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IGF-I slightly improves nuclear maturation and cleavage rate of bovine oocytes exposed to acute heat shock *in vitro*

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Summary

An *in vitro* model of embryo production was used to examine the effects of insulin-like growth factor (IGF)-I on maturation and developmental competence of oocytes exposed to heat shock. Cumulus–oocyte complexes were matured at 38.5°C or exposed to acute heat shock (HS; 41.5°C), with or without 100 ng/ml IGF-I, for 22 h through *in vitro* maturation. The experimental groups were control (C), C + IGF-I, HS, and HS + IGF-I. Oocytes were fertilized at the end of maturation, and the proportion of cleaved embryos was recorded 44 h later. HS during maturation increased the proportion of TUNEL-positive oocytes ($P < 0.05$). HS did not have any effect on cortical granule translocation but impaired resumption of meiosis, expressed as a decreased proportion of oocytes with nuclei in metaphase I ($P < 0.05$) and metaphase II (MII; $P < 0.05$). HS decreased the proportion of oocytes that cleaved ($P < 0.05$), in particular those oocytes that further developed to 4-cell-stage embryos ($P < 0.05$). IGF-I alleviated, to some extent, the deleterious effects of HS on the oocytes as reflected by a reduced proportion of TUNEL-positive oocytes ($P < 0.03$). While not significant, IGF-I tended to increase the proportion of MII-stage oocytes ($P < 0.08$) and 4-cell-stage cleaved embryos ($P < 0.06$). Further examination is required to explore whether IGF-I also affects the developmental competence of oocytes exposed to HS.

Keywords: Bovine, Cleavage rate, Heat shock, IGF-I, Oocyte maturation

Introduction

In vitro and *in vivo* studies have indicated that both germinal vesicle (GV)- and metaphase II (MII)-stage oocytes are highly sensitive to elevated temperature. Exposure of bovine to elevated temperatures can potentially impair the ovarian pool of GV-stage oocytes, leading to oocytes with reduced competence to undergo maturation, fertilization and development to blastocysts (de S. Torres-Júnior *et al.*, 2008; Roth, 2008). Heat stress also compromises oocyte maturation, exposing lactating cows to heat stress between estrus and insemination (i.e. the estimated time of oocyte maturation *in vivo*) increased the proportion of abnormal embryos (Puteny *et al.*, 1988). *In vitro* studies showed that heat shock (HS) impairs intracellular events and is associated with alterations in both nuclear and cytoplasmic maturation, such as translocation of cortical granules to the oolemma (Payton *et al.*, 2004), cytoskeletal rearrangement (Roth & Hansen, 2007) and spindle formation (Ju *et al.*, 2005). In addition, exposure of bovine oocytes to HS increases the proportion of oocytes that undergo apoptosis (Roth & Hansen, 2004a,b), and that show impaired mitochondrial activity (Paula-Lopes *et al.*, 2013). Given that a multifactorial mechanism is involved, development of a new method to reduce the negative effects of HS on oocyte competence and to improve bovine reproductive performance in hot weather is a big challenge.

Insulin-like growth factor (IGF)-I plays multiple roles in cellular metabolism, proliferation, growth and differentiation. Exposure of dairy cows to heat stress

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decreased plasma IGF-I concentration in association with reduced oocyte quality (de Rensis & Scaramuzzi, 2003). Previous studies have suggested that IGF-I is a survival factor for preimplantation bovine embryos exposed to HS (Jousan *et al.*, 2008) as it can block HS-induced apoptosis and reduced blastocyst development (Jousan & Hansen, 2007). It is therefore possible that nutritional or hormonal administration to increase IGF-I might mitigate the deleterious effect of HS on fertility. IGF-I receptors are expressed in ovarian follicles and immature oocytes) Lorenzo *et al.*, 1994; Yoshida *et al.*, 1998; Guler *et al.*, 2000; Lonergan *et al.*, 2000), enabling direct effects on the oocyte or indirect effects via the surrounding cumulus cells. Under normothermal conditions, IGF-I has been shown to have an anti-apoptotic effect, as maturation of oocytes with IGF-I reduces apoptotic DNA degeneration (Wasielak & Bogacki, 2007). In addition, IGF-I seems to have a long-lasting effect on developing embryos as culture of preantral follicles with IGF-I subsequently improved embryonic development in mice (Demeestere *et al.*, 2004). Nevertheless, other studies have reported that IGF-I does not affect oocyte maturation in sheep (Gomez *et al.*, 1993), cattle (Rieger *et al.*, 1998) or pigs (Reed *et al.*, 1993). Zhandi *et al.* (2009) reported that under normothermic conditions, addition of 100 ng/ml IGF-I to the maturation medium increases the proportion of oocytes that undergoes nuclear maturation and reduces the proportion of TUNEL-positive oocytes. In contrast, under HS, IGF-I has been found to have a negative effect since it increases the proportion of TUNEL-positive oocytes and decreases oocytes' developmental competence (Zhandi *et al.*, 2009). On the other hand, Paula-Lopes *et al.* (2013) reported recently that 100 ng/ml IGF-I reduces the detrimental effect of HS on mitochondrial activity and DNA fragmentation, but not on blastocyst formation. Taken together, the effects of IGF-I on developmental competence oocyte exposed to thermal stress and its mode of action remain unclear.

The present study examined the effects of IGF-I on both cytoplasmic and nuclear maturation of bovine oocytes exposed to HS, and its ability to improve the development of early-cleaved embryos. It was hypothesized that under extreme conditions IGF-I will act as survival factor. Accordingly, we induced an acute HS (41.5°C), hyperthermia that non-cooled lactating cows might develop through a part of the day during the summer. Those conditions were previously found in our system to induce apoptosis in oocytes without being absolutely lethal (Kalo & Roth, 2011). Given the relationship between early-cleaved embryos and developmental competence of *in vitro*-derived embryos (Dinnyés *et al.*, 1999; Lonergan *et al.*, 1999), we used the rate of the two first embryonic divisions (i.e., cleavage to 2-cell and 4-cell embryos) to estimate oocyte developmental competence. Considering that under stress, apoptosis has an essential role in oocyte function, we also examined if IGF-I acts as an anti-apoptotic factor in oocytes exposed to HS.

Materials and methods

Materials

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (Rehovot, Israel). The ovine pituitary extracts, Ovagen and follicle stimulating hormone (FSH), were from ICPbio (Auckland, New Zealand). Dulbecco's phosphate-buffered saline (PBS) and RQ1 RNase-free DNase I were from Promega (Madison, WI, USA). Paraformaldehyde (16%) was from Electron Microscopy Sciences (Hatfield, PA, USA). Essential amino acids and non-essential amino acids were from Life Technologies (Carlsbad, CA, USA). Fluoromount medium was from Diagnostic Biosystems (Pleasanton, CA, USA). The culture medium HEPES Tyrode's lactate (TL), sperm (SP)-TL and *in vitro* fertilization (IVF)-TL were prepared in our laboratory (Gendelman *et al.*, 2010): HEPES-TL was supplemented with 0.3% (w/v) bovine serum albumin (BSA), 0.2 mM sodium pyruvate, and 0.75 mg/ml gentamicin (HEPES-TALP); SP-TL was supplemented with 0.6% BSA, 1 mM sodium pyruvate, and 0.2 mg/ml gentamicin (SP-TALP); IVF-TL was supplemented with 0.6% essential fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.05 mg/ml gentamicin and 0.01 mg/ml heparin (IVF-TALP). Oocyte maturation medium (OMM) consisted of TCM-199 with Earle's salts supplemented with 10% (v/v) heat-inactivated fetal calf serum (Bio-Lab, Jerusalem, Israel), 0.2 mM sodium pyruvate, 50 μg/μl gentamicin, 1.32 μg/ml FSH and 2 μg/ml estradiol. Potassium simplex optimization medium (KSOM) was prepared in our laboratory (Gendelman *et al.*, 2010) and contained 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.8% (v/v) sodium lactate, 0.2 mM sodium pyruvate, 0.2 mM D(+)-glucose, 25 mM $NaHCO_3$, 1 mM L-glutamine, 0.01 mM ethylene diamine tetraacetic acid (EDTA) and 0.01 mM phenol red supplemented with 1.7 mM $CaCl_2 \cdot 2H_2O$, 0.1 mg/ml polyvinylalcohol, 10 μl/ml essential amino acids, 5 μl/ml non-essential amino acids, 100 U/ml penicillin-G and 0.1 mg/ml streptomycin. Primary stock of IGF-I (Cat. No. I1271) was dissolved in 10 mM HCl (1 mg/ml).

Experimental design

The study was performed in the winter to eliminate any previous effect of summer thermal stress. Oocytes

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were matured for 22 h in OMM with or without 100 ng/ml IGF-I at 38.5°C (control; C) or at 41.5°C (HS) in an atmosphere of 5% or 7% CO_2 in humidified air, respectively (the percentage of CO_2 was adjusted to maintain pH at 7.4). The experimental groups were: C, C + IGF-I, HS, and HS + IGF-I. After maturation, oocytes were fertilized and cultured for 2 days as described in section ***In vitro* production of embryos**. The percentages of oocytes that cleaved and developed to 2- and 4-cell-stage embryos (i.e., study end point) were recorded 44 h post-insemination. The ratio between 2-cell and 4-cell-stage embryos was calculated and further used to estimate embryonic developmental competence (Arav *et al.*, 2008).

A subgroup of oocytes was collected at the end of 22 h maturation, denuded of cumulus cells, fixed and subjected to terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), Hoechst 33342 or fluorescein isothiocyanate (FITC)–LCA (*Lens culinaris* agglutinin) labelling as described in sections **Fluorescein isothiocyanate (FITC)–*Lens culinaris* agglutinin (LCA) staining of cortical granules** and **Nuclear staining and TUNEL procedure**.

In vitro production of embryos

In vitro production of bovine embryos was performed as previously described by Gendelman *et al.* (2010). Ovaries were obtained from multiparous Holstein cows at the local abattoir and transported to the laboratory in physiological saline solution (0.9% w/v NaCl at 37°C with 50 μg/ml penicillin–streptomycin) within 60–90 min. Cumulus–oocyte complexes (COCs) were aspirated from 3–8-mm follicles, and groups of 10 oocytes were transferred to 50-μl droplets of OMM overlaid with mineral oil and incubated in humidified air with 5% CO_2 for 22 h at 38.5°C. At the end of maturation, COCs were fertilized with ~1 × 10^6 Percoll-purified spermatozoa from frozen–thawed semen for 18 h at 38.5°C in a humidified atmosphere with 5% CO_2. After fertilization, putative zygotes were removed from the fertilization wells, denuded of cumulus cells and randomly placed in groups of 10 in a 25-μl KSOM droplet overlaid with mineral oil for 44 h in an atmosphere of humidified air with 5% CO_2 and 5% O_2.

Fluorescein isothiocyanate (FITC)–*Lens culinaris* agglutinin (LCA) staining of cortical granules

Cortical granule distribution was examined according to Izadyar *et al.* (1998). Briefly, after maturation, oocytes were denuded of cumulus cells by gentle vortexing in HEPES-TALP containing 1000 U/ml hyaluronidase. Then the oocytes were treated with 0.4% (w/v) pronase E to remove the zona pellucida, washed three times in PBS with 1 mg/ml polyvinylpyrrolidone (PBS–PVP) and transferred to 4.0% (v/v) paraformaldehyde in PBS for 15 min at room temperature. Following fixation, cells were washed five times (30 min each) in blocking solution (PBS that contained 1.0% BSA, 0.1 M L-glycine and 0.05% v/v Triton X-100), and washed three times in PBS–PVP. Oocytes were then transferred to FITC conjugated to LCA (10 μg/ml) in PBS for 30 min in the dark at room temperature. Labelled oocytes were washed in PBS–PVP, incubated in Hoechst 33342 (1 μg/ml) for 15 min in the dark, washed another four times in PBS/PVP and then transferred to fluoromount media drops and evaluated under an epifluorescence microscope. The oocytes were classified into three types according to the observed distributional pattern of the cortical granules as defined by Izadyar *et al.* (1998): type I – large aggregates of cortical granules distributed over the entire cytoplasm; type II – cortical granules localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates; type III – cortical granules more or less evenly dispersed in the cortical cytoplasm aligned with the oolemma (Fig. 1*A*, *a–c*, respectively).

Nuclear staining and TUNEL procedure

After maturation, oocytes were denuded and fixed with 4% paraformaldehyde For TUNEL and Hoechst labelling as described previously (Kalo & Roth, 2011). Briefly, oocytes were transferred to a 50-μl drop of PBS–PVP and then permeabilized in 0.1% Triton X-100 containing 0.1% (w/v) sodium citrate for 30 min at room temperature. Oocytes were then washed and incubated in a 25-μl drop of Hoechst 33342 (1 μg/ml) for 15 min in the dark. Following another washing, oocytes were transferred to the fluoromount media drops and evaluated for Hoechst 33342 (blue) and TUNEL (green) labelling of nuclei under an epifluorescence microscope. The proportion of oocytes in each meiotic stage – GV, GV breakdown (GVBD), metaphase I (MI), anaphase I (Ana. I), telophase I (Telo. I) and MII (Fig. 2*A*, *a–f*, respectively), as well as the proportion of TUNEL-negative and -positive oocytes (Fig. 3*A*) were recorded.

Statistical analysis

Data were analyzed with JMP-7 software (Statistical Analysis System (SAS) Institute Inc., Cary, NC, USA). Overall comparison of treatments for incidence data was performed by chi-squared test followed by Pearson test. After significance was established, pairs of treatments were also compared by Fisher's exact test, isolating the relevant data. Variables were: oocyte distribution into cortical granule types (I–III), meiotic stages (GV, GVBD, MI, Ana. I, Telo. I, and MII) and apoptotic status (TUNEL-positive and

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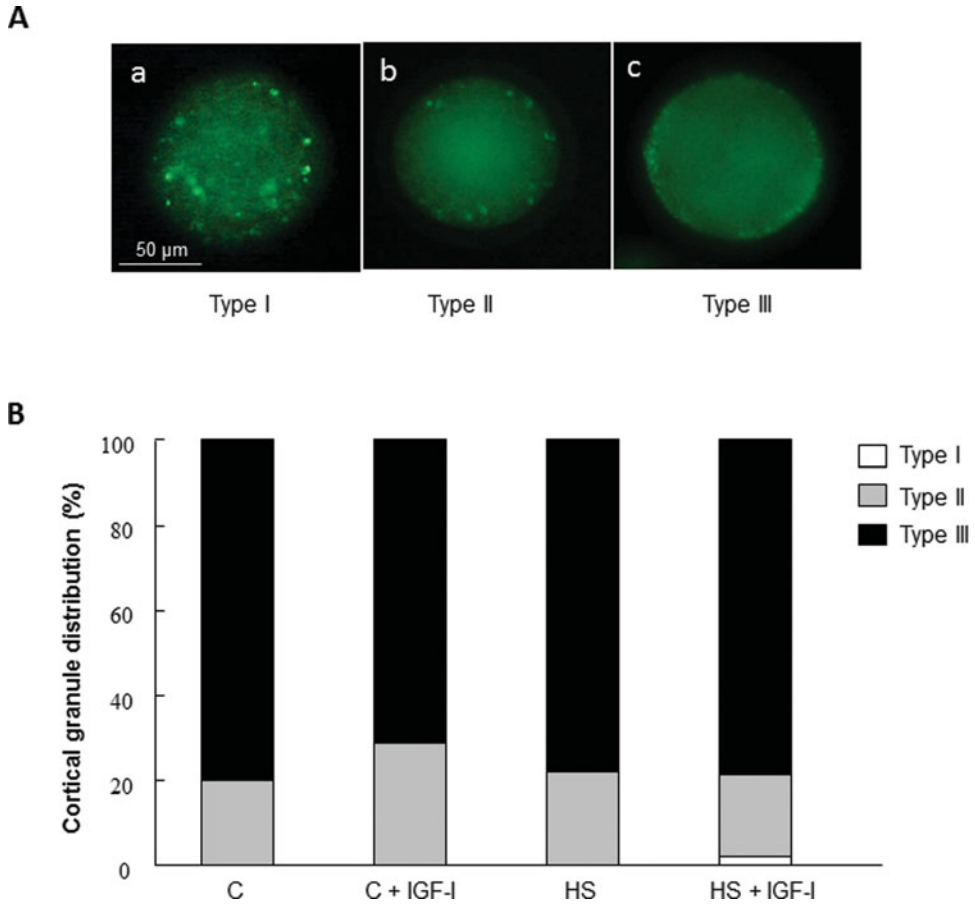

Figure 1 (*A*) Fluorescence microscopic images of typical cortical granules labelled with FITC–LCA. Type I (a), Type II (b) and Type III (c). (*B*) Distribution of cortical granule types (I, II and III) in control (C) and heat-shocked (HS) oocytes matured with or without 100 ng/ml insulin-like growth factor (IGF)-I. Data are presented as percentage of total oocytes.

TUNEL-negative oocytes). Data are presented as percentage of total oocytes.

To examine differences in the proportion of cleaved oocytes, 2-cell and 4-cell-stage embryos and the ratio between them, data were subjected to one-way analysis of variance (ANOVA) followed by Tukey–Kramer test. Before analysis, percentage data were arcsine-transformed. Data are presented as mean ± SEM. For all analyses, $P < 0.05$ was considered significant. *P*-values between 0.05 and 0.1 were also reported as trends that may be real and worthy of note.

Results

Cortical granule translocation in oocytes subjected to HS

Cytoplasmic maturation was evaluated by examining cortical granule distribution in immature oocytes immediately after their aspiration and at the end of maturation. The proportion of types I and II oocytes in the C group was high before maturation and low at the end of it ($P < 0.05$; Table 1), indicating that most oocytes in our system underwent cytoplasmic maturation. The opposite pattern was found for type III oocytes, with a low proportion before maturation and a high proportion after 22 h of maturation ($P < 0.05$; Table 1). Cortical granule migration was not impaired by HS, and IGF-I did not have any effect on cortical granule distribution (Fig. 1*B*).

Nuclear maturation in oocytes subjected to HS

Nuclear maturation was evaluated by meiotic status at the end of oocyte maturation. Findings revealed different distribution patterns between groups ($P < 0.0001$; Table 2). The percentage of MII-stage oocytes in the C groups was around 60, similar to the percentage that generally matures in our system. On the other hand, exposure of oocytes to HS deleteriously affected the nuclear maturation process, as reflected by a higher proportion of oocytes exhibiting MI-stage nuclei in

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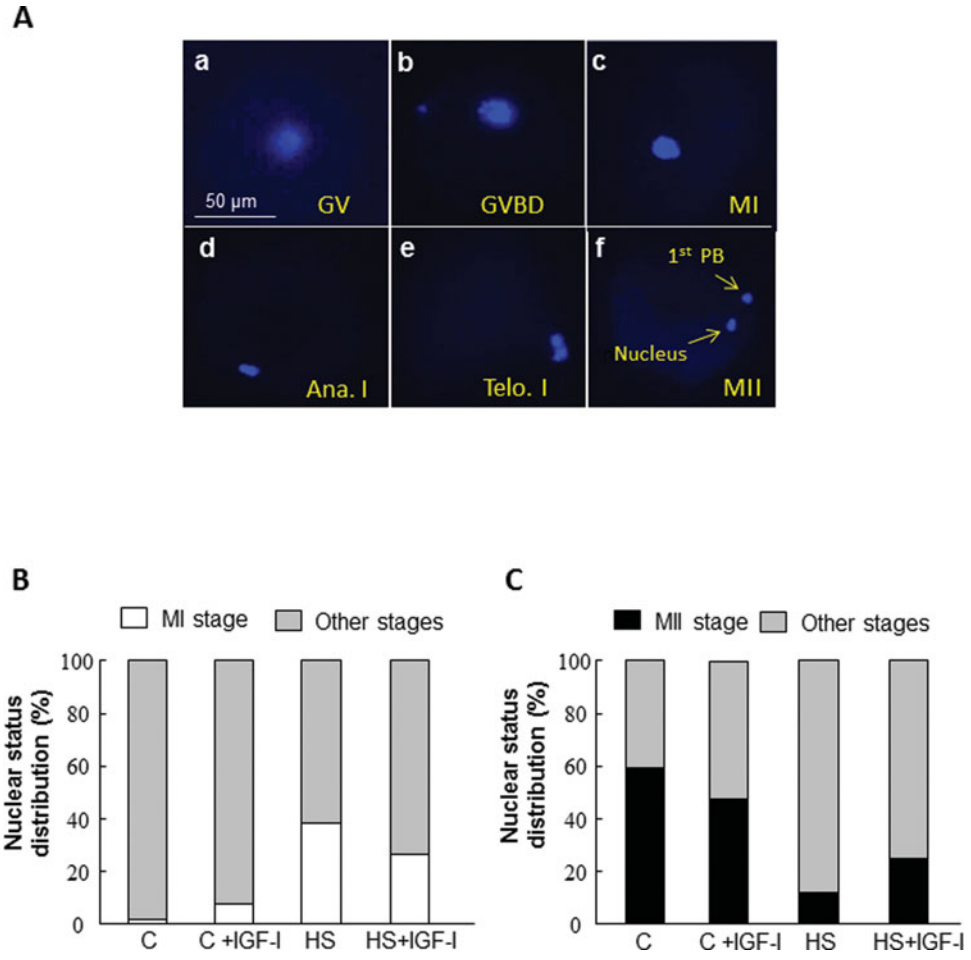

Figure 2 (*A*) Representative images of nuclear stages of bovine oocytes during maturation. Germinal vesicle (a, GV), germinal vesicle breakdown (b, GVBD), metaphase I (c, MI), anaphase I (d, Ana. I), telophase I (e, Telo. I), metaphase II (f, MII). PB, polar body. Proportion of oocytes at the MI stage (*B*) and MII stage (*C*) relative to the other nuclear stages in control (C) and heat-shocked (HS) oocytes matured with or without 100 ng/ml insulin-like growth factor (IGF)-I. Data are presented as percentage of total oocytes.

the HS group relative to the C and C + IGF-I groups ($P < 0.0002$, Fig. 2*B*). On the other hand, the proportion of MII-stage oocytes was lower in the HS group than in the C and C + IGF-I groups at the end of maturation ($P < 0.0001$, Fig. 2*C*).

IGF-I did not have any effect on the proportion of MI-stage oocytes in the C versus HS group ($P = 0.17$). Although not significant ($P = 0.08$, Fig. 2*C*), IGF-I increased to some extend the proportion of MII-stage oocytes in the HS group.

Apoptotic status in oocytes subjected to HS during maturation

The percentage of TUNEL-positive (i.e. apoptotic) oocytes was higher in the HS group than in the C group ($P < 0.05$, Fig. 3*B*). IGF-I did not affect the nuclei's apoptotic status when oocytes were matured at 38.5°C. However, it reduced ($P < 0.03$) the proportion of TUNEL-positive oocytes in the HS + IGF-I versus HS group, to a level similar to that of the C group.

Cleavage rate of oocytes subjected to HS during in vitro maturation

Exposure of oocytes to HS during maturation reduced the proportion of oocytes that cleaved 44 h post-fertilization relative to the C group ($P < 0.05$; Fig. 4*A*). Their distribution into 2-cell and 4-cell-stage embryos differed between groups, with a lower proportion at the 4-cell stage in the HS versus C group ($P < 0.05$, Fig. 4*B*). This situation was also reflected by a higher ratio of 2-cell to 4-cell-stage embryos in the HS versus C group, suggesting that HS during maturation further impairs the second embryonic division (Fig. 4*C*). IGF-I tended to increase the proportion of oocytes that cleaved 44 h post-fertilization (Fig. 4*A*); this tendency

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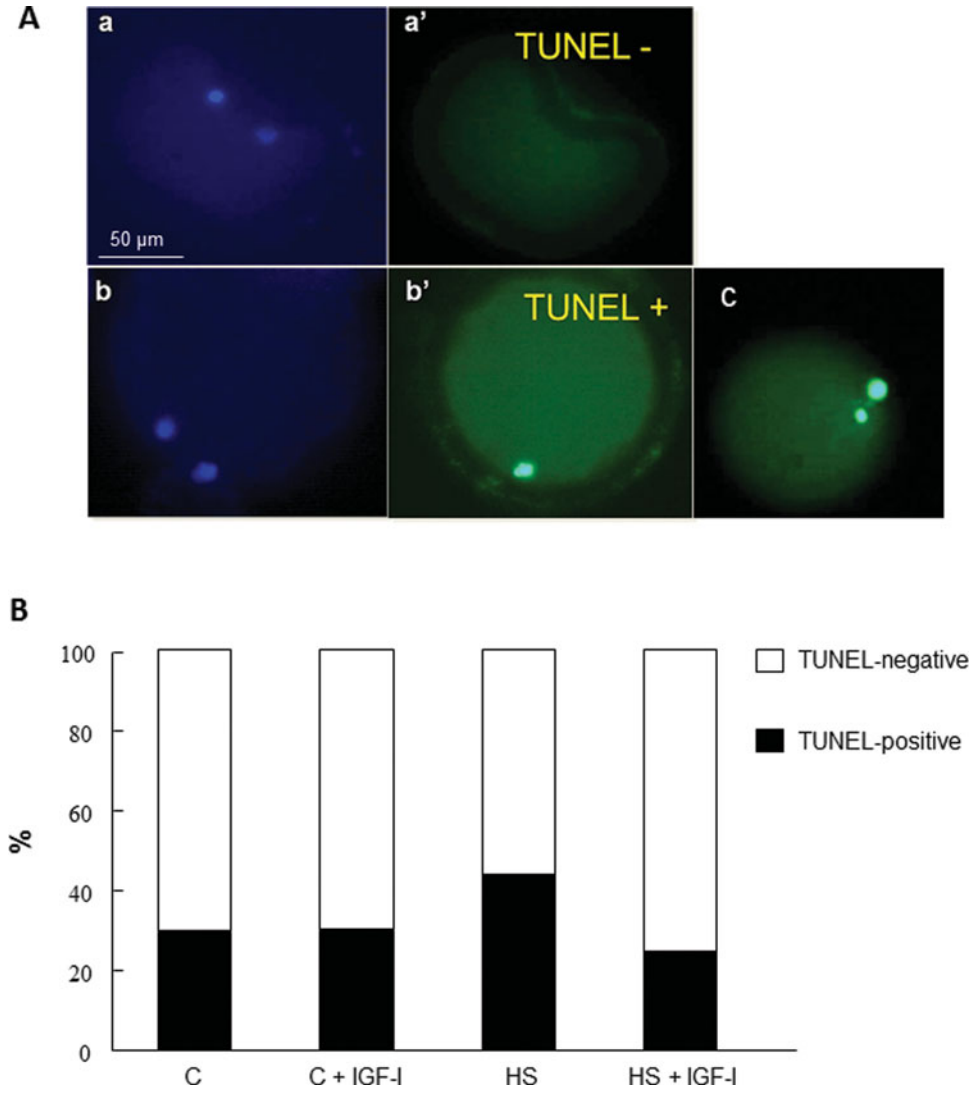

Figure 3 (*A*) Representative images of Hoechst-stained (a, b), and TUNEL-negative (a′) and TUNEL-positive (b′) oocytes. Panel Ab′ shows an oocyte with apoptotic MII-plate fluorescing green. (*B*) Proportion of TUNEL-negative and TUNEL-positive oocytes in control (C) and heat-shocked (HS) oocytes matured with or without 100 ng/ml insulin-like growth factor (IGF)-I. Data are presented as percentage of total oocytes.

was also expressed by a slight decrease in the ratio of 2-cell to 4-cell-stage embryos (Fig. 4C).

Discussion

In vivo and *in vitro* studies have shown that bovine oocytes are highly susceptible to elevated temperatures (Roth, 2008). In support of this fact, the findings of the current study indicated that exposure of bovine oocytes to HS during maturation impairs nuclear maturation in association with an increased proportion of TUNEL-positive oocytes and reduced cleavage rate. It should be noted, however, that whereas some studies have reported that HS affects oocyte cleavage (Roth & Hansen, 2004a,b; Kalo & Roth, 2011; current study), others have reported only a minimal or no effect (Edwards & Hansen, 1996; Dinnyés *et al.*, 1999; Edwards *et al.*, 2005; de Castro e Paula *et al.*, 2007). Nevertheless, further analysis revealed that HS impaired the ratio of 2-cell to 4-cell-stage embryos, with a prominent reduction in the proportion of 4-cell-stage embryos, suggesting impairment in the second embryonic division. Similar seasonal effects have been recently reported by Gendelman *et al.* (2010), with a lower cleavage rate and lower proportion of 4-cell-stage oocytes in the cold season relative to the hot season.

Time of cleavage and first mitotic division is considered indicative of embryo quality, in particular of whether the embryo is transferable or competent to survive cryopreservation (Hasler *et al.*, 1995; Shoukir

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Table 1 Distribution of cortical granule types (I, II and III) in bovine oocytes before and after maturation at 38.5ºC for 22 h

Treatment	Oocytes (*n*)	Cortical granule (%)		
		Type I	Type II	Type III
Before maturation	41	41.46 ± 9.76^{a}	56.09 ± 11.03^{a}	2.44 ± 2.69^{a}
After maturation	187	0.54 ± 0.55^{b}	22.46 ± 3.11^{b}	77.01 ± 3.49^{b}

Data are presented as mean ± standard error of the mean (SEM).
[a,b]Different superscript letters within a column represent significant differences at $P < 0.05$.

Table 2 Distribution of nuclear stages during maturation in control (C) and heat-shocked (HS) oocytes matured with or without 100 ng/ml insulin-like growth factor (IGF)-I

Treatment	Oocytes (*n*)	GVBD (%)	MI (%)	Ana. I (%)	Telo. I (%)	MII (%)
C	54	3.70 ± 2.21	1.85 ± 21.77	18.52 ± 5.99	16.67 ± 9.44	59.26 ± 13.10
C + IGF-I	53	1.89 ± 2.61	7.55 ± 10.43	13.21 ± 5.53	30.18 ± 4.78	47.17 ± 12.75
HS	50	0	37.99 ± 5.68	33.99 ± 11.59	15.99 ± 3.94	11.99 ± 3.39
HS + IGF-I	53	0	26.42 ± 6.92	33.96 ± 7.89	15.09 ± 13.35	24.53 ± 4.16

Model *P*-value < 0.05. Data are presented as mean ± standard error of the mean (SEM).

et al., 1997; Sakkas *et al.*, 1998; Van Montfoort *et al.*, 2004). A relationship between early-cleaved embryos and developmental competence has been documented for *in vitro*-derived bovine embryos (Dinnyés *et al.*, 1999; Lonergan *et al.*, 1999). Moreover, time of first cleavage has been related to the expression level of IGF-I (Lonergan *et al.*, 2000). Nevertheless, whereas HS-induced alterations in the two first divisions might explain, in part, the reduced developmental competence of HS oocytes (Roth & Hansen, 2004a,b, 2005), IGF-I did not counteract these effects, as only slight improvement in the ratio between 2-cell and 4-cell-stage embryos was found. To date, the protective effect of IGF-I reported for 5-day-old embryos exposed to HS (Jousan & Hansen, 2007) has not been clearly observed in bovine HS oocytes.

Paula-Lopes *et al.* (2013) have suggested that using an IGF-I concentration higher than the physiological level found in the follicular fluid obscures its protective effect. Maturation of heat-shocked oocytes with 100 ng/ml IGF-I, similar to that used in the current study, counteracted the deleterious effect of HS on oocyte mitochondrial function, but did not improve developmental competence. On the other hand, physiological doses similar to that in the preovulatory follicle (i.e. 12.5 ng/ml) can alleviate the effects of HS on both oocyte maturation and developmental competence. In support of this assumption, *in vitro* culture of preantral follicles with high doses (1000 ng/ml) of human recombinant IGF-I has a detrimental effect on bovine oocyte competence (Thomas *et al.*, 2007). Similarly, intraovarian administration of 1 μg IGF-I reduced oocyte developmental competence and embryo viability (Velazquez *et al.*, 2011). Taking together, the findings suggest the importance of IGF-1 administration at physiologic dose to achieve a positive response. Moreover, using non-physiological doses under HS conditions might even cause negative effects (Zhandi *et al.*, 2009).

Previous studies have reported the effects of HS on nuclear (Roth & Hansen, 2005) and cytoplasmic maturation (Payton *et al.*, 2004) in bovine oocytes. Most bovine oocytes exposed to temperatures of 40 or 41ºC during the first 12 h of *in vitro* maturation did not complete nuclear maturation, as reflected by the high proportion of oocytes arrested at the MI stage (Roth & Hansen, 2005). However, other studies have reported no effect (Edwards *et al.*, 2005; Zhandi *et al.*, 2009). In that respect, IGF-I has been shown to enhance nuclear maturation in oocytes surrounded by compact cumulus cells in both bovines (Lorenzo *et al.*, 1994) and humans (Gomez *et al.*, 1993). IGF-I is also known to stimulate maturation of *Xenopus* (Hainaut *et al.*, 1991), rabbit (Lorenzo *et al.*, 1996) and buffalo (Pawshe *et al.*, 1998) oocytes. Demeestere *et al.* (2004) reported reduced oocyte degeneration post-fertilization when mouse follicles were cultured and the oocytes were matured with IGF-I. Nevertheless, in the current study, whereas HS caused clear impairments in nuclear maturation, IGF-I had only a modest effect, reflected by a slight increase in the proportion of MII-stage oocytes.

Previous studies have shown that HS during maturation impairs cortical granule distribution (Payton *et al.*, 2004) and accelerates their translocation to the oolemma, suggesting oocyte ageing (Edwards *et al.*, 2005). The cortical granules migrate from the Golgi apparatus to the vitelline surface (Ducibella

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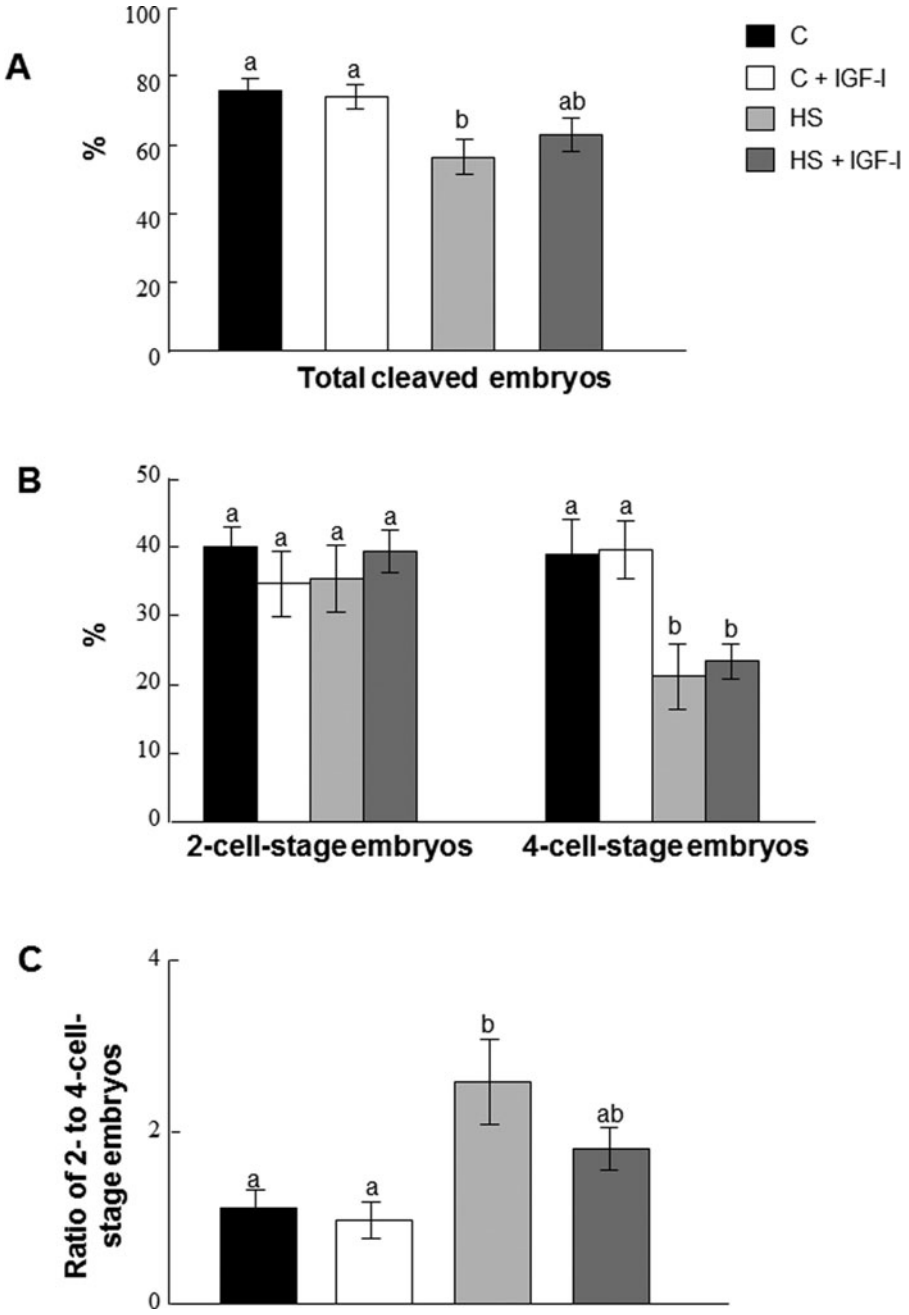

Figure 4 Oocytes were matured at 38.5°C (C) or 41.5°C (HS) for 22 h in oocyte maturation medium (OMM) with or without 100 ng/ml insulin-like growth factor (IGF)-I. Presented values are percentage of total cleaved oocytes (*A*), cleavage rates into 2-cell or 4-cell-stage embryos (*B*), and ratio of 2-cell to 4-cell-stage embryos (*C*) 44 h post-fertilization. Data are mean ± standard error (SE). [a,b]Columns headed by different letters differ at $P < 0.05$.

& Buetow, 1994). This translocation is an essential event for the success of fertilization processes such as oocyte membrane fusion and blockage of polyspermia (Damiani *et al.*, 1996; Carneiro *et al.*, 2002). In the current study, in contrast with its effects on nuclear maturation, HS did not have any effect on cytoplasmic maturation: both C and HS oocytes expressed the same cortical granule distribution and a similar proportion of type III oocytes at the end of maturation. Given that in the current study COCs were exposed to severe HS (41.5°C, 22 h) relative to that induced by Payton *et al.* (2004; 41°C, 12 h), one might expect a stronger effect of HS on cortical granule translocation. While not entirely clear, it is possible that the source of the oocytes or the culture medium, or both, rather than HS condition, underlies the differences between studies. It should be noted, however, that in the current study, IGF-I did not have any beneficial effect on cortical granule

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distribution under either thermoneutral conditions or HS.

IGF-I has been suggested as a survival factor for cells exposed to stress, and has been shown to block apoptosis in many cell types (Peruzzi *et al.*, 1999; Su *et al.*, 2003), including preimplantation embryos (Makarevich & Markkula, 2002; Fabian *et al.*, 2004) in particular those exposed to HS (Jousan & Hansen, 2007). In this respect, Wasielak and Bogacki (2007) reported that adding IGF-I to the maturation medium reduced apoptotic DNA degeneration after oocyte recovery, suggesting that IGF-I serve as an anti-apoptotic factor in oocytes. Heat-induced apoptosis is one of the mechanisms suggested to be involved in the reduced developmental competence of bovine oocytes exposed to elevated temperature (Roth & Hansen, 2004a,b; Ispada *et al.*, 2010). In the current study, we found a higher proportion of TUNEL-positive oocytes in the HS versus C group, and IGF-I decreased the proportion of TUNEL-positive oocytes to the level of the C group. In support of these findings, Ispada *et al.* (2010, 2011) reported that IGF-I reversed HS-induced apoptosis in bovine oocytes. In contrast, Zhandi *et al.* (2009) reported that IGF-I increased the proportion of TUNEL-positive oocytes when matured at 41°C but not at 38.5°C.

In summary, the present study indicated that exposure of bovine oocytes to HS impairs nuclear maturation and increases apoptosis. The findings support and extend our previous observations, in which exposure to HS reduced the cleavage rate of preimplantation embryos, in particular the second division to the 4-cell stage. It seems that IGF-I has an anti-apoptotic effect on HS oocytes, however it did not counteract the deleterious effects of HS on early divisions. The results are not clear cut, and further exploration of the effects of IGF-I on the developmental competence of oocytes exposed to HS is warranted.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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