

Research Article

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Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during *in vitro* maturation

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Summary

Heat shock may disrupt oocyte function by increasing the generation of reactive oxygen species (ROS). We evaluated the capacity of the antioxidant melatonin to protect oocytes using two models of oxidative stress – heat shock and the pro-oxidant menadione. Bovine cumulus–oocyte complexes (COC) were exposed in the presence or absence of 1 µM melatonin to the following treatments during maturation: 38.5°C, 41°C and 38.5°C + 5 µM menadione. In the first experiment, COC were matured for 3 h with 5 µM CellROX® and analyzed by epifluorescence microscopy to quantify production of ROS. The intensity of ROS was greater for oocytes exposed to heat shock and menadione than for control oocytes. Melatonin reduced ROS intensity for heat-shocked oocytes and oocytes exposed to menadione, but not for control oocytes. In the second experiment, COC were matured for 22 h. After maturation, oocytes were fertilized and the embryos cultured for 7.5 days. The proportion of oocytes that cleaved after fertilization was lower for oocytes exposed to heat shock and menadione than for control oocytes. Melatonin increased cleavage for heat-shocked oocytes and oocytes exposed to menadione, but not for control oocytes. Melatonin tended to increase the developmental competence of embryos from heat-shocked oocytes but not for embryos from oocytes exposed to menadione or from control oocytes. In conclusion, melatonin reduced production of ROS of maturing oocytes and protected oocytes from deleterious effects of both stresses on competence of the oocyte to cleave after coincubation with sperm. These results suggest that excessive production of ROS compromises oocyte function.

Introduction

Exposure of cows to thermal stress at oestrus can reduce oocyte competence for subsequent development after fertilization (Putney *et al.*, 1988). This action of heat stress may involve direct effects of elevated body temperature on the function of the maturing oocyte. Exposure of oocytes during maturation to elevated culture temperatures (i.e. heat shock) can disrupt mitochondrial function (Rodrigues *et al.*, 2016; Payton *et al.*, 2018) and reduce oocyte competence to complete nuclear maturation (Roth & Hansen, 2005; Nabenishi *et al.*, 2012; Cebrian-Serrano *et al.*, 2013; Meiyu *et al.*, 2015), be fertilized and undergo cleavage (Roth & Hansen, 2004a, 2005; de Castro e Paula & Hansen, 2007; Meiyu *et al.*, 2015). Moreover, the competence of the resultant embryos to develop to the blastocyst stage can be compromised (Roth *et al.*, 2004a, b; Nabenishi *et al.*, 2012; Rodrigues *et al.*, 2016).

Heat shock may disrupt oocyte function, at least in part, by increasing the generation of reactive oxygen species (ROS). Production of ROS is increased by heat shock (Nabenishi *et al.*, 2012; Ispada *et al.*, 2018) and antioxidants such as retinol (Lawrence *et al.*, 2004), cysteine (Nabenishi *et al.*, 2012) and astaxanthin (Ispada *et al.*, 2018) can reduce the negative consequences of heat shock on the oocyte. Moreover, the function of the maturing oocyte can be disrupted by other oxidative stresses, as shown for the nitric oxide donor sodium nitroprusside

in the bovine (Soto *et al.*, 2003; Cheuquemán *et al.*, 2015) and hydrogen peroxide in the mouse (Tamura *et al.*, 2008) and pig (Yazaki *et al.*, 2013).

One molecule that may exert protective effects on the oocyte is the multifunctional hormone melatonin (*N*-acetyl-5-methoxytryptamine). Administration of melatonin to heat-stressed females improved the embryo competence for development in mice (Matsuzuka *et al.*, 2005) and fertility in lactating cows (Garcia-Ispierto *et al.*, 2013). Melatonin affects cellular function by interacting with membrane and nuclear receptors and by functioning as an antioxidant (Tan *et al.*, 2002; Mayo *et al.*, 2017). Melatonin improved oocyte maturation in the pig (Shi *et al.*, 2009), sheep (Tian *et al.*, 2017) and bovine (El-Raey *et al.*, 2011; Tian *et al.*, 2014; Marques *et al.*, 2018).

Experiments to test whether melatonin protects oocytes from heat shock have yielded inconclusive results, however. Effects of melatonin in the pig were evaluated in heat-shocked oocytes but not in oocytes cultured at normal temperature (Li *et al.*, 2015, 2016). Protective effects of high concentrations of melatonin (10 mM) on bovine oocytes cultured at 41.5°C were difficult to interpret because 10 mM melatonin reduced oocyte competence in the absence of heat shock (Cebrian-Serrano *et al.*, 2013).

In the current experiment, we evaluated the capacity of melatonin to protect oocytes using two models of oxidative stress. In addition to heat shock, cytoprotective properties of melatonin against the pro-oxidant menadione were examined. Menadione (2-methyl-1,4-naphthoquinone) is a vitamin K precursor that can react with a single electron to produce a free radical derivative that can cause superoxide anion formation (Comporti, 1989). Menadione can reduce developmental capacity of the pre-implantation bovine embryo (Moss *et al.*, 2009) and act on bovine spermatozoa to reduce fertilizing ability and compromise developmental competence of the resultant embryos (Hendricks & Hansen, 2010).

Materials and methods

Oocyte collection and maturation

Ovaries were obtained from a local abattoir from cows of a variety of genotypes including *Bos taurus* and admixtures of *B. taurus* and *B. indicus*. Ovaries were transported within 10 h to the laboratory at 23°C in a solution of 0.9% (w/v) NaCl. Cumulus – oocyte complexes (COCs) were harvested from follicles 2 to 8 mm in diameter by cutting the surface of the ovary with a scalpel and swirling the ovary in BoviPRO™ Oocyte Washing Medium (with BSA) (MOFA Global, Verona, WI, USA). Only COCs with at least three or four layers of compact cumulus and with an oocyte with a uniform cytoplasm were selected for maturation. Selected COC were washed and matured in 6-well plates in groups of 25–30 in 300 µl BO-IVM oocyte maturation medium (IVF Bioscience, Falmouth, UK) that was prepared ± 1 µM melatonin (Santa Cruz Biotechnology, Dallas, TX, USA) and with either 5 µM menadione (Sigma-Aldrich, St. Louis, MO, USA) or an equivalent volume of vehicle. Melatonin was prepared as described by Ortega *et al.* (2016) and menadione as described by Moss *et al.* (2009). Depending on the experiment, maturation was carried out for 3 or 22 h at 38.5°C or 41°C under an atmosphere of either 5% (v/v) CO₂ in humidified air (38.5°C) or 7% (v/v) CO₂ in humidified air (41°C). The higher CO₂ for maturation at 41.0°C was used to maintain pH at 7.4 in the face of lower solubility of CO₂ at the higher temperature. The control

temperature of 38.5°C was chosen because it is similar to the body temperature of the cow in the absence of heat stress. Heat shock was performed at 41°C because cows subjected to heat stress often experience rectal temperatures of 41°C or higher (Dikmen & Hansen, 2009). Moreover, exposure of bovine oocytes during maturation to 41°C disrupted mitochondrial function (Rodrigues *et al.*, 2016; Payton *et al.*, 2018), increased apoptosis (Roth & Hansen, 2004a; Rodrigues *et al.*, 2016) and reduced oocyte competence to complete nuclear maturation (Roth & Hansen, 2005) and be fertilized, undergo cleavage and develop to the blastocyst stage (Roth & Hansen, 2004a, 2005; de Castro e Paula & Hansen, 2007; Rodrigues *et al.*, 2016).

Production of ROS

An experiment was conducted to determine whether heat shock and menadione would increase the production of ROS by oocytes at the beginning of exposure to stress and whether increased production would be blocked by melatonin. For each replicate, COCs (~100) were randomly assigned to one of six treatments in a 3 × 2 factorial arrangement with the main effects of stress (control, heat shock, or menadione) and melatonin (0 or 1 µM). Controls were cultured at 38.5°C, heat shock was 41°C and menadione involved culture at 38.5°C in BO-IVM containing 5 µM menadione. This concentration of menadione was chosen because treatment of bovine embryos at day 6 after insemination with 5 µM menadione increased ROS production and apoptosis, while almost completely blocking development to the blastocyst stage (Moss *et al.*, 2009). For all treatments, medium contained 5 µM CellROX® Green, a cell-permeant dye that exhibits bright green photostable fluorescence upon oxidation and subsequent binding to DNA.

After 3 h of maturation, COCs were denuded of cumulus cells by vortexing groups of 25 to 30 for 5 min in 200 µl hyaluronidase as described earlier (Ortega *et al.*, 2017). Oocytes were then washed three times in 50 µl droplets of Dulbecco's phosphate-buffered saline (DPBS) containing 1% (w/v) polyvinylpyrrolidone (PVP), fixed in 4% (w/v) paraformaldehyde in DPBS, washed three more times in DPBS-PVP and mounted in groups of 10 oocytes on microscope slides using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA, USA). Oocytes were examined individually for fluorescence within 10 h after labelling using fluorescence microscopy and a green emission filter with a Zeiss Axio Plan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Digital images of each oocyte were acquired using AxioVision software (v.4.8.2; Zeiss) and a high-resolution black and white Zeiss AxioCam MRM digital camera. Analysis of the images was performed using ImageJ software v.1.48 (National Institutes of Health, Bethesda, MD, USA). Net fluorescent intensity was calculated by obtaining the average pixel intensity of each oocyte (obtained after manually drawing a boundary around the oocyte) and subtracting the background intensity obtained from a region of the image not containing the oocyte. The experiment was replicated four times using 41–72 oocytes per treatment ($n = 326$ total).

Competence of oocytes to cleave and develop after fertilization

An experiment was conducted to determine whether: (1) heat shock and menadione would alter competence of matured oocytes to cleave after fertilization and alter ability of the resultant

embryos to develop to the blastocyst stage; and (2) if actions of heat shock and menadione would be blocked by melatonin. For each replicate ($n = 12$ replicates total), COCs (~200) were randomly assigned to one of six treatments in a 3×2 factorial arrangement with main effects of stress (control, heat shock, or menadione) and melatonin (0 or $1 \mu\text{M}$). Control COCs were matured for 22 h at 38.5°C , heat-shocked COCs were matured for 14 h at 41.0°C and for 8 h at 38.5°C and menadione-treated COCs were matured for 22 h at 38.5°C in medium containing $5 \mu\text{M}$ menadione.

After maturation, COCs were washed three times in HEPES-TALP (Tyrode's albumin – lactate – pyruvate) and placed in wells of 6-well plates containing $425 \mu\text{l}$ fertilization medium [*in vitro* fertilization-TALP; see Ortega *et al.* (2017) for recipes for TALP medium] and $1 \times 10^6 \text{ ml}^{-1}$ spermatozoa. For each replicate, fertilization was performed with semen pooled from three individual bulls of various taurine breeds; the total number of bulls used in the experiment was 17. Sperm were purified from frozen – thawed straws of extended semen using an Isolate® gradient [Irvine Scientific, Santa Ana, CA USA; 50% (v/v) and 90% (v/v) Isolate]. In addition, $20 \mu\text{l}$ of penicillamine – hypotaurine – epinephrine solution (Ortega *et al.*, 2017) was added to each fertilization well to improve sperm motility. Fertilization proceeded for 14 to 16 h at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air.

Putative zygotes (i.e. oocytes exposed to sperm) were denuded from the surrounding cumulus cells at the end of fertilization (14 to 16 h) by vortexing groups of 25 to 30 zygotes for 5 min in $200 \mu\text{l}$ of HEPES-TALP containing 10 000 U/ml of hyaluronidase. Embryos were cultured in groups of 25–30 in $50 \mu\text{l}$ drops of culture medium (SOF-BE2; Ortega *et al.*, 2017) that were covered with mineral oil. Embryos were cultured at 38.5°C in a humidified atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2 with the balance N_2 . The percentage of oocytes that cleaved was determined at day 3.5 of development (day 0 = day of fertilization) and the per cent of cleaved embryos that became blastocysts was determined at day 7.5 of development.

The experiment was replicated 12 times with the total number of COCs ranging from 299 to 444 per treatment (total number = 2491).

Statistical analysis

Data were analyzed using SAS v.9.4 (SAS Institute Inc., Cary, NC, USA). Data on ROS production were analyzed by analysis of variance using the MIXED procedure. The model included main effects of stress and melatonin and the interaction of stress and melatonin as fixed effects and replicate as a random effect. Effects of stress and melatonin on the proportion of oocytes cleaving and on the proportion of cleaved embryos becoming blastocysts was evaluated using the GLIMMIX procedure. Each embryo was considered an observation with binary response (0 = not developed to blastocyst; 1 = developed to blastocyst) and analysis was performed by logistic regression fitting binary data distribution. The statistical model included the fixed effects of stress, melatonin, stress \times melatonin interaction and random effect of replicate.

For both analyses, two sets of orthogonal contrasts were used to make individual degree-of-freedom comparisons of resolve multilevel effects of stress and interactions of stress and melatonin. In the first set of contrasts, differences between types of stress (control, heat shock and menadione) were evaluated by two orthogonal contrasts: (1) the comparison of control *v.* heat

shock + menadione; and (2) the comparison of heat shock *v.* menadione. In the second set of contrasts, differences in the effect of melatonin for each stress were evaluated by comparisons of: (1) control *v.* control + melatonin; (2) heat shock *v.* heat shock + melatonin; and (3) menadione *v.* menadione + melatonin.

Results

Production of ROS

Representative images of labelling of oocytes using CellROX are shown in Fig. 1. Intensity of fluorescence was higher for oocytes exposed to heat shock (41°C) or $5 \mu\text{M}$ menadione than for oocytes matured at 38.5°C (compare Fig. 1C and Fig. 1E with Fig. 1A). Addition of $1 \mu\text{M}$ melatonin reduced fluorescence intensity under all culture conditions (compare Fig. 1A, C and E with Fig. 1B, D and F).

Results of quantification of ROS labelling are shown in Fig. 2. The intensity of ROS was greater ($P = 0.0577$) for oocytes exposed to heat shock and menadione than for control oocytes. Overall, melatonin reduced ROS intensity ($P = 0.0002$) and there was no interaction between stress and melatonin ($P = 0.4806$). However, analysis of the effects of melatonin for each stress indicated that melatonin reduced ROS intensity for heat-shocked oocytes ($P = 0.0305$) and oocytes exposed to menadione ($P = 0.0007$) but not for control oocytes ($P = 0.2002$).

Competence of oocytes to cleave and develop after fertilization

Results on cleavage of oocytes after fertilization are presented in Fig. 3A. The proportion of oocytes that cleaved after fertilization was lower ($P < 0.0001$) for oocytes exposed to heat shock and menadione than for control oocytes. Overall, melatonin increased cleavage ($P = 0.0041$). While the interaction between stress and melatonin was not significant ($P = 0.2944$), analysis of the effects of melatonin for each stress indicated that melatonin increased cleavage for heat-shocked oocytes ($P = 0.0305$) and oocytes exposed to menadione ($P = 0.0122$) but not for control oocytes ($P = 0.6675$).

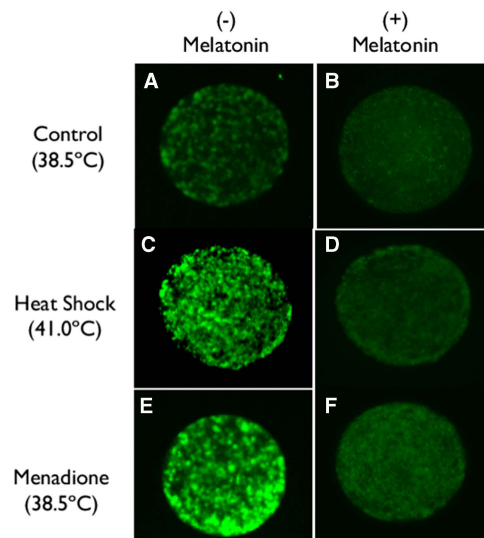


Figure 1. (A–F) Representative images of oocytes labelled with CellROX (Thermo Fisher Scientific, Waltham, MA, USA) to assess the production of ROS as affected by incubation temperature, menadione and melatonin.

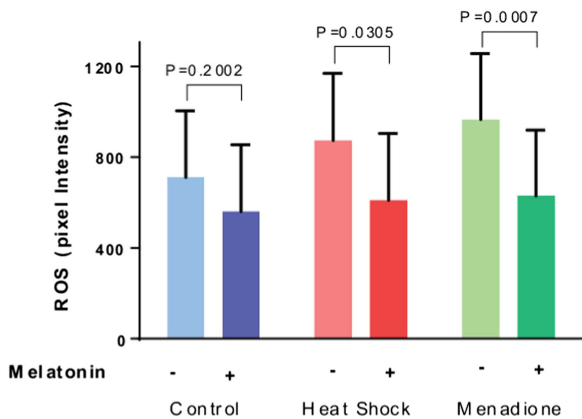


Figure 2. Effects of melatonin (1 μ M) on the production of reactive oxygen species (ROS) by maturing oocytes exposed to control conditions (38.5°C), heat shock (41.0°C) and menadione (5 μ M at 38.5°C). Data are least-squares means \pm standard error of the mean (SEM) of pixel intensity. Overall, the intensity of ROS was greater ($P=0.0577$) for oocytes exposed to heat shock and menadione than for control oocytes. Probability values for the effect of melatonin for each stress are indicated above the bars.

As shown in Fig. 3B, the proportion of cleaved embryos that became blastocysts was not affected by stress (heat shock + menadione *v.* control, $P=0.7871$) but was increased by melatonin ($P=0.0634$). Despite a lack of a stress \times melatonin interaction ($P=0.5991$), analysis of effects of melatonin for each stress indicated that melatonin tended to increase development for embryos from heat-shocked oocytes ($P=0.0702$) but not for embryos from oocytes exposed to menadione ($P=0.5426$) or from control oocytes ($P=0.4384$).

Discussion

Present results indicate that melatonin can reduce ROS production by the bovine oocyte exposed to conditions that promote production of ROS and partially preserve developmental competence of the oocyte exposed to those stresses. These results confirm the importance of oxidative stress for damaging the oocyte and show how administration of an antioxidant can block that effect.

It has been repeatedly demonstrated, both in the present experiments and by others, that heat shock increases ROS production by the bovine oocyte (Nabenishi *et al.*, 2012; Ispada *et al.*, 2018) and reduces the percentage of oocytes that cleave after coincubation with spermatozoa (Roth & Hansen, 2004a; de Castro e Paula & Hansen, 2007). In some cases, the deleterious actions of heat shock on the oocyte also compromise the ability of the subsequent embryo to develop to the blastocyst stage (Roth *et al.*, 2004a,b; Nabenishi *et al.*, 2012; Rodrigues *et al.*, 2016) although this consequence of heat shock has not always been observed (de Castro e Paula & Hansen, 2007; Cebrian-Serrano *et al.*, 2013). In the present experiment, the per cent of cleaved embryos becoming blastocysts for oocytes cultured without melatonin was 26.7% for control oocytes and 22.0% for heat-shocked oocytes. Therefore, the primary defect caused by heat shock here was the competence of the oocyte to cleave after fertilization. Treatment of the oocyte with the prooxidant menadione also reduced per cent of oocytes that cleaved while not significantly affecting subsequent development of cleaved embryos. Previous experiments with heat shock would indicate that reduced competence for cleavage is due to disruption of nuclear maturation (Roth &

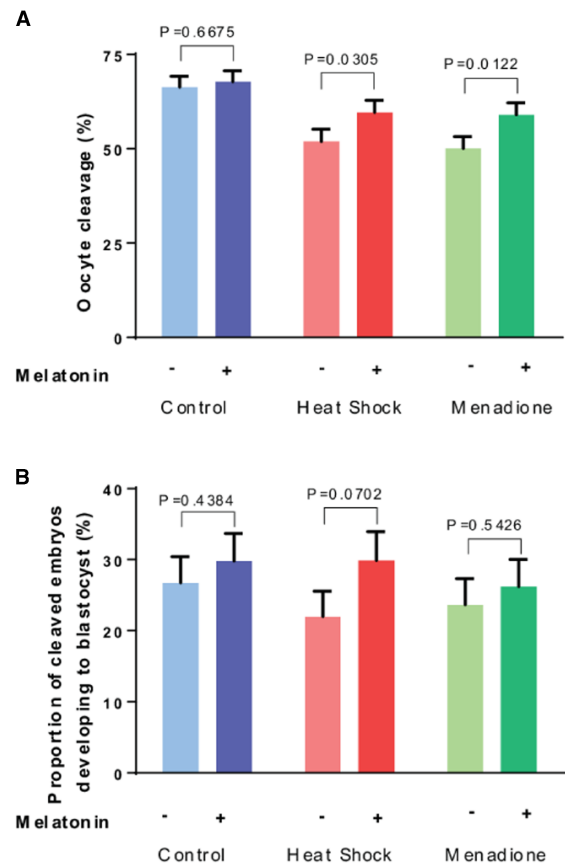


Figure 3. Effects of melatonin (1 μ M) on developmental competence of oocytes exposed to control conditions (38.5°C), heat shock (41.0°C) and menadione (5 μ M at 38.5°C) during *in vitro* maturation. Data are least-squares means \pm standard error of the mean (SEM) of the proportion of oocytes that cleaved after fertilization (A) and the proportion of cleaved embryos developing to the blastocyst stage (B). Overall, the proportion of oocytes that cleaved after fertilization was lower ($P<0.0001$) for oocytes exposed to heat shock and menadione than for control oocytes. The proportion of cleaved embryos that became blastocysts was not affected by stress (heat shock + menadione *v.* control, $P=0.7871$). Probability values for the effect of melatonin for each stress are indicated above the bars.

Hansen, 2005; Nabenishi *et al.*, 2012; Sebrían-Serrano *et al.*, 2013; Meiyu *et al.*, 2015) as well as disruption of mitochondrial function (Rodrigues *et al.*, 2016; Payton *et al.*, 2018). Induction of oocyte apoptosis is also very important: inhibition of apoptosis can block anti-developmental effects of heat shock (Roth & Hansen, 2004a, 2005). The observation that melatonin was more effective at blocking effects of heat shock on competence of cleaved embryos to become blastocysts than the effect of menadione probably reflects ROS-independent actions of menadione on cellular function. For example, menadione can block Siah2 ubiquitin ligase through actions not involving ROS (Shah *et al.*, 2009).

Deleterious effects of both heat shock and menadione on per cent of oocytes that cleaved were reduced by melatonin. Moreover, there was a tendency for melatonin to increase the competence of embryos derived from heat-shocked oocytes to develop to the blastocyst stage. Earlier experiments to evaluate the thermoprotective effect of melatonin on the oocyte have been difficult to interpret either because of lack of control oocytes not exposed to heat shock (Li *et al.*, 2015, 2016) or the high concentrations of melatonin (10 mM) required to protect oocytes from heat shock reduced oocyte competence in the absence of heat shock (Cebrian-Serrano *et al.*, 2013). The experiments conducted here did not allow the determination of whether the cytoprotective

effects of melatonin were mediated by reducing ROS production or through changes in cellular function mediated by activation of melatonin receptors. The former explanation seems more likely because addition of melatonin caused a large reduction in ROS generation, melatonin was protective against two different stresses (heat shock & menadione) that both increased ROS production and there were little effects of melatonin on oocyte competence in the absence of heat shock or menadione. There are reports of the existence of melatonin receptors or their mRNA in the bovine oocyte and cumulus cell (El-Raey *et al.*, 2011; Tian *et al.*, 2014) and further studies are necessary to resolve the mechanism of action of the cytoprotective effects of melatonin on the oocyte.

An additional indication of the mechanism of action of melatonin is the effective concentration of the hormone. The concentration of melatonin used here, 1 μM , is much higher than the reported KD of membrane melatonin receptors, having values of $\sim 30 - 225 \text{ pM}$ (Poon *et al.*, 1994; Kobayashi *et al.*, 2003; Liu *et al.*, 2013). In an earlier study, there was no thermoprotective effect of 1 pM or 1 nM melatonin on bovine oocytes exposed to heat shock (Cebrian-Serrano *et al.*, 2013; Ahmed *et al.*, 2016).

There is a report that administration of melatonin to cows via subcutaneous implants can improve fertility of heat-stressed cows (Garcia-Ispuerto *et al.*, 2013). In that study, concentrations of melatonin in peripheral blood of treated cows peaked at 60–70 pg/ml (i.e. 260–300 pM). Therefore, beneficial effects of melatonin in that study may have involved receptor-mediated actions of melatonin on one or more components of the reproductive system rather than a direct cytoprotective action of the molecule.

The finding that 1 μM melatonin reduced effects of heat shock and melatonin on the oocyte is in contrast with recent results with the bovine 2-cell embryo. Culture of embryos at this stage of development causes an increase in ROS production and a decrease in the proportion of embryos that develop to the blastocyst stage (Ortega *et al.*, 2016). In that study, melatonin blocked the increase in ROS production caused by heat shock but did not rescue the ability of 2-cell embryos to develop to the blastocyst stage. Perhaps, heat shock causes deleterious changes in cellular function of the 2-cell embryo that are independent of ROS production (and of the protective action of melatonin), whereas ROS production is the major proximate cause of damage to the oocyte caused by heat shock. Consistent with this idea is the fact that heat shock disrupts developmental competence of the 2-cell embryo to a greater degree than the oocyte (Edwards & Hansen, 1997) and that oxygen concentration used for culture is not an important determinant of the magnitude of effects of heat shock on the zygote or 2-cell embryo (Rivera & Hansen, 2001; Sakatani *et al.*, 2012). Experiments using caspase inhibitors (Roth & Hansen, 2004a) or sphingosine 1-phosphate (Roth & Hansen, 2004b) indicated that the induction of apoptosis by heat shock is a major cause of decreased developmental competence of the oocyte. Such a mechanism for damage by heat shock is not operational in the 2-cell embryo because apoptosis responses in the preimplantation embryo are blocked until the 8- to 16-cell stages (Hansen & Fear, 2011).

While it is clear that heat stress can reduce oocyte competence *in vivo* (Putney *et al.*, 1988), an important question is the importance of the direct effect of elevated temperature on the oocyte as a cause of compromised oocyte function. As stated in the previous paragraph, the oocyte is more resistant to disruption by heat shock than the early cleavage-stage embryo. Moreover, there is recent evidence in the cow that intrafollicular temperature

of the ovulatory follicle is about 0.9 to 1.1°C lower than rectal temperature (López-Gatius & Hunter, 2019a, b). Thus, the actual degree of heat shock at the level of the oocyte may be less than the temperature used here. Additionally, recent data indicate that follicular fluid contains molecules that can protect the oocyte from heat shock (Rodrigues *et al.*, 2019). Although melatonin is present in follicular fluid, at concentrations ranging from 10^{-11} to 10^{-9} M (Shi *et al.*, 2009; Tong *et al.*, 2017), it is not responsible for the protective properties of follicular fluid because concentrations are too low to block effects of heat shock (Cebrian-Serrano *et al.*, 2013; Ahmed *et al.*, 2016). At least some of the protective activity is associated with the exosome fraction of follicular fluid (Rodrigues *et al.*, 2019).

In the absence of heat shock or menadione, there was no effect of melatonin on competence of oocytes to cleave or of the resultant embryos to develop to the blastocyst stage. Similar results have been obtained by others (Farahavar *et al.*, 2010; Rodrigues-Cunha *et al.*, 2016). The absence of beneficial effects of melatonin on performance of *in vitro* fertilization procedures is in contrast with studies in cattle (Zhao *et al.*, 2015; Yang *et al.*, 2017; Pang *et al.*, 2018) and goats (Soto-Heras *et al.*, 2018) in which melatonin improved one or more indices of oocyte function. Except for the study of Pang *et al.* (2018), the studies in which melatonin exhibited positive effects used oocytes that were compromised in some way, either being classified as being of poor quality based on morphological criteria (Zhao *et al.*, 2015; Yang *et al.*, 2017) or being from immature animals (Soto-Heras *et al.*, 2018). It may be, therefore, that melatonin can be a useful additive to oocyte maturation medium when oocyte competence is compromised.

In conclusion, melatonin reduced the production of ROS by maturing oocytes, especially when they were exposed to heat shock or menadione and protected oocytes from deleterious effects of both stresses on competence to cleave after coincubation with sperm. These results suggested that excessive production of ROS compromises oocyte function. Damage to the oocyte caused by ROS may, therefore, be important for effects of elevated temperature on the oocyte during maturation (Putney *et al.*, 1988), at least in cases in which oocyte temperature rises sufficiently to trigger increased ROS production.

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Conflicts of interest. The authors have no conflict to declare.

Ethical standards. Not applicable.

References

Ahmed JA, Dutta D and Nashiruddin N (2016) Comparative efficacy of antioxidant retinol, melatonin, and zinc during *in vitro* maturation of

- bovine oocytes under induced heat stress. *Turkish J Vet Anim Sci* **40**, 365–73.
- Cebrian-Serrano A, Salvador I, Raga E, Dinnyes A and Silvestre MA** (2013) Beneficial effect of melatonin on blastocyst *in vitro* production from heat-stressed bovine oocytes. *Reprod Domest Anim* **48**, 738–46.
- Cheuquemán C, Loren P, Arias M, Risopatrón J, Felmer R, Álvarez J, Mogas T and Sánchez R** (2015) Effects of short-term exposure of mature oocytes to sodium nitroprusside on *in vitro* embryo production and gene expression in bovine. *Theriogenology* **84**, 1431–7.
- Comporti M** (1989) Three models of free radical-induced cell injury. *Chem Biol Interact* **72**, 1–56.
- de Castro e Paula LA and Hansen PJ** (2007) Interactions between oxygen tension and glucose concentration that modulate actions of heat shock on bovine oocytes during *in vitro* maturation. *Theriogenology* **68**, 763–70.
- Dikmen S and Hansen PJ** (2009) Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? *J Dairy Sci* **92**, 109–16.
- Edwards JL and Hansen PJ** (1997) Differential responses of bovine oocytes and preimplantation embryos to heat shock. *Mol Reprod Dev* **46**, 138–45.
- El-Raey M, Geshi M, Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE, Sosa GA, El-Roos ME and Nagai T** (2011) Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation *in vitro* in cattle. *Mol Reprod Dev* **78**, 250–62.
- Farahavar A, Shahane AZ, Kohram H and Vahedi V** (2010) Effect of melatonin on *in vitro* maturation of bovine oocytes. *African J Biotechnol* **9**, 2579–83.
- García-Ispuerto I, Abdelfatah A and López-Gatius F** (2013) Melatonin treatment at dry-off improves reproductive performance postpartum in high-producing dairy cows under heat stress conditions. *Reprod Domest Anim* **48**, 577–83.
- Hansen PJ and Fear JM** (2011) Cheating death at the dawn of life: developmental control of apoptotic repression in the preimplantation embryo. *Biochem Biophys Res Commun* **413**, 155–8.
- Hendricks KE and Hansen PJ** (2010) Consequences for the bovine embryo of being derived from a spermatozoon subjected to oxidative stress. *Aust Vet J* **88**, 307–10.
- Ispada J, Rodrigues TA, Risolia PHB, Lima RS, Gonçalves DR, Rettori D, Nichi M, Feitosa WB and Paula-Lopes FF** (2018) Astaxanthin counteracts the effects of heat shock on the maturation of bovine oocytes. *Reprod Fertil Dev* [Epub ahead of print]
- Kobayashi Y, Itoh MT, Kondo H, Okuma Y, Sato S, Kanishi Y, Hamada N, Kiguchi K and Ishizuka B** (2003) Melatonin binding sites in estrogen receptor-positive cells derived from human endometrial cancer. *J Pineal Res* **35**, 71–4.
- Lawrence JL, Payton RR, Godkin JD, Saxton AM, Schrick FN and Edwards JL** (2004) Retinol improves development of bovine oocytes compromised by heat stress during maturation. *J Dairy Sci* **87**, 2449–54.
- Li Y, Zhang Z, He C, Zhu K, Xu Z, Ma T, Tao J. and Liu G** (2015) Melatonin protects porcine oocyte *in vitro* maturation from heat stress. *J Pineal Res* **59**, 365–75.
- Li Y, Wang J, Zhang Z, Yi J, He C, Wang F, Tian X, Yang M, Song Y, He P and Liu G** (2016) Resveratrol compares with melatonin in improving *in vitro* porcine oocyte maturation under heat stress. *J Anim Sci Biotechnol* **7**, 33.
- Liu XY, Xu YT, Shi Q, Lu QS, Ma SR, Xu XY and Guo XZ** (2013) Alterations of reproductive hormones and receptors of male rats at the winter and summer solstices and the effects of pinealectomy. *Neuroendocrinol Lett* **34**, 143–53.
- López-Gatius F and Hunter RHF** (2019a) Pre-ovulatory follicular cooling correlates positively with the potential for pregnancy in dairy cows: Implications for human IVF. *J Gynecol Obstet Hum Reprod* [Epub ahead of print]
- López-Gatius F and Hunter RHF** (2019b) Pre-ovulatory follicular temperature in bi-ovular cows. *J Reprod Dev* **65**, 191–4.
- Marques TC, da Silva Santos EC, Diesel TO, Leme LO, Martins CF, Dode M, Alves BG, Costa F, de Oliveira EB and Gambarini ML** (2018) Melatonin reduces apoptotic cells, SOD2 and HSPB1 and improves the *in vitro* production and quality of bovine blastocysts. *Reprod Domest Anim* **53**, 226–36.
- Matsuzuka T, Sakamoto N, Ozawa M, Ushitani A, Hirabayashi M and Kanai Y** (2005) Alleviation of maternal hyperthermia-induced early embryonic death by administration of melatonin to mice. *J Pineal Res* **39**, 217–23
- Mayo JC, Sainz RM, González-Menéndez P, Hevia D and Cernuda-Cernuda R** (2017) Melatonin transport into mitochondria. *Cell Mol Life Sci* **74**, 3927–40.
- Meiyu Q, Liu D and Roth Z** (2015) IGF-I slightly improves nuclear maturation and cleavage rate of bovine oocytes exposed to acute heat shock *in vitro*. *Zygote* **23**, 514–24.
- Moss JI, Pontes E and Hansen PJ** (2009) Insulin-like growth factor-1 protects preimplantation embryos from anti-developmental actions of menadione. *Arch Toxicol* **83**, 1001–7.
- Nabenishi H, Ohta H, Nishimoto T, Morita T, Ashizawa K and Tsuzuki Y** (2012) The effects of cysteine addition during *in vitro* maturation on the developmental competence, ROS, GSH and apoptosis level of bovine oocytes exposed to heat stress. *Zygote* **20**, 249–59.
- Ortega MS, Rocha-Frigoni NAS, Mingoti GZ, Roth Z and Hansen PJ** (2016) Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in *HSPA1L*. *J Dairy Sci* **99**, 9152–64.
- Ortega MS, Wohlgenuth S, Tribulo P, Siqueira LG, Cole JB and Hansen PJ** (2017) A single nucleotide polymorphism in *COQ9* affects mitochondrial and ovarian function and fertility in Holstein cows. *Biol Reprod* **96**, 652–63.
- Pang Y, Zhao S, Sun Y, Jiang X, Hao H, Du W and Zhu H.** (2018) Protective effects of melatonin on the *in vitro* developmental competence of bovine oocytes. *Anim Sci J.* **89**, 648–60.
- Payton RR, Rispoli LA, Nagle KA, Gondro C, Saxton AM, Voy BH and Edwards JL** (2018) Mitochondrial-related consequences of heat stress exposure during bovine oocyte maturation persist in early embryo development. *J Reprod Dev* **64**, 243–51.
- Poon AM, Liu ZM, Pang CS, Brown GM and Pang SF** (1994) Evidence for a direct action of melatonin on the immune system. *Biol Signals* **3**, 107–17.
- Putney DJ, Drost M and Thatcher WW** (1988) Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. *Theriogenology* **30**, 195–209.
- Rivera RM and Hansen PJ** (2001) Development of cultured bovine embryos after exposure to high temperatures in the physiological range. *Reproduction* **121**, 107–15.
- Rivera RM, Dahlgren GM, De Castro E Paula LA, Kennedy RT and Hansen PJ** (2004) Actions of thermal stress in two-cell bovine embryos: oxygen metabolism, glutathione and ATP content, and the time-course of development. *Reproduction* **128**, 33–42.
- Rodrigues TA, Ispada J, Risolia PH, Rodrigues MT, Lima RS, Assumpção ME, Visintin JA and Paula-Lopes FF** (2016) Thermoprotective effect of insulin-like growth factor 1 on *in vitro* matured bovine oocyte exposed to heat shock. *Theriogenology* **86**, 2028–39.
- Rodrigues TA, Tuna KM, Alli AA, Tribulo P, Hansen PJ, Koh J and Paula-Lopes FF** (2019) Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survival to heat shock. *Reprod Fertil Dev* **31**, 888–97.
- Rodrigues-Cunha MC, Mesquita LG, Bressan F, Collado MD, Balieiro JC, Schwarz KR, de Castro FC, Watanabe OY, Watanabe YF, de Alencar Coelho L and Leal CL.** (2016) Effects of melatonin during IVM in defined medium on oocyte meiosis, oxidative stress, and subsequent embryo development. *Theriogenology* **86**, 1685–94.
- Roth Z and Hansen PJ** (2004a) Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. *Biol Reprod* **71**, 1898–906.
- Roth Z and Hansen PJ** (2004b) Sphingosine 1-phosphate protects bovine oocytes from heat shock during maturation. *Biol Reprod* **71**, 2072–8.
- Roth Z and Hansen PJ** (2005) Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction* **129**, 235–44.
- Sakatani M, Alvarez NV, Takahashi M and Hansen PJ** (2012) Consequences of physiological heat shock beginning at the zygote stage on embryonic development and expression of stress response genes in cattle. *J Dairy Sci* **95**, 3080–91.

- Shah M, Stebbins JL, Dewing A, Qi J, Pellecchia M and Ronai ZA** (2009) Inhibition of Siah2 ubiquitin ligase by vitamin K3 (menadione) attenuates hypoxia and MAPK signaling and blocks melanoma tumorigenesis. *Pigment Cell Melanoma Res* **22**, 799–808.
- Shi JM, Tian XZ, Zhou GB, Wang L, Gao C, Zhu SE, Zeng SM, Tian JH and Liu GS** (2009) Melatonin exists in porcine follicular fluid and improves *in vitro* maturation and parthenogenetic development of porcine oocytes. *J Pineal Res* **47**, 318–23.
- Soto P, Natzke RP and Hansen PJ** (2003) Identification of possible mediators of embryonic mortality caused by mastitis: actions of lipopolysaccharide, prostaglandin F_{2α}, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. *Am J Reprod Immunol* **50**, 263–72.
- Soto-Heras S, Roura M, Catalá MG, Menéndez-Blanco I, Izquierdo D, Fouladi-Nashta AA and Paramio MT** (2018) Beneficial effects of melatonin on *in vitro* embryo production from juvenile goat oocytes. *Reprod Fertil Dev* **30**, 253–261.
- Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ and Sugino N** (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res* **44**, 280–7.
- Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M and Hardeland R** (2002) Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem* **2**, 181–97.
- Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, Xu J, Ji P and Liu G** (2014) Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. *J Pineal Res* **57**, 239–47.
- Tian X, Wang F, Zhang L, He C, Ji P, Wang J, Zhang Z, Lv D, Abulizi W, Wang X, Lian Z and Liu G** (2017) Beneficial effects of melatonin on the *in vitro* maturation of sheep oocytes and its relation to melatonin receptors. *Int J Mol Sci* **18**, E834.
- Tong J, Sheng S, Sun Y, Li H, Li WP, Zhang C and Chen ZJ** (2017) Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve. *Reproduction* **153**, 443–51.
- Yang M, Tao J, Chai M, Wu H, Wang J, Li G, He C, Xie L, Ji P, Dai Y, Yang L and Liu G** (2017) Melatonin improves the quality of inferior bovine oocytes and promoted their subsequent IVF embryo development: mechanisms and results. *Molecules* **22**, 2059.
- Yazaki T, Hiradate Y, Hoshino Y, Tanemura K and Sato E** (2013) l-Carnitine improves hydrogen peroxide-induced impairment of nuclear maturation in porcine oocytes. *Anim Sci J* **84**, 395–402.
- Zhao XM, Min JT, Du WH, Hao HS, Liu Y, Qin T, Wang D and Zhu HB** (2015) Melatonin enhances the *in vitro* maturation and developmental potential of bovine oocytes denuded of the cumulus oophorus. *Zygote* **23**, 525–36.