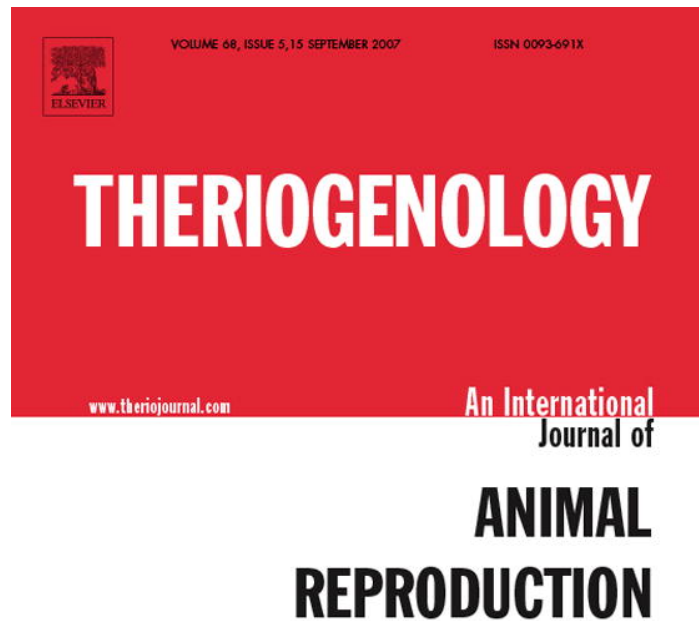


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Interactions between oxygen tension and glucose concentration that modulate actions of heat shock on bovine oocytes during in vitro maturation

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Abstract

Exposure of oocytes to elevated temperature (i.e. heat shock) during maturation can reduce fertilization rate and development of the resultant embryos. Given the possible role of free radicals in actions of heat shock on cellular function, we tested the hypothesis that a high oxygen environment exacerbates the magnitude of deleterious effects of heat shock on in vitro maturation of bovine oocytes. A preliminary experiment was performed to establish conditions for oocyte maturation that would be independent of oxygen concentration. Oocytes were matured in a modified tissue culture medium-199 (mTCM-199) or modified synthetic oviduct fluid (mSOF) containing 5.6 or 20 mM glucose and under either high (atmospheric oxygen, ~21%, v/v) or low oxygen (5%, a value approximating oxygen content of the follicle). For oocytes matured in mTCM-199, development was greater in high oxygen than in low oxygen, whereas development was unaffected by oxygen using mSOF (medium \times oxygen, $P < 0.05$). Accordingly, mSOF was used as the maturation medium in a second study to test the effect of oxygen concentration on the magnitude of actions of heat shock during maturation. Maturation was at 38.5 °C for 22 h (control) or 41 °C for 12 h and 38.5 °C for 10 h (heat shock). Heat shock slightly decreased cleavage rate, regardless of the maturation conditions, and decreased blastocyst development under all maturation conditions except for the group matured under high oxygen and high glucose (temperature \times glucose for oocytes under low oxygen, $P < 0.05$). The percentage of oocytes becoming blastocysts for control and heat shocked oocytes was 25.9% versus 22.5% (low oxygen—5.6 mM glucose), 41.6% versus 34.9% (low—20 mM), 41.7% versus 35.0% (high—5.6 mM), and 37.6% versus 37.5% (high—20 mM). In conclusion, under an oxygen tension that approached physiological conditions, heat shock during in vitro maturation reduced oocyte competence for fertilization and subsequent development.

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Keywords: Oocyte; Heat shock; Oxygen; Glucose; Bovine

1. Introduction

Oocyte viability is modified by the microenvironment in which the oocyte undergoes growth and maturation. One environmental determinant of oocyte viability is heat stress. Exposure of lactating dairy cows

to environmental conditions leading to hyperthermia during summer can alter the temperature at which the oocyte undergoes growth and maturation. Heat stress also alters secretion of hormones involved in follicular function (e.g. steroids, LH, FSH and inhibin [1–3]). That oocytes can be compromised by heat stress is indicated by reduced capacity for fertilization in vitro [4] and in vivo [5] and reduced capacity for development of embryos derived from fertilized or chemically activated oocytes [6,7].

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Experiments evaluating restoration of follicular and oocyte function after heat stress are indicative that heat stress affects the follicle 30–40 days before estrus or earlier [8,9]. The period during which the oocyte is susceptible to disruption by heat stress extends through the processes of maturation when the oocyte completes cytoplasmic and nuclear maturation. Indeed, heat stress applied for 10 h beginning at estrus caused a reduction in the proportion of oocytes that became normal embryos later in development [10]. Effects of heat stress during maturation could reflect either direct effects of elevated temperature on function of the oocyte and its supporting cumulus cells, alterations in blood flow to the ovary (as shown in the rabbit [11]), or altered hormone secretion [3]. Direct effects of elevated temperature on oocyte maturation have been demonstrated *in vitro* using experiments where cumulus oocyte complexes (COCs) were matured in incubators under temperatures similar to those experienced by cows during heat stress. Heat shock during maturation also induced oocyte apoptosis [12,13], disrupted chromosome alignment [14,15], and decreased the number of oocytes reaching metaphase II [15,16]. The proportion of oocytes that become blastocysts after insemination was also reduced by heat shock during maturation [12–14,16,17]. This reduction was associated with a reduced fertilization rate in some experiments [12,13] and with a reduced proportion of cleaved embryos that developed to the blastocyst stage in other studies [14,16,17].

One caveat with relating results of most *in vitro* studies of heat shock to the situation *in vivo* is that culture experiments are usually carried out in a gaseous environment high in oxygen (~21%, v/v). This oxygen tension is higher than encountered by the COCs *in vivo*, which has been estimated in follicular fluid as 5–9% in humans [18,19] and from <5 to 14% in cows [20]. Effects of heat shock on preimplantation bovine embryos were greater when culture was performed in atmospheric oxygen than when performed in 5% oxygen [21]; perhaps the effects of heat shock on oocytes *in vitro* would not be duplicated in the lower oxygen environments *in vivo*.

The objective of the present study was to test the hypothesis that effects of heat shock during maturation on oocyte competence for fertilization and development would be reduced in a low oxygen environment. To test this hypothesis, it was first necessary to establish maturation conditions where oxygen concentration would have little effect on the outcome. Using a modified synthetic oviduct fluid (SOF) as maturation medium, Hashimoto et al. [22] reported that optimal

maturation of oocytes under 5% O₂ required raising glucose concentration from 5.6 to 20 mM. Therefore, an initial experiment was performed to determine effects of oxygen environment during maturation as performed in two media (modified TCM-199 versus modified SOF) and under two glucose concentrations (5.6 mM versus 20 mM). Once conditions for maturation that were unaffected by oxygen concentration were determined, a second experiment was performed to determine effect of oxygen concentration on actions of heat shock during maturation. Heat shock was applied during the first 12 h of maturation, because oocytes were more sensitive to heat shock during this period than during the last 12 h of maturation [23].

2. Materials and methods

2.1. Preparation of media

Oocyte collection medium (OCM) was tissue culture medium-199 (TCM-199) with Hank's salts and without phenol red (Atlanta Biologicals, Norcross, GA, USA) that was supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA) containing 2 U/mL heparin. Medium was also supplemented with 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Two media were used for oocyte maturation: a modified TCM-199 (mTCM-199) and a modified SOF (mSOF). The TCM-199 contained Earle's salts and was purchased from BioWhittaker (Walkersville, MD, USA). The SOF was prepared based on the formulation described by Fischer-Brown et al. [24] by the Specialty Media Division of Millipore (Billerica, MA, USA). Both media were modified by adding 10% (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine FSH (Folltropin-V; Vetepharm Canada, Belleville, ON, Canada), 50 µg/mL gentamicin sulfate, and 1 mM glutamine. In addition, mSOF contained 1× basal medium eagle amino acids (Sigma, St. Louis, MO, USA) as used by Hashimoto et al. [22]. The mTCM-199 was also supplemented with 22 µg/mL sodium pyruvate. The media HEPES-Tyroses lactate (TL), *in vitro* fertilization (IVF)-TL, and Sperm-TL were purchased from Specialty Media and used to prepare HEPES-tyroses albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described [25]. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Potassium simplex optimized medium (KSOM) that contained 1 mg/mL bovine serum albumin was obtained from Specialty Media). Essentially fatty-acid free bovine steer albumin was from Sigma. On the day of use, KSOM was

modified for bovine embryos to produce KSOM-BE2, as described elsewhere [26].

2.2. *In vitro* production of embryos

Cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL, USA). Those COCs with at least two complete layers of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50- μ L drops of either mTCM-199 or mSOF, depending on the experiment, overlaid with mineral oil, and matured for 20–22 h. Temperature and oxygen concentration varied between treatments and experiments and are described in detail in the next section. Matured COCs were washed once in HEPES-TALP and transferred (in groups of 30) to 4-well plates containing 600 μ L of IVF-TALP and 25 μ L of PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μ M epinephrine in 0.9% (w/v) NaCl] per well and fertilized with $\sim 1 \times 10^6$ Percoll-purified spermatozoa. In each replicate, the sperm represented a pool of frozen-thawed semen from three bulls (donated by Southeastern Semen Services, Wellborn, FL, USA) and a different pool was used for each replicate. After 10–12 h at 38.5 °C in an atmosphere of 5% CO₂ in humidified air (i.e. 21% oxygen), putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 mL of 1000 U/mL hyaluronidase in HEPES-TALP, and placed in groups (up to 30) in 50- μ L drops of KSOM-BE2. Within a replicate, approximately equal (± 1) numbers of embryos were added for all treatments. All drops of embryos were overlaid with mineral oil and cultured at 38.5 °C in a humidified atmosphere of 5% O₂, 5% CO₂, and the balance N₂. Embryos were cultured until Day 8 after insemination (Day 0 = day of insemination).

2.3. Comparison of mTCM-199 versus mSOF as maturation media in high and low oxygen environments

Oocytes were collected during September. The experimental design was a $2 \times 2 \times 2$ factorial with main effects of maturation medium, glucose concentration, and oxygen environment. Cumulus-oocyte complexes were collected and placed in 50 μ L drops of mTCM-199 or mSOF that were formulated to contain 5.6 or 20 mM glucose. Maturation was in either 5% CO₂ in humidified air (high oxygen) or a humidified

environment of 5% O₂ and 5% CO₂ with the balance N₂ (low oxygen). Fertilization and embryo culture were performed as described earlier. Development was ascertained once, on Day 8 after insemination, to determine the percentage of embryos becoming blastocysts. The experiment was replicated six times, using a total of 2020 oocytes. All eight treatments were represented in each replicate.

2.4. Effect of oxygen concentration on heat shock effects during oocyte maturation

Oocytes were collected during the months of October through February. The design was a $2 \times 2 \times 2$ factorial, with main effects of temperature, glucose concentration and oxygen environment. Cumulus-oocyte complexes were collected and matured for 22 h at either 38.5 °C for 22 h or at 41 °C for 12 h followed by 38.5 °C for 10 h. Maturation was performed in 50 μ L drops of mSOF containing 5.6 or 20 mM glucose and in a high or low oxygen atmosphere. The CO₂ environment was maintained at 5% (v/v) for maturation at 38.5 °C and was raised to 6% for maturation at 41 °C to correct for reduced solubility of CO₂ and to maintain the pH of the medium at a value similar to that for maturation at 38.5 °C (pH \sim 7.4) [27]. Fertilization and embryo culture were performed as described earlier. Embryos were examined for development twice. Cleavage rate was recorded on Day 3 after insemination and the percentage of oocytes and cleaved embryos becoming blastocysts was recorded on Day 8 after insemination. The experiment was replicated nine times using a total of 3215 oocytes. All eight treatments were represented in each replicate.

2.5. Statistical analysis

Data on percentage of oocytes and cleaved embryos becoming blastocysts were calculated for all oocytes and cleaved embryos treated alike for each replicate. These percentages calculated for each replicate were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC, USA). Percentage data were analyzed without transformation and after performing arcsin transformation. Similar results were obtained for both variables; untransformed data are reported in this manuscript.

In the first experiment, main effects included medium (mTCM-199 and mSOF), glucose concentration (5.6 and 20 mM) and oxygen environment (high and low). The mathematical model for the overall

analysis included the main effects and interactions. To determine interactions between two main effects within one factor of a third main effect, the third main effect was fixed using the Sort procedure from SAS. For example, to determine if there was an interaction between medium and glucose under high or low oxygen, the Sort procedure was used to determine effects of medium, glucose and their interaction for each oxygen treatment separately.

In the second experiment, main effects included temperature (38.5 and 41 °C), glucose concentration (5.6 and 20 mM) and oxygen environment (high and low). The mathematical model for the overall analysis included the main effects and interactions, as mentioned above. The Sort procedure was also used as described above.

All values reported are least-squares means \pm S.E.M. from non-transformed data. In the results section, it is noted whether values refer to the overall analysis or sorted analysis.

3. Results

3.1. Comparison of mTCM-199 versus mSOF as maturation media in high and low oxygen environments

The proportion of oocytes that became blastocysts (Fig. 1) was not affected by glucose concentration ($P > 0.1$) or medium ($P > 0.1$). However, there was a medium \times oxygen interaction ($P < 0.05$). For oocytes matured in mTCM-199, development was greater in high oxygen than in low oxygen. For oocytes matured in mSOF, in contrast, the percentage of oocytes that became blastocysts was not affected by oxygen.

Although the overall glucose \times oxygen interaction was not significant ($P > 0.1$), sorted analysis for each glucose concentration revealed that development of oocytes to the blastocyst stage was higher in high oxygen compared to low oxygen when 5.6 mM glucose was used ($41.4 \pm 3.3\%$ versus $25.5 \pm 3.3\%$; $P < 0.05$) but not when 20 mM glucose was used ($30.9 \pm 2.8\%$ versus $30.5 \pm 2.8\%$). Data were also analyzed separately for each oxygen concentration. For oocytes matured in high oxygen, those cultured in 5.6 mM glucose yielded a higher percentage of blastocysts than those cultured in 20 mM glucose ($41.4 \pm 3.0\%$ versus $30.9 \pm 3.0\%$; $P = 0.06$). The percentage of oocytes that became blastocysts was not affected by glucose concentration when oocytes were in low oxygen ($25.5 \pm 2.1\%$ for 5.6 mM versus $30.5 \pm 2.1\%$ for 20 mM; $P > 0.1$).

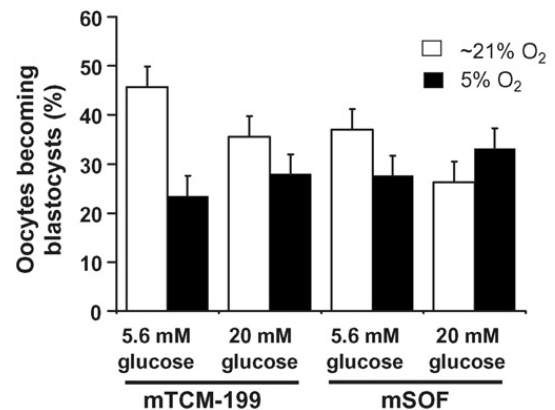


Fig. 1. Least-squares means (\pm S.E.M.) for percentage of bovine oocytes developing to the blastocyst stage on Day 8 after fertilization, as determined by maturation medium, glucose concentration, and oxygen environment. Oocytes were matured for \sim 22 h in modified TCM-199 or modified SOF in the presence of 5.6 or 20 mM glucose in either high (\sim 21%) or low (5%) oxygen environment. The percentage of oocytes becoming blastocysts was affected by the medium \times oxygen interaction ($P < 0.05$) because oxygen was a greater determinant of development to the blastocyst stage for mTCM-199. Also, blastocyst development was higher in high oxygen compared to low oxygen when 5.6 mM glucose was used ($P < 0.05$), but not when 20 mM glucose was used ($P > 0.10$). Oocytes cultured in 5.6 mM glucose yielded a higher percentage of blastocysts than those cultured in 20 mM glucose when culture was in high oxygen ($P = 0.06$), but not when culture was in low oxygen.

3.2. Effect of oxygen and glucose concentration on heat shock effects during oocyte maturation

In this experiment, mSOF was used for maturation because there was no effect of oxygen on the proportion of oocytes becoming blastocysts for this medium in the first experiment. Overall, heat shock during maturation slightly but significantly ($P < 0.05$) reduced cleavage rate ($69.1 \pm 1.2\%$ versus $65.6 \pm 1.2\%$ for 38.5 and 41 °C, respectively) and the percentage of oocytes becoming blastocysts ($36.7 \pm 1.1\%$ versus $32.5 \pm 1.1\%$; $P < 0.05$; Fig. 2). The percentage of cleaved embryos becoming blastocysts was also reduced by heat shock, but this effect was of a lower magnitude and only approached significance ($52.9 \pm 1.5\%$ versus $48.9 \pm 1.5\%$; $P < 0.1$). Oocytes matured in the presence of 5.6 mM glucose had decreased development in low oxygen compared to high oxygen but development was not affected by oxygen when 20 mM glucose was used (oxygen \times glucose for percentage of oocytes becoming blastocysts and percentage of cleaved embryos becoming blastocysts, $P < 0.01$).

Although the overall effect of temperature was significant and there were no interactions with temperature, there was no numerical difference in cleavage or blastocyst development between 38.5 and

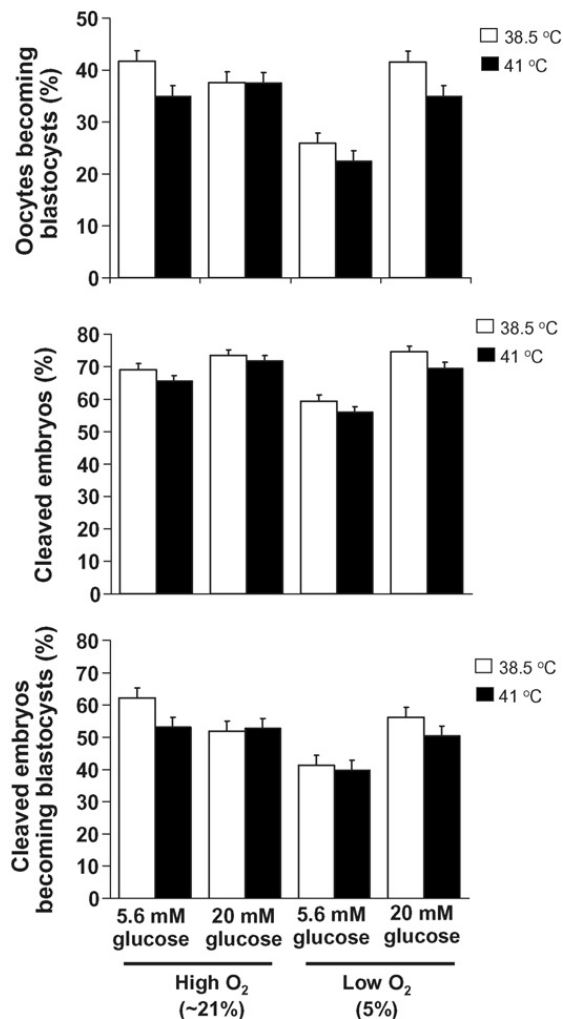


Fig. 2. Least-squares means (\pm S.E.M.) for the percentage of oocytes developing to the blastocyst stage on Day 8 after fertilization (A), percentage oocytes cleaving (B) and percentage of cleaved embryos becoming blastocysts (C), as determined by incubation temperature, glucose concentration, and oxygen environment. Oocytes were heat shocked, or not, for the first 12 h of maturation in modified SOF in the presence of 5.6 or 20 mM glucose in either a high or low oxygen environment. Overall, heat shock during maturation reduced ($P < 0.05$) the percentage of oocytes becoming blastocysts (A) and cleavage rate (B). Heat shock also tended ($P < 0.1$) to reduce the percentage of cleaved embryos becoming blastocysts (C). Oocytes matured in the presence of 5.6 mM glucose had decreased development in low oxygen compared to high oxygen, but development was not affected by oxygen when 20 mM glucose was used [oxygen \times glucose for percentage of oocytes becoming blastocysts (A) and percentage of cleaved embryos becoming blastocysts (C), $P < 0.01$]. Based on separate analyses for oocytes in high oxygen, the percentage of oocytes that cleaved (A) was not affected by heat shock in either 5.6 or 20 mM glucose and the percentage of oocytes becoming blastocysts (B) was reduced when oocytes were matured in 5.6 mM glucose but not when oocytes were matured in 20 mM glucose (temperature \times glucose, $P < 0.07$). Results were similar for percentage of cleaved embryos becoming blastocysts (C) (temperature \times glucose, $P < 0.05$). For oocytes matured in low oxygen, heat shock tended to decrease ($P = 0.07$) the percentage that cleaved (B), decreased ($P = 0.05$) the percentage of oocytes that developed

to the blastocyst stage (A), and did not significantly affect the percentage of cleaved embryos becoming blastocysts (C). For oocytes in low oxygen, there were no significant temperature \times glucose interactions.

41 °C when 20 mM glucose was used in high oxygen. To address this observation, further analysis was performed by determining how glucose concentration altered the magnitude of heat shock effects at each oxygen concentration. In high oxygen, the percentage of oocytes that cleaved was not affected by heat shock when oocytes were matured in either 5.6 or 20 mM glucose (see Fig. 2; temperature, $P > 0.1$). The percentage of oocytes becoming blastocysts was reduced when oocytes were matured in 5.6 mM glucose, but not when oocytes were matured in 20 mM glucose (temperature \times glucose, $P < 0.07$). Results were similar for percentage of cleaved embryos becoming blastocysts (temperature \times glucose, $P < 0.05$). For oocytes matured in low oxygen, heat shock tended to decrease the percentage that cleaved ($P = 0.07$) and decreased the percentage of oocytes that developed to the blastocyst stage ($P = 0.05$). However, there were no significant temperature \times glucose interactions for either of these variables, i.e. the effect of heat shock was independent of glucose concentration. The proportion of cleaved embryos becoming blastocysts was not affected by temperature or temperature \times glucose.

4. Discussion

Based on the first experiment, we concluded that effectiveness of culture systems for supporting oocyte maturation depended upon interactions between medium, oxygen tension and glucose concentration. In general, blastocyst yield was reduced if maturation was performed under a low oxygen environment, with the only exception being maturation in mSOF in 20 mM glucose. This effect of oxygen and its modulation by glucose concentration for mSOF was apparent in both experiments. The superiority of a high oxygen environment is likely related to oxygen availability to the oocyte and cumulus cells. When embryoid bodies were cultured under high oxygen, cells from the center of the bodies had about 30% less available oxygen than cells in the periphery [28]. Therefore, when the number of cells is high and oxygen is limiting, i.e. as under the low oxygen atmosphere used in the present studies, it is likely that cumulus cells in close contact with the oocyte and the oocyte itself experience a deficit in oxygen availability. Hypoxia results in uncoupling of electron transfer and oxidative phosphorylation in the mitochondria, accumulation of pyruvate, and a shift to glycolysis

to the blastocyst stage (B), and did not significantly affect the percentage of cleaved embryos becoming blastocysts (C). For oocytes in low oxygen, there were no significant temperature \times glucose interactions.

[29,30]. Because glycolysis is less efficient than oxidative phosphorylation for ATP production, it is likely that ATP production by the oocyte becomes insufficient in oxygen-restricted environments. Indeed, oocyte ATP content was reduced in oocytes matured in 5% oxygen as compared to those matured in atmospheric oxygen [22].

Under low oxygen conditions, raising glucose concentration from 5.6 to 20 mM increased the proportion of oocytes that became blastocysts when the maturation medium was mSOF. There were no deleterious effects of maturing oocytes under low oxygen when the maturation medium was mSOF containing 20 mM glucose. Hashimoto et al. [22] also reported that increasing glucose concentration in an SOF-based maturation medium from 1.5 to 20 mM increased ATP content and the proportion of oocytes that reached metaphase II. The addition of glucose at 20 mM did not reverse the detrimental effects of maturation in low oxygen when mTCM-199 was the maturation medium. It was unclear why raising glucose concentration did not improve oocyte competence for development for oocytes matured with mTCM-199, but it may be related to the redox status of the oocyte. Under high oxygen conditions, raising glucose concentration in maturation medium can increase production of reactive oxygen species and decrease oocyte glutathione content [31]. Indeed, under high oxygen conditions, high glucose concentrations were detrimental to oocyte maturation as determined by subsequent blastocyst yield in the present study. Perhaps production of reactive oxygen species was higher for oocytes matured with mTCM-199 than for oocytes matured with mSOF; therefore, beneficial effects of raising glucose concentrations under low oxygen on ATP synthesis were counteracted by effects on free radical metabolism.

Effects of oxygen and glucose concentrations on the magnitude of effects of heat shock were tested using mSOF, as oxygen effects were less when compared to oxygen effect using mTCM-199. Using this experimental model, previous observations that heat shock during maturation reduces oocyte competence for fertilization and development [12–14,16,17,23] were confirmed. In particular, heat shock reduced the proportion of oocytes that cleaved and the proportion of cleaved embryos that became blastocysts. Furthermore, the effects of heat shock did not depend upon oxygen concentration and glucose concentration reduced effects of heat shock only in a high oxygen environment.

That heat shock reduced oocyte competence under conditions of high or low oxygen indicated that damage

to the cultured oocyte by heat shock does not require a high-oxygen environment. In contrast, in preimplantation embryos, the effects of heat shock were exacerbated when culture is in high oxygen [21]. One explanation for this difference is that the cell density is greater in cultures of maturing oocytes than for cultures of preimplantation embryos (because of the presence of cumulus cells in the former); this increased density may make the generation of free radicals by heat shock less dependent upon oxygen concentration.

One surprising observation was that effects of heat shock on oocyte competence were eliminated when oocytes matured in high oxygen (but not low oxygen) were cultured with 20 mM glucose. One should be careful in ascribing a thermoprotective effect to high glucose, however, because culture in 20 mM glucose tended to reduce oocyte competence for blastocyst development in high oxygen. Perhaps the oocyte populations being damaged by heat shock were the same ones damaged by elevated glucose concentrations so that no additive effects were seen when both stresses were combined.

Except for oocytes matured using 20 mM glucose in high oxygen, where no reduction was observed, the magnitude of reduction in blastocyst development caused by heat shock (expressed as a percentage of development at 38.5 °C) ranged from 14 to 17%. This magnitude of heat shock effect was less than for many studies in which a similar heat shock was applied during development. We hypothesize that the magnitude of heat shock effects during maturation on oocyte competence depends upon the suitability of the culture system for supporting development. Effects of heat shock were reduced in systems where the proportion of oocytes becoming blastocysts was high. In the present study, the proportion of oocytes that developed to the blastocyst stage at 38.5 °C was high, ranging from 38 to 42% for all treatments but one (5.6 mM glucose in low oxygen) and the magnitude of effects of heat shock were low. Similarly, Edwards and Hansen [32], using a similar exposure to heat shock, found that 46% of non heat-shocked oocytes became blastocysts and the reduction in development caused by heat shock was 11%. In studies in which the percentage of oocytes that became blastocysts in the absence of heat shock ranged from only 20 to 30%, the reduction in development caused by heat shock was approximately 40 to 65% [12,16,17,23].

Based on the present study, heat shock can damage oocyte competence for development to the blastocyst stage after insemination under an oxygen tension similar to that in the follicle and oviduct. Whether such

damage occurs in vivo may depend on other factors that affect embryonic capacity for development. In particular, heat stress may be more likely to compromise the process of oocyte maturation when either (1) the oocyte was exposed before maturation to conditions that reduce oocyte potential for development (including heat stress and other factors such as low nutrition [4–7,33]), or (2) the subsequent embryo is exposed to conditions after fertilization that limit embryonic development.

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