

Mitogenic and anti-apoptotic activity of insulin on bovine embryos produced *in vitro*

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Insulin improves development of mammalian preimplantation embryos and, in addition to the regulation of glucose transport, it exerts mitogenic and anti-apoptotic activities. The expression of glucose transporters (Glut) mediating the uptake of this essential energy substrate is critical for embryo survival. An impaired expression of Glut leads to an increase in apoptosis at the blastocyst stage and involves Bax. The various effects of insulin were unravelled by supplementing the *in vitro* culture medium with insulin ($1.7 \mu\text{mol l}^{-1}$) and (i) the rates of cleavage and blastocyst development were recorded; (ii) mitogenic activity was studied by determining the total number of blastocyst cells and the ratio between trophectoderm and inner cell mass (ICM) cells; (iii) the frequency of apoptosis in blastocysts was determined by the TdT-mediated dUTP nick-end labelling (TUNEL) assay and by quantification of the relative amounts of mRNA for Bax and Bcl-X_L; and (iv) expression for Glut1,

Glut3 and Glut8 transcripts was compared between embryos cultured in the presence or absence of insulin. Insulin increased rates of cleavage (81.2 ± 2.2 (control) to 86.0 ± 2.5) and blastocyst development (24.7 ± 1.9 to 31.3 ± 1.2), and number of blastocyst cells (123.7 ± 6.0 to 146.3 ± 6.6); the increase in the number of blastocyst cells was due to a significantly higher number of trophectoderm cells (82.3 ± 5.0 versus 100.3 ± 5.5). Blastocysts derived from cultures supplemented with insulin showed a significant decrease in apoptosis as determined by the TUNEL assay (14.8 ± 0.9 to 12.2 ± 0.7). No effects of insulin on the mRNA expression of Glut isoforms and Bax and Bcl-X_L were found. These results demonstrate that the mitogenic and anti-apoptotic effects of insulin on bovine preimplantation embryos did not correlate with changes in the amounts of mRNA for the glucose transporter isoforms Glut1, -3 and -8, or transcripts for Bax and Bcl-X_L.

Introduction

Despite significant progress in the production of embryos *in vitro* (IVP), *in vitro* embryos differ from embryos produced *in vivo* with regard to morphology, metabolism (Khurana and Niemann, 2000) and gene expression (Niemann and Wrenzycki, 2000). Advancements in the understanding of the development of embryos produced *in vitro* have been achieved using defined culture media that enable the specific effects of a variety of media supplements, such as growth factors including insulin, to be studied (Kaye and Harvey, 1995; Diaz-Cueto and Gerton, 2001).

As a potent anabolic hormone in differentiated cells (for a review, see Saltiel and Kahn, 2001), insulin stimulates glucose (Summers *et al.*, 1999) and amino acid transport, and RNA, protein and glycogen synthesis (McGowan *et al.*, 1995), and has mitogenic and

anti-apoptotic properties (Alessi and Cohen, 1998; Downward, 1998; Dalle *et al.*, 2001). Studies in preimplantation embryos demonstrated improved embryo development after supplementation of culture media with insulin and insulin-like growth factor I (IGF-I) (mouse: Harvey and Kaye, 1990; Rao *et al.*, 1990; Gardner and Kaye, 1991; Dunlison and Kaye, 1993; Heyner, 1997; rabbit: Herrler *et al.*, 1998; cow: Matsui *et al.*, 1995a; pig: Lewis *et al.*, 1992; human: Spanos *et al.*, 2000). Similar to differentiated cells, insulin increased glucose uptake, stimulated RNA and protein synthesis (Harvey and Kaye, 1988, 1991; Lewis *et al.*, 1992; Dunlison and Kaye, 1993), and had mitogenic and anti-apoptotic activities in murine and rabbit embryos (Harvey and Kaye, 1990; Herrler *et al.*, 1998). Experiments performed *in vivo* have shown that insulin improved rates of embryo development and pregnancy in diabetic animals (mouse: Diamond *et al.*, 1989; Moley *et al.*, 1991; rat: De Hertogh *et al.*, 1992), rescuing embryos from the detrimental effects of maternal hyperglycaemia. However, the exact mechanism by which insulin influences this broad range of developmental effects in preimplantation embryos is not clear.

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Glucose is an essential energy substrate for development of mammalian blastocysts (mouse: Chatot *et al.*, 1994; Martin and Leese, 1995; Biggers and McGinnis, 2001; sheep: McGinnis and Youngs, 1992; pig: Hagen *et al.*, 1991; cow: Renard *et al.*, 1980; Dorland *et al.*, 1992; Brackett *et al.*, 1997; Iwata *et al.*, 1998). Glucose also becomes the predominant energy source after activation of the bovine embryonic genome (Javed and Wright, 1991; Khurana and Niemann, 2000). Glucose transport across plasma membranes is mediated by an extended family of sugar transport facilitators (Glut) (Joost and Thorens, 2001; Joost *et al.*, 2002). The different Glut isoforms are characterized by their tissue or cell-specific expression, substrate specificity and kinetics of transport. Preimplantation embryos express different Glut isoforms (Pantaleon and Kaye, 1996, 1998; Carayannopoulos *et al.*, 2000; Augustin *et al.*, 2001). Normal glucose uptake by blastocysts is accomplished by Glut3 and Glut1 (Pantaleon and Kaye, 1996, 1998). Expression of Glut8 was recently reported to be responsive to insulin in blastocysts (Carayannopoulos *et al.*, 2000). Glut3 and Glut8 are critical for development of mouse blastocysts (Pantaleon *et al.*, 1997; Carayannopoulos *et al.*, 2000; Pinto *et al.*, 2002). The process of controlled cell death occurs, as a physiological phenomenon, in early embryo development (for a review, see Hardy, 1999). However, different *in vitro* culture conditions affect the rate of apoptosis in preimplantation embryos (Byrne *et al.*, 1999). Impaired glucose transport can increase apoptosis in cell lines (Kan *et al.*, 1994), as well as in preimplantation embryos, of rats (Leunda-Casi *et al.*, 2002) and mice (Moley *et al.*, 1998a). Exposure of mouse preimplantation embryos to high glucose results in downregulation of Glut expression (Moley *et al.*, 1998b) and increased apoptosis (Moley *et al.*, 1998a). Apoptosis in embryos induced by high glucose is Bax dependent (Moley *et al.*, 1998a) and *in vivo* and *in vitro* mouse and rat experimental models for maternal pre-conceptional diabetes have shown that it can be reversed in part by insulin treatment (Pampfer, 2000). These data indicate a link between insulin, glucose transport or metabolism and apoptosis in developing embryos.

Expression of receptors and ligands of the insulin-IGF family (IR, IGF-IR, IGF-IIR (Schultz *et al.*, 1992; Yaseen *et al.*, 2001), insulin-like growth factor binding proteins (IGFBPs: Winger *et al.*, 1997; Prella *et al.*, 2001) and glucose transporters, including the insulin responsive isoforms Glut4 and Glut8 (Navarrete Santos *et al.*, 2000; Augustin *et al.*, 2001), in bovine preimplantation embryos is indicative of the presence of an active insulin-IGF-I signalling pathway, and points to glucose transporters as a possible link. This contention prompted the present authors to investigate the effects of insulin on bovine preimplantation embryos with respect to its mitogenic and anti-apoptotic activities, and in relation to expression of glucose transporters. Bovine embryos produced *in vitro* were exposed to insulin throughout the culture

period and several factors were examined: (i) rates of cleavage and blastocyst development, total number of blastocyst cells and the ratio of trophectoderm to inner cell mass (ICM) cells; (ii) apoptosis using the TUNEL assay and determination of the relative amounts of mRNA for the pro- and anti-apoptotic genes *Bax* and *Bcl-X_L*; and (iii) mRNA expression of the glucose transporter isoforms Glut1, Glut3 and Glut8.

Materials and Methods

In vitro production (IVP) of bovine embryos

Unless otherwise stated, the biochemicals used for IVP of bovine embryos were purchased from Sigma (St Louis, MO). Ovaries were collected from a local abattoir and were transported to the laboratory within 2 h in Dulbecco's phosphate buffered saline (DPBS), supplemented with 10 000 iu penicillin, 10 mg streptomycin and 250 mg amphotericin B l⁻¹, maintained at 32–34°C. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles by slicing with razor blades (Eckert and Niemann, 1995) in modified DPBS with 2 iu heparin and 0.1% (w/v) BSA Fraction V. COCs were selected in Hepes buffered (25 mmol l⁻¹) TCM199 and only COCs with at least three layers of compact cumulus cells and a homogeneous cytoplasm were considered to be suitable for maturation and were used for the following experiments. Maturation medium was TCM199, supplemented with 0.68 mmol L-glutamine l⁻¹, 25 mmol NaHCO₃ l⁻¹, 10% fetal calf serum, 10 iu eCG ml⁻¹ and 5 iu hCG ml⁻¹ (Suigonan; Intervet, Wiesbaden) and 1 µg oestradiol ml⁻¹. Groups of 30–40 COCs were matured in 500 µl maturation medium. Incubation was performed for 24 h in four-well dishes (Nunc, Roskilde) at 39°C in a humidified atmosphere of 5% CO₂ in air. After maturation, the COCs were washed twice in Tyrode's albumin lactate pyruvate medium (TALP; Bavister *et al.*, 1983) supplemented with 0.6% (w/v) fatty acid free BSA and 20 mmol Hepes l⁻¹ (H-TALP) and groups of 25–35 COCs were transferred to 300 µl fertilization medium (TALP medium supplemented with 0.6% (w/v) fatty acid free BSA, 10 mg heparin ml⁻¹, 20 mmol penicillamine l⁻¹, 1 mmol adrenalin l⁻¹ and 100 mmol hypotaurine l⁻¹ (Bavister, 1989)). A straw containing frozen spermatozoa was thawed in a waterbath at 34°C for 1 min, and the cells were layered on to a 45–90% (v/v) Percoll gradient in modified TALP medium. After centrifugation for 30 min at 600 g, the resulting motile sperm fraction was washed once in TALP medium, counted and diluted to a final concentration of 1 × 10⁶ spermatozoa ml⁻¹ in fertilization medium. Co-incubation of oocytes and spermatozoa was performed in four-well dishes for 18 h at 39°C in a humidified atmosphere of 5% CO₂ in air.

Presumptive zygotes were denuded from cumulus cells by vortexing in H-TALP for 3 min. After washing

in synthetic oviductal fluid (SOF; Tervit *et al.*, 1972), groups of 8–10 embryos were placed in 30 μl droplets of SOF medium under oil supplemented with 0.8% (w/v) BSA, glutamine (0.33 mmol l^{-1}), and essential and non-essential amino acids. Embryos were cultured in an incubation chamber (Billups Rothenberg, del Mar, CA) under 5% O_2 , 5% CO_2 and 90% N_2 at 39°C. For insulin-supplemented *in vitro* culture, insulin was used at a concentration of 1.7 $\mu\text{mol l}^{-1}$ in SOF. Presumptive zygotes had been washed in the same medium before they were placed into microdroplets for culture. This insulin concentration was selected on the basis of previous results describing an increased number of blastocyst cells and proportion of morulae grown in mSOF + polyvinylalcohol (PVA) supplemented with 1 $\text{mmol glucose l}^{-1}$ and amino acids (Matsui *et al.*, 1995a). Analyses were performed at the end of culture at 186 h after insemination.

Differential staining of blastocysts: determination of trophectoderm cell:ICM ratio

The differential staining of ICM and trophectoderm cells was performed according to Thouas *et al.* (2001) with minor modifications. In brief, expanded blastocysts were incubated in 500 μl Hepes-buffered SOF medium with 1% (v/v) Triton-X100 and 100 μg propidium iodide ml^{-1} for 30 s by carefully moving the embryos by pipetting to ensure equal permeabilization of the trophectoderm cells. Immediately afterwards, the embryos were transferred into 500 μl fixation medium consisting of 100% ethanol and 25 $\mu\text{g ml}^{-1}$ of Hoechst 33 258, and were incubated for 20 min at room temperature (21°C). The blastocysts were mounted directly in glycerol droplets on to a microscope slide and flattened gently with a coverslip using silicone as a spacer. Samples were examined under a Nikon Diaphot microscope equipped with epifluorescence.

TUNEL assay

The embryos were washed three times in PBS supplemented with PVA and fixed in 4% (w/v) PBS-buffered paraformaldehyde (PFA) for 20 min. The embryos were incubated for 10 min in 0.5% (v/v) Triton-X100 in PBS for membrane permeabilization. After washing the embryos in PBS, the TdT-mediated dUTP nick-end labelling (TUNEL) assay was performed according to the users' manual (TUNEL apoptosis detection kit; Boehringer Mannheim, Mannheim). In brief, the embryos were incubated for 1 h at 37°C in 30 μl droplets of the TUNEL reaction mixture under oil. The reaction was stopped by transferring the blastocysts into PBS supplemented with BSA (0.5% (w/v)) for 10 min in the dark. The nuclei were stained with Hoechst (10 $\mu\text{g ml}^{-1}$ diluted in 2.3% (w/v) sodium citrate solution) and the embryos were mounted

in glycerol as described for the differential staining procedure. Fixed blastocysts were treated with DNase (5 $\mu\text{l ml}^{-1}$ in cacodylate buffer) for 1 h at 37°C before the TUNEL reaction as positive controls. The specificity of the TUNEL assay was verified by omitting the TUNEL reagent as described in the manufacturer's instructions. Samples were examined under a Nikon Diaphot microscope equipped with epifluorescence and apoptosis was determined as the percentage of apoptotic bodies out of the total number of cells.

Gene expression analysis: Poly(A)+ RNA extraction and semiquantitative RT-PCR

Poly(A)⁺RNA from single blastocysts was extracted as described by Wrenzycki *et al.* (1999) and Augustin *et al.* (2001) using the Dynabeads mRNA direct Kit[®]. Single blastocysts were lysed for 10 min in 20 μl lysis/binding buffer (0.1 mol Tris-HCl l^{-1} , pH 7.5; 0.5 mol LiCl l^{-1} ; 10 mmol EDTA l^{-1} , pH 8.0; 1% (w/v) lithium dodecylsulphate (LiDS); 5 mmol dithiothreitol l^{-1}). As a control to monitor the efficiency of mRNA extraction, 0.1 pg rabbit globin mRNA (Invitrogen, Karlsruhe) was included in each sample as an exogenous standard. An aliquot (5 μl) of prewashed Dynabeads oligo (dT)25[®] was added and after 5 min of hybridization, the beads were separated from the lysis buffer using the Dynal[®] magnetic separator. The beads were washed once with 30 μl buffer A (10 mmol Tris-HCl l^{-1} , pH 7.5; 0.15 mol LiCl l^{-1} ; 1 mmol EDTA l^{-1} ; 0.1% LiDS) and twice in the same volume with buffer B (10 mmol Tris-HCl l^{-1} , pH 7.5; 0.15 mmol LiCl l^{-1} ; 1 mmol EDTA l^{-1}). The poly(A)⁺RNA was eluted from the beads with 11 μl diethylpyrocarbonate-treated water and incubated at 65°C for 2 min. The resulting poly(A)⁺RNA was reverse-transcribed in a total volume of 20 μl using 2.5 mmol random hexamer primers l^{-1} , 10 \times PCR buffer, 20 iu RNase inhibitor, 50 iu MuLV reverse transcriptase (Perkin Elmer, Weiterstadt), 5 mmol $\text{MgCl}_2 \text{l}^{-1}$ and 1 mmol each dNTP l^{-1} . The reverse transcription reaction was performed by incubating the samples for 10 min at 25°C, for 1 h at 42°C and 5 min at 99°C. Samples were overlaid with oil to prevent evaporation during the last incubation step. Possible carryover of genomic DNA during the extraction process was controlled by the use of exon-intron spanning Bcl-X PCR primers. On the basis of the human Bcl-X sequence the PCR primer pair matches exons 2 and 4 of the human gene (Z23115, Z23116) and results in the amplification of a 505 bp and a 316 bp fragment that correspond to the Bcl-X_L and Bcl-X_S isoforms, respectively. PCR amplification of the blastocyst cDNA was performed as described by Augustin *et al.* (2001). For each gene, the primer combinations, the expected size of the amplification product, the number of cycles and the amount of cDNA used to determine relative mRNA expression in the linear range of the PCR are shown (Table 1). As a result of

Table 1. Primer combinations, size of amplification product, number of PCR cycles and amounts of cDNA used for semi-quantitative RT-PCR

Gene	Primer sequence 5'–3'	Number of PCR cycles	cDNA in embryo equivalent	Product size (bp)	Accession no. (EMBL)
Actin	TGAACCCTAAGGCCAACCGTG TGTAGCCACGCTCGGTCAGG	28	1/20	268	K00622 BC016624
Glut1	AGCGTCATCTTCATCCCAGC CCACAATGCTCAGGTAGGAC	34	2/20	544	M60448
Glut3	ATGGCAGTGGCTGGTTGTTC GCTCCTCATGTCAGAGATCTTC	38	4/20	508	AF308829
Glut8	GCTCCTCATGTCAGAGATCTTC GGCTGTGATTGTCCAGAGTC	34	2/20	235	AF321324
Bax	TGCTTCAGGGTTTCATCCAG GTGTCCCAAAGTAGGAGAGG	32	2/20	427	U92569
Bcl-X _L	GGATAGCCCTGCTGTGAATGG TGAGCCCAGCAGAACCACAC	32	2/20	505	AF245489

its low expression, transcripts of the insulin-regulated Glut4 were not quantified (Augustin *et al.*, 2001). In total, 18 embryos per group from at least three different culture experiments were used for the semiquantitative RT-PCR. Two expanded blastocysts for the control and the insulin group were processed in parallel for RNA extraction, RT-PCR and agarose gel electrophoresis, and were considered as a single assay. The effect of the assay was included as a factor in the two-way ANOVA statistical analysis. As the total number of blastocyst cells was found to be significantly different between the two groups, the housekeeping gene actin (the endogenous standard) rather than the exogenous control globin was used to standardize gene expression. Although actin mRNA expression varies during embryo development (Robert *et al.*, 2002), this housekeeping gene is an accepted standard to quantify gene expression at the blastocyst stage (Lonergan *et al.*, 2000; Prella *et al.*, 2001). The PCR products were analysed by agarose gel electrophoresis using a digital gel documentation system (Bioprint; LTF-Labortechnik, Wasserburg) and relative amounts were determined as absorbance and analysed using the Bio-1-D program (LTF).

Statistical analysis

The rates of cleavage and blastocyst development were evaluated by the chi-squared test. The number of cells, the index of apoptosis and the data for mRNA expression were compared between the two groups using the SPSS 10.0 software package applying the two-way ANOVA. For comparison of the number of blastocyst cells and the apoptotic index between the two groups, the effect of the independent culture experiments (replicate) was included as the second factor in the analysis. For analysis of mRNA expression, the effect of the assay was tested as the second factor. Data are presented as mean \pm standard error of the means (SEM) and the level of significance was $P < 0.05$.

Table 2. Effects of insulin on embryo development and number of blastocyst cells

	Control	Insulin supplementation
Total number of oocytes	484	527
Cleavage (% of oocytes)	81.2 \pm 2.2 ^a	86.0 \pm 2.5 ^b
Day 7 blastocysts (% of oocytes)	22.8 \pm 2.1 ^a	30.4 \pm 1.7 ^b
Number of blastocyst cells	123.7 \pm 6.0 ^a	146.3 \pm 6.6 ^b
Ratio of trophectoderm : inner cell mass (ICM) cells	2.0 \pm 0.1 ^a	2.3 \pm 0.1 ^a
Number of trophectoderm cells	82.4 \pm 5.0 ^a	100.3 \pm 5.5 ^b
Number of ICM cells	41.1 \pm 2.2 ^a	45.9 \pm 2.4 ^a

Total numbers of oocytes used, percentage of cleaved zygotes and the proportion of embryos that developed to the blastocyst stage calculated from ten individual culture experiments over the total number of oocytes exposed per group.

Number of blastocyst cells and the ratio of trophectoderm:ICM cells were determined for expanded blastocysts from the control (49 blastocysts) and insulin-treated (42 blastocysts) groups.

^{ab}Insulin significantly increased the number of blastocyst cells as a result of a significant increase in the number of trophectoderm cells, as indicated by different superscripts ($P < 0.05$).

Results

Effects of insulin on embryonic development

Insulin significantly increased the rate of cleavage ($P < 0.05$), as well as the proportion of blastocysts ($P < 0.05$) at 186 h after insemination (Table 2).

Effects of insulin on total number of blastocyst cells and trophectoderm cell : ICM ratio

Blastocysts derived from insulin-supplemented *in vitro* cultures showed a significant increase in total number of cells ($P < 0.01$) compared with the control group. This increase was due to a significantly higher number

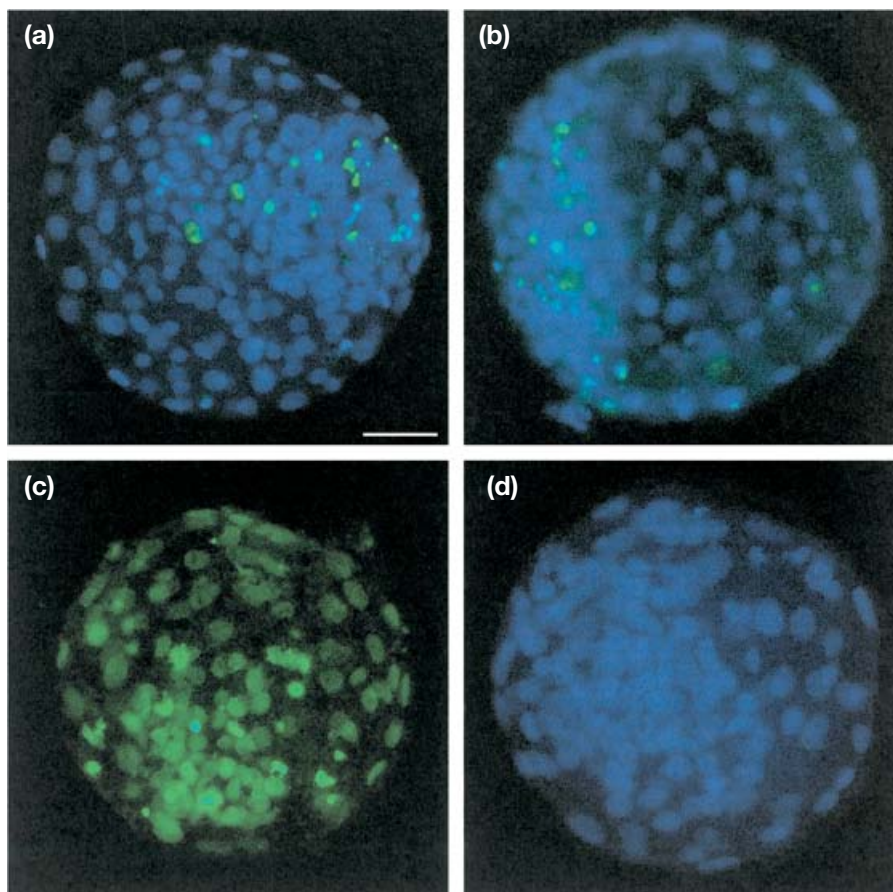


Fig. 1. Representative images of bovine blastocysts at 186 h after insemination subjected to TUNEL staining to determine apoptosis. Blastocysts representing embryos from (a) the control group, (b) the insulin-treated group, (c) a positive control and (d) a negative control for the TUNEL assay are shown. Green staining indicates fragmented DNA of cells undergoing apoptosis detected by fluorescein isothiocyanate (FITC)-labelled dUTP, whereas intact cell nuclei are stained blue with Hoechst dye. Scale bar represents 50 μm .

of trophectoderm cells ($P < 0.01$), whereas the ratio of trophectoderm:ICM cells was not different between the groups (Table 2).

Effects of insulin on apoptosis

TUNEL assay. The TUNEL assay (Fig. 1) revealed that blastocysts grown in the presence of insulin showed a significantly ($P < 0.05$) lower number of apoptotic bodies compared with the control group. Insulin decreased the apoptotic index from 14.8 ± 0.9 for controls ($n=53$) to 12.2 ± 0.7 ($n=65$) in the insulin-treated group. Embryos from six independent culture experiments were compared.

Bax and Bcl-X_L mRNA. The relative amounts of mRNA from insulin-treated and non-treated blastocysts were compared based on standardization using beta-actin mRNA. Insulin supplementation did not affect expression of Bax (1.02 ± 0.12 -fold) and Bcl-X_L (1.00 ± 0.09 -fold) mRNA compared with control blastocysts.

Effects of insulin on Glut1, 3 and 8 mRNA. The relative amounts of Glut transcripts in blastocysts derived from insulin-supplemented IVP did not differ significantly for Glut1 (0.96 ± 0.12 -fold), Glut3 (0.94 ± 0.12 -fold) and Glut8 (1.13 ± 0.08 -fold) compared with the control group.

Discussion

In the present study, the potential mitogenic and anti-apoptotic effects of insulin on development of bovine preimplantation embryos and on expression of Glut isoforms were examined. Although there has been progress in the average efficiency of IVP of bovine embryos over the last few decades, embryos produced *in vitro* differ from embryos produced *in vivo* in many aspects (Thompson, 1997; Niemann and Wrenzycki, 2000; Dieleman *et al.*, 2002; Holm *et al.*, 2002). Beneficial effects of various growth factors, such as insulin-IGF-I,

transforming growth factor beta (TGF- β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and growth hormone (GH), on embryo development *in vitro* have been reported in several species (Kaye, 1997). Growth factors of the insulin-IGF-I family have proliferative, mitogenic and anti-apoptotic effects on embryo development in several species (cow: Matsui *et al.*, 1995a,b; Byrne *et al.*, 2002; mouse: Harvey and Kaye, 1990; rabbit: Herrler *et al.*, 1998; man: Spanos *et al.*, 2000). Mice with gene knockouts for the receptors and ligands of the insulin-IGF-I growth factor family are viable with respect to preimplantation embryo development (for a review, see Nakae *et al.*, 2001). However, these animals are characterized by substantial pre- and postnatal growth retardation (Liu *et al.*, 1993; Kaye and Gardner, 1999).

The present data indicate that insulin can exert mitogenic and anti-apoptotic activities in bovine preimplantation embryos. The results of the present study show that insulin increases rates of cleavage and blastocyst development by 6 and 33%, respectively, as well as the total number of blastocyst cells by 18%. These results are in agreement with the findings of Matsui *et al.* (1995a), whereas Mihalik *et al.* (2000a) did not find any significant effects of insulin on blastocyst development. With the exception that the embryos were cultured under reduced O₂ concentration using a premixed triple gas (5% CO₂, 5% O₂ and 90% N₂) in the present study, the media composition in both studies (mSOF with either PVA (Matsui *et al.*, 1995a) or BSA (Mihalik *et al.*, 2000)) was similar to the medium used here. Therefore, IVP culture procedures rather than basic culture media composition might be responsible for the different experimental findings.

In the present study, the trophectoderm:ICM ratio was determined to obtain more information about the effect of insulin on blastocyst quality. The increased number of cells in embryos cultured in the presence of insulin was mainly due to an increased number of trophectoderm cells. The trophectoderm:ICM ratio was not significantly different between the two groups. These findings are similar to a study in which bovine embryos were grown in the presence of IGF-I (Prelle *et al.*, 2001). In contrast, in mouse blastocysts cultured with insulin supplementation the higher number of blastocyst cells was selectively caused by a significant increase in ICM cells (Harvey and Kaye, 1990; Smith *et al.*, 1993). The reasons for these divergent findings need further clarification.

The beneficial effect of insulin supplementation on blastocyst development *in vitro* is further supported by the present findings on the decreased rate of apoptosis. The difference in apoptosis between the groups cultured with and without insulin, represented by the TUNEL index, is comparable with investigations on the effects of IGF-I (Makarevich and Markkula, 2002) or serum

(Byrne *et al.*, 1999) on apoptosis in bovine embryos. In a recent study investigating the mitogenic and anti-apoptotic effects of insulin, IGF-I and IGF-II on bovine preimplantation embryos, a significant decrease in the apoptotic index was found at insulin concentrations of 1.8 nmol l⁻¹ (Byrne *et al.*, 2002). However, in contrast to the findings of the present study, no differences in rates of blastocyst development and numbers of cells were noted (Byrne *et al.*, 2002). Further investigations to elucidate the role of insulin and IGF-I on preimplantation development, in particular on number of cells, cell allocation and apoptosis, need to include embryos grown *in vivo*, thereby allowing a more conclusive view on the effects of insulin-IGF-I observed in IVP systems.

The anti-apoptotic Bcl-X_L and the death-promotor Bax proteins are known to form homodimers and heterodimers (Sedlak *et al.*, 1995). The shift in the ratio of Bax:Bcl-X_L at the RNA or protein level determines survival and the degree of apoptosis in various cell systems (Yang and Korsmeyer, 1996; Weinmann *et al.*, 1999; Kim *et al.*, 2001). In the present study, a comparison of the Bax and Bcl-X_L mRNA between insulin versus control embryos did not reveal significant differences. This finding might indicate that, as apoptosis in bovine embryos occurs at the 8–16-cell stage (Byrne *et al.*, 1999), a change in mRNA expression for these genes as a result of insulin activity might occur earlier in development, thereby preceding the decrease in DNA fragmentation observed at the blastocyst stage.

No significant changes in the relative amounts of mRNA for Glut1, Glut3 and Glut8 were found between the two groups. The composition of the culture medium significantly affects the expression of glucose transporter mRNA and protein in mouse (Moley *et al.*, 1998; Leppens-Luisier *et al.*, 2001) and bovine (Wrenzycki *et al.*, 1998, 1999) preimplantation embryos. Recent data provide evidence that glucose transporter expression is crucial for development (mouse: Pantaleon *et al.*, 1997; Leppens-Luisier *et al.*, 2001) and is altered in bovine embryos grown *in vitro* compared with those derived *in vivo* (Wrenzycki *et al.*, 2001; Lazzari *et al.*, 2002). Depending on the composition of the culture media, changes in Glut1 mRNA expression in bovine embryos were observed at the 8–16-cell (Wrenzycki *et al.*, 1999) and morula stages (Wrenzycki *et al.*, 2001). Differences were less pronounced in blastocysts (Wrenzycki *et al.*, 2001). In a preliminary study investigating the effects of a short-time exposure of expanded day 7 bovine blastocysts to insulin (10 μ g ml⁻¹) for 1, 2 and 4 h, a significant increase in Glut3 and Glut8 mRNA was found after 1 h of treatment (R. Augustin, P. Pocar and B. Fischer, unpublished). As a result of these findings, it is concluded that differences in the expression of Glut genes among bovine embryos derived from different IVP procedures (for example, control versus insulin-treated group) might be more pronounced at the earlier stages of development or at shorter periods after exposure to insulin.

Insulin and IGF-I crossreact with the corresponding receptors. Therefore, the question arises as to whether the observed effects of insulin on bovine embryos were mediated by the insulin or IGF-I receptor. In mouse blastocysts, the effects of insulin are transmitted by both receptors but for different functions. Insulin stimulation of protein synthesis and mitogenesis is mediated by the insulin receptor (Harvey and Kaye, 1988; Harvey and Kaye, 1992), whereas insulin-stimulated glucose uptake in blastocysts is accomplished by the IGF-I receptor (Pantaleon and Kaye, 1996). Recent data for bovine embryos indicate a proliferative, mitogenic and anti-apoptotic activity for IGF-I (Prelle *et al.*, 2001; Makarevich and Markkula, 2002). With respect to the action of insulin, two observations merit attention. First, Matsui *et al.* (1995a,b) concluded that the action of insulin in bovine embryos is mediated by the IGF-I receptor, as it was blocked by an anti-IGF-I receptor antibody (alphaIR-3) (Matsui *et al.*, 1997). Second, the beneficial effects of insulin on development of bovine embryos were observed only when the culture medium was supplemented with glucose (Matsui *et al.*, 1995a,b). As the action of insulin is strongly related to the presence of glucose in the medium (Pantaleon *et al.*, 1997), absence of glucose in the medium might be one of the underlying reasons for the controversial experimental findings in bovine IVP embryos too.

Although the specificity in ligand binding and signalling by insulin and IGF-I receptors is not fully understood (Nakae *et al.*, 2001; Siddle *et al.*, 2001), recent data show that IRS-1 (Amoui *et al.*, 2001) and the PI3-kinase–Akt pathway mediate insulin-specific signalling that results in metabolic (Ueki *et al.*, 1998), as well as mitogenic (Conejo and Lorenzo, 2001) and anti-apoptotic (Barber *et al.*, 2001; Iida *et al.*, 2002; Tseng *et al.*, 2002) activity. A key mediator in insulin–IGF-I signalling leading to its anti-apoptotic activity and regulation of glucose transport is protein kinase B (PKB or Akt) (Datta *et al.*, 1999; Hajduch *et al.*, 2001; Lawlor and Alessi, 2001). Preliminary data from the authors' laboratory showing that PKB mRNA is expressed in bovine oocytes and preimplantation embryos indicate that bovine embryos may be able to react specifically to insulin via an active insulin–IGF-I signalling pathway.

In conclusion, the present data provide evidence that insulin exerts a mitogenic as well as an anti-apoptotic activity on bovine embryos *in vitro* resulting in improved blastocyst development and, probably, quality. These activities could not be correlated with changes in the expression of glucose transporters Glut1, Glut3 and Glut8 by insulin.

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