Retinol Improves Development of Bovine Oocytes Compromised by Heat Stress During Maturation

J. L. Lawrence, R. R. Payton, J. D. Godkin, A. M. Saxton,
F. N. Schrick, and J. L. Edwards

Department of Animal Science, The University of Tennessee,
Knoxville 37996-4574

ABSTRACT

The objectives of this study were to evaluate: 1) effects of a physiologically relevant elevated temperature on in vitro development of maturing oocytes, 2) effects of retinol on in vitro development of maturing oocytes, and 3) effects of retinol to improve development of oocytes compromised by an elevated temperature. Bovine oocytes were matured for 24 h at 38.5 or 41.0°C (first 12 h) in 0 or 5 μM retinol. After insemination, cleavage and blastocyst development were assessed on d 3 and 8, respectively. Temperature, retinol, and their interaction were included in the statistical model. Culture of oocytes at 41.0°C decreased the proportion of 8- to 16-cell embryos and increased that of 2-cell embryos. In addition, culture at 41.0°C decreased the ability of oocytes to develop to the blastocyst stage. Blastocysts derived from oocytes cultured at 41.0°C had fewer total nuclei. In 3 of the 7 experimental replicates, effects of 41.0°C to reduce blastocyst development were minimal (difference in the development of the control vs. heat stress group was <20%). To provide a more precise test of our hypothesis (retinol administration may improve development of oocytes compromised by heat stress), data were analyzed, including only those replicates ($n = 4$) in which heat stress reduced development to blastocyst >20%. When this was done, a significant temperature $\times$ retinol interaction was noted. The addition of retinol to the maturation medium prevented heat-induced reductions in development of oocytes to blastocyst stage. Results indicate that retinol may protect oocytes from some of the deleterious effects of heat stress.

(Key words: heat stress, reproduction, bovine oocyte maturation, retinol)

Abbreviation key: FBS = fetal bovine serum, IVF = in vitro fertilization, KSOM = potassium simplex optimized medium, NEAA = nonessential amino acids,

OMM = oocyte maturation medium, PZ = presumptive zygotes.

INTRODUCTION

Seasonal periods of heat stress reduce milk yields and fertility in lactating dairy cows (Hansen and Aréchiga, 1999). Exposure of Holstein heifers to elevated ambient temperatures during estrus increased the proportion of retarded and degenerated embryos recovered from the uterus 7 d after superovulation and insemination (Putney et al., 1989). These results indicated that the oocyte is very susceptible to heat stress while preparing for fertilization. Negative effects of heat stress to decrease continued development of the oocyte are in large part due to direct effects of elevated temperatures. Edwards and Hansen (1996, 1997) cultured maturing bovine oocytes at 41.0°C and noted direct effects of elevated temperature to reduce blastocyst development. Mechanisms responsible for heat-induced reductions in the development of maturing oocytes are poorly defined. However, reduced intracellular protein synthesis (Edwards and Hansen, 1996, 1997), altered cortical granule types (Payton et al., 2003b), and increased glutathione content (Payton et al., 2003a) suggest possible alterations in the cytoskeleton and increases in free radical production.

Vitamin A and other natural retinoids have been shown to improve reproduction in heat-stressed dairy cows and the development of otherwise developmentally incompetent oocytes (Livingston et al., 2002). For example, supplementing postpartum lactating dairy cow diets with β -carotene during the summer increased pregnancy rates in the fall (Aréchiga et al., 1998). Injections of retinol palmitate (a metabolite of β -carotene) in combination with superovulation (which has been shown to reduce the developmental competence of oocytes; Merton et al., 2003) increased the mean number of quality grade 1 and 2 bovine embryos (Shaw et al., 1995). Further, administering retinol to ewes during superovulation followed by natural service increased the ability of recovered embryos to develop in vitro (Eberhardt et al., 1999). Livingston et al. (2002) reported

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Corresponding author: J. L. Edwards; e-mail: jedwards@utk.edu.

that retinol administration during maturation improved the competence of otherwise developmentally challenged oocytes to develop to the blastocyst stage (development of control oocytes to blastocyst after IVF was less than 20%). Taken together, the working hypothesis of this study was that retinol administration during maturation might improve development of oocytes compromised by heat stress. The objectives of this study were to evaluate: 1) effects of an elevated temperature, observed in heat-stressed dairy cows, on the development of maturing oocytes; 2) effects of retinol on the development of maturing oocytes; and 3) effects of retinol to improve development of oocytes compromised by elevated temperature.

MATERIALS AND METHODS

Materials

All reagents and chemicals used for in vitro production of bovine embryos were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Modified Tyrode's Albumin Lactate Pyruvate (HEPES-TALP, in vitro fertilization [IVF]-TALP, and SPERM-TALP [Parrish et al., 1988]), and potassium simplex optimized medium (**KSOM**; Biggers et al., 2000) were prepared in the laboratory or purchased from Specialty Media, Inc. (Phillipsburg, NJ). Harrogate Genetics, Inc. (Harrogate, TN) donated frozen semen. Bovine ovaries, from which oocytes were obtained, were purchased from an abattoir (Gaffney, SC) between the months of March and May. *Bos indicus* cattle represented a small proportion (<1%) of the females processed. Medium-199, gentamicin, penicillin-streptomycin, and nonessential amino acids (**NEAA**) were purchased from Specialty Media, Inc. (Phillipsburg, NJ). Fetal bovine serum (**FBS**) was obtained from BioWhittaker (Walkersville, MD). Follicle stimulating hormone (Folltropin-V) was obtained from VetrepHarm, Canada, Inc. (London, Ontario). Luteinizing hormone was generously provided by the USDA (Beltsville, MD). All *trans*-retinol was purchased from Sigma and was solubilized in ethanol (Aaper Alcohol Ethyl Alcohol USP Absolute 200 Proof) before storing at -80°C as a 20 mM stock. Concentration of each stock (range 12 to 24 mM) was verified using a UV-spectrometer and reading the absorbance in triplicate (wavelength = 325 nm). Calculations to dilute the retinol stock to 5 μM were based on the average absorbance.

In Vitro Production of Embryos

In vitro production of embryos was performed as previously described by Edwards and Hansen (1996) with some modifications. Oocytes were harvested from an-

tral follicles ranging from 3 to 8 mm in diameter by making checkerboard incisions approximately 2 mm in length and width on the ovarian surface. After evaluation, only oocytes having an evenly dark and granulated ooplasm and compact cumulus were selected for maturation. Oocytes were cultured in an oocyte maturation medium (**OMM**) consisting of Medium-199 containing Earle's salts, 10% FBS, 50.0 $\mu\text{g}/\text{mL}$ gentamicin, FSH (5.0 $\mu\text{g}/\text{mL}$), LH (0.3 $\mu\text{g}/\text{mL}$), 0.2 mM Na pyruvate, and 2 mM L-glutamine for 24 h at 38.5°C in 5.5% CO_2 in humidified air. Oocytes, presumed mature, were fertilized with frozen-thawed Percoll-prepared sperm (750,000 sperm/mL; pool of 2 individual bulls) and cultured at 38.5°C in 5.5% CO_2 in humidified air. Pooled semen from the same 2 bulls was used for IVF in each experimental replicate. Approximately 13 to 17 h after addition of sperm, presumptive zygotes (**PZ**) were denuded of cumulus and associated spermatozoa by vortexing. Presumptive zygotes were cultured in KSOM containing 0.5% BSA, 1 $\times$ NEAA, penicillin (50 U/mL), and streptomycin (50 $\mu\text{g}/\text{mL}$) at 38.5°C in 5.5% CO_2 , 7.0% O_2 , and 87.5% N_2 in humidified air for 8 d after IVF. The ability of PZ to cleave and develop to the blastocyst stage was evaluated on d 3 and 8, respectively (d 0 = day of IVF). The ability of the oocyte to cleave after IVF was assessed by recording the number of 1-, 2-, 4-, and 8- to 16-cell embryos present on d 3 (70 to 75 h postinsemination). Blastocyst stage embryos (d 8; 187 to 195 h postinsemination) were stained with Hoechst 33342 (5 $\mu\text{g}/\text{mL}$; 15 min), mounted on a glass slide (50% glycerol), and exposed to UV light directed through a DAPI filter (excites at a wavelength of 330 to 380 nm) to enumerate the total number of nuclei contained within each embryo.

Developmental Competence of Bovine Oocytes Matured at an Elevated Temperature in the Presence of Retinol

For this study, oocytes were matured at 38.5°C for 24 h or 41.0°C (first 12 h, 38.5°C thereafter) in 0 or 5 μM retinol. Ethanol was added to the OMM at the same volume as the retinol stock to provide a diluent control (0.03%, vol/vol, final concentration). Use of 41.0°C was appropriate, as rectal temperatures of lactating dairy cows may reach or exceed this temperature (Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993).

Variables of interest that were recorded throughout the experiment included number of PZ recovered after denuding oocytes of cumulus and associated spermatozoa, number of PZ that were visibly lysed after denuding, and number of PZ that cleaved and developed to blastocyst stage. Number of nuclei in resulting blasto-

cysts also was recorded. Two independent evaluators, blind to treatments, assessed variables of interest.

To examine effects of elevated temperature in combination with retinol during maturation to activate oocytes in the absence of sperm (induce parthenogenesis), 2 additional subsets of oocytes were cultured concurrent with remaining treatments: 1) oocytes were matured at 38.5°C for 24 h in OMM ($n = 153$), and 2) oocytes were matured at 41.0°C (first 12 h of culture; 38.5°C thereafter) in combination with 5 μM retinol ($n = 145$). Thereafter, oocytes were treated in a manner similar to those mentioned previously, with the exception that they were not inseminated. Development to blastocyst stage was recorded.

Statistical Analyses

Data were analyzed as a randomized block design, blocked on day of oocyte collection, using mixed models (SAS, 2001). Fixed effects of temperature, retinol, and their interaction were evaluated. The experimental unit was a group of oocytes receiving the same treatment within replicate, and block $\times$ treatment interactions were used as the correct error term. This experiment was replicated on 7 different days (a total of 323 to 345 oocytes were included in each of the 4 treatments). Data were tested for normality (Shapiro-Wilk test; $W \geq 0.90$). Protected least significant differences were used for separating least square means.

RESULTS

The culture of oocytes at 41.0°C did not affect proportions of PZ recovered after removal of associated cumulus and spermatozoa (94.4 and 89.8% for 38.5 and 41.0°C, respectively; SEM = 2.2; $P > 0.15$) or those that had visibly lysed (1.6 and 2.1% for 38.5 and 41.0°C, respectively; SEM = 0.8; $P > 0.60$). In addition, the ability of oocytes to cleave after the addition of sperm was not compromised (SEM = 3.8; $P > 0.2$; Figure 1) when matured at 41.0°C during the first 12 h of culture. However, oocyte culture at 41.0°C increased proportions of 2-cell embryos (SEM = 1.3; $P < 0.04$; Figure 1), but decreased the proportion of 8- to 16-cell (SEM = 4.7; $P < 0.05$; Figure 1) and blastocyst-stage embryos (SEM = 2.2; $P < 0.007$; Figure 1). Moreover, blastocysts derived from heat-stressed oocytes had fewer total nuclei (SEM = 6.7; $P < 0.005$; Figure 1).

The addition of 5 μM retinol to the OMM did not alter the proportion of PZ recovered after removal of associated cumulus and spermatozoa (93.1 and 91.1% for 0 and 5 μM ; SEM = 2.2; $P > 0.54$) or those that had visibly lysed (1.5 and 2.2% for 0 and 5 μM ; SEM = 0.8; $P > 0.55$). The ability of PZ to cleave (SEM = 3.8; $P >$

Figure 1. Development (%) of oocytes matured at 38.5°C (control, □) or 41°C (heat stress, ■). After in vitro fertilization, ability of oocytes to cleave was assessed between 70 to 75 h postinsemination. Presence of more than one cell was indicative of cleavage, and the proportion of 2-, 4-, and 8- to 16-cell embryos was recorded. Between 187 and 195 h postinsemination, proportions of oocytes that developed to blastocyst were recorded. Thereafter, total number of cells was estimated in individual blastocysts by counting the number of Hoechst-stained nuclei. * $P < 0.05$.

0.9; Figure 2), develop to the 8- to 16-cell (SEM = 4.7; $P > 0.6$; Figure 2) or blastocyst (SEM = 2.2; $P > 0.5$; Figure 2) stages after IVF was similar for control and retinol-treated oocytes. Further, the number of nuclei was similar within blastocysts derived from oocytes cultured in retinol and the diluent control (SEM = 6.7; $P > 0.7$; Figure 2).

Overall there was no temperature $\times$ retinol interaction detected ($P > 0.9$). In 3 of the experimental replicates, however, effects of 41.0°C to reduce blastocyst development were minimal (the percent difference in the control vs. heat stress treatment was $<20\%$). To have a more precise test of our hypothesis (retinol ad-

Figure 2. Development (%) of oocytes matured in 0 μM (control, □) or 5 μM retinol (■). After in vitro fertilization, the ability of oocytes to cleave was assessed between 70 to 75 h postinsemination. Presence of more than one cell was indicative of cleavage, and the proportion of 2-, 4- and 8- to 16-cell embryos were recorded. Between 187 and 195 h postinsemination, the proportion of oocytes developing to blastocyst stage and the number of nuclei (or cell number) was recorded.

Figure 3. The proportion (%) of oocytes developing to blastocyst stage after culture at 38.5 or 41.0°C without (0) or with 5 μ M all trans-retinol. *Temperature $\times$ retinol interaction; * $P < 0.05$.

ministration during oocyte maturation may improve development of oocytes compromised by heat stress), only those experimental replicates ($n = 4$) in which percentage blastocyst difference in the control vs. heat-stressed oocytes was $>20\%$ were included in the statistical analysis. When this was done, a significant temperature $\times$ retinol interaction was noted ($n = 4$ replicates; 189 to 197 oocytes per treatment; SEM = 2.4; $P < 0.03$; Figure 3).

Culture of oocytes at 41.0°C with 5 μ M retinol did not increase parthenogenic activation. Blastocyst development of unfertilized oocytes cultured at 41.0°C with 5 μ M retinol ($4.1 \pm 1.6\%$) was similar to those cultured at 38°C in OMM for 24 h ($2.6 \pm 1.3\%$).

DISCUSSION

Culture of bovine oocytes at an elevated temperature observed in heat-stressed dairy cows (41.0°C; Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993) disrupted further development after IVF by increasing proportions of embryos arresting at the 2-cell stage and decreasing proportions of embryos that developed to the 8- to 16-cell and blastocyst stages. Moreover, blastocyst-stage embryos derived from oocytes that were heat-stressed during maturation had fewer total nuclei. Retinol administration during oocyte maturation ameliorated the negative effects of heat stress to reduce embryo development after fertilization.

Heat-induced reductions in oocyte development are in agreement with previous studies. Edwards and Han-

sen (1996, 1997) showed that culture of bovine oocytes at 41.0°C during the first 12 h of maturation did not alter their ability to cleave into more than one cell, but reduced continued development to the blastocyst stage after IVF by 50 to 65%. Reduced cell number in blastocysts derived from oocytes exposed to a physiologically relevant elevated temperature has been reported previously (Putney et al., 1989), documenting that heat-induced perturbations occurring in the oocyte carry over to the early embryo.

The magnitude of the effects of heat stress to reduce oocyte development varied among individual replicates (7.1 to 53.4%), with negative effects being less prominent when the development of control oocytes was greater than usual ($>34\%$). The explanation for the disparate effects of heat stress may be related to inherent differences among individual batches of oocytes related to genotype (Rocha et al., 1998), individual donors (Tammassia et al., 2003), physiological status of donor cows (Armstrong et al., 2003), subtle differences in ovary collection, shipment and oocyte evaluation, and/or other unidentified factors.

Specific mechanisms accounting for heat-induced reductions in oocyte development remain unclear. Oocytes compromised by heat stress progress to metaphase II at a rate comparable to controls (Payton et al., 2003b) but synthesize fewer intracellular proteins (Edwards and Hansen, 1996, 1997), have increased glutathione content (Payton et al., 2003a), and undergo cytoplasmic maturation faster than controls (cortical granule translocation to oolemma; Payton et al., 2003b), suggesting possible alterations in the cytoskeleton as well as increased free radical production.

Heat-induced reductions in the development of oocytes were no longer evident when 5 μ M retinol was added to the OMM. Beneficial effects of retinol were noted only when the effects of heat stress were pronounced enough to reduce continued development of oocytes by at least 20% of controls. Beneficial effects of adding retinol to maturation medium for increasing development of otherwise "stressed" oocytes were observed previously (i.e., when development of control oocytes was $<20\%$; Livingston et al., 2002).

Specific mechanisms through which retinoids increased development of oocytes compromised by heat stress remain unclear. Retinoids may act as antioxidants by scavenging free radicals and preventing lipid peroxidation (Kantha and Krishnamurthy, 1977; Ciaccio et al., 1993). Ciaccio et al. (1993) showed that administration of vitamin A to rats increased concentrations of retinol and retinyl esters in cell membranes of the brain and heart, and doing so was associated with heightened resistance of membrane lipids to oxidative stress. In other cell types, retinoid binding to retinoic

acid receptors upregulates the transcription of antioxidant genes, including superoxide dismutase (Ahlemeyer et al., 2001) and glutathione S-transferase (Lo and Ali-Osman, 1997).

Retinoid receptors are present in the cumulus oocyte complex (retinoic acid receptor and retinoid X receptor; Mohan et al., 2001, 2002). Additional analyses have shown that both types of nuclear receptors, as well as retinal-binding proteins, are localized predominantly within the cumulus cells surrounding the oocyte (Mayer, personal communication). Cumulus cells are transcriptionally active (Tirone et al., 1997) and project through the zona pellucida as well as the oolemma as a means of establishing an intercellular bidirectional form of communication between the oocyte and the cumulus cells (reviewed by Eppig, 1994). As such, positive effects of retinol to improve the development of oocytes compromised by heat stress may be receptor mediated. Mechanistic effects thereafter are unclear.

Determining whether heat stress induces parthenogenesis was important because Komar (1973) reported that murine oocytes exposed to extreme elevations in temperature (44.0 to 45.0°C) underwent parthenogenesis. Results from our study showed that oocyte culture at 41°C during retinol administration did not increase parthenogenesis.

CONCLUSIONS

Retinol administration during oocyte maturation may protect bovine oocytes from harmful effects of heat stress to reduce oocyte quality. Further study on the mechanisms of retinol to improve development of oocytes compromised by heat stress is warranted. Doing so could lead to the development of therapeutic strategies for dairy producers faced with problems of heat stress to decrease oocyte quality.

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