

Insulin-like Growth Factor-I as a Survival Factor for the Bovine Preimplantation Embryo Exposed to Heat Shock¹

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ABSTRACT

Insulin-like growth factor-I (IGF-I) is a survival factor for preimplantation mammalian embryos exposed to stress. One stress that compromises preimplantation embryonic development is elevated temperature (i.e., heat shock). Using bovine embryos produced *in vitro* as a model, it was hypothesized that IGF-I would protect preimplantation embryos by reducing the effects of heat shock on total cell number, the proportion of blastomeres that undergo apoptosis, and the percentage of embryos developing to the blastocyst stage. In experiment 1, embryos were cultured with or without IGF-I; on Day 5 after insemination, embryos ≥ 16 cells were cultured at 38.5°C for 24 h or were subjected to 41°C for 9 h followed by 38.5°C for 15 h. Heat shock reduced the total cell number at 24 h after initiation of heat shock and increased the percentage of blastomeres that were apoptotic. Effects of heat shock were less for IGF-I-treated embryos. Experiment 2 was conducted similarly except that embryos were allowed to develop to Day 8 after insemination. The percentage reduction in blastocyst development for heat-shocked embryos compared with those maintained at 38.5°C was less for embryos cultured with IGF-I than for control embryos. Heat shock reduced the total cell number in blastocysts and increased the percentage of blastomeres that were apoptotic, whereas IGF-I-treated embryos had increased total cell number and a reduced percentage of apoptosis. Taken together, these results demonstrate that IGF-I can serve as a survival factor for preimplantation bovine embryos exposed to heat shock by reducing the effects of heat shock on development and apoptosis.

apoptosis, early development, embryo, environment, growth factors

INTRODUCTION

The fate of the developing preimplantation embryo depends on its ability to adapt to the microenvironment established by the mother. In some instances, such as when the female has experienced heat stress, the embryo might not be able to successfully adjust to the increased reproductive tract temperature and embryonic development can become terminated [1, 2]. In cattle, studies using *in vitro*-produced embryos demonstrated that the oocyte and early

preimplantation embryo are very sensitive to detrimental effects of heat shock and that the embryo acquires some ability to protect itself from heat shock as it progresses in development [3–6].

A cell's resistance to stress also depends upon the array of extracellular signals that can modify cytoprotective responses. Several molecules have been demonstrated to be beneficial for protecting preimplantation embryos from deleterious effects of stress, including transforming growth factor- α [7] and epidermal growth factor [8, 9]. Among the survival factors, insulin-like growth factor-I (IGF-I) has been widely studied for its effects on embryonic development in the presence and absence of stress. In numerous species (human [10, 11], rabbit [12], mouse [13, 14], and bovine [15–18]), development to the blastocyst stage was increased when embryos were stimulated with IGF-I. In addition, IGF-I increased embryonic inner cell mass number [10, 14] and total cell number [12, 13, 16–18].

Addition of IGF-I to culture medium reduced the incidence of spontaneous apoptosis in embryos of various species, including human [10], rabbit [12], mouse [19], and bovine [17, 20]. In addition, IGF-I blocked apoptosis in preimplantation embryos induced by ultraviolet radiation [12], camptothecin and actinomycin D [19], and tumor necrosis factor- α [21]. Addition of IGF-I to culture medium also reduced the effect of hydrogen peroxide on development of mouse preimplantation embryos [9].

For the current experiments, it was hypothesized that IGF-I serves as a survival factor for bovine preimplantation embryos subjected to heat shock by reducing the effect of heat shock on embryo cell number, the proportion of blastomeres that undergo apoptosis, and the percentage of embryos developing to the blastocyst stage.

MATERIALS AND METHODS

Materials

The media HEPES-Tyrodes Lactate (TL), IVF-TL, and Sperm-TL were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described [22]. Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hanks salts without phenol red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 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 (Bio-Whittaker, Walkersville, MD) with Earle salts supplemented with 10% (v/v) bovine steer serum, 2 μ g/ml estradiol 17- β , 20 μ g/ml bovine FSH (Folltropin-V; Vetrepharm Canada, London, ON), 22 μ g/ml sodium pyruvate, 50 μ g/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL). Potassium simplex optimized medium (KSOM) that contained 1 mg/ml BSA was obtained from Cell and Molecular Technologies (Lavallete, NJ). Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). Recombinant human IGF-I was purchased from Upstate Biotechnology (Lake Placid, NY). On the day of use, KSOM was modified

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for bovine embryos to produce KSOM-BE2 as described elsewhere [23]. For the addition of IGF-I to KSOM-BE2, a vial containing 25 μg of lyophilized IGF-I was rehydrated with 100 μl 0.1 M acetic acid. This solution was then stored at -20°C in 5- μl aliquots until use, when a single aliquot of IGF-I was diluted with KSOM-BE2 to a concentration of 100 ng/ml.

The In Situ Cell Death Detection Kit (fluorescein) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Hoechst 33342 and glycerol were purchased from Sigma. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

In Vitro Production of Embryos

Embryo production was performed as previously described [2, 24] except for the culture medium used in the current study. Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer 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 20–22 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 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 spermatozoa from a pool of frozen-thawed semen from three bulls. After 8–10 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1000 U/ml hyaluronidase in HEPES-TALP, and placed in groups of 30 in 50- μl drops of KSOM-BE2. Approximately half of the putative zygotes were cultured in KSOM-BE2 containing 100 ng/ml IGF-I for the entire culture period, whereas the remaining zygotes were cultured in KSOM-BE2. All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air until selected for treatment at Day 5 after insemination. At this time, embryos ≥ 16 cells were harvested from culture drops according to the specific experimental design. In addition, each replicate contained an undisturbed drop of 15 embryos cultured \pm IGF-I that was used to assess cleavage rates at Day 3 after insemination and development to the blastocyst stage at Day 8 after insemination.

TUNEL and Hoechst 33342 Labeling

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group characteristic of DNA strand breaks. Embryos were removed from KSOM-BE2 \pm IGF-I and washed two times in 50- μl drops of 10 mM KPO_4 pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml PVP (PBS-PVP) by transferring the embryos from drop to drop. 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 600 μl of PBS-PVP at 4°C until the time of assay. All steps of the TUNEL assay were conducted using microdrops in a humidified box.

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) at 37°C in the dark for 1 h. 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 h 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 33342 (1 $\mu\text{g}/\text{ml}$) for 12 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4- μl drops of glycerol, and coverslips were placed on the slides. Labeling of TUNEL and Hoechst 33342 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 $20\times$ objective. Digital images were acquired using AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Experiments

Protective effect of IGF-I on heat-shock induced apoptosis. Embryos ≥ 16 cells were collected on Day 5 after insemination and transferred to a fresh drop of KSOM-BE2 \pm IGF-I. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 9 h followed by culture at 38.5°C for 15 h. All harvested embryos were fixed on Day 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated seven times using 86–100 embryos per treatment.

Developmental competence of IGF-I treated embryos subjected to heat shock. Bovine embryos ≥ 16 cells were collected on Day 5 after insemination and transferred to a fresh drop of KSOM-BE2 \pm IGF-I. Embryos were maintained at 38.5°C or were heat-shocked at 41°C for 9 h followed by culture at 38.5°C until Day 8 when development to the blastocyst stage was assessed. All blastocysts were fixed and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 11 times using 191–236 embryos per treatment.

Developmental capacity of undisturbed embryos cultured continuously with or without IGF-I. For each replicate of the above-mentioned experiments to test IGF-I effects on resistance to heat shock, an additional, undisturbed drop of 15 embryos were cultured in KSOM-BE2 \pm IGF-I (100 ng/ml) for assessment of cleavage and blastocyst development rates on Days 3 and 8 after insemination, respectively. A total of 18 replicates were completed using 270 embryos per treatment.

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS [25]. Percentage data were transformed by arcsin transformation before analysis. Independent variables included IGF-I, heat shock treatment, and replicate. The mathematical model included main effects and all interactions. Tests of significance for IGF-I and heat shock were performed using IGF-I \times replicate and heat shock \times replicate as the error terms, respectively. 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

Cleavage and Development as Modified by IGF-I

Culture of embryos continuously in the presence of IGF-I beginning after fertilization increased the percentage of oocytes that cleaved on Day 3 after fertilization ($P < 0.01$) as well as the percentage of oocytes that developed to the blastocyst stage on Day 8 after fertilization ($P < 0.06$) (Fig. 1). There was no significant difference in the percentage of cleaved embryos that became blastocysts.

Protective Effect of IGF-I on Induction of Apoptosis by Heat Shock

Representative fluorescent images illustrating the effect of IGF-I on the frequency of apoptotic nuclei for Day 5 embryos ≥ 16 cells exposed to 38.5°C for 24 h or to 41°C for 9 h and 38.5°C for 15 h are shown in Figure 2. In the absence of IGF-I, heat shock caused a reduction in the total number of nuclei and an increase in the proportion of nuclei that were apoptotic (compare Fig. 2A for an embryo cultured continuously at 38.5°C with Fig. 2B for an embryo exposed to 41.0°C). These deleterious effects of heat shock were not apparent in embryos cultured with IGF-I (compare Fig. 2C of an embryo cultured continuously at 38.5°C and Fig. 2D of an embryo exposed to 41.0°C). Quantitative analysis of total cell number and percentage of cells that were TUNEL-positive are illustrated in Figure 3. Exposure

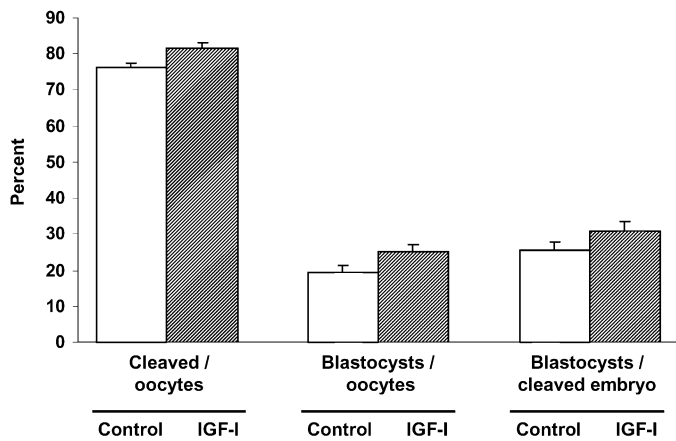


FIG. 1. Cleavage rates and blastocyst development on Days 3 and 8 after insemination, respectively, for embryos cultured with and without 100 ng/ml IGF-I. Blastocyst development was expressed as the proportion of oocytes and cleaved embryos that became blastocysts. The experiment was replicated 18 times using 270 embryos per treatment. Results are least-squares means \pm SEM. The percentage of oocytes that cleaved ($P < 0.01$) as well as the percentage of oocytes that developed to the blastocyst stage ($P < 0.06$) were affected by IGF-I. There was no significant treatment effect on the percentage of cleaved embryos that developed to the blastocyst stage.

of embryos to 41°C for 9 h on Day 5 after insemination reduced total cell number (Fig. 3A; $P < 0.05$) and increased the percentage of nuclei that were TUNEL-positive (Fig. 3B; $P < 0.001$) on Day 6 after insemination. Effects of heat shock were less for IGF-I-treated embryos for both total cell number (heat shock \times IGF-I, $P = 0.07$) and for the percentage of nuclei that were TUNEL-positive (heat shock \times IGF-I, $P < 0.01$).

Reduction by IGF-I of the Heat-Shock-Induced Inhibition in Blastocyst Development

IGF-I increased the percentage of embryos ≥ 16 cells at Day 5 after insemination that developed to the blastocyst stage ($P < 0.001$; Fig. 4A). Heat shock caused a reduction in the percentage of embryos that developed to the blastocyst stage for both control and IGF-I treated embryos ($P < 0.001$; Fig. 4A). As shown in Figure 4B, however, the reduction in development caused by heat shock, expressed as a percentage of development for embryos not subjected to heat shock, was less ($P < 0.05$) for embryos cultured in the presence of IGF-I (19.4%) than for embryos cultured without IGF-I (46.4%).

Properties of blastocysts as affected by IGF-I and heat shock treatments are summarized in Figure 5. Heat shock reduced total blastocyst cell number ($P < 0.01$; Fig. 5A) and increased the percentage of blastomeres that were TUNEL-positive ($P < 0.05$; Fig. 5B). The lack of a significant heat shock \times IGF-I interaction indicated that effects of heat shock were apparent for embryos with or without IGF-I. However, IGF-I-treated blastocysts had increased total cell number ($P < 0.001$; Fig. 5A) and a reduced percentage of blastomeres that were TUNEL-positive ($P < 0.01$; Fig. 5B), and these effects of IGF-I occurred regardless of heat shock (i.e., the heat shock \times IGF-I interaction was not significant).

DISCUSSION

Results from the current study implicate IGF-I as a survival factor for preimplantation embryos exposed to heat shock. In particular, IGF-I blocked the effects of heat shock on cell number and development to the blastocyst stage and prevented induction of apoptosis in response to elevated temperature. These results have implications for under-

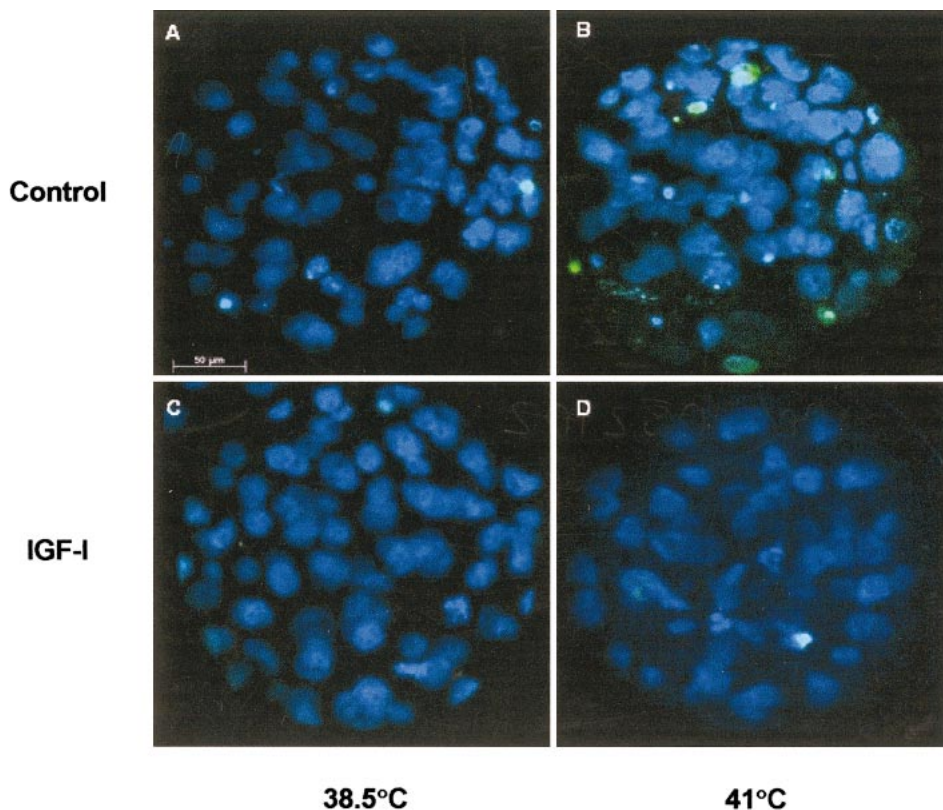


FIG. 2. Representative images illustrating the effect of IGF-I on frequency of apoptotic nuclei in embryos ≥ 16 cells that were cultured continuously at 38.5°C (A, C) or were subjected to heat shock for 9 h at 41°C (B, D) on Day 5 after insemination. Labeling at 15 h after the end of heat shock was performed for nuclei using Hoechst 33342 (blue) and for apoptotic nuclei using the TUNEL reaction and fluorescein isothiocyanate-conjugated dUTP (green). Bar = 50 μ m.

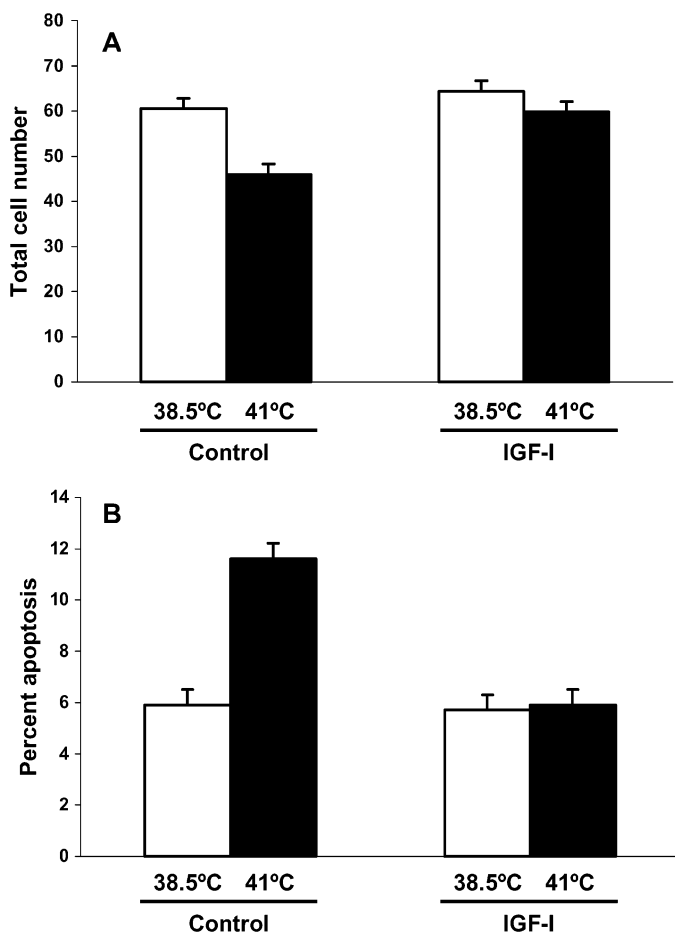


FIG. 3. Inhibition of heat-shock-induced apoptosis and growth retardation in embryos ≥ 16 -cells by IGF-I. Open bars represent embryos maintained continuously at 38.5°C, while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C for 15 h. The experiment was replicated 7 times using 86–100 embryos per treatment. Results are least-squares means \pm SEM. Heat shock reduced the total cell number ($P < 0.05$; **A**) and increased the percentage of nuclei that were TUNEL-positive ($P < 0.001$; **B**). Effects of heat shock were less for IGF-I-treated embryos as determined by heat shock \times IGF-I effects for total cell number ($P = 0.07$; **A**) and the percentage of nuclei that were TUNEL-positive ($P < 0.01$; **B**).

standing the range of biological systems that regulate embryonic responses to stress and point the way to possible therapeutic interventions for enhancing embryonic survival during stress.

There could be several mechanisms whereby IGF-I blocked effects of heat shock on cell number 24 h after heat shock and on the proportion of embryos that became blastocysts on Day 8 after insemination. A direct mitogenic action of IGF-I is possible but not likely, because IGF-I did not increase the proportion of blastomeres positive for proliferating nuclear cell antigen in bovine embryos [20]. Also, there was no major change in percent development to the blastocyst stage in embryos cultured continuously with IGF-I. The slight increase in development to the blastocyst stage caused by IGF-I, although nonsignificant, is similar in magnitude to that seen in an earlier study in our laboratory [16]. It is more likely that the inhibition of heat-shock induced apoptosis caused by IGF-I prevented the reduction in cell number caused by heat shock and allowed for a greater proportion of embryos to successfully develop to the blastocyst stage. Indeed, IGF-I has been demonstrated to block apoptosis in many cell types [26, 27], including

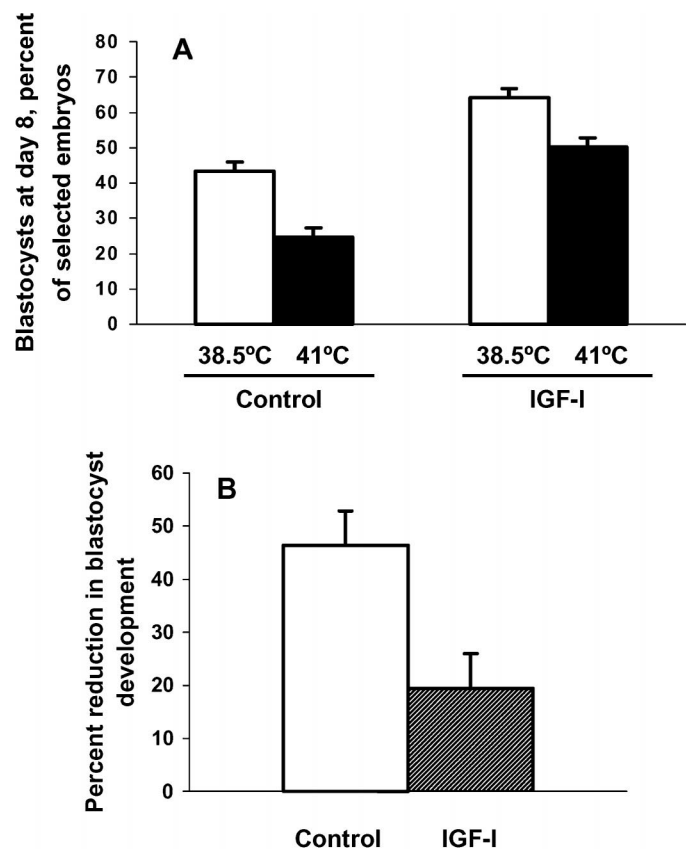


FIG. 4. Inhibition of the deleterious effects of heat shock on development of embryos ≥ 16 cells to the blastocyst stage by IGF-I. The experiment was replicated 11 times using 191–236 embryos per treatment. Results are least-squares means \pm SEM. For **(A)**, open bars represent embryos maintained continuously at 38.5°C, while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C until Day 8 after insemination. Development was affected by the presence of IGF-I ($P < 0.001$) and heat shock ($P < 0.001$). **(B)** Percentage reduction in blastocyst development for embryos caused by heat shock expressed as a percentage of development for nonshocked embryos [100 – (percent development at 41°C/percent development at 38.5°C)]. The percent reduction in blastocyst development caused by heat shock was less ($P < 0.05$) for embryos cultured in the presence of IGF-I than for embryos cultured without IGF-I.

preimplantation embryos [10, 11, 19–21]. Inhibition of apoptosis by IGF-I is mediated through activation of Akt by phosphoinositide 3-kinase and subsequent phosphorylation and inactivation of proapoptotic proteins such as Bad, which cause mitochondrial membrane depolarization and release of cytochrome c [28].

It is conceivable, however, that the survival function of IGF-I occurred independently of its actions on apoptosis. Inhibition of apoptosis using the caspase inhibitor z-DEVD-fmk actually increased susceptibility of Day 5 bovine preimplantation embryos to heat shock as determined by their capacity to develop into blastocysts [29]. These data were interpreted to mean that a limited degree of apoptosis was beneficial for an embryo at this stage exposed to stress because apoptosis removed those cells most damaged by heat shock. One possibility is that actions of IGF-I that are proliferation- and apoptosis-independent enhanced developmental competence and made these embryos more likely to survive stress. For example, IGF-I up-regulated glucose uptake in preimplantation mouse embryos [30], and such an effect might make embryos more able to survive cellular damage caused by heat shock. IGF-I has been reported to

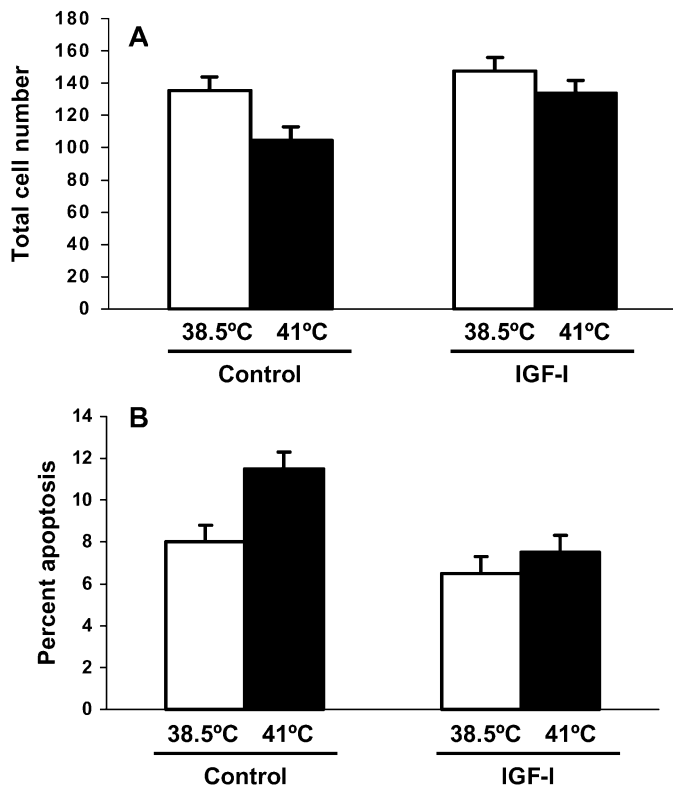


FIG. 5. Cell number and apoptosis in Day 8 blastocysts as affected by heat shock on Day 5 after insemination and IGF-I. Open bars represent embryos maintained continuously at 38.5°C, while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C until Day 8 after insemination. The experiment was replicated 11 times using 191–236 blastocysts per treatment. Results are least-squares means \pm SEM. Heat shock reduced total cell number ($P < 0.01$; **A**) and increased the percentage of nuclei that were TUNEL-positive ($P < 0.05$; **B**). However, IGF-I-treated blastocysts had increased total cell number ($P < 0.001$; **A**) and a lower percentage of nuclei that were TUNEL-positive ($P < 0.01$; **B**).

reduce effects of hydrogen peroxide on development of mouse preimplantation embryos [9], and given the induction of peroxide production by heat shock in bovine embryos [31], it is possible that IGF-I protected embryos from heat shock in the current study by blocking the deleterious actions of free radicals.

Bovine embryos collected on Day 5 after insemination have been demonstrated to be more resistant to heat shock than embryos collected at earlier stages [3, 5, 6, 31]. Heat shock at this stage sometimes [32], but not always [3, 5, 6], reduced the potential for subsequent development to the blastocyst stage. In the present study, heat shock at this stage did reduce the proportion of embryos that developed to the blastocyst stage, but IGF-I reduced this effect. In the absence of IGF-I, moreover, blastocyst cell number was lower and the proportion of blastomeres classified as apoptotic was higher for blastocysts formed from heat-shocked embryos. Thus, it is likely that the potential for these blastocysts to continue development and establish pregnancy was compromised by the earlier heat shock. However, blastocyst cell number and apoptosis for IGF-I treated embryos exposed to heat shock was similar to values for control embryos not exposed to heat shock. These results imply that IGF-I might increase blastocyst development and pregnancy rates of cows exposed to periods of heat stress during early embryonic development. Pregnancy rates of embryo-transfer recipients in the summer were higher for cows that

received embryos cultured with IGF-I than for cows that received control embryos [16]. Additional studies to evaluate the effects of IGF-I on fertility of heat-stressed cows are warranted.

One implication of the present results is that the IGF-I status within the reproductive tract is likely to be an important criterion for embryonic survival. Both the oviduct and uterus produce IGF-I [33] and content of IGF-I in the uterine lumen (but not the oviduct) was increased through injection of somatotropin [34]. Consistent with the idea of IGF-I as a determinant of embryonic survival are findings that fertility of lactating dairy cows, which have reduced concentrations of IGF-I in circulation, can be increased by treatment with bovine somatotropin [35, 36]. Perhaps manipulation of the IGF-I system with somatotropin could enhance embryonic survival in females exposed to heat stress or other stresses. In such a scheme, effects of somatotropin on hyperthermia [37] would have to be overcome.

In conclusion, IGF-I protected the preimplantation embryo from the detrimental effects of heat shock. This protective action of IGF-I, which was associated with an increase in total cell number and a reduction in the number of blastomeres that become apoptotic, may involve inhibition of apoptosis. These results imply that manipulation of the IGF-I system may enhance embryonic survival in females exposed to heat stress or other stresses. Additional studies are needed to characterize the mechanistic function whereby IGF-I enhances the survival of embryos exposed to heat shock.

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