouse embryos isolated from females after the two-cell block (50-51 h after hCG administration).

In vitro studies of preimplantation embryos from a number of mammalian species have shown that the oviduct and uterus contain growth factors that stimulate cellular proliferation and differentiation of preimplantation embryos. It is known that insulin and insulin-like growth factors are important for embryonic growth and metabolism. Imunocytochemical studies using gold-labeled insulin have provided evidence that insulin is internalized by preimplantation mouse embryos by means of receptor-mediated endocytosis (Heyner *et al.* 1989). Moreover, the insulin receptor on early mouse embryo, investigated immunohistochemically, was present on compacting 8-cell embryos and on morulae and blastocysts (Harvey and Kaye 1991). Recently, we have detected marked changes in preimplantation embryo development in diabetic ICR mice (200 $mg.kg^{-1}$ streptozotocin) and this process was partly reversible by insulin treatment (Rehák *et al.* 1996). Furthermore, moderately impaired maternal insulin secretion during the pre- and earliest post-conception stage had detrimental effects on embryo development which continued despite their removal from maternal diabetic milieu and culture *in vitro* (Mihalik *et al.* 1998). Together with previous studies (Harvey and Kaye 1988) showing a 90 % increase in endogenous protein synthesis in blastocysts following short-term stimulation with insulin, the results of Dunglison and Kaye (1993) suggest that insulin increase the endogenous protein reserves in the embryo. It was demonstrated that insulin promotes cell proliferation and morphological development of preimplantation mouse embryos. Harvey and Kaye (1990) showed 9 % stimulation of blastocyst cell number by insulin that was entirely due to a 23 % increase in ICM cell number. Protein synthesis in preimplantation 8-cell mouse embryos was influenced by insulin and IGFs and furthermore, each growth factor affected specific proteins (Shi *et al.* 1994). Gardner and Kaye (1991) reported that insulin promoted cleavage by 16-20 % when added into the common basal medium for *in vitro* culture of 2-cell mouse embryos to morulae, blastocysts and expanded blastocysts. A similar response was observed after insulin addition at concentrations 1.7 and 170 $\mu mol.l^{-1}$, respectively. Pantaleon and Kaye (1996) demonstrated that insulin could also stimulate glucose uptake in preimplantation mouse embryos.

According to Zhang and Armstrong (1990), the addition of insulin alone to the medium does not enhance rat embryo development *in vitro*, but acts synergistically with amino acids to improve embryo development. The beneficial effect of free amino acids could not be duplicated by bovine serum albumin. Koo *et al.* (1997) demonstrated that insulin and amino acids synergistically enhance the incidence of blastocoel formation and hatching of 1-2 cell porcine embryos *in vitro*. Similarly, our results indicate marked effects of insulin on mouse embryo cell proliferation and blastocysts *in vitro* development in media supplemented with amino acids and glucose.

Interestingly, we were not able to prove any significant influence of insulin on bovine preimplantation embryo development *in vitro* despite the fact that insulin receptor mRNA is present in bovine preimplantation embryos from 1-cell stage (Schultz *et al.* 1992). Matsui *et al.* (1995a) reported that the addition of 1.7 $mmol.l^{-1}$ insulin to mSOF media increased the percentage of bovine morulae on Day 5 (5 days after *in vitro* fertilization) and also the cell number of blastocysts. However, the supraphysiological insulin concentration used was 10 times higher in comparison to our experiment, raising thus the question of its biological significance. Using high (1.7 $mmol.l^{-1}$) insulin

concentration Bowles and Lishman (1998) observed the significant improvement of bovine oocyte *in vitro* maturation, but no significant changes in embryo cleavage and blastocyst production. However, Matsui *et al.* (1995b) reported an increase in the number of bovine blastocyst cells during cultivation in the mSOF medium supplemented with 1 $mmol.l^{-1}$ glucose and 170 $\mu mol.l^{-1}$ insulin. It seems likely that the insulin effect on bovine preimplantation embryo development is essentially mediated through its influence on glucose metabolism and to a lesser extent on proteosynthesis.

Our findings show that *in vitro* development of mouse embryos could be enhanced by the insulin addition to the culture medium and further improved by the addition of glucose. In contrast to this, our results indicate that insulin has no detectable beneficial effect on the preimplantation development of bovine embryos in mSOF medium. Further studies of the growth factor effects are required to improve the culture systems so as to minimize the developmental lag of embryos *in vitro* behind their *in vivo* counterparts.

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