Thermoprotective effect of insulin-like growth factor 1 on *in vitro* matured bovine oocyte exposed to heat shock

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ABSTRACT

The role of insulin-like growth factor 1 (IGF1) on cellular function and developmental capacity of heat-shocked oocytes has not been completely understood. Therefore, the objective of this study was to determine the effect of IGF1 on apoptosis, mitochondrial activity, cytoskeletal changes, nuclear maturation, and developmental competence of bovine oocytes exposed to heat shock. Cumulus–oocyte complexes were submitted to control (38.5 °C for 22 hours) and heat shock (41 °C for 14 hours followed by 38.5 °C for 8 hours) in the presence of 0 or 100 ng/mL IGF1 during IVM. Heat shock increased the percentage of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)–positive oocyte and reduced oocyte mitochondrial activity. However, addition of 100 ng/mL IGF1 minimized these deleterious effects of temperature. Caspase activity was affected neither by heat shock nor IGF1. Exposure of bovine oocytes to 41 °C during the first 14-hour IVM affected cortical actin localization and microtubule organization at the meiotic spindle and reduced the percentage oocytes that reached the metaphase II stage. However, in the presence of IGF1, cortical actin and percentage of metaphase II oocytes were not different between control and heat-shocked oocytes, suggesting a partial beneficial effect of IGF1. There was no effect of IGF1 on microtubule organization. Heat shock also reduced the percentage of oocytes that reached the blastocyst stage, blastocyst cell number, and increased the percentage of TUNEL-positive blastomeres. However, there was no effect of 100 ng/mL IGF1 on oocyte development to the blastocyst stage and blastocyst quality. Therefore, 100 ng/mL IGF1 prevented some heat shock–induced cellular damage in bovine oocytes but had no effect on oocyte developmental competence. In contrast, a low IGF1 concentration (25 ng/mL) had a thermoprotective effect on oocyte developmental competence to the blastocyst stage. In conclusion, IGF1 prevented part of the damage induced by heat shock on oocyte function. This effect was modulated by IGF1 concentration.

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1. Introduction

Exposure of lactating Holstein cows to elevated temperature and humidity causes hyperthermia leading to maternal heat stress. It has been known that heat stress

affects fertility of lactating dairy cows inducing deleterious effects on follicular growth [1,2], hormonal secretion [2,3], endometrial function [4], uterine blood flow [5], oocyte competence [6,7], and preimplantation embryonic development [8,9].

Despite the multiple deleterious effects of heat stress on fertility, there is increasing evidence that oocytes are major targets of maternal heat stress. For instance, heat stress during the periovulatory period increased the proportion of retarded embryos in heifers suggesting the negative effect of elevated temperature on oocyte maturation [10]. *In vitro* studies also reported the direct effects of heat shock on oocyte maturation. Exposure of bovine oocytes to severe (42 °C) or moderate (40 °C–41 °C) heat shock during the first 12-hour IVM (0–12 hours IVM) reduced oocyte ability to develop to the blastocyst stage [11,12]. Moreover, oocyte exposure to 43 °C for 45 and 60 minutes during IVM reduced blastocyst and expanded blastocyst rates [13]. The cellular mechanisms triggered by heat shock on that effect are not completely understood. There is evidence that exposure of bovine oocytes to heat shock during IVM affected cytoskeleton organization [14–16], altered cortical granule distribution [17], chromosome separation during fertilization and cleavage [16,18], decreased the proportion of oocytes that reached the MII stage after IVM [15], and increased oocyte apoptosis [12].

Although different oocyte cellular compartments have been shown to be affected by elevated temperature [19], nuclear transfer experiments found that exposure of donor cell nuclei to heat shock did not affect subsequent embryonic development in either *Bos indicus* or *Bos taurus* oocytes [20]. However, exposure of receptor ooplasm to heat shock decreased developmental competence of *Bos taurus* oocytes [20], indicating greater cytoplasmic susceptibility to elevated temperature.

It has been shown that exposure of dairy cows to heat stress reduced serum insulin-like growth factor 1 (IGF1) levels leading to impairment of oocyte quality [21]. Cumulus cells and oocytes express IGF1 receptor mRNA [22–24]. Indeed, several members of the IGF system have been reported in bovine oocytes and blastocysts. IGF1 mRNA was greater in heat-shocked embryos compared with control [25]. Bovine oocytes expressed mRNA for IGF1 and IGF2, IGF1 and IGF2 receptor (IGF1R and IGF2R), IGF-binding protein 2 and 4 (IGFBP2 and IGFBP4), and pregnancy-associated plasma protein A [25]. There is evidence that free IGF1 in follicular fluid can bind to oocyte IGFR activating phosphatidylinositol 3-kinase/protein kinase A (PI3K/AKT), and mitogen-activated protein kinases 3/1 (MAPK3/1) pathways [26]. In addition, activation of MAPK leads to phosphorylation of various subtracts like phospholipases, transcription factors, and cytoskeletal proteins [27]. PI3K/AKT activation is known to be involved in oocyte meiotic maturation [28] and activation of MAPK pathways by IGF1 promotes blastocyst development [29].

IGF1 can modulate effects of elevated temperature on cellular function. Addition of 100 ng/mL IGF1 to *in vitro* culture medium improved preimplantation embryo resistance to heat shock preventing the deleterious effect of temperature on development to the blastocyst stage and blastocyst apoptosis [30,31]. Similarly, heat-stressed lactating dairy cows had higher pregnancy rates after transfer of IGF1-treated embryos compared with control embryos [32]. However, the effects of IGF1 during IVM of heat-shocked bovine oocytes are still controversial. In a study conducted by Zhandi et al. [33], addition of 100 ng/mL IGF1 during IVM enhanced heat shock–induced apoptosis and compromised developmental competence of heat-shocked oocytes. In contrast, Meiyu et al. [34] found that addition of 100 ng/mL IGF1 during IVM prevented heat shock–induced apoptosis in bovine oocytes. Therefore, the objective of this study was to determine the role of IGF1 on nuclear maturation, apoptosis, mitochondrial activity, cytoskeleton organization, and developmental competence of bovine oocytes exposed to heat shock during IVM.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA). Antibodies were purchased from Molecular Probes (Eugene, OR, USA). Recombinant human IGF1 expressed in *E. coli* was purchased from Upstate Biotechnology (Lake Placid, NY, USA), *In Situ* Cell Detection Kit Fluorescein was from Boehringer Mannheim/Roche Diagnostics (Penzberg in Upper Bavaria, Germany), and PhiPhiLux-G_1D_2 was from OncoImmunin, Inc. (Gaithersburg, MD, USA). MitoTracker Red CMX-Ros was purchased from Invitrogen (Carlsbad, CA, USA), VEC-TASHIELD Mounting Medium was from Vector Laboratories, Inc. (Burlingame, CA, USA), and goat serum from Cripion (Andradina, São Paulo, Brazil).

Oocyte collection medium was tissue culture medium-199 (TCM-199) containing L-glutamine and phenol red (GIBCO, Grand Island, NY, USA) supplemented with 2.2 mg/mL sodium bicarbonate, 1% (vol/vol) fetal bovine serum (FBS) (GIBCO) (containing 2 U/mL heparin), 0.01 µg/mL streptomycin, and 0.01 U/mL penicillin-G. Pre-IVM medium was TCM-199 HEPES with Hanks salts (GIBCO), supplemented with 10% (vol/vol) FBS, 50 µg/mL gentamicin, and 0.2 mM sodium pyruvate. Oocyte maturation medium was TCM-199 sodium bicarbonate with earle's salts (GIBCO) supplemented with 10% (vol/vol) FBS, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 1 µg/mL estradiol 17-β, 10 µg/mL FSH (FOLL-TROPIN–V from Bioniche Animal Health Canada Inc., Bellevile, Ontario, Canada), and 10 µg/mL LH (Chorulon from Intervet Schering Plow, Roseland, NJ, USA). Pre-IVF medium was TCM-199 HEPES (GIBCO) supplemented with 3 mg/mL bovine serum albumin fraction V (BSA-V), 50 µg/mL gentamicin, and 0.2 mM sodium pyruvate. IVF medium was a modified Tyrode's albumin-lactate pyruvate (TALP) medium [35] containing 6 mg/mL essentially fatty acid–free BSA, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 100 µg/mL heparin, and 41.66 µL/mL PHE (penicillamine 2.7 µg/mL, hipotaurine 1 µg/mL, epinephrine 0.33 µg/mL in 0.9% [wt/vol] NaCl). SP-TALP medium used during sperm purification was as described by Parrish et al. [35]. Frozen semen from Nelore was acquired from CRV Lagoa (Sertãozinho, São Paulo, Brazil). *In vitro* culture medium was potassium simplex optimized medium (KSOM, MR-107-D; Millipore, Darmstadt,

Germany) supplemented with 10% FBS, 0.25 μg/mL gentamicin, and 0.5% 100× non-essential amino acids.

2.2. Methods

2.2.1. In vitro maturation, fertilization, and culture

Slaughterhouse ovaries from crossbred *Bos indicus* cattle were transported to the laboratory in sterile saline (0.9% [wt/vol] NaCl containing 100 U/mL penicillin-G, and 100 μg/mL streptomycin) at 37 °C during 2 hours. Cumulus–oocyte complexes (COCs) were collected by slicing 2 to 8 mm follicles in oocyte collection medium [36]. Grade I and II COCs were selected as previously described [37] and washed once in pre-IVM medium. Groups of 10 COCs were transferred to 50 μL drop of oocyte maturation medium with or without IGF1 overlaid with mineral oil for 22 to 24 hours at 38.5 °C in an atmosphere of 5% CO_2 (vol/vol) in humidified air. After IVM, COCs were washed once in pre-IVF medium. Groups of 10 COCs were transferred to 90 μL drop IVF-TALP as described by Parrish et al. in 1988. Frozen-thawed spermatozoa from two Nelore bulls were centrifuged in Percoll gradient (90% and 45%) for 5 minutes at $9.000 \times g$ followed by centrifugation for 2 minutes at $9.000 \times g$ on SP-TALP [35]. For fertilization, it was used an insemination dose of 1×10^6 sperm/mL for 18 hours at 38.5 °C and 5% (vol/vol) CO_2 in humidified air. Presumptive zygotes were removed from fertilization drops, denuded of cumulus cells by repeated pipetting in Pre-IVF medium, and washed once in modified KSOM. Groups of 25 to 30 presumptive zygotes were transferred to 50 μL drop of modified KSOM overlaid with mineral oil at 38.5 °C and 5% CO_2 (vol/vol) in humidified air. The proportion of oocytes that cleaved and developed to the blastocyst stage was determined at days 3 and 7 to 9 after fertilization, respectively.

2.2.2. TUNEL labeling

TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) was similar to previously described [36,38]. COCs were denuded by repeated pipetting 22 to 24 hours after beginning of IVM. Embryos or denuded oocytes were fixed in 100 μL drop of 4% (wt/vol) paraformaldehyde in 0.2 M PBS for 1 hour at room temperature (RT), washed three times in 10 mM PBS containing 1 mg/mL PVP (PBS-PVP), and stored in PBS-PVP at 4 °C until TUNEL assay. For TUNEL staining, samples were permeabilized in 0.5% (vol/vol) Triton X-100 in 0.1% (wt/vol) sodium citrate for 1 to 2 hours (for oocytes and embryos, respectively) at RT and washed three times in PBS-PVP. Positive and negative controls were incubated in 50 U/mL RQ1 RNase-free DNase at 37 °C for 1 hour. Samples were washed three times in PBS-PVP and incubated with TUNEL reaction mixture (fluorescein isothiocyanate–conjugated dUTP and terminal deoxynucleotidyl transferase) as recommended by the manufacturer for 1 hour at 37 °C in the dark. Although positive control and experimental groups were incubated in TUNEL solution, negative control was incubated in labeling solution 2-deoxyuridine-5-triphosphate with FITC (dUTP-FITC). All groups were washed three times in PBS-PVP and counterstained with 5 μg/mL Hoechst 33342 in 10 mM PBS for 15 minutes at RT in the dark. Oocytes/embryos were washed in PBS-PVP for 15 minutes and transferred to 10% (vol/vol) poly-L-lysine–coated slides mounted with coverslips. Samples were analyzed in Olympus IX81 epifluorescence microscope with filter sets for FITC (emission 520 nm and excitation 460–490 nm) and 4′,6-diamidino-2-phenylindole (DAPI, emission 420 and excitation 330–385 nm). The percentage of embryo apoptosis was determined by counting the total number of blastomeres and total number of TUNEL-labeled cells per blastocyst. The percentage of apoptotic oocytes was determined by counting the total number of oocytes and total number of TUNEL-labeled oocytes.

2.2.3. Group II caspase activity

Group II caspases include caspase-3, -2, and -7. COCs were denuded by repeated pipetting 14 hours after beginning of IVM. Groups of 30 denuded oocytes were incubated in 15 μL of TCM-199 HEPES supplemented with 1 mg/mL polyvinyl acetate (PVA) (TCM-199 HEPES-PVA) and 5 μM PhiPhiLux-G_1D_2 during 40 minutes at 39 °C in humid chamber in the dark. Negative control oocytes were incubated in the absence of PhiPhiLux-G_1D_2. Oocytes were washed three times in 50 μL drop TCM-HEPES-PVA, transferred to poly-L-lysine–coated slides, and mounted with a coverslip. Caspase assay was conducted immediately after the end of 14-hour heat shock using an Olympus IX81 fluorescence microscope equipped with FITC filter (emission 520 nm and excitation 460–490 nm). Digital images from each oocyte were obtained by Image Plus software and analyzed using Image J, version 1.44. The area from each oocyte was manually circled using a circular tool to delimitate the region of interest, and the pixel intensity per unit area was determined. Oocyte pixel fluorescence intensity (PI) was divided into two categories according with mean PI: high (PI > 23.071) and low (PI < 23.071) pixel intensity.

2.2.4. MitoTracker red CMX-ROS assay

COCs were denuded by repeated pipetting 22 to 24 hours after beginning of IVM and submitted to Mito-Tracker assay to evaluate mitochondrial activity. Denuded oocytes were incubated in 500 μL TCM-199 HEPES containing 1 mg/mL PVA and 50 nM/mL MitoTracker Red CMX-ROS during 15 minutes in water bath at 37 °C in the dark. Oocytes were washed three times in PBS-PVP, incubated in 5 μg/mL Hoechst 33342 for 15 minutes at RT, and washed three times in PBS-PVP for 15 minutes in each drop. Oocytes were placed on 10% (vol/vol) poly-L-lysine–coated slides mounted with coverslips. Oocytes were evaluated under Olympus IX81 epifluorescence microscope equipped with texas red filter (emission 590 nm and excitation 510–550 nm) for mitochondrial activity and DAPI filter (emission 420 nm and excitation 330–385 nm). Digital images from each oocyte were analyzed by Image J, version 1.43. A circular draw function was manually performed for each region of interest (oocyte), and pixel fluorescence intensity per unit area was determined.

2.2.5. Microfilament and microtubule localization

COCs were denuded by repeated pipetting 22 hours after the beginning of IVM. Denuded oocytes were fixed in 3.7% (vol/vol) formaldehyde. Zona pellucida was removed in 0.1% (wt/vol) protease for 5 minutes at 37 °C. For

microfilament localization, oocytes were permeabilized in 0.1% (vol/vol) Triton X-100 in 10 mM PBS for 30 minutes at RT. Nonspecific binding sites were blocked in 100 μL PBS-PVP supplemented with 10% (vol/vol) goat serum for 30 minutes at RT. Oocytes were incubated in 5 UI/mL Alexa Fluor 568 phalloidin for 1 hour at RT and washed three times in 50 μL PBS-PVP. Samples were transferred to poly-L-lysine–coated slides and mounted with VECTASHIELD Mounting Medium.

For microtubule localization, oocytes were permeabilized in 0.5% (vol/vol) Triton X-100 for 1 hour at RT. Nonspecific binding sites were blocked in 100 μL PBS-PVP supplemented with 5% (vol/vol) goat serum for 30 minutes at RT. Oocytes were incubated in 2 μg/mL mouse anti-bovine α-tubulin for 1 hour at 37 °C. Oocytes were incubated with secondary antibody Alexa Fluor 488 goat anti-mouse IgG (H + L) (10 μg/mL) for 1 hour at RT in the dark. For chromatin evaluation, oocytes were incubated in Hoechst 33342 (5 μg/mL) for 10 minutes and washed three times in PBS-PVP. Samples were transferred to poly-L-lysine–coated slides and mounted with VECTASHIELD Mounting Medium. Samples were analyzed in epifluorescence microscope Olympus IX81 or Leica DM2500 equipped with the following filters: DAPI (emission 420 and excitation 330–385), texas red for actin (emission 590 nm and excitation 510–550 nm), and FITC for tubulin (emission 520 nm and excitation 460–490 nm).

Samples were first subjected to morphologic evaluation. Digital images from each oocyte were then evaluated using Image J, version 1.43. To quantify cortical actin pixel intensity (CAPI), a circular draw function was manually performed in the entire oocyte and its internal area (70% of total area in the center of the oocyte). Total actin pixel intensity (TAPI) and internal pixel intensity (IPAI) were used to calculate cortical actin [CAPI = (TAPI − 0.7 × IAPI)/0.3]. Cortical actin PI was normalized by the ratio cortex/internal PI. To quantify tubulin fluorescence, total fluorescence and microtubule fluorescence were measured. Microtubule fluorescence was normalized by total oocyte fluorescence.

2.3. Experimental design

Experiments 1 to 7 were designed as a 2 × 2 factorial arrangement of treatments to determine the effects of temperature and IGF1 during oocyte maturation. For all these experiments, COCs were subjected to control (38.5 °C for 22 hours) or heat shock (41 °C for 14 hours followed by 38.5 °C for 8 hours) in the presence of 0 or 100 ng/mL IGF1 during IVM. For experiment 8, COCs were subjected to heat shock (41 °C for 14 hours followed by 38.5 °C for 8 hours) in the presence of 0 or 25 ng/mL IGF1 and control (38.5 °C for 22 hours) treatments during IVM.

2.3.1. Experiment 1: the effect of IGF1 on heat shock–induced DNA fragmentation during oocyte maturation

The objective of this study was to determine the effect of IGF1 and heat shock in the induction of apoptosis in bovine oocytes. After 22- to 24-hour maturation, COCs were denuded by repeated pipetting and fixed in 4% (vol/vol) paraformaldehyde for TUNEL analysis. This experiment was replicated four times using 95 to 105 oocytes/treatment.

2.3.2. Experiment 2: the effect of IGF1 on heat shock–induced caspase activity during oocyte maturation

The objective of this study was to determine the effect of IGF1 on heat shock–induced caspase activity during IVM. COCs were denuded by repeated pipetting and submitted to group II caspase activity assay immediately after the first 14-hour IVM. This experiment was replicated five times using 50 to 85 oocytes/treatment.

2.3.3. Experiment 3: the effect of IGF1 on heat shock–induced mitochondrial activity during oocyte maturation

The objective of this study was to determine the effect of IGF1 on heat shock–induced mitochondrial activity during oocyte maturation. After 22- to 24-hour maturation, COCs were denuded by repeated pipetting and submitted to MitoTracker Red. This experiment was replicated six times using 97 to 204 oocytes/treatment.

2.3.4. Experiments 4 and 5: the effect of IGF1 on heat shock-induced cytoskeletal changes during oocyte maturation

The objectives of these studies were to determine the effect of IGF1 on heat shock–induced microfilament (experiment 4) and microtubule (experiment 5) organization during oocyte maturation. After 22- to 24-hour maturation, COCs were denuded by repeated pipetting and fixed in 3.7% formaldehyde for microfilament (N = 5 replicates using 30–43 oocytes/treatment) and microtubule (N = 5 replicates using 44–67 oocytes/treatment) localization.

2.3.5. Experiment 6: the effect of IGF1 on nuclear maturation of heat-shocked oocytes

The objective of this study was to determine the effect of IGF1 on the proportion of heat-shocked oocytes that reached MII stage during IVM. After 22- to 24-hour maturation, COCs were denuded by repeated pipetting and incubated with Hoechst 33342. Oocytes from experiments 1 and 5 stained with Hoechst 33342 were pooled to determine meiotic progression. This experiment was replicated nine times using 160 to 194 oocytes/treatment.

2.3.6. Experiment 7: the effect of IGF1 on developmental competence of bovine oocytes exposed to heat shock during oocyte maturation

The objective of this study was to determine the effect of IGF1 on developmental competence of bovine oocytes exposed to heat shock during IVM. After 22- to 24-hour maturation, oocytes were subjected to IVF and culture. Cleavage rate was assessed at Day 3 and development to the blastocyst stage at days 7 to 9 post-insemination. This experiment was replicated seven times using 266 to 280 oocytes/treatment. Blastocysts from five replicates (19–40 blastocyst/treatment) were harvested at Day 9 and fixed in 4% paraformaldehyde for TUNEL analysis and determination of total cell number (Hoechst 33342).

2.3.7. Experiment 8: the thermoprotective effect of low IGF1 concentration on developmental competence heat-shocked oocytes

The objective of this study was to determine whether a low IGF1 concentration (25 ng/mL IGF1) could have a

thermoprotective effect on developmental competence of bovine oocytes exposed to heat shock during IVM. COCs were subjected to heat shock (41 °C for 14 hours followed by 38.5 °C for 8 hours) in the presence of 0 or 25 ng/mL IGF1 and control (38.5 °C for 22 hours) treatments during IVM. After 22 to 24 hours maturation, oocytes were subjected to IVF and *in vitro* culture. Cleavage rate was assessed at Day 3 and development to the blastocyst stage at Days 7 to 9 post-insemination. This experiment was replicated three times using 99 to 115 oocytes/treatment.
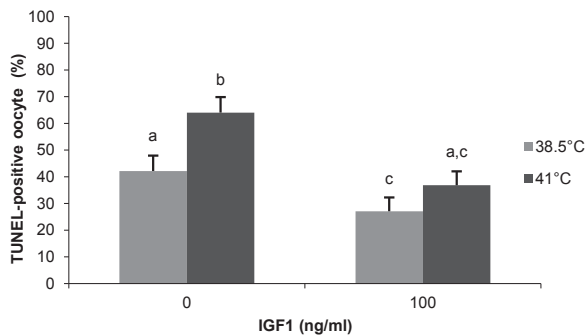
2.4. Statistical analysis

ANOVA assumptions (normally distributed data and homogeneity of variance) were initially determined by SAS [39]. Parametric data were analyzed by least-squares ANOVA using general linear model and mixed-model procedures of SAS [39]. Dependent variables were percentage of TUNEL-positive oocytes, percentage of oocytes that cleaved, blastocyst total cell number, cortical actin, and percentage of cleaved embryos that reached the blastocyst stage. Independent variables were temperature, IGF1, and replicates. The statistical model considered all the main effects and all possible interactions. PDIFF procedure of SAS was used to establish significant comparisons among means. Nonparametric data (TUNEL-positive blastomeres, percentage of MII oocytes, microtubule PI, oocytes with high caspase activity, mitochondrial activity, and percentage of oocytes that reached the blastocyst stage) were analyzed by Wilcoxon test. Data were shown as least-squares means ± SEM.

3. Results

3.1. The effect of IGF1 on heat shock–induced DNA fragmentation during oocyte maturation

Exposure of bovine oocytes to heat shock during the first 14-hour IVM (41 °C, 0 ng/mL IGF1) increased ($P < 0.01$) the proportion of TUNEL-positive oocytes compared with control (38.5 °C, 0 ng/mL IGF1; Fig. 1). However, addition of 100 ng/

Table 1
The lack of temperature and the effect of IGF1 on the percentage of oocytes with high caspase activity.[a]

Temperature (°C)	IGF1 (ng/mL)	High caspase activity oocytes (%)
38.5	0	21.6 ± 17.5
38.5	100	10.3 ± 15.1
41	0	29.1 ± 17.5
41	100	31.7 ± 15.1

Abbreviation: IGF1, insulin-like growth factor 1.
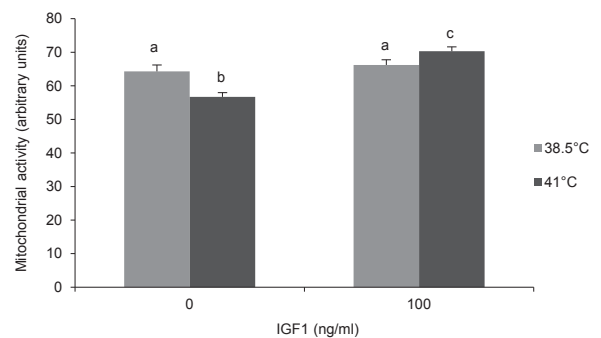[a] Data represent least-squares means ± SEM.

mL IGF1 during oocyte maturation reduced (IGF1 $P < 0.01$) the percentage of heat-shocked TUNEL-positive oocytes (41 °C, 100 ng/mL IGF1) compared with heat-shocked oocytes cultured without IGF1 (41 °C, 0 ng/mL IGF1). Moreover, the percentage of TUNEL-positive oocytes was similar between control and heat-shocked oocytes matured in 100 ng/mL IGF1 (Fig. 1). There was a temperature × IGF1 ($P = 0.07$) effect on the percentage of TUNEL-positive oocyte.

### 3.2. The effect of IGF1 on heat shock–induced caspase activity during oocyte maturation

This experiment determined the effect of temperature (38.5 °C and 41 °C) and IGF1 (0 and 100 ng/mL) on bovine oocyte caspase activity. Oocytes were evaluated with Phi-PhiLux-G_1D_2 immediately after the end of heat shock to determine caspase activity. Oocyte PI was divided into two categories according with mean PI: high ($PI > 23.071$) and low ($PI < 23.071$) pixel intensities. There was no effect of temperature or IGF1 on the percentage of oocyte with high caspase activity (Table 1).

3.3. The effect of IGF1 on heat shock–induced mitochondrial activity during oocyte maturation

Exposure of bovine oocytes to heat shock during the first 14-hour IVM (41 °C, 0 ng/mL IGF1) reduced mitochondrial activity ($P < 0.001$) compared with control (38.5 °C, 0 ng/mL IGF1; Fig. 2). However, IGF1 prevented

Fig. 1. Effect of IGF1 on heat shock–induced apoptosis in bovine oocytes. Results are least-squares means ± SEM of four replicates using 95 to 105 oocytes/treatment. There was a temperature ($P < 0.01$), IGF1 ($P < 0.05$), and temperature × IGF1 ($P = 0.07$) effect on the percentage of TUNEL-positive oocyte. Different letters in each bar represents significant difference ($P < 0.05$). IGF1, insulin-like growth factor 1; TUNEL, terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling.

Fig. 2. IGF1 reduced the deleterious effect of heat shock on bovine oocyte mitochondrial activity. Results are least-squares means ± SEM of six replicates using 97 to 204 oocytes/treatment. There was a temperature ($P < 0.01$) and temperature × IGF1 ($P < 0.01$) effect on oocyte mitochondrial activity. Different letters in each bar represents significant difference ($P < 0.01$). IGF1, insulin-like growth factor 1.
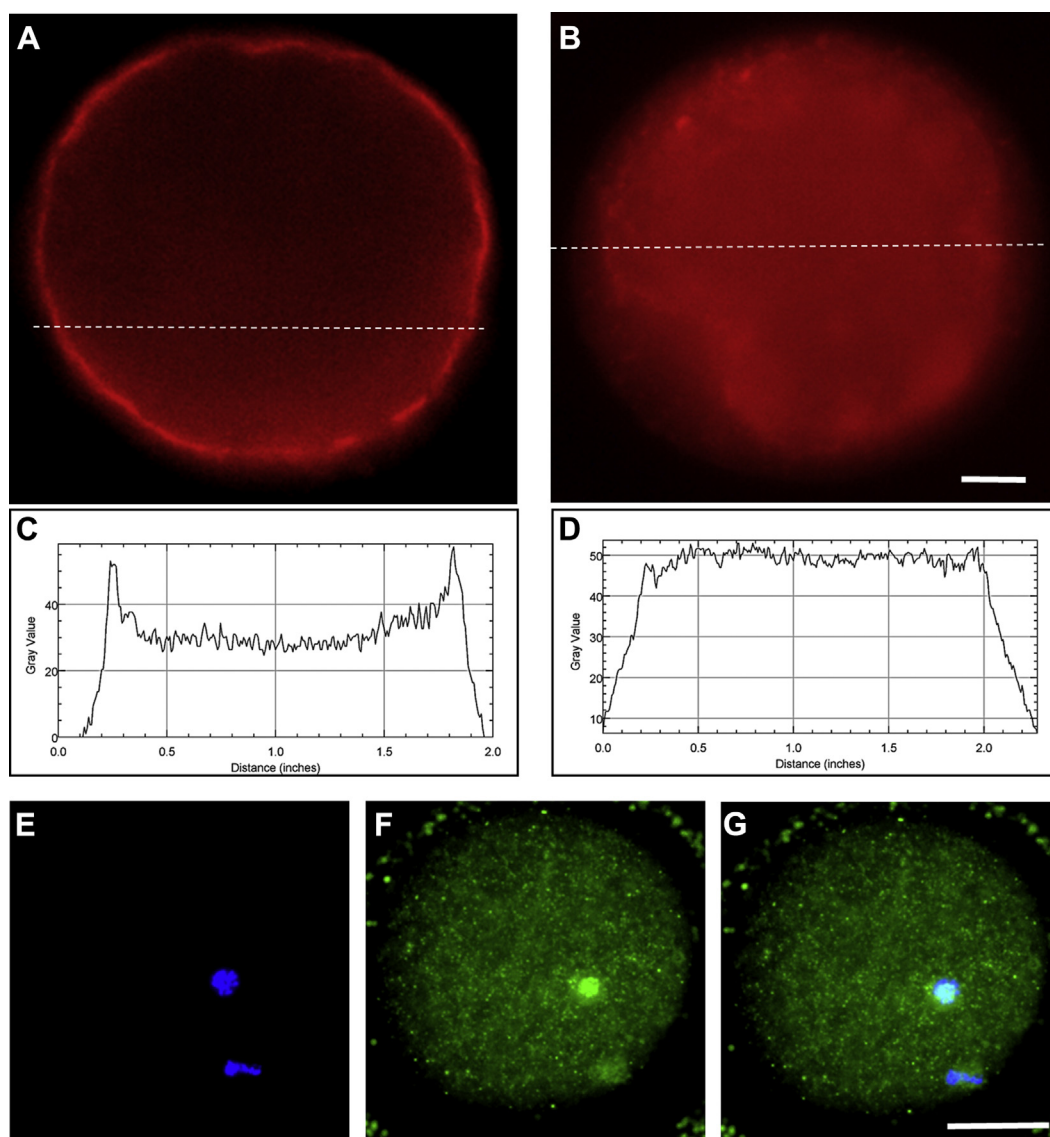
Fig. 3. Representative digital images illustrating microfilament (F-actin) and microtubule (α-tubulin) localization in bovine oocytes. Panels (A) and (B) illustrate control (A) and heat-shocked oocyte (B) microfilament organization, whereas panels (C) and (D) illustrate control (C) and heat-shocked oocyte (D) microfilament fluorescence intensity profile. Panels (E), (F), and (G) illustrate DNA (Hoechst 33342), tubulin organization, and merged image, respectively. The white bar represents the range of 20 μm.

this effect of temperature (temperature × IGF1 P < 0.001). Mitochondrial activity of heat-shocked oocytes was enhanced (P < 0.001) with 100 ng/mL IGF1 compared with all the other treatments (Fig. 2).

3.4. The effect of IGF1 on heat shock–induced cytoskeletal changes during oocyte maturation

Representative digital images of bovine oocytes submitted to microfilament localization are shown on Figure 3. Oocytes matured at 38.5 °C showed an intense actin ring in the cortical region (Fig. 3A). In contrast, heat-shocked oocytes had a more homogenous cytoplasmic actin distribution (Fig. 3B). Representative cortical actin fluorescence intensity profile is reported in Figure 3C, D. Exposure of

bovine oocytes to heat shock during the first 14-hour IVM (41 °C, 0 ng/mL IGF1) reduced (P < 0.001) cortical actin organization compared with control (38.5 °C, 0 ng/mL IGF1; Fig. 4A). However, IGF1 minimized this effect of temperature (temperature × IGF1, P = 0.06). Although cortical actin fluorescence intensity was similar between heat-shocked oocytes regardless of IGF1, control oocytes matured with IGF1 (38.5 °C, 100 ng/mL IGF1) was not different from heat-shocked oocytes matured with IGF1 (41 °C, 100 ng/mL IGF1).

Tubulin fluorescence was located adjacent to meiotic spindle and polar body DNA. Representative digital images of bovine oocytes submitted to tubulin localization are shown in Figure 3F, G. Tubulin fluorescence intensity was quantified at the metaphase plate and normalized by total
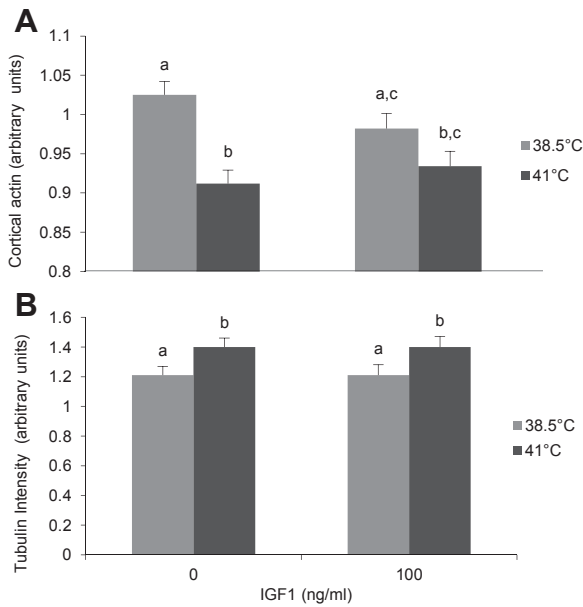
Fig. 4. Effect of IGF1 on cytoskeleton organization of heat-shocked bovine oocytes. Results are least-squares means ± SEM. (A) Cortical actin fluorescence intensity (n = 5 replicates using 30–43 oocytes/treatment) was affected by temperature (P < 0.001) and temperature × IGF1 (P = 0.06). (B) Microtubule fluorescence intensity (n = 5 replicates using 44–67 oocytes/treatment) was affected by temperature (P < 0.01). Different letters in each bar represents significant difference (P < 0.05). IGF1, insulin-like growth factor 1.

oocyte tubulin fluorescence. Polar body was analyzed separately. MII plate oocyte tubulin fluorescence intensity was stronger (P < 0.01) in heat-shocked oocytes compared with control regardless of IGF1 (Fig. 4B). There was also no effect of temperature or IGF1 on polar body tubulin fluorescence intensity.

3.5. The effect of IGF1 on nuclear maturation of heat-shocked oocytes

Heat shock reduced (P < 0.01) the percentage of bovine oocytes at MII stage. The percentage of heat-shocked oocytes treated with 100 ng/mL IGF1 that reached MII stage
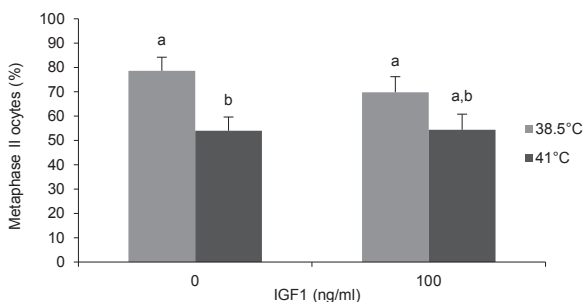
Fig. 5. Effect of IGF1 and heat shock on the percentage of oocytes that reached the metaphase II stage. Results are least-squares means ± SEM of nine replicates using 160 to 194 oocytes/treatment. There was a temperature effect (P < 0.05). Different letters in each bar represents significant difference (P < 0.01). IGF1, insulin-like growth factor 1.

was similar to all other treatments. Moreover, there was no effect of IGF1 on control oocytes (Fig. 5).

3.6. The effect of IGF1 on developmental competence of bovine oocytes exposed to heat shock during oocyte maturation

There was no effect of heat shock and IGF1 on cleavage rate (Fig. 6A). However, heat shock during IVM reduced the proportion of oocytes that reached the blastocyst stage at Day 9 post-insemination (P < 0.05; Fig. 6B), reduced blastocyst total cell number (P < 0.001; Fig. 7A), and increased the proportion of TUNEL-positive blastomeres (P < 0.01; Fig. 7B). Representative digital images of blastocysts stained with Hoechst 33342 and TUNEL are shown in Figure 8.

The percentages of Day 9 blastocyst (Fig. 6B) and TUNEL-positive blastomeres (Fig. 7B) were similar between control and heat-shocked oocytes matured in 100 ng/mL IGF1. There was no effect of IGF1 on total blastocyst cell number in heat-shocked oocytes (Fig. 7A). Addition of 100 ng/mL IGF1 during oocyte maturation at 38.5 °C did not affect percentage of blastocyst (Fig. 6B) or blastocyst total cell number (Fig. 7A).

3.7. The thermoprotective effect of low IGF1 concentration on developmental competence heat-shocked oocytes

Exposure of bovine oocytes to heat shock reduced cleaved rate (P < 0.05) and the percentage of oocytes/cleaved embryos (P < 0.001) that developed to the blastocyst stage (Table 2). However, addition of 25 ng/mL IGF1
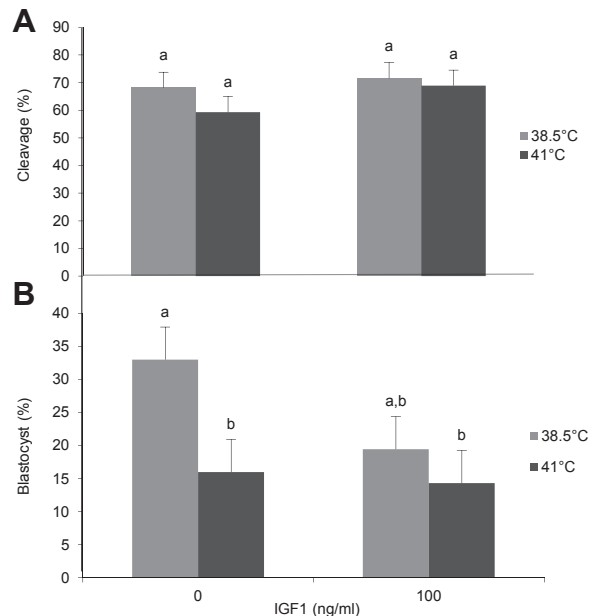
Fig. 6. Effect of IGF1 and heat shock on the percentage of oocytes that cleaved (A) and developed to the blastocyst stage (B). Results are least-squares means ± SEM of seven replicates using 266 to 280 oocytes/treatment. There was a temperature effect (P < 0.05). Different letters in each bar represents significant difference (P < 0.05). IGF1, insulin-like growth factor 1.
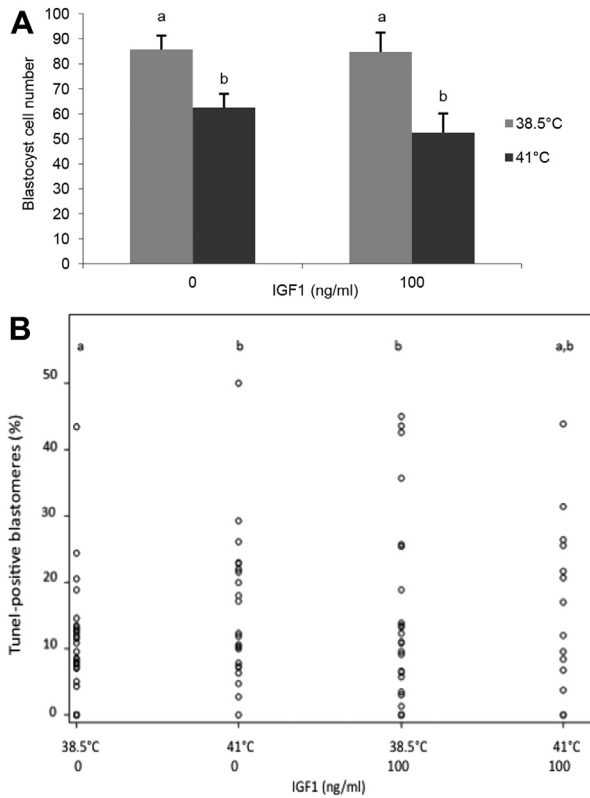
Fig. 7. Effect of IGF1 and heat shock on total blastocyst cell number (A) and the percentage of TUNEL-positive blastomeres (B). Results are least-squares means ± SEM of five replicates using 19 to 40 oocytes/treatment. There was a temperature effect (P < 0.05). Different letters in each bar represents significant difference (P < 0.05). IGF1, insulin-like growth factor 1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

had a protective effect. This low IGF1 concentration prevented the negative effect of temperature on the percentage of oocytes and cleaved embryos that reached the blastocyst stage.

4. Discussion

The present study demonstrated the beneficial effect of IGF1 preventing the deleterious effect of heat shock on oocyte function during oocyte maturation. For example, IGF1 reduced the magnitude of heat shock–induced damage on oocyte mitochondrial activity, cell death, and developmental competence.

Oocyte mitochondrial activity was reduced after exposure to 41 °C heat shock during the first 14-hour IVM. Previous studies have also reported that oocyte mitochondrial membrane potential (MMP) was reduced by heat shock during 21-hour IVM [40] and a 20-hour heat shock scheme mimicking the temperature fluctuation of lactating dairy cows (39.5 °C for 5 hours, 40 °C for 5 hours, 40.5 °C for 6 hours, and 40 °C for 4 hours; total 20 hours) [41]. In contrast, *in vivo* seasonal heat stress did not affect oocyte MMP but altered oocyte mitochondrial distribution [42].

Heat shock–induced reduction on oocyte mitochondrial activity indicated that high temperature directly compromised mitochondrial function and/or triggered activation of the mitochondrial apoptotic pathway. Indeed, heat shock increased the proportion of TUNEL-positive oocytes, suggesting that mitochondrial damage triggered the apoptotic cascade. Stress-induced alteration of anti-apoptotic (Bcl2 and BclxL) and pro-apoptotic (Bax, Bak, Bad, Bid, and Bim) proteins regulate external mitochondrial membrane integrity [43,44] releasing small pro-apoptotic molecules [45,46] to the cytosol followed by caspase-dependent or caspase-independent apoptosis cascade [47]. Previous studies found that anti-apoptotic peptides Bcl2 homology domain 4 of BclxL (TAT-BH4) and Bax inhibitor peptide (BIP) suppressed heat shock–induced increase on chromatin damage but did not restore oocyte MMP [40], suggesting that anti-apoptotic peptides may act downstream to mitochondrial damage.

Although heat shock increased oocyte DNA fragmentation, there was no effect on group II caspase activity (-3, -2, and -7). In contrast, heat shock–induced caspase activity and DNA fragmentation have been reported in bovine oocytes exposed to 12-hour shock during IVM [12]. Although in the present study caspase activity was evaluated 14 hours after the beginning of heat shock, in the study conducted by Roth and Hansen [12] caspase activity was evaluated 22 hours after the beginning of heat shock. Therefore, it is possible that the 14-hour interval was not long enough to induce a caspase activity peak.

Heat shock also compromised cytoskeleton organization. Heat-shocked oocytes presented a homogenous microfilament pattern throughout the oocyte cytoplasm, whereas oocytes matured at 38.5 °C presented a clear actin ring in the cortical region. Roth and Hansen [15] reported that control oocytes had an intense actin ring under the plasma membrane, whereas heat shock lead to a more distinct cytoplasmic distribution. Ju and Tseng [14] also found that cortical microfilament fluorescence intensity was greater in control oocytes compared with oocytes exposed to 2 or 4 hours of heat shock. The mechanism by which heat shock compromises cortical actin distribution is unknown. However, heat shock–induced intracellular calcium increase in oocytes [48] could promote gelsolin activation leading to microfilament breakdown and disorganization as observed in the present study. Activated gelsolin can laterally bind to G-actin monomers leading to filament breakdown or can bind to the actin filament–positive end blocking elongation of a new fragment [49].

In the present study, exposure of bovine oocytes to 41 °C heat shock during 14-hour IVM increased tubulin fluorescence intensity at the metaphase II plate. It is possible that heat shock caused microtubule hyperpolarization. There is evidence that heat shock triggers a rapid increase in heat shock protein (HSP), such as HSP90 [50], leading to tubulin stabilization and polymerization. This event is mediated by HSP90 cochaperone (FKBP51) that increases tubulin polymerization [51]. It has also been shown that the effect of heat shock on porcine oocyte microtubule pattern varied according to the intensity of stress. For example, porcine oocyte tubulin fluorescence intensity was enhanced as the intensity of stress increased [14].
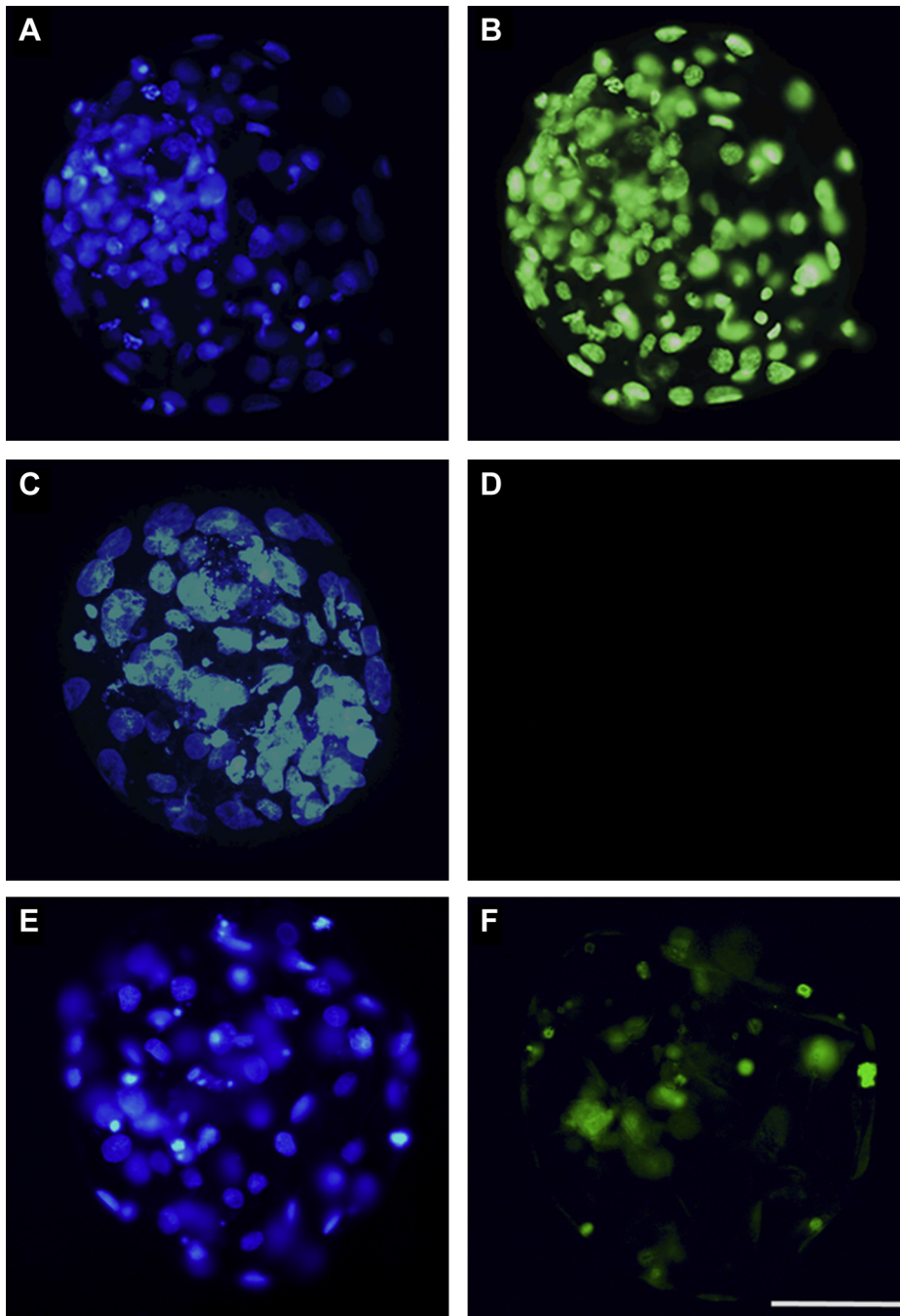
Fig. 8. Representative digital images illustrating positive control (A and B) blastocysts and blastocyst derived from control (C and D) and heat-shocked oocytes (E and F). Blastocysts were stained with Hoechst 33342 (A, C, and E) and TUNEL assay (B, D, and F). The white bar represents the range of 50 μm.

Microtubules and microfilaments are essential cytoskeletal structures for meiotic division because spindle modifications limit chromosomal segregation [52,53]. Heat shock–induced changes in cytoskeleton organization were followed by a reduction in the proportion of bovine oocytes that reached the MII stage. These oocytes remained at intermediate stages of meiosis (condensed nuclei, germinal vesicle, metaphase I, anaphase I, and telophase I, data not shown), indicating that heat shock during IVM delayed or blocked meiotic progression. Another study found that heat-shocked oocytes had abnormal spindles leading to a decrease in MII rate. However, heat-shocked oocytes that reached MII had a morphology similar to control oocytes where the meiotic spindle were well organized with defined structures and with chromosomes aligned along the midline [15]. There is

Table 2
Effect of IGF1 on developmental competence of heat-shocked bovine oocytes.[d]

Temperature (°C)	IGF1 (ng/mL)	Cleavage (%)	Blastocyst per oocytes (%)	Blastocyst per cleaved embryos (%)
38.5	0	83.3 ± 3.4[a]	20.5 ± 1.1[a]	24.4 ± 1.1[a]
41	0	62.5 ± 3.4[b]	7.9 ± 1.1[b]	12.7 ± 1.1[b]
41	25	69.2 ± 3.4[b]	15.3 ± 1.1[c]	22.9 ± 1.1[a]

Different letters (a, b, and c) in the same column represents significant difference (P < 0.05).
Abbreviation: IGF1, insulin-like growth factor 1.
[d] Data represent least-squares means ± SEM.

evidence that elevated temperature can cause a diversity of direct or indirect alterations on oocyte meiotic progression. Exposure to *in vivo* heat stress affected meiotic spindle morphology [54] and caused abnormal chromatin condensation [16] in different species. Similarly, exposure of oocytes to heat shock during IVM reduced nuclear maturation and lead to chromosome aggregation or condensation [14,16].

Cellular oocyte alterations induced by heat shock culminated with a reduction in oocyte developmental competence as reflected by a decrease in the proportion of oocytes that reached the blastocyst stage and total blastocyst cell number and an increase in the proportion of TUNEL-positive blastomeres. This reduction in oocyte developmental potential may be associated with the decrease in mitochondrial activity also observed in heat-shocked oocytes. The amount of active mitochondria has been positively related to oocyte developmental potential to the blastocyst stage [55,56]. Previous studies reported that heat shock reduced blastocyst rate [12], increased in TUNEL-positive blastomeres, and reduced oocyte mitochondrial potential [40]. Moreover, the effect of heat shock on cleavage rate was controversial. Although in experiment 7 the heat-shock reduction in cleavage rate did not reach statistical significance, this effect was accentuated in experiment 8. Such variation has been previously reported *in vitro* [12,33], whereas *in vivo* studies reported that heat stress delayed initial cleavage divisions [57].

The present study reported the beneficial effect of IGF1 on oocyte survival after 14 hours of heat shock. Addition of IGF1 to maturation medium decreased heat shock–induced apoptosis in bovine oocytes as indicated by a reduction in the proportion of TUNEL-positive oocytes. The mechanisms by which IGF1 acts as a survival factor involves activation of PI3K/AKT pathway increasing Bcl2 levels and phosphorylation of the pro-apoptotic factor Bad [58]. However, the thermoprotective role of IGF1 has been controversial. Although 100 ng/mL IGF1 reduced the proportion of TUNEL-positive oocytes after 22 hours of heat shock [34], there is also evidence that IGF1 increased oocytes that were TUNEL-positive [33]. Other studies reported that 100 ng/mL IGF1 caused a slight reduction on oocyte caspase activity 24 hours after IVM [59] and reduced cumulus cells [60] and oocyte apoptosis [59]. In the present study, there was no effect of IGF1 on caspase activity, reinforcing the idea that the interval for caspase evaluation was too short.

IGF1 minimized the negative effect of heat shock on oocyte mitochondrial activity. There is evidence that IGF1 improved mitochondrial function through PI3K/Akt pathway reducing mitochondrial generation of reactive oxygen species [61]. Moreover, IGF1 can preserve MMP and maintain cytochrome c at the mitochondrial inter-membrane space reducing apoptosis in endothelial cells [62].

IGF1 partially reversed the deleterious effects of heat shock on cortical actin organization. Addition of IGF1 preserved part of the pericellular actin ring morphologic structure. Actin filament stabilization depends on proteins, such as tropomodulin, which attaches to the actin filament negative end [63]. IGF1 binding to the receptor causes phosphorylation of intracellular subtracts like insulin receptor substrates 1 to 4 [64]. This activates kinase p70[S6], protein kinase B, PI3K, and pyruvate dehydrogenase kinase that control transcription, metabolism, apoptosis, translation, and cellular growth [65]. It is possible that IGF1 activated kinase p70[S6] enhancing tropomodulin production and actin filaments stability.

In the present study, IGF1 had only a partial effect minimizing the deleterious effects of heat shock on nuclear maturation. Similarly, it has been shown that heat-shocked oocytes treated with IGF1 had a slight increase on nuclear maturation [34]. Although meiotic progression is dependent of microtubule and microfilament interaction [66], there was no effect of IGF1 on microtubule organization of heat-shocked oocytes. One possibility is that the slight beneficial effect of IGF1 on microfilament organization contributed to meiotic progression in heat-shocked oocytes. Microfilaments are important during maturation and fertilization; they mediate peripheral nucleus migration, cortical spindle anchorage, homologous chromosome separation, cortex development/maintenance, polarity establishment, and first polar body emission during oocyte maturation and peripheral cortical granule migration [67].

Although addition of 100 ng/mL IGF1 to maturation medium prevented different cellular alterations induced by temperature, these modifications were not accompanied by an increase on oocyte developmental competence after heat shock. This lack of IGF1-positive effect on heat-shocked oocytes has been previously shown in experiments using 100 ng/mL IGF1 [33,34], suggesting this concentration may not be optimal for the oocyte. There is evidence that heat-stressed dairy cows respond to recombinant bovine somatotropin by increasing plasma IGF1 concentration to 100 ng/mL few days after treatment. However, such increased IGF1 concentration did not improve fertility in heat-stressed cows [31]. It has also been shown that supraphysiological concentration of IGF1 increased blastocyst cell death [68]. High IGF1 concentration causes IGF-1R complex internalization decreasing IGF-1R number [69,70]. Therefore, an experiment was conducted to determine whether a low IGF concentration would exert a thermoprotective effect in heat-shocked oocytes. In this study, 25 ng/mL IGF1 minimized the deleterious effect of heat shock on the ability of the oocyte and the cleaved embryo to form a blastocyst after fertilization. These results indicate that IGF1 concentration modulated

the effect of heat shock on oocyte developmental competence. Free IGF1 concentration found in the follicular fluid of preovulatory follicles is approximately 14.3 ng/mL [71], suggesting that 25 ng/mL IGF1 is a closer physiological model mimicking the oocyte microenvironment.

4.1. Conclusions

The bovine oocyte is susceptible to elevated temperature during oocyte maturation. Oocytes responded to heat shock by altering cytoskeletal organization, reducing meiotic progression to metaphase II and mitochondrial activity, triggering cell death, and compromising subsequent preimplantation embryonic development. *In vitro* studies indicated that IGF1 can modulate oocyte response to heat shock depending on the concentration. IGF1 exerted a thermoprotective effect preventing some of the deleterious effects of heat shock in bovine oocytes.

Acknowledgments

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