

Effects of Insulin-Like Growth Factor-1 on Cellular and Molecular Characteristics of Bovine Blastocysts Produced In Vitro

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ABSTRACT Addition of insulin-like growth factor-1 (IGF-1) to culture medium increases the proportion of bovine embryos that develop to the blastocyst stage and increases embryo survival following transfer to heat-stressed, lactating dairy cows. The objective of the present study was to determine molecular and cellular correlates of these actions of IGF-1. Embryos were produced in vitro and cultured for 7 days with or without 100 ng/ml IGF-1. On d 7 after insemination, grade 1 expanded blastocysts were harvested and used to determine total cell number, percent apoptosis, cell allocation to the inner cell mass and trophectoderm, and the relative abundance of several developmentally important gene transcripts. There was no significant effect of IGF-1 treatment on blastocyst cell number, the proportion of blastomeres that were apoptotic, or the number of cells in the inner cell mass and trophectoderm. However, differences in the relative abundance of several mRNA transcripts were observed between control and IGF-1 treated embryos. Addition of IGF-1 increased ($P < 0.02$) amounts of mRNA for IGF binding protein-3 and desmocollin II and tended ($P < 0.08$) to increase amounts of mRNA for Na/K ATPase and Bax. Moreover, IGF-1 treatment decreased ($P < 0.05$) steady-state amounts of transcripts for heat shock protein 70 and tended ($P < 0.08$) to reduce amounts of IGF-1 receptor mRNA. In conclusion, increased survival of embryos treated with IGF-1 does not appear due to effects on cell number, percent apoptosis, or cell allocation. Addition of IGF-1 to culture can, however, alter expression of several transcripts which may be important for embryo development and survival following transfer. *Mol. Reprod. Dev.* 75: 895–903, 2008. © 2007 Wiley-Liss, Inc.

Key Words: insulin-like growth factor-1; in vitro fertilization; gene expression; bovine

INTRODUCTION

The production of bovine embryos in vitro is associated with altered metabolism (Khurana and Niemann, 2000), gene expression (Bertolini et al., 2002; Lazzari et al., 2002; Lonergan et al., 2003), and cryo-survival

(Enright et al., 2000; Rizos et al., 2003) compared to embryos produced following superovulation. Differences are also manifested during post-culture development in that the transfer of in vitro produced embryos is associated with reduced embryo survival (Farin and Farin, 1995; Hasler et al., 1995; Drost et al., 1999), fetal and neonatal overgrowth (Lazzari et al., 2002) and increased fetal and placental abnormalities (van Wageningen-de Leeuw et al., 1998, 2000; Farin et al., 2006).

The addition of growth factors to culture medium is one potential strategy to improve embryo development and survival. In vivo, the oviduct, uterus and the early developing embryo express an array of growth factors including epidermal growth factor, insulin-like growth factor-1 (IGF-1), IGF-II, platelet derived growth factor, transforming growth factor- α , and fibroblast growth factor (Kane et al., 1997; Diaz-Cueto and Gerton, 2001; Yaseen et al., 2001; Hardy and Spanos, 2002). Moreover, in many cases, the embryo has been shown to express the receptor for these growth factors so that these molecules may exert paracrine and autocrine functions in early embryo development (Kane et al., 1997; Diaz-Cueto and Gerton, 2001; Yaseen et al., 2001; Hardy and Spanos, 2002).

One of the most studied growth factors is IGF-1. Insulin-like growth factor-1 can affect bovine embryo development in vitro in several ways. Addition of IGF-1 to culture has been reported to stimulate development of bovine embryos to the blastocyst stage (Herrler et al., 1992; Palma et al., 1997; Prella et al., 2001; Moreira et al., 2002; Block et al., 2003; Sirisathien et al., 2003), increase blastocyst cell number (Moreira et al., 2002; Byrne et al., 2002a; Sirisathien and Brackett, 2003;

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Sirisathien et al., 2003) and glucose transport (Pantaleon and Kaye, 1996), and reduce the proportion of blastomeres that are apoptotic (Makarevich and Markkula, 2002; Byrne et al., 2002a; Sirisathien and Brackett, 2003). Moreover, treatment of embryos during culture with IGF-1 increases post-transfer survival of those embryos when transferred into heat stressed, lactating dairy cows (Block et al., 2003; Block and Hansen, 2007).

The objective of the present experiment was to determine molecular and cellular actions of IGF-1 that could explain the increased potential for embryonic survival after transfer (Block et al., 2003; Block and Hansen, 2007). Focus was placed on effects of IGF-1 on cell number, cell allocation, and apoptosis and the relative abundance of several developmentally important mRNA transcripts.

MATERIALS AND METHODS

All materials were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) unless specified otherwise.

Culture Medium

Sperm-Tyrode's Lactate, IVF-Tyrode's Lactate, and Hepes Tyrode's Lactate were purchased from Caisson Laboratories, Inc. (Logan, UT). These media were used to prepare Sperm-Tyrode's Albumin Lactate Pyruvate (TALP), IVF-TALP, and Hepes-TALP as described previously (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) and supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR), 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (Invitrogen, Carlsbad, CA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-β, 20 µg/ml bovine FSH (Folltropin-V; Bioniche, Bellevue, Ontario, Canada), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Potassium simplex optimized medium (KSOM) that contained 1 mg/ml BSA was from Caisson. On the day of use, KSOM was modified to produce KSOM-BE2 as described previously (Soto et al., 2003).

In Vitro Embryo Production

Embryos were produced in vitro as described previously (Soto et al., 2003). Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (predominantly beef cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with multiple layers of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-µl drops of OMM overlaid with mineral oil and matured for 21–24 hr at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups

of 30- to 4-well plates containing 600 µl of IVF-TALP and 25 µl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with $\sim 1 \times 10^6$ Percoll-purified (Amersham Pharmacia Biotech, Uppsala, Sweden) spermatozoa from a pool of frozen-thawed semen from three bulls of various breeds. A different pool of semen was used for each replicate. Depending on the experiment, COCs and spermatozoa were allowed to coincubate for 20–24 hr at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. After fertilization, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in HEPES-TALP containing 1,000 U/ml hyaluronidase, and randomly placed in groups of 25 in 50-µl drops of either KSOM-BE2 or KSOM-BE2 containing 100 ng/ml IGF-1 (Upstate Biotech, Lake Placid, NY) as described previously (Block et al., 2003). All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. The proportion of cleaved oocytes was recorded on day 3 after insemination and the proportion of blastocysts and advanced blastocysts was recorded on day 7.

TUNEL assay

The TUNEL assay was performed as described previously (Jousan and Hansen, 2004) using an in situ cell death detection kit (Roche, Indianapolis, IN). Embryos were removed from culture and washed two times in 50-µl drops of 10 mM KPO₄ pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml PVP (Eastman Kodak, Rochester, NY; PBS-PVP). Zona pellucida-intact embryos were fixed in a 50-µl drop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 500 µl of PBS-PVP at 4°C until the time of assay.

On the day of the TUNEL assay, embryos were transferred to a 50-µl drop of PBS-PVP and then permeabilized in 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 10 min at room temperature. Controls for the TUNEL assay were incubated in 50 µl of RQ1 RNase-free DNase (50 U/ml; Promega, Madison, WI) at 37°C in the dark for 1 hr. Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 µl of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by and following the guidelines of the manufacturer) for 1 hr at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS-PVP and incubated in a 25-µl drop of Hoechst 33258 (1 µg/ml) for 15 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33258, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4-µl drops of glycerol, and the slides affixed with coverslips. Labeling of TUNEL and Hoechst 33258 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive

blastomeres (green nuclei) with DAPI and FITC filters, respectively, using a 20× objective.

Differential Staining

Zona-intact embryos were removed from culture and washed three times in 50 µl drops of PBS-PVP. To label trophectoderm cells (TE), embryos were placed into 500 µl of PBS-PVP containing 0.5% Triton X-100 and 100 µg/ml propidium iodide for 30 sec at 37°C. Embryos were then washed immediately through 3 wells of a 4-well plate containing 500 µl of PBS-PVP each. To fix embryos and stain inner cell mass cells (ICM), embryos were then incubated in a 50 µl drop of PBS-PVP containing 4% paraformaldehyde and 10 µg/ml Hoechst 33258 for 15 min at room temperature. Embryos were then washed three times in PBS-PVP, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4-µl drops of glycerol, and then covered with coverslips. Labeling of propidium iodide and Hoechst 33258 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for the number of ICM (blue nuclei), the number of TE cells (pink nuclei), and total cell number (blue + pink nuclei) with a DAPI filter using a 20× objective.

RT-PCR

The relative abundance of 14 gene transcripts was determined using semi-quantitative RT-PCR as described previously (Wrenzycki et al., 2001b). Primer sequences, annealing temperatures, fragment sizes, and accession numbers for sequences are summarized in Table 1. The PCR primers were designed from the coding regions of each gene sequence using the OLIGO program National Biosciences, Plymouth, USA.

Harvested embryos were washed three times in PBS-PVP and then stored at -80°C until further processing. Poly(A)⁺ RNA was isolated from single blastocysts as previously described (Wrenzycki et al., 1999) and was used immediately for reverse transcription (RT) that was carried out in a total volume of 20 µl using 2.5 µM random hexamers (GeneAmp[®] RNA PCR Kit components, Applied Biosystems, CA, Perkin-Elmer, Wellesley, MA). Prior to RNA isolation, 1 pg of rabbit globin RNA (BRL, Gaithersburg, MD) was added as an external standard. The reaction mixture consisted of 1× RT buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4; Invitrogen 10 mM Tris-HCl, pH 8.3; Perkin-Elmer), 5 mM MgCl₂ (Invitrogen), 1 mM of each dNTP (Amersham, Brunswick, Germany), 20 IU RNase inhibitor (GeneAmp[®] RNA PCR Kit components, Applied Biosystems, Perkin-Elmer), and 50 IU murine leukemia virus reverse transcriptase (GeneAmp[®] RNA PCR Kit components, Applied Biosystems, Perkin-Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT reaction was carried out at 25°C for 10 min, 42°C for 1 hr followed by a denaturation step at 99°C for 5 min and flash cooling on ice. Polymerase chain reaction (PCR) was performed with cDNA equivalents as described in Table 1 from individual embryos as well as 50 fg of globin RNA in

a final volume of 50 µl of 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4; Invitrogen, Karlsruhe, Germany) 20 mM Tris-HCl, pH 8.4, 50 mM KCl; Gibco BRL, Eggenstein, Germany), 1.5 mM MgCl₂ (Invitrogen, Karlsruhe, Germany), 200 µM of each dNTP, 1 0.5 µM of each sequence-specific primer (globin: 0.5 µM). The PCR reactions were performed using a PTC-200 thermocycler (MJ Research, Watertown, MA). To ensure specific amplification, a hot start PCR was employed by adding 1 IU Taq DNA polymerase (Invitrogen, Karlsruhe, Germany; Gibco) at 72°C. The PCR program employed an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by different cycle numbers (see Table 1) of 15 sec each at 95°C for DNA denaturation, 15 sec at different temperatures for annealing of primers, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C. As negative controls, tubes were prepared in which RNA or reverse transcriptase was omitted during the RT reaction.

The RT-PCR products were subjected to electrophoresis on a 2% (w/v) agarose gel in 1× TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 µg/ml ethidium bromide. The image of each gel was recorded using a charge-coupled device camera (Quantix, Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band was assessed by densitometry using an image analysis program (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each transcript by the intensity of the globin band for each embryo. To circumvent the problem that the differences in the relative abundance of the transcripts were due to different cell numbers of the blastocysts analyzed, the relative abundance of each transcript for each embryo was divided by the mean total cell number for that treatment and multiplied by 100. The value for mean total cell number for embryos in the replicates used for RNA analysis were 131.8 cells (n=96) for control embryos and 117.7 cells (n=76) for IGF-1 treated embryos. For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of cycle number over which linear amplification occurred and the number of PCR cycles was kept within this range (Wrenzycki et al., 1999). Because the total efficiency of amplification for each set of primers during each cycle is not known, such an assay can only be used to compare relative abundances of one mRNA among different samples (Temeles et al., 1994).

Experiment 1: Effect of IGF-1 on Total Cell Number, Apoptosis and Cell Allocation

Grade 1 expanded blastocysts (Robertson and Nelson, 1998) were harvested on day 7 after fertilization. For seven replicates, harvested embryos were used to determine the proportion of apoptotic nuclei with the TUNEL assay. There were between 71 and 84 embryos for each treatment. For an additional seven replicates,

TABLE 1. Primers Used for RT-PCR

Genes	Primer sequences and positions	Annealing temp (°C) × cycle number and embryo equivalent	Fragment size (bp)	EMBL accession no.
Glucose transporter 1 (Glut1) SLC2A1	5' primer: (894-914) = CAG GAG ATG AAG GAG GAG AGC; 3' primer: (1,131-1,151) = CAC AAA TAG CGA CAC GAC AGT	59°C × 32, 0.05	257	M60448
Glucose transporter 3 (Glut3) SLC2A3	5' primer: (1,095-1,118) = CCT TGG AGG GAT GGC TTT TTG TTC; 3' primer: = CGT GGC TGA GGG GAA GAG CAG TCC	59°C × 32, 0.1	259	NM_174603
Glucose transporter 8 (Glut8) SLC2A8	5' primer: (711-730) = CCT CGC TTC CTG CTG TCT CA; 3' primer: (935-954) = CCT CCT CAA AGA TGG TCT CC	58°C × 34, 0.2	244	AY208940.1
Bax	5' primer: (227-249) = TGC AGA GGA TGA TCG CAG CTG TG; 3' primer: (402-424) = CCA ATG TCC AGC CCA TGA TGG TC	60°C × 32, 0.1	197	NM_173894.1
Bcl-xL (Bcl)	5' primer: (197-221) = ATG GAG CCA CTG GCC ACA GCA GAA G; 3' primer: (479-503) = GTT GCG ATC CGA CTC ACC AAT ACC T	60°C × 32, 0.2	307	NM_001077486
HSP70.1 (Hsp70)	5' primer: (844-864) = GGG GAG GAC TTC GAC AAC AGG; 3' primer: (1,068-1,088) = CCG AAC AGG TCG GAG CAC AGC	60°C × 32, 0.2	245	NM_174550.1
Na ⁺ /K ⁺ -ATPase (NaK) ATPA1	5' primer: (2,884-2,905) = ACC TGT TGG GCA TCC GAG AGA C; 3' primer: (3,198-3,219) = AGG GGA AGG CAC AGA ACC ACC A	58°C × 31, 0.1	336	NM_001076798
E-cadherin (Ecad) CDH1	5' primer: (1,486-1,515) = CTC AAG CTC GCG GAT AAC CAG AAC AAA GAC; 3' primer: (1,785-1,814) = AGG CCC CTG TGC AGC TGG CTC AAA TCA AAG	56°C × 34, 0.2	332	X06339
Desmocollin 2 (Dc II)	5' primer: (918-942) = TGC CAA CAT TCA CCC GTT CTT CTT A; 3' primer: (1,335-1,359) = CCT GTT TCC GGG TCG TAT GCT TTA T	56°C × 34, 0.2	442	M81190.1
Plakophilin (Plako) PKP1	5' primer: (1,337-1,361) = CCC GTG GAC CCC GAG GTC TTC TTC A; 3' primer: (1,580-1,604) = CCG TGT AGG CGT TGC GGG CGT TGT A	64°C × 35, 0.4	268	Z37975
Insulin-like growth factor-1 receptor (IGF1R)	5' primer: (186-212) = CAT CTC CAA CCT CCG GCC TTT TAC TCT; 3' primer: (695-722) = CCC AGC CTG CTG CTA TTT CTT TTT CTA T	59°C × 37, 0.3	538	X54980
IGF binding protein-2 (IGFBP2)	5' primer: (594-614) = TCC AGG CCG AGG TGA TGT TTC; 3' primer: (394-414) = AGC GCC AGC CCC GAG CAG GTT	61°C × 33, 0.2	221	NM_174555.1
IGF binding protein-3 (IGFBP3)	5' primer: (714-735) = AAC TTC TCC TCT GAG TCC AAG C; 3' primer: (904-924) = CGT ACT TAT CCA CAC ACC AGC	56°C × 35, 0.2	210	M76478
IGF binding protein-5 (IGFBP5)	5' primer: (403-423) = GGC AGT CGT GCG GCG TCT ACA; 3' primer: (667-686) = CTT TCT GCG GTC CTT CTT CA	61°C × 35, 0.2	284	XM_878464.1

harvested embryos were used to determine cell allocation to the ICM and TE using differential staining. There were between 146 and 163 embryos for each treatment. For all 14 replicates, harvested embryos were used to evaluate total cell number.

Experiment 2: Effect of IGF-1 on the Relative Abundance of Developmentally Important Genes

Grade 1 expanded blastocysts (Robertson and Nelson, 1998) were harvested on day 7 after insemination. Approximately half of the selected embryos (Control $n = 104$ and IGF-1 $n = 93$) were then randomly assigned to evaluate the relative abundance of mRNA transcripts for IGF-1 receptor (IGF-1R), IGF binding protein-2 (IGFBP2), IGF binding protein-3 (IGFBP3), IGF binding protein-5 (IGFBP5), glucose transporter-1 (Glut1), Glut3, Glut8, heat shock protein 70.1 (Hsp70), Bax, Bcl-1(Bcl), desmocollin-II (Dc II), E-cadherin (Ecad), and plakophilin (Plako). For each transcript, assays were performed on 7–22 embryos per treatment. The remaining embryos (Control $n = 96$ and IGF-1 $n = 76$) were used to determine total cell number.

Statistical Analysis

Data were analyzed by analysis of variance using the GLM procedure of SAS (SAS for Windows, version 9.0, SAS Inst., Inc., Cary, NC). Percentage data were transformed by arcsin transformation before analysis. Independent variable for the following variables were IGF-1 treatment and replicate: cleavage rate, blastocyst development, total cell number, percent apoptosis, the number of ICM and TE cells, and the ratio of TE cells to ICM cells. For gene transcripts, treatment was the only independent variable included in the model. All values reported are least-squares means \pm SEM. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

RESULTS

Among grade 1 expanded blastocysts selected on day 7 after fertilization, treatment with IGF-1 did not affect total cell number or the proportion of blastomeres that were apoptotic (Table 2). There was also no effect of IGF-1 treatment on the number of cells in the TE or the ratio of TE:ICM. There was, however, a tendency

($P < 0.06$) for IGF-1 treated embryos to have less cells in the ICM than controls (Table 2).

Results on relative abundance of the 14 gene transcripts are presented in Figure 1. Among transcripts involved in cell to cell adhesion and blastocyst expansion, treatment with IGF-1 tended ($P < 0.08$) to increase relative abundance of NaK transcripts and increased ($P < 0.01$) relative abundance of Dc II transcripts. There was no effect of IGF-1 on relative abundance of transcripts for Ecad or Plako. Of the two genes examined that are involved in apoptosis, IGF-1 tended to increase ($P < 0.06$) relative abundance of Bax transcripts and had no effect on amounts of Bcl transcript. In addition, IGF-1 treatment reduced ($P < 0.05$) the relative abundance of Hsp70 transcripts. For transcripts of genes involved with insulin-like growth factor, IGF-1 tended ($P < 0.07$) to reduce abundance of IGF1R mRNA and increased ($P < 0.02$) abundance of IGFBP3 transcripts. There was no effect of IGF-1 treatment on the relative abundance of transcripts for IGFBP2 and IGFBP5. There was also no effect of IGF-1 on the relative abundance of Glut1, Glut3, or Glut8 mRNA.

DISCUSSION

Insulin-like growth factor-1 can change the physiology of the bovine embryo so that, at least under some conditions, it is more likely to establish pregnancy when transferred to recipients (Block et al., 2003; Block and Hansen, 2007). The objective of the present study was to determine molecular and cellular actions of IGF-1 that could explain the increased potential for embryonic survival after transfer. Current results indicate that among the changes in embryo physiology caused by IGF-1 at the blastocyst stage are increases in the relative abundance of transcripts for Dc II, Na/K, and Bax and IGFBP3 and a decrease in amounts of Hsp70 transcripts. In contrast, there was no effect of IGF-1 treatment on cell number, allocation to the ICM and TE, or the proportion of blastomeres undergoing apoptosis. Thus, effects of IGF-1 on subsequent survival in vivo are more likely the result of differences in gene expression rather than in changes in cell number, allocation or apoptosis.

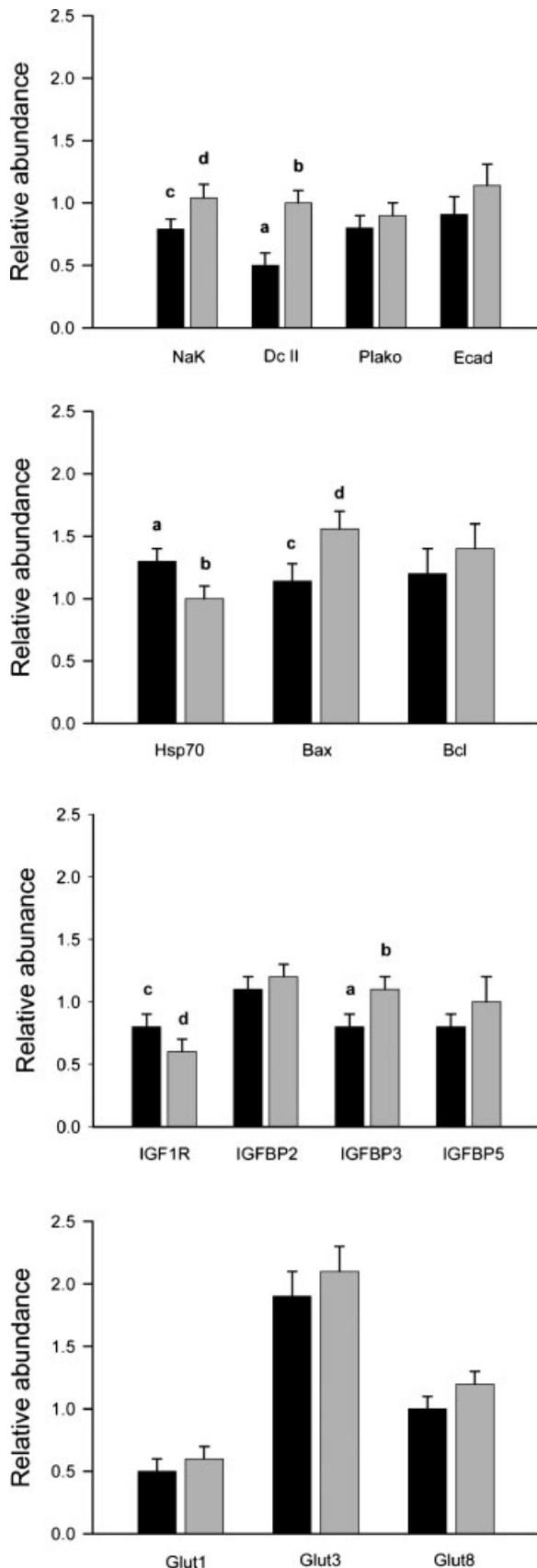
Among the transcripts elevated by IGF-1 were Dc II and Na/K. Both of these genes are involved with blastocyst formation. Desmocollin II is involved in the

TABLE 2. Effect of IGF-1 on Cleavage Rate, Blastocyst Development, Cell Number, Cell Allocation and Apoptosis

Variable	Control	IGF-1
Cleavage rate day 3 (%)	85.3 \pm 1.4%	81.9 \pm 1.4%
Blastocysts/oocytes day 7 (%)	27.9 \pm 1.3%	29.4 \pm 1.3%
Advanced blastocysts/oocytes day 7 (%)	20.9 \pm 1.0%	21.4 \pm 1.0%
Total cell number	127.2 \pm 2.8	124.7 \pm 2.9
Number of inner cell mass cells	47.7 \pm 1.4	44.0 \pm 1.5 [†]
Number of trophectoderm cells	83.0 \pm 2.8	79.8 \pm 3.1
Ratio trophectoderm:inner cell mass	2.1 \pm 0.1	2.1 \pm 0.1
Apoptotic blastomeres/total cells (%)	2.1 \pm 0.3%	2.2 \pm 0.3%

Data are least-squares means \pm SEM.

[†] $P < 0.06$.



formation of desmosomes and these play a critical role in stabilizing the TE during blastocyst formation and expansion (Fleming et al., 1991; Collins et al., 1995). In addition, Na/K regulates the accumulation of fluid in the blastocoel (Watson and Barcroft, 2001) as well as the formation of tight junctions during blastocyst expansion (Violette et al., 2006). Such differences in mRNA for Dc II and Na/K may indicate that IGF-1 treated embryos were at a more advanced stage of blastocyst expansion than controls even though all embryos were similar in terms of gross morphology. In addition, IGF-1 treated embryos may possess a more effective TE with respect to ion and water movement.

Compared to embryos produced following superovulation, embryos produced in vitro under sub-optimal culture conditions have an increased abundance of Hsp70 mRNA (Wrenzycki et al., 2001a; Lazzari et al., 2002; Sagirkaya et al., 2006). In the present study, IGF-1 reduced Hsp70 transcript abundance. One possibility for this finding is that IGF-1 makes embryos more resistant to one or more stresses associated with culture. Treatment of cultured embryos with IGF-1 reduced the effect of hydrogen peroxide (Kurzawa et al., 2002) and heat shock (Jousan and Hansen, 2004, 2007).

One of the actions of Hsp70 is to block apoptosis (Garrido et al., 2001, 2003). The fact that Hsp70 transcripts were reduced by IGF-1 implies that effects on Hsp70 synthesis are not involved in the anti-apoptotic effects of IGF-1 on apoptosis induced spontaneously during culture (Herrler et al., 1998; Lighten et al., 1998; Byrne et al., 2002a; Fabian et al., 2004) or by ultraviolet radiation (Herrler et al., 1998), tumor necrosis factor- α (Byrne et al., 2002b), or heat shock (Jousan and Hansen, 2004). There was also no effect of IGF-1 on transcript abundance for the anti-apoptotic gene, Bcl. Moreover, relative abundance of transcripts for the pro-apoptotic gene Bax was increased by IGF-1. This is somewhat surprising given that increased abundance of Bax might make embryos more susceptible to apoptosis. In addition, IGF-1 reduced abundance of the Bax gene transcript in porcine embryos (Kim et al., 2006). The increased abundance of Bax coupled with no effect of IGF-1 treatment on the abundance of the anti-apoptotic gene Bcl may help to explain why IGF-1 treated embryos in the present study did not have reduced levels of apoptosis.

The IGFBPs regulate the activity of IGF-1 in several ways, including extending the half-life of IGF-1, transporting and localizing IGF-1 to specific cell types and tissues, and stimulating and/or inhibiting IGF-1 actions at the cellular level (Jones and Clemmons, 1995; Clemmons, 1997; Cohick, 1998; Mohan and Baylink,

Fig. 1. Effect of IGF-1 on the relative abundance of developmentally important gene transcripts in grade 1 expanded blastocysts harvested on day 7 after insemination. Black bars represent control embryos and gray bars represent embryos treated with IGF-1 during culture. For each transcript, data are least-squares means \pm SEM of results for 7–22 embryos per treatment. Bars for each transcript with different superscripts were statistically different (a:b $P < 0.05$ or less) or tended to be statistically different (c:d $P < 0.08$ or less).

2002). While the precise role of IGFBPs in early embryo development is not fully understood, IGF-1 can alter the expression of IGFBPs by the early embryo (Prelle et al., 2001) and IGFBPs can modulate the effects of IGF-1 on early embryo development (Lin et al., 2003). In the present study, IGF-1 treatment increased the abundance of IGFBP3 transcripts. The majority of IGF-1 in the circulation is bound by IGFBP3 (Jones and Clemmons, 1995) and IGF-1 has been reported to increase circulating levels of IGFBP3 in vivo (Zapf et al., 1989; Camacho-Hubner et al., 1991a; Liao et al., 2006) as well as mRNA and protein levels in vitro (Bale and Conover, 1992; Camacho-Hubner et al., 1991b; Fleming et al., 2005). Treatment with IGF-1 also reduced transcripts for IGF1R, as has been found previously for bovine embryos (Prelle et al., 2001) and other cells (Hernandez-Sanchez et al., 1997). Taken together, it appears that one of the embryonic responses to IGF-1 is to dampen embryonic responses to IGF-1 through increased sequestration (via IGFBP3) and receptor downregulation.

Addition of IGF-1 to embryo culture in the present study did not affect total cell number, the allocation of cells to the ICM and TE, or the percent of blastomeres that were apoptotic. The literature is inconsistent regarding effects of IGF-1 on these characteristics in bovine embryos. Some reports indicate IGF-1 can increase cell number (Byrne et al., 2002a; Moreira et al., 2002; Sirisathien and Brackett, 2003; Sirisathien et al., 2003), increase the number of cells in the ICM (Sirisathien et al., 2003) and decrease the percent of blastomeres that were apoptotic (Byrne et al., 2002a; Sirisathien and Brackett, 2003). However, Sirisathien and Brackett (2003) reported a positive effect of IGF-1 on cell number and apoptosis for embryos collected at d 8 but not for embryos collected at d 7. In addition, Prelle et al. (2001) reported no effect of IGF-1 on total cell number or cell allocation to the ICM and TE. Culture conditions or timing of development may dictate the nature of the effect of IGF-1 on these characteristics of blastocysts.

In contrast to previous reports from our laboratory (Moreira et al., 2002; Block et al., 2003; Block and Hansen, 2007), as well as others (Palma et al., 1997; Prelle et al., 2001; Sirisathien et al., 2003), there was no effect of IGF-1 on development to the blastocyst stage. Differences between the present study and others may be related to differences in culture conditions because these have been reported to affect whether IGF-1 stimulates embryo development (Herrler et al., 1992; Palma et al., 1997). It may be that IGF-1 is more effective at increasing blastocyst development when the culture system results in a low yield of blastocysts. In the present study, the proportion of oocytes that developed to the blastocyst stage in the control group on d 7 was quite high ($27.9 \pm 1.3\%$). In previous reports where IGF-1 stimulated embryo development, blastocyst development in the control groups ranged between 9% and 19% on day 7 (Byrne et al., 2002a; Block et al., 2003; Block and Hansen, 2007) and between 10.5% and

28.5% on d 8 (Moreira et al., 2002; Block et al., 2003; Sirisathien et al., 2003).

The fact that IGF-1 did not affect blastocyst development does not imply that it would not affect embryonic survival. Culture conditions in the present study were the same as those used previously in our laboratory in which IGF-1 treatment enhanced embryo survival after transfer while causing only a slight increase in blastocyst yield (Block et al., 2003; Block and Hansen, 2007). Another report that illustrates the lack of relationship between blastocyst yield and post-transfer survival was recently made by Merton et al. (2007). Placement of a carbon activated air filtration system in the incubator used for embryo culture did not affect embryo development to the blastocyst stage but the resultant embryos were more likely to establish pregnancy after transfer to recipients than control embryos.

In conclusion, treatment of cultured bovine embryos with IGF-1 increased or tended to increase the relative abundance of certain mRNA transcripts, including Na/K, Dc II, Bax, and IGFBP3, and decreased or tended to decrease transcripts for Hsp70 and IGF1R. There was no effect of IGF-1 on the proportion of embryos developing to the blastocyst stage, cell number, cell allocation, or apoptosis. The alteration of steady state levels of certain gene transcripts by IGF-1 treatment may be important for the improved survival of IGF-1 treated embryos reported previously (Block et al., 2003; Block and Hansen, 2007). An increase in Dc II and Na/K may improve blastocyst expansion and development after hatching. Homologous recombination experiments in mice indicate that Dc III, another member of the desmocollin family, is required for preimplantation development (Den et al., 2006). The reduced abundance of Hsp70 transcripts is consistent with the idea that IGF-1 reduced cellular stress and such an effect could also contribute to higher survival.

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