To the Graduate Council:

I am submitting herewith a thesis written by Janelle L. Lawrence entitled "Retinol Improves Development of Heat-Stressed Oocytes During Maturation". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

J. Lannett Edwards, Major Professor

We have read this thesis and recommend its acceptance:

F. Neal Schrick

James D. Godkin

Acceptance for the Council:

Vice Provost and Dean of Graduate Studies

RETINOL IMPROVES DEVELOPMENT OF HEAT-STRESSED OOCYTES DURING MATURATION

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Janelle Leigh Lawrence
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DEDICATION

This thesis is dedicated to my parents Janine and Gene Lawrence, my brother Jordan, my grandparents Ferne and Bernard Savoie, and Mary and Richard Lawrence and the rest of my family for supporting and believing in me daily.

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ABSTRACT

Addition of retinol may improve development of maturing oocytes stressed by culture. Objective was to evaluate retinol for improving development of heat-stressed oocytes. Cumulus oocyte complexes (COC) were matured for 24 h at 38.5 or 41.0°C (first 12 h) in 0 or 5 μM all trans-retinol. Ability of putative zygotes (268-297/treatment) to cleave and develop to blastocyst was assessed on days 3 and 8 after fertilization, respectively. Fixed effects in statistical model included temperature, retinol, and the interaction. Data were tested to ensure normality. Culture of COC at 41.0°C did not affect lysis (1.6 and 2.1% for 38.5 and 41.0°C; SEM=0.8) or cleavage after fertilization (75.4 and 72.2% for 38.5 and 41.0°C; SEM=3.8). Within cleaved embryos however, culture of COC at 41.0°C increased 2-cell (10.6 versus 6.2% for 41.0 and 38.5°C; SEM=1.3; $P<0.04$) but decreased 8-16 cell (41.4 versus 51.1% for 41.0 and 38.5°C, SEM=4.7; $P<0.05$) embryos and development to blastocyst (20.0 vs 27.5% for 41.0 and 38.5°C, SEM=2.2; $P<0.007$). Blastocysts derived from heat-stressed COC had fewer nuclei (85.3 versus 94.0 for 41.0 and 38.5°C; SEM=6.7; $P<0.005$; Hoechst staining). Addition of retinol during maturation did not improve development of COC regardless of culture temperature. In three replicates, effects of 41.0°C for reducing development of COC to blastocyst were minimal. When these replicates were deleted from analysis, a significant temperature x retinol interaction was observed (n=4 reps; 189-197 COC/treatment) for development to blastocyst ($P<0.01$) and number of nuclei ($P<0.004$). Addition of retinol to COC compromised by heat stress during maturation prevented heat-induced reductions in development to blastocyst (24.6 versus 14.7% for 38.5 and 41.0° C; and 23.2 and 22.0% for 38.5 and 41.0°C cultured in presence of 5 μM retinol;

SEM=2.4). Also, culture of COC at 38.5°C in 5 μM retinol increased number of nuclei in blastocysts (102.6 versus 98.7 for 38.5 C and 41.0°C; and 120.5 versus 95.1 for 38.5 and 41.0°C cultured in 5 μM retinol; SEM=5.0). Retinol may protect oocytes that are heat-stressed during maturation from the deleterious effects of elevated temperature.

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NOMENCLATURE

°C	Celsius
d	days
h	hour(s)
kDa	kilodalton
kg	kilogram
l	liter
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
mg	milligram
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
nmol	nanomol
rpm	revolutions per minute
U	unit

Abbreviations

BSO	buthionine sulfoximine
bST	bovine somatotropin
Ca^{2+}	calcium
CG	cortical granule
cAMP	cyclic adenosine monophosphate
CO_2	atmospheric carbon dioxide
COC	cumulus oocyte complexes
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HEPES	N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid
hCG	human choronic gonadotropin
H_2O_2	hydrogen peroxide
HSP	heat stress proteins
IU	international units
IVM	in vitro maturation
IVF	in vitro fertilization
KSOM	potassium simplex optimized medium
LH	luteinizing hormone

MI	metaphase I
MII	metaphase II
MAP	mitogen-activated protein kinase
MPF	maturation promoting factor
mRNA	messenger ribonucleic acid
NEAA	non-essential amino acids
OMM	oocyte maturation medium
O_2	atmospheric oxygen
O_2^-	superoxide anion radical
PZ	presumptive zygotes
RA	retinoic acid
RAR	retinoic acid receptor
RH	relative humidity
RNA	ribonucleic acid
RXR	retinoid X receptor
PMSG	pregnant mares serum gonadotropin
SEM	standard error of the means
TALP	Tyrode's Albumin Lactate Pyruvate

CHAPTER 1

INTRODUCTION

Seasonal periods of heat stress are one of the central causes of depressed milk yields (Roman-Ponce et al., 1977) and reduced fertility in lactating dairy cattle (Badinga et al., 1985, du Preez et al., 1991). Reduced fertility is of particular concern for lactating dairy cows (Badinga et al., 1985) as ambient temperatures increase from 20-35°C (Cavestany et al., 1985). Some of the negative effects of heat stress may be due to heat-induced elevations in body temperatures (39.6-41.0°C; Monty and Wolff, 1974; Collier et al., 1982; Wise et al., 1988; Wilson et al., 1998). Elevated rectal temperatures have been negatively correlated with fertility (Dunlap and Vincent, 1971). In fact, reduced fertility has been reported with a rise in temperature of 1°C (Ulberg and Burfening, 1967). The negative effects of heat stress on reducing reproduction occur very early (periestrual period; Putney et al., 1989). Specifically, exposure of heifers to high ambient temperatures during oocyte maturation increased proportion of retarded embryos recovered 7 days after insemination (Putney et al., 1989). Some of the negative effects of heat stress for reducing reproduction in dairy cattle may be due to direct effects of elevated temperature on the maturing oocyte. Maturing oocytes at 41.0°C for the first 12 hours of maturation reduced protein synthesis, (Edwards and Hansen, 1996), altered cortical granule types (Payton et al., 2003d), increased glutathione content (Payton et al., 2003b) and decreased subsequent development to blastocyst after fertilization (Edwards and Hansen, 1996, 1997). Given that some of the negative effects of elevated temperature may be to increase free radical production within cells (Loven, 1988),

administration of antioxidants may ameliorate some of the reported negative effects on maturing oocytes or preimplantation embryos. The use of retinoids as antioxidants has been well-documented (reviewed by Palace et al., 1999). Retinol palmitate (a metabolite of β-carotene) injected 5-7 days prior to induced estrus in beef cattle in combination with superovulation increased the number of high quality embryos collected on day 8 (Shaw et al., 1995). Administration of all trans-retinol to ewes during superovulation followed by natural service improved ability of recovered embryos to continue in development from the 1-4 cell and morula stages to blastocyst and hatched blastocyst stages in vitro (Eberhardt et al., 1999). The presence of all trans-retinol during maturation improved developmental competence of oocytes "stressed" by culture environment (Livingston et al., 2002). Interestingly, retinoids may be beneficial when given during a time when animals are experiencing a stress such as high environmental temperature. Specifically, lactating Holstein cows fed supplemental β-carotene (400 mg/d/cow) for at least 90 d beginning approximately 15-30 days after calving during the summer and early fall had a higher pregnancy rates at 120 d postpartum than control cows (Aréchiga et al., 1998a). This indicates that heat-stressed lactating dairy cows fed diets supplemented with β-carotene have improved fertility. The *central hypothesis* of the proposed research is that administration of retinol at the time of heat shock may protect oocytes from negative effects of elevated temperature. To test the central hypothesis and complete the overall objective of this thesis, the following *research objective* was pursued:

- ***To evaluate development of oocytes matured at an elevated temperature in the presence of retinol.*** Based on previous work (Edwards and Hansen, 1996;

Eberhardt et al., 1999; Livingston et al., 2002; Shaw et al., 1995), the working hypothesis for this objective is that administration of retinol during maturation may improve developmental competence of heat-stressed oocytes.

This research will utilize procedures of in vitro production of bovine embryos including in vitro maturation and fertilization of oocytes, and culture of resulting embryos as a means of examining the role of retinol on the development bovine of oocytes cultured at an elevated temperature. Specifically, COC will be matured in the presence (5 μM) or absence (0; diluent control) of all trans-retinol and cultured at 38.5 or 41.0°C (heat shock to be administered during the first 12 h of maturation only). The proposed research is expected to yield the following outcomes: 1) to further characterize the direct effects of elevated temperature on maturing oocytes, 2) to evaluate direct effects of retinol on maturing oocytes, and 3) to determine if there is a beneficial effect of retinol on development of heat-stressed oocytes. Information gained from this study may lead to a preventative strategy for ultimately reducing the negative effects of heat stress in dairy cattle related to quality of oocytes.

Rationale and significance

Rationale of the research

The ***rationale*** for this research is that it provides a means of determining if there is a beneficial effect of retinol for improving development of heat-stressed oocytes. Previous studies have indicated that elevated temperature is detrimental to the development of maturing oocytes both in vivo and in vitro. Exposure of the maturing

oocyte to elevated temperature results in negative changes which occur primarily within the cytoplasm. Production of free radicals may increase within the cytoplasm of maturing oocytes exposed to elevated temperature. The use of retinoids may reduce free radical production. Administration of retinoids has been shown to improve fertility in vivo. Specifically, cows fed supplemental β-carotene during the summer and early fall have higher pregnancy rates (Aréchiga et al., 1998a). Similar retinoid supplementation has been used to improve development of maturing oocytes while in vitro. However, it is still unknown if retinoids can be beneficial to heat-shocked maturing oocytes. Information gained from this study may lead to a preventative strategy for ultimately reducing the negative effects of heat stress in dairy cattle.

Significance of the research

Cows calving in summer months are an economic risk for dairy producers (Britt, 1975). In fact, a cow not pregnant by 90 days postpartum may cost dairy producers greater than $4.00/cow/day (Britt, 1975). Reductions in fertility during hot months of the year may be due to several factors including irregular estrus, improper regulation of hormones, and extended follicular waves (reviewed by Rensis and Scaramuzzi, 2003). In addition, the reductions in fertility during summer months may be due to direct effects of elevations in body temperature above 41.0°C (Gaalaas, 1945; Wegner et al., 1973; Monty and Wolff, 1974; Roman-Ponce et al., 1977; Turner, 1982; Putney et al., 1988, 1989; Elvinger et al., 1991, 1992; Ealy et al., 1993; Wolfenson et al., 1993).

Specifically, oocytes exposed to elevated temperatures during maturation have reduced developmental competence. Heifers exposed to hyperthermic chambers (42°C at

75% relative humidity (RH)) between the onset of estrus and insemination (i.e. when the oocyte is preparing for fertilization) had elevated body temperatures of greater than 41.0°C for 6 hours and after insemination had reduced numbers of good to excellent quality embryos (Putney et al., 1989). However, in vivo studies do not allow separation of maternal and direct effects. Some of the negative effects of heat-stress may be due to direct effects of elevated temperature on the maturing oocyte. Edwards and Hansen (1996) demonstrated that maturing oocytes at 41.0 C for the first 12 hours of maturation reduced protein synthesis and decreased subsequent development to blastocyst after fertilization. Other in vitro studies showed that elevated temperature was detrimental to morphology, cytoplasm and nuclear progression of the maturing oocyte. Given that some of the negative effects of elevated temperature may result in increased free radical production within cells (Loven, 1988), administration of antioxidants may ameliorate some of the reported negative effects on maturing oocytes. Retinoids have been described as antioxidants (reviewed by Palace et al., 1999) and utilized both in vivo and in vitro for improving development of the maturing oocyte. It is still unknown if retinoids improve development of heat stressed maturing oocytes.

Despite the known effects of heat-stress during oocyte maturation on reducing dairy reproduction, a preventative strategy has to yet to be designed to ultimately reduce the negative effects of heat-stress in dairy cattle. The outcome of the proposed research will determine if there is a beneficial effect of retinol for improving development of heat-stressed oocytes. *The proposed research is significant, because incorporation of the resultant new knowledge may lead to the development of therapeutic strategies for dairy producers faced with problems of heat stress for decreasing oocyte quality.*

CHAPTER 2

REVIEW OF LITERATURE

Effects of heat stress on reproduction

During periods of elevated temperature, it is difficult for females to become pregnant (reviewed by Rensis and Scaramuzzi, 2003). Females exposed to elevated temperature likely have multiple factors contributing to decreased fertility. In the summer, decreased fertility in lactating dairy cattle could be due to a shortened estrus period (8.2 versus 14.0 hours; hot season and cool season, respectively; Wolff and Monty, 1974) or an estrus lacking visible signs essential for proper breeding (reviewed by Rensis and Scaramuzzi, 2003). Inaccurate detection of estrus leads to longer days open (days to first service) and lowered conception rates (reviewed by Rensis and Scaramuzzi, 2003). Another contributor to lowered reproduction in heat-stressed dairy cattle may be improper regulation of hormones (FSH, LH, inhibin, estradiol, progesterone) within the hypothalamic-hypophyseal-ovarian axis (reviewed by Rensis and Scaramuzzi, 2003). In addition, waves of follicular development in summer months are longer, which exposes an immature oocyte (within the dominant follicle) to elevated temperature for a longer period of time (reviewed by Rensis and Scaramuzzi, 2003).

There are negative effects of elevated temperature on the development of gametes and embryos within the female, which can affect premature losses (reviewed by Rensis and Scaramuzzi, 2003). A female may have elevated body temperatures above 41.0°C if exposed to natural hyperthermic conditions (Gaalaas, 1945; Monty and Wolff, 1974; Roman-Ponce et al., 1977; Turner, 1982; Elvinger et al., 1991, 1992; Ealy et al., 1993) or

artificial heat stress (environmental chambers; Wegner et al., 1973; Putney et al., 1988, 1989; Wolfenson et al., 1993). Body temperatures of females may be further elevated with the use of bovine somatotropin (bST; heat stress, 40.5°C and heat stress plus bST, 41.1°C; Elvinger et al., 1992). Prolonged exposure of females to elevated temperature may elevate body temperatures to greater than 42.0°C in a natural environment (42.8°C; Elvinger et al., 1991) as well as in artificially imposed environment (43.3°C; Wegner et al., 1973). Body temperature can exceed 41°C in as little as 4 hours in an artificially imposed environment (Putney et al., 1989) and within 12 hours under natural heat stress (Roman-Ponce et al., 1977). Furthermore, temperatures may stay elevated for 6 hours in heifers (Putney et al., 1989) and for 12 hours in cows (Wegner et al., 1973).

An additional series of events that may be contributing to decreased fertility in heat-stressed dairy cattle may relate to nutrition. Dry matter intake is decreased in heat-stressed cattle, which results in a longer period of negative energy balance (reviewed by Rensis and Scaramuzzi, 2003). Negative energy balance may also alter factors (insulin, glucose and IGF-1) contributing to events related to infertility (reviewed by Rensis and Scaramuzzi, 2003). In summary, infertility during warm summer months may be a combination of several factors within the dairy cow (reviewed by Rensis and Scaramuzzi, 2003). The following sections will discuss in detail the effects of elevated temperature on the female gamete (germinal vesicle-stage oocyte and maturing oocyte) and the effect of elevated temperature on other cell types that may be accounting for decreased fertility in cows during hot months of the year.

Effects of heat stress on germinal vesicle-stage oocytes

Some of the reductions in reproductive capability of heat-stressed animals may be due to effects on the immature (GV-stage) oocytes. Heat stressed GV-stage oocytes exhibit decreased quality and development (Curci et al., 1987, 1991; Rocha et al., 1998; Rutledge et al., 1999; Zeron et al., 2001; Al-Katanani et al., 2002; Payton et al., 2003a, 2003c). The following section is a review of the literature describing the effects of elevated temperatures on altering GV-stage oocytes and their development.

Seasonal changes impact the quality of GV-stage oocytes. The morphology of GV-stage oocytes differs by season. Oocytes during summer months contain non-homogenous cytoplasm with dark regions (~65%) compared to oocytes during the winter in which the cytoplasm is more homogenous and dark (~85%; Zeron et al., 2001). In addition, GV-stage oocytes from summer months differ from winter months when comparing lipid profiles of the biological membranes (Zeron et al., 2001). Specifically, the composition of saturated fatty acids within oocytes was higher and percentage of polyunsaturated fatty acids was lower during summer months compared to winter (Zeron et al., 2001). Furthermore, immature oocytes from ovarian follicles of heat-stressed cattle exhibit reduced embryonic development beginning at the 2-cell stage through the blastocyst stage (Rocha et al., 1998; Rutledge et al., 1999; Zeron et al., 2001; Al-Katanani et al., 2002).

Determination of direct effects of elevated temperature on GV-stage oocytes is possible with the use of meiotic inhibitors. In vitro studies are able to isolate the direct effects of elevated temperature on morphology, cytoplasm and nuclear progression of GV-stage oocytes. The following studies by Curci et al. (1987, 1991) used dibutyryl

cAMP to inhibit meiosis. Murine oocytes cultured at an elevated temperature (40-45°C for 5 to 9 minutes; cultured in a plastic tube in a water bath; recovered at 37°C for 15 minutes) are not grossly affected when evaluating oocyte morphology at 40-42°C, but oocyte shape is distorted at 43°C (depending on the duration of heat shock) when collected from superovulated mice (5 I.U. PMSG; oocytes collected 46-48 hours later; Curci et al., 1987). Interestingly, distorted oocytes regain normal shape in 15 to 30 minutes at a thermoneutral temperature (37°C; Curci et al., 1987). Murine preovulatory oocytes incubated at an elevated temperature exhibit decreased protein synthesis with increased heat shock temperatures (43°C; Curci et al., 1987) and lack the ability to synthesize heat shock proteins (70- and 80-kDa polypepetides; Curci et al., 1987, 1991). In addition, as incubation temperature of GV-stage murine oocytes increased from 40-41°C to 43°C, resumption of meiosis was also inhibited (Curci et al., 1987).

The effects of elevated temperature on GV-stage oocytes are also evident in the bovine. Elevated temperature (41°C; first 12 hours of culture) on GV-stage oocytes accelerated cortical granule migration (Type III) in GV-stage oocyte prior to IVM, increased number of sperm/oocyte but did not alter penetration, pronuclear formation, monospermic or putative embryos (Payton et al., 2003c). Ability of oocytes heat-shocked (41°C; 5 hours) at GV stage to cleave decreased 8-16 cell embryos and increased the proportion of 4-cell embryos (Payton et al., 2003a). Moreover, GV-stage oocytes cultured at 41°C for the first 12 hours reduced development of embryos to blastocysts (Payton et al., 2003a).

Effects of heat stress on the maturing oocyte

Heat stress during the time of oocyte maturation has been shown to be detrimental to the reproductive performance of an animal. Exposure of females to heat stress conditions while the oocyte is preparing for fertilization (oocyte maturation) may decrease oocyte quality, embryo development, successful implantation and gestational success. The following section is an extensive review of the literature describing the immediate and delayed effects of heat stress on the maturing oocyte as occurring within the maternal environment (in vivo) or in vitro (direct effects of elevated temperature).

Oocytes removed from heat-stressed mice have altered morphology. Studies on mice exposed to an extreme treatment of elevated temperature (44.0-45.0°C; heat shock applied 8-12 hours after ovulation by immersing externalized ovaries and fallopian tubes in hot water for 5-10 minutes) during oocyte maturation resulted in degenerate oocytes (from mice killed 4-5 hours after heat shock) and gross fragmentation (collected at 48-70 hours after heat shock; Braden and Austin, 1954). Furthermore, exposure of synchronized mice (3 I.U. Pregnant Mares Serum Gonadotropin (PMSG) followed 48 hours later by 3 I.U. human chronic gonadotropin (hCG)) to a more realistic heat stress (35±1°C; 65±3% RH; 15.5 hours) prior to fertilization resulted in loosening of cumulus cells and partial disruption and granularity of the zona pellucida (Baumgartner and Chrisman, 1981b).

The effects of elevations in temperature on the maturing murine oocyte (within the maternal Graffian follicle) are also evident at the cytoplasmic level. Oocytes from mice exposed to elevated temperature (44.0-45.0°C; 5-10 minutes) during oocyte maturation exhibited forms of cleavage (resembling 2-cell and 4-cell embryos; Braden

and Austin, 1954). In addition, collecting matured oocytes from mice exposed to an extended period of heat stress (during oocyte maturation; 15.5 h) further compromised the cytoplasm by increasing fragmentation (bicellular and tricellular) when compared to controls (monocellular; Baumgartner and Chrisman, 1981b).

The maturing murine oocyte has been shown to be altered at the nuclear level when exposed to elevations in temperature (within the maternal Graffian follicle). Mice introduced to temperatures above body temperature for 5-10 minutes (44.0-45.0°C) during oocyte maturation resulted in reformation of nuclei, which resembled pronuclei of fertilized eggs (Braden and Austin, 1954). Exposure of synchronized mice (3 I.U. PMSG followed 48 hours later by 3 I.U. hCG) to heat stress (35±1°C; 65±3% RH; 15.5 hours) during oocyte maturation caused disruption of meiotic maturation (25% of analyzable ova), arrest at MI (12.3% of the oocytes), and an increased number of oocytes with prophase-like nuclei or subnuclei (Baumgartner and Chrisman, 1981a). Furthermore, Baumgartner and Chrisman (1987) exposed mice to the same protocol as described above (Baumgartner and Chrisman, 1981a) for 12.5 hours (during the time of oocyte maturation) to study the effects of heat stress on oocytes. Oocytes matured within a heat-stressed maternal environment had uncharacteristic nuclear configurations (dispersed chromatin, micronuclei or degenerating chromatin), disrupted meiotic maturation and retained polar body chromosomes (Baumgartner and Chrisman, 1987).

Other studies have shown detrimental effects of heat stress applied during murine oocyte maturation on events following early embryo development. Synchronized mice (3 I.U. PMSG followed 48 hours later by 3 I.U. hCG) were exposed to elevated temperature (35±1° C; 65±3% RH) immediately after injection of hCG for 12.5 hours (during the time

of oocyte maturation; Baumgartner and Chrisman, 1988). After 12.5 hours of heat stress, females were paired with males of proven fertility and on day 9 of gestation, reproductive tracts were removed to collect embryos (Baumgartner and Chrisman, 1988). Maternal heat stress increased the number of pre-implantation losses (6.0 versus 0.7 %; heat stress and control, respectively) and increased the number of implantation sites lacking embryos (1.6 versus 0.5%; heat-stress and control, respectively) for reproductive tracts (day 9) collected from dams heat-stressed during oocyte maturation. Development was delayed up to 48 hours in embryos collected from heat-stressed (during oocyte maturation) dams (Baumgartner and Chrisman, 1988). Baumgartner and Chrisman (1987) reported on day 19 of gestation that maternal heat stress caused a significant decrease in average number of pups (8.8 versus 13.2; heat stress and control, respectively) due to preimplantation loss, early post-implantation loss and mid-gestation mortality.

Other species, such as the rat, are affected by elevated temperature during the time of oocyte maturation. Rats exposed to elevated temperature (44.5-45.5°C; heat shock applied after mating and ovulation, but before fertilization by immersing externalized ovaries and fallopian tubes in hot water for 8-12 minutes) during oocyte maturation resulted in oocytes exhibiting no polar body (inhibition of the second polar body meiosis) and a high rate of polyspermy (Austin and Braden, 1954).

The bovine species is also affected by elevated temperature during the time of oocyte maturation. Several studies indicated that the negative effects of elevated temperature are most prominent at or during the time of estrus (during oocyte maturation; Fallon, 1962; Stott and Williams, 1962; Long et al., 1969; Gwazdauskas et al., 1973; Putney et al., 1989). Putney et al. (1989) exposed superovulated heifers to elevated

temperature (42.0°C; 75% RH for 10 hours; environmental chamber treatment) beginning at the onset of estrus until insemination (during oocyte maturation). Embryos collected on day 7 were of reduced quality (4.9 and 2.9 for heat stress and control, respectively; quality classification score 1 through 6; 1=excellent, perfect embryo through 6=unfertilized ova), embryo stage (1.4 and 3.1 for heat stress and control, respectively; based on international embryo transfer society codes) and had decreased cell numbers (10.9 and 41.9 for heat stress and control, respectively; Putney et al., 1989). In the bovine, an attempt to lessen the negative effects of elevated temperature on the maturing oocyte (beginning at the onset of estrus until insemination) for reducing reproduction was made by Stott and Wiersma (1976). Specifically, lactating cows exposed to cooled climatic-control stanchions (27°C and 40% RH; late July through September) during estrus had improved pregnancy rates (38%) compared to a treatment imposed at other times (Stott and Wiersma, 1976).

Many have utilized in vitro culture systems (outside the maternal environment) to examine the direct effects of elevated temperature on the maturing oocyte. Oocytes exposed to elevated temperature during in vitro maturation had decreased viability and alterations in oocyte morphology. Exposing maturing murine oocytes to heat shock (42.5-43.0°C in water bath) for 1 hour decreased viability (determined by fluorescein diacetate assay; Hahnel et al.,1986). Oocytes collected from oviducts (16 hours post-hCG) of superovulated (5 I.U. PMSG followed by 5 I.U. hCG) mice were heat-shocked (42-43°C for 1 hour) and initial observations determined the oocytes to be nonviable, fragmented, and composed of a granulated cytoplasm (Hendrey and Kola, 1991). After 3 hours in culture at 42-43°C, maturing oocytes exposed to elevated temperature became

morphologically abnormal (fragmentation, degeneration, severe shape abnormalities, oocyte lysis, and very granulated ooplasms; Hendrey and Kola, 1991).

Maturing murine oocytes at an elevated temperature has been shown to directly alter the components within the cytoplasm of the oocyte. Early studies by Komar (1973) reported potential parthenogenetic activation and development of oocytes exposed to elevated temperature and found that culturing oocytes at 44.0-45.0°C (for 5-10 minutes) elicited immediate cleavage. A subsequent study by Hahnel et al. (1986) characterized an extended duration (1 hour) of heat shock (42.5-43.0°C; in water bath) on oocytes from superovulated (with hCG) mice and observed decreased protein synthesis (measured by incorporation of radiolabeled amino acids into acid-insoluble protein). Despite the decreased synthesis of proteins, there was translation of a 35-kD protein, but no detection of heat shock proteins (80-100 kD, 68-74 kD or 15-30 kD) in heat-shocked oocytes (Hendrey and Kola, 1991). Oocytes incubated at an elevated temperature (42-43°C) did not show an increase in 70 kD protein (Hsp70; Hendrey and Kola, 1991), confirming previous findings by Hahnel et al. (1986), but were benefited by injection of Hsp70 mRNA by acquiring the ability of maturing oocytes to tolerate elevations in temperature (Hendrey and Kola, 1991). Although Hendry and Kola (1991) reported that Hsp70 was not transcribed by the maturing oocyte in response to heat exposure, Kim et al. (2002) advanced the understanding of maturing oocyte expression of Hsps by evaluating Hsp25. Hsp25 was detected in mouse GV-stage oocytes and levels increased up to MII by 2.5-fold under both acute (for 30 minutes and 1 hour) and chronic (up to 4 hours) heat stress (43.0°C) and was found distributed throughout the entire cytoplasm (Kim et al., 2002).

The comparison of acute versus chronic heat stress revealed that short-term heat shock (30 minutes and 1hour) might accelerate GVBD (Kim et al., 2002).

In addition, maturing murine oocytes at elevated temperature in vitro altered nuclear progression. The effects of elevated temperature (38.0-41.0°C) for the entire period of maturation (17 hours) were studied in the mouse by evaluating meiotic maturation and chromosome spread (Fiorenza and Mangia, 1992). Oocyte maturation was blocked at the MI stage (38.5-40.0°C) and at chromatin I and II (41.0°C) at different culture temperatures. Interestingly, Fiorenza and Mangia (1992) reported that culturing oocytes at 40.5°C during the first 8 hours of maturation was more detrimental to oocyte maturation than exposing oocytes to hyperthermic conditions during the later 8 hours. In addition, abnormal chromosome features were observed in both thermoneutral and stressed oocytes, although heat-shocked oocytes matured at 40.0°C exhibited increased sister chromatid separation and parthenogenesis (Fiorenza and Mangia, 1992). Maturing oocytes at elevated temperatures (44.0-45.0°C; 5-10 minutes) without subsequent fertilization resulted in nuclear conformations within activated eggs such as; (1) second polar body extrusion, one haploid pronucleus; (2) second polar body suppression, one diploid pronucleus; (3) second polar body suppression, two haploid pronuclei; and (4) nuclear material in the form of large numbers of nuclei (Komar, 1973).

The effects of elevated temperature have been studied in the rabbit. Chang (1952) heat shocked (45°C; placed in a small test tube kept in a container) matured oocytes collected from superovulated adult rabbits, transferred the oocytes back into recipient rabbits and recovered the oocytes 32-48 hours later to subsequently examine. Oocytes treated at 45°C had scattered chromosomes at the center of the cytoplasm and the absence

of chromatin. In addition, oocytes cultured at 45°C for 10-30 minutes, transferred back into a recipient, and recovered 32-48 hours after transfer exhibited no cleavage and no activation of nuclear material (Chang, 1952).

Effects of elevated temperature on the maturing oocyte are also evident in the bovine. The effects of elevated temperature on the maturing oocyte can be observed when evaluating the morphology. Exposure of cumulus oocyte complexes to elevated temperature (41.0°C) inhibited cumulus expansion and decreased polar body extrusion (Lenz et al., 1983). Culture of cumulus oocyte complexes at elevated temperature (41 or 42°C; Edwards and Hansen, 1996; 41°C; Edwards and Hansen, 1997) during the first or last 12 hours of maturation did not alter membrane integrity regardless of heat shock temperature.

Culture of maturing oocytes at an elevated temperature alters cytoplasmic processes. Exposing cumulus oocyte complexes to heat shock temperatures (41 or 42°C) during the first or last 12 hours of maturation reduced the total amount of intracellular protein but did not alter the amount of inducible Hsp70 (Edwards and Hansen, 1996). Interestingly, in a subsequent study by Edwards and Hansen (1997), heat shock (41.0°C for the first 12 hours of maturation) did not decrease the amount of intracellular protein or overall heat shock proteins when compared to controls. Furthermore, exposure of maturing oocytes to elevated temperature altered distribution of cortical granule types (larger proportion of Type III; Payton et al., 2003d). In addition, heat shock of maturing oocytes (during the first 12 hours) increased glutathione content (Payton et al., 2003b).

Culture of maturing oocytes at an elevated temperature in vitro has also been evaluated at the nuclear level. Exposing bovine oocytes to 41.0°C during maturation (24

hours) decreased the number progressing to MII (considered mature only after extruding a polar body; Lenz et al., 1983). In contrast, Payton showed that greater that 70% of heat stressed oocytes not having a polar body had in fact progressed to MII (Payton et al., 2003d). This study (Payton et al., 2003d) indicated that elevated temperature (41.0°C) may accelerate degeneration of the polar body, but not necessarily decrease progression to MII. In addition, maturation of bovine oocytes at an elevated temperature (41.0°C) during the first or last 12 hours of maturation did not alter nuclear maturation (MII; Dorado et al., 2001; Payton et al., 2003d). The previous studies on nuclear progression indicate that reductions in development seen in bovine oocytes during oocyte maturation may not be due to nuclear alterations, but may due to changes within the cytoplasm.

Finally, maturing bovine oocytes at an elevated temperature alters cleavage and embryonic development. Exposure of oocytes to elevated temperature (41.0°C) during the first or last 12 hours of maturation did not affect cleavage, although culture at 41.0°C during the first 12 hours of maturation reduced development to blastocyst (Edwards and Hansen, 1996, 1997). In contrast to results seen at 41.0°C, culturing oocytes at 42.0°C reduced the number of oocytes cleaving and developing to blastocyst for heat shock during the first and last 12 hours of maturation (Edwards and Hansen, 1996, 1997). In a subsequent study by Edwards and Hansen (1997), heat-shocking (41.0°C for the first 12 hours of maturation) oocytes decreased the number developing to blastocyst when cultured in BSO (an inhibitor of glutathione). An additional study was performed to characterize effects of heat treatment (42.0°C) for different durations (0, 1, 2, and 4 hours) on maturing bovine oocytes (Ju et al., 1998). Results indicated that maturation of bovine oocytes at an elevated temperature for 4 hours did not alter fertilization or

cleavage but reduced blastocyst formation, number of trophoblast cells, total average cell number and average blastocyst cell number (Ju et al., 1998). A follow up experiment by Ju et al. (1999) characterized effects of 4-hour heat shock (40.5-43°C with recovery incubations; 39°C) prior to fertilization. Results indicated no differences in cleavage or blastocyst formation when oocytes were exposed to 40.5 to 41.5°C for 30 or 60 minutes. However, when heat shock temperature was increased to 43.0°C for up to 60 minutes, there was a reduction in blastocyst formation (Ju et al., 1999).

In conclusion, as duration and severity of heat stress increase, the negative effects of elevated temperature for decreasing development increase. Heat stress also has differing effects on the maturing oocyte depending on the species in question. Elevated temperatures were more extreme in murine studies than bovine studies so comparison among species may be biased.

Effects of heat stress on other cell types

Effects of heat stress have been reported on a variety of other cell types. Reports of elevated temperature on cells are extensive and differ with severity and duration. Specific effects include alterations in membrane integrity, cytoplasm and nucleus.

Cell membrane integrity is altered by heat. Alterations in the plasma membrane in response to elevated temperature include reductions in transmembrane potential (41°C), alterations in membrane permeability (41.5°C), decreased fluidity of the plasma membrane (41-45°C) and decreased cell surface charge (43°C; reviewed by Leyko and Bartosz, 1986). Carcinoma cells exposed to 43°C had cell body shrinkage and a decreased cell number (Huang et al., 1999). In addition, cells cultured at an elevated

temperature lacked proper ability to plate on a substrate (43°C; reviewed by Leyko and Bartosz, 1986; 45°C; Wang et al., 1998). Heat-stressed cells exhibited inactivation of membrane receptors (45°C) and alterations in second messengers (45°C; reviewed by Leyko and Bartosz, 1986). In addition, elevated temperature altered cell surface appearance by increasing blebs and ruffles at the cellular surface (42 or 43°C; reviewed by Leyko and Bartosz, 1986; 45°C; Funk et al., 1999), causing rupture of the cell membrane (42 or 43°C), and inducing appearance of a cobblestone-like surface (47°C; reviewed by Leyko and Bartosz, 1986).

Elevated temperature also alters components within cytoplasm. Foremost, cytoskeleton (composed of three major filaments; microfilaments, microtubules and intermediate filaments) integrity is affected by heat (Welch and Suhan, 1985; Wang et al., 1998; Huang et al., 1999). Microfilaments (formed from actin) from cells exposed to elevated temperature had an increased number of stress fibers (42-43°C; Welch and Suhan, 1985), progressive depolarization and disassembly of the central focal adhesions (43°C; Huang et al., 1999), almost complete elimination of microfilaments within the cytoplasm and were detected within the nucleus (45°C; Wang et al., 1998). Microtubules within cells exposed to heat were unaffected at 42-43°C (Welch and Suhan, 1985), depolymerized at 43°C (Huang et al., 1999) and retracted from the cell periphery at 45°C (Wang et al., 1998). Intermediate filaments within heat-shocked cells were tightly associated with the nuclear membrane (42-43°C; Welch and Suhan, 1985; 45°C; Wang et al., 1998), reduced in number and depolymerized (43°C; Huang et al., 1999). A cell exposed to elevated temperature may be delayed (45°C; Vidair et al., 1993) or blocked in mitosis (40.5-42°C; Bergan, 1960) based on the rise above normal body temperature.

The centrosome is a cellular structure that may be contributing to the mitotic abnormalities seen within heat- shocked cells (37°C; Debec and Marcaillou, 1997; Barrau et al., 1978).

Within the cytoskeleton of a cell exposed to elevated temperature, several organelles are altered (Barrau et al., 1978; Welch and Suhan, 1985; Collier et al., 1993; Vidair et al., 1993; Debec and Marcaillou, 1997; Wang et al., 1998; Funk et al., 1999). Mitochondria within heat-shocked cells have been shown to invade the central portion of the cells (42-43°C; Welch and Suhan, 1985; 45°C; Collier et al., 1993; 37°C; Debec and Marcaillou, 1997; 45°C; Wang et al., 1998) and have changed morphology (45°C; Funk et al., 1999). The Golgi apparatus was altered in heat-shocked cells as it separated and formed small vesicles near the nucleus (45°C; Wang et al., 1998). The endoplasmic reticulum within cells seems unaffected by elevated temperature (45°C; Collier et al., 1993; 45°C; Wang et al., 1998). Embryos exposed to elevated temperature (41°C) have organelles that have moved away from the peripheral cortex and have distributed within the central portion of the blastomere (Rivera et al., 2003). In fact, protein synthesis can be reduced or inhibited with elevated temperature (>43°C; Duncan and Hershey, 1989).

Nuclear components within cells are affected by elevated temperature. Within the nucleus of heat-stressed cells, the nuclear envelope was disrupted (37°C; Drosophila cells; Debec and Marcaillou, 1997) and the compaction of the nucleoli was relaxed and thought to be unraveling (42-43°C; rat fibroblasts; Welch and Suhan, 1985). Heat stress may (41-46°C; human white blood cells; Mitchel and Birnboim, 1985) or may not (42.5-43.5°C; HL-60 Cells; Richards et al., 1988; 45°C; HeLa Cells; Slater et al., 1981) alter cellular DNA.

Lastly, cells exposed to heat shock have increasing levels of reactive oxygen radicals such as O_2^- and H_2O_2 which can cause cell damage and death by affecting the entire cellular composition (Loven, 1988). Reactive oxygen species cause cellular injuries by damaging DNA and RNA, lipid peroxidation (affecting membrane structure and function) and also damaging proteins.

Effects of retinoids on reproduction

Retinoids have been shown to be essential for reproduction. A deficiency in retinoids decreases reproduction whereas supplementation of retinoids may improve reproduction.

Female farm animals deficient in vitamin A have delayed puberty (reviewed by Smith and Akinbamijo, 2000) and lowered conception rates (reviewed by Hurley and Doane, 1989). In dairy cattle, the negative effects of diets deficient in vitamin A may not be seen until after establishment of pregnancy. Reproductive disorders in vitamin A deficient cattle include embryonic losses (reviewed by Smith and Akinbamijo, 2000) and high perinatal mortality associated with dead, blind and weak offspring (reviewed by Hurley and Doane, 1989) in addition to retained placentas (reviewed by Hurley and Doane, 1989).

In contrast, animals injected with or fed a diet supplemented with retinoids have reproductive success. Reported effects of vitamin A and/or β-carotene on reproduction include increased estrus intensity, improved conception rates, decreased services per conception, decreased days open, and decreased occurrence of cystic ovaries (Ascarelli et al., 1985; reviewed by Hurley and Doane, 1989). After the establishment of pregnancy,

the different roles that β-carotene and/or vitamin A have on the animal consist of decreased embryonic losses (Brief and Chew, 1985; reviewed by Hurley and Doane, 1989) and increased number of offspring (Brief and Chew, 1985). There are also studies that report no effects of vitamin A and β-carotene on reproduction (Bindas et al., 1984; Rakes et al., 1985; Akordor et al., 1986; Aréchiga et al., 1998b; Wang et al., 1982; reviewed by Hurley and Doane, 1989; Besenfelder et al., 1996; Pusateri et al., 1999). Discrepancies may be due to the use of the specific retinoid derivative, dose, duration and mode of administration or age and environment of the animal during the trial.

Retinoids may be beneficial when given during a time when animals are experiencing a stress such as high environmental temperature. Specifically, lactating Holstein cows fed supplemental β-carotene (400 mg/d/cow) for at least 90 d beginning approximately 15-30 days after calving during the summer and early fall had a higher pregnancy rates (35.4 versus 21.1% for β-carotene and control, respectively) at 120 d postpartum than control cows (Aréchiga et al., 1998a). In contrast, lactating cows fed supplemental β-carotene (500 mg/d/cow) for ≥60 d during the winter and early spring did not have higher pregnancy rates when comparing to controls (Aréchiga et al., 1998a). This indicates that heat-stressed lactating dairy cows fed diets supplemented with β-carotene have improved fertility.

Specific mechanisms through which retinoids may be having a beneficial effect by alleviating the deleterious effects of heat stress for decreasing reproduction are unclear. Retinoids may be acting through transcriptionally or non transcriptionally mediated mechanisms. Retinoids are composed of conjugated double bonds, which aid in antioxidant activity through a non-transcriptionally-mediated mechanism (Mascio et al.,

1991; Sies and Stahl, 1995) acting as hydrophobic non-enzymatic scavengers originating in lipoproteins and membranes (Chaudière and Ferrari-Iliou, 1999). Retinoids work as antioxidants through mechanisms to bind free radicals that may cause cellular damage (Bendich and Olson, 1989; Chaudière and Ferrari-Iliou, 1999; reviewed by Paiva and Russell, 1999; Palace et al., 1999). A carotenoid acts to bind free radicals resulting in an excited carotenoid that scatters its energy and then restores its ability to bind free radicals (reviewed by Paiva and Russell, 1999). Studies thus far have confirmed that carotenoids, such as β-carotene, are involved in two pathways that result in binding free radicals (Chaudière and Ferrari-Iliou, 1999). The first pathway involves electron transfer to produce a cation radical and the second pathway adds a free radical to the polyenic chain (Chaudière and Ferrari-Iliou, 1999). In addition, carotenoids and vitamin A may act as antioxidants by scavenging lipid peroxyl radicals, but only at low oxygen pressure (Chaudière and Ferrari-Iliou, 1999). Vitamin A (retinol) and its active metabolites may also affect reproduction through transcription (reviewed by Mehta, 2003). Cellular retinol binding proteins (CRBP) may be involved in translocation to the cell nucleus, but this is not certain. CRBP is involved in retinoid storage, esterification and presentation for metabolism (reviewed by Napoli, 1996). Once inside the cell, retinol is converted into its active form, retinoic acid (RA), which acts on the nuclear retinoic acid receptors (RAR) or retinoid X receptors (RXR) depending on the conformation of the acid. Retinoic acid receptors are activated by both all-trans-RA and 9-cis-RA, while 9-cis RA is the only RA activating the RXRs within the cell nucleus (Chambon, 1996). Furthermore, RARs have subgroups (RARα, RARβ, RARγ) as do RXRs (RXRα, RXRβ, RXRγ) which can be activated or suppressed by RA (reviewed by Mehta, 2003). Most

recently, RARα, RARγ, RXRα, RARβ and retinaldehyde dehydrogenase 2 mRNA were found within in vitro produced bovine embryos from the oocyte to hatched blastocysts (Mohan et al., 2001, 2002). In addition, proteins for RARα and RARγ-2 (Mohan et al., 2001) and RXRβ (Mohan et al., 2002) were found within the inner cell mass and trophectoderm of intact and hatched bovine blastocysts. Within the cell nucleus, retinoic acid may activate gene transcription by binding to specific elements within the promoter region of target genes (reviewed by Mehta, 2003).

Effects of retinoids on oocyte and embryo development

Vitamin A and/or other retinoids can influence reproductive efficiency. Effects of retinoids change throughout the reproductive cycle of an animal. The following section is a review of the literature describing the effects vitamin A and/or other retinoids at various developmental stages (GV-stage oocyte, maturing oocyte, pre-implantation embryo (blastocyst) and post-implantation embryo) within the maternal environment (indirect effect; in vivo) and/or direct effect (in vitro).

Initial studies to determine effects of retinoids on GV-stage oocytes contained within growing antral follicles were conducted in vivo with variable results depending on the specific parameter evaluated. Swine injected with retinol palmitate (1,000,000 I.U.) on day 16 of the estrous cycle (estrous cycle approximately 21 days for swine) exhibited increased follicular diameter when evaluated on day 18 or 19 of the same estrous cycle (Robertson et al., 1997). Whaley et al. (1994) injected gilts (fed a high energy diet before treatment to reduce embryonic survival) with retinol palmitate (1,000,000 I.U.) 6 days before observed estrus and found that retinol-treated gilts (laparotomized during 24-28,

28-32, 32-36 or 36-40 h after onset on estrus) had developmental stages (from oocytes and embryos collected) that were slightly (not statistically) more advanced (control, 4.12±0.21 and treated, 4.52±0.17; 4 = MI and 5 = telophase I) and more centrally distributed than in control gilts (injected with corn oil). In a subsequent study, Whaley et al. (2000) injected gilts (fed a high energy diet before treatment to reduce the number of embryo survival) with retinol palmitate (1,000,000 I.U.) 6 days before observed estrus and recovered (laparotomized during 24-28, 28-32, 32-36 or 36-40 h after onset on estrus) an increased average number of oocytes and embryos with more advanced stage of development (control, 2.74 versus treated, 3.04; 2 = GVBD, 3 = Ovulated and Unfertilized, 4 = Ovulated and Fertilized, sperm in cytoplasm, no pronucleus). Work done by Whaley et al. (2000) indicated an effect of retinol palmitate at the nuclear level in the GV-stage oocytes to accelerate subsequent development. Whaley et al. (1997) used the same protocol as previously described (Whaley et al., 1994) to assess effects of retinol palmitate on embryonic development (days 11 and 12 after estrus) in gilts and found that retinol palmitate was numerically, but not statistically beneficial for the number of embryos and embryonic survival. The percentage of spherical embryos (size $\geq$ 1 mm and $\leq$ 7.5 mm in diameter) was different between retinol palmitate-treated gilts and controls (Whaley et al., 1997). Vitamin A also increased the number of large embryos recovered (5.0-5.5 mm size; Whaley et al., 1997). In another study, Coffey et al. (1993) injected β-carotene (0, 50, 100, or 200 mg) at weaning and the percentage of small litters ($\leq$ 7 pigs) was reduced. In addition, injection of 200 mg of β-carotene at weaning increased the percentage of large litters and 50 mg of β-carotene reduced the number of stillborns (Coffey et al., 1993).

Sheep reproduction is also affected by retinoids. Sexually mature ewes were synchronized (progestin implant (Synchromate B) on day 0; 6 days later received 2 Lutalyse (15 mg) injections 12 days apart), superovulated (total of 24 units of FSH over 3 days in decreasing doses – beginning 9-11 days after implant) and injected with various retinoids (500,000 I.U. all-trans retinol; 15 mg all-trans RA; 15 mg 9-cis RA; corn oil (control)) on the first and last day of FSH treatments (Eberhardt et al., 1999). Use of all trans-retinol (500,000 I.U.) in combination with superovulation increased blastocyst formation when compared to control animals (injected with corn oil) when embryos were collected at 84 hours (1-4 cell stage embryo) or 144 hours (morula-stage embryo) post-implant removal and cultured in vitro for assessment to blastocyst (Eberhardt et al., 1999). In contrast, the injection of other retinoids (15 mg all trans-RA and 15 mg 9-cis RA) did not improve embryonic development to the blastocyst when embryos were collected at 144 hours (morula-stage embryo) subsequently cultured in vitro (Eberhardt et al., 1999).

Effects of retinoids on GV-stage bovine oocytes have also been studied (Shaw et al. 1995). In beef cattle, both nonlactating cows and heifers were synchronized (2 doses of PGF2α; 25 mg IM, 11 days apart), superovulated (32.5 or 27 units of FSH in decreasing doses over 4 days; 8-10 days after synchronization of estrus; luteolysis on 4th day of FSH treatment) and injected with retinol palmitate (1,000,000 I.U.) on the first day of FSH injection (Shaw et al., 1995). Retinol-treated females had an increased number of high quality (Quality 1,2) and transferable embryos (Quality 1,2 and 3; recovered day 7 post-estrus) compared to controls (injected with corn oil; Shaw et al., 1995). Duque et al. (2002a) evaluated effects of 9-cis RA (5 nmol/l) on GV-stage oocytes at the end of pre-

maturation culture (within a basic medium and roscovitine; 25μmol/l for 24 h) and after a 24-hour maturation period. Nuclear status was similar, but cortical granule (CG) patterns for control and retinoic acid -treatments differed in GV-stage and matured oocytes both treated with retinoid acid during pre-maturation (Duque et al., 2002a). Assessment of nuclear status and CG pattern of GV-stage and matured oocytes cultured in 9-cis RA during pre-maturation infer the possibility that RA may accelerate CG migration, but limitations within the experiment include the low numbers of oocytes utilized and the interpretations the authors had of the data as reported (Duque et al., 2002a). Bovine GV-stage oocytes (within a basic medium and roscovitine; 25μmol/l for 24 h) cultured 9-cis RA developed significantly better than controls when evaluating cleaved embryos, morula-stage embryos, and development to blastocyst (Duque et al., 2003a). Lastly, Duque et al. (2002a) evaluated the effect of culturing GV-stage oocytes in 9-cis RA by exposing subsequent blastocysts (d 7) to a freezing and thawing stressor which concluded that 9-cis RA was benefical for improving survival and hatchability although the low number of experimental units (16-21 blastocysts/treatment) makes the data somewhat questionable.

Retinoids also exert their effects on the maturing oocyte (oocyte preparing for fertilization). Studies thus far have exposed the maturing oocyte to retinoids (all trans-retinol, 9-cis-RA) in vitro and have had varied effects when evaluating the oocyte for cytoplasmic and nuclear progression and the subsequent embryo for development and/or total cell counts. Duque et al. (2002a) matured bovine oocytes in 9-cis RA (5 nmol/l; previously held at GV-stage with 25 μmol/l roscovitine) and found no effect on CG type or nuclear status, although caution must be taken to formulate any inferences due to the

limited low numbers of oocytes utilized (n=5 oocytes/treatment). Maturing bovine oocytes in the presence of all trans-retinol (Duque et al., 2002b; Livingston et al., 2002) or 9-cis RA (Duque et al., 2002a, 2002b) during maturation had no effect on cleavage. While bovine oocytes matured in retinol (5.0 μM; Livingston et al., 2002) had improved blastocyst rates, studies by Duque et al. (0.5 μM; 2002b) had blastocyst development similar to controls. These discrepancies may be attributed to varied retinol concentrations or other differences in experimental design. In contrast, 9-cis RA (5 nmol/l; oocytes previously held at GV-stage with 25 μmol/l roscovitine; Duque et al., 2002a) had no effect on subsequent development of maturing oocytes. Hidalgo et al. (5 nmol/l 9-cis RA; 2003) and Duque et al. (5 nmol/l 9-cis retinoic acid; 2002b) observed a beneficial effect of retinoids on maturing oocytes only for day 7 blastocysts and the percentage of hatched blastocysts. Blastocysts derived from bovine oocytes matured in all trans-retinol (5 μM; Livingston et al., 2002) or 9-cis RA (5 nmol/l; Duque et al., 2002a) had no effect on total cell numbers within blastocysts, but culture with 9-cis RA (5 nmol/l; Hidalgo et al., 2003) substantially decreased total cell numbers. The previously described studies of Livingston et al. (2002) research was significant in the literature because it revealed that when blastocyst development was below 20% in the control treatment, the addition of 5μM all trans-retinol dramatically improved blastocyst development (21.8 versus 12.5 for retinol and control) indicating more of a beneficial effect of retinol.

Retinoids can also affect reproductive efficiency of an animal by impacting development of pre-implantation-stage embryos (blastocyst). In mice, retinoids had no effect on early (orally administered all trans-RA (50 mg/kg or 100 mg/kg; on the morning

of day 1 and 2 of gestation) or late pre-implantation embryos (orally administered all trans-RA (50 mg/kg or 100 mg/kg; night of day 2 and morning of day 3) when evaluating all endpoints (mean number of embryos, number of expanded blastocysts, early blastocyst or morula, or degenerative embryos) on the afternoon of day 3 of gestation (Huang and Lin, 2001a). Mouse expanded blastocysts exposed directly to 10 μmol all trans-RA for the first 2 days of culture (subsequently cultured for remaining 6 days in basic medium) impaired development (Huang et al., 2001b). The previous study by Huang et al. (2001b) increased the knowledge of direct effects of RA on blastocysts and its immediate and delayed teratogenic effects. Exposing murine blastocysts to RA (0.1 μmol/l or 10 μmol/l; for 24 hours) has been shown to be detrimental to subsequent embryo attachment (post-implantation; Huang et al., 2001b). In addition, there was a 17% reduction in trophectoderm lineage in blastocysts exposed to 10 μmol/l RA for 24 hours (Huang et al., 2003). Finally, blastocysts exposed to RA (10 μmol/l) for 24 hours had a 2-3 fold increase in cell death (apoptosis) compared to controls (Huang et al., 2003). An apoptotic effect of RA on blastocysts is intriguing because induced effects of RA at this stage of development and the subsequent post-implantation development had not been previously shown (Huang et al., 2003).

Post-implantation development is affected by exposure of embryos to retinoids. Specifically, Huang et al. (2001b) evaluated long-term (8 days) exposure of murine oocytes to retinoids on embryonic development throughout post-implantation stages and found that increased dose of all trans-RA (0.001; 0.1 and 10 μmol/l), increased detrimental effects on embryonic development. Embryos cultured in 10 μmol/l all trans-RA failed to survive past day 4 (Huang et al., 2001b). Culture of murine embryos in 10

μmol/l all trans-RA (at any defined stage in post-implantation development) reduced embryonic survival (Huang et al., 2003).

Although there are several studies describing the beneficial effect of retinoids on animal reproduction, the administration of retinoids either by feeding or injection may also be harmful if there is an excessive amount of vitamin A (hypervitaminosis). There are multiple retinoid-induced defects on the early postimplantation embryo and during organogenesis (Tzimas and Nau, 2001).

In summary, the use of retinoids in reproduction during the pre-embryonic stages may have positive effects, but when used in subsequent embryo development (post-fertilization) may have teratogenic effects. A thorough understanding of retinoids and the reproductive cycle stage is essential.

Oocyte maturation

Oocytes within a follicle at first are not developmentally competent, but must grow to be able to resume nuclear maturation, fertilization and subsequent cleavage (Picton et al., 1998). The growing oocyte accumulates water, ions and lipids causing an increase in cell volume (Picton et al., 1998). In the cytoplasm of the growing oogonia, there is an accumulation of organelles (mitochondria, ribosomes, Golgi apparatus, endoplasmic reticulum) and the zona pellucida is secreted by the oogonia along the cortex (Picton et al., 1998). Oocytes are held in meiotic arrest by low levels of cytoplasmic Ca^{2+} (He et al., 1997), preventing a decrease in cyclic AMP (Sun et al., 1999) and maintaining low levels of maturation promoting factor (MPF) and mitogen-activated protein kinases (MAP kinases; Fissore et al., 1996). There are increasing levels

of mRNA and synthesis of proteins within the nucleus and mitochondria (Picton et al., 1998). After the bovine oocyte has reached 110 μm (Hyttel et al, 1997), it is able to undergo germinal vesicle breakdown (GVBD; first step in oocyte maturation; Hyttel et al., 1997).

Oocyte maturation begins with GVBD at the LH surge in vivo (reviewed by van Soom and Kruif, 1996) or release of the oocyte from the ovarian follicle in vitro (Wassarman et al., 1976). The oocyte is heavily influenced by gonadotropins (Dominko and First, 1997). The timing of meiotic progression is delayed with FSH-supplemented medium compared to LH-supplemented medium (Dominko and First, 1997). The cumulus cells, cytoplasm and nucleus all change during oocyte maturation. Cumulus cells are intimately associated with the oocyte through gap junctions and undergo morphological changes during maturation (Loos et al., 1992). The close interaction between the two cell types maintains meiotic arrest (Fissore et al., 1996; reviewed by van Soom and Kruif, 1996). Epidermal growth factor has been found to mediate and induce bovine cumulus expansion and resumption of meiosis (reviewed by van Soom and Kruif, 1996). Upon completion of oocyte maturation, cumulus cells expand completely (break down of gap junctions) in vivo, but have variable levels of expansion in vitro (Loos et al., 1992).

Cytoplasmic maturation

Cytoplasmic maturation involves alterations in organelle arrangement (Loos et al., 1992; Hyttel et al., 1997), cortical granule types (Loos et al., 1992), intracellular stores of Ca^{2+} (Eppig, 1996; He et al., 1997), protein synthesis (Sirard et al., 1989; Kastrop et al.,

1990; Eppig, 1996; Tomek et al., 2002), cyclic AMP (Sun et al., 1999), MAPK cascade (Fissore et al., 1996) and maturation-promoting factor (MPF or histone 1; composed of a complex cyclin B-p34^{cdc2}; Eppig, 1996; Fissore et al., 1996). At the completion of oocyte maturation, organelles have moved away from the cortex region in vivo, while within in vitro systems, matured oocytes have organelles arranged throughout the entire cytoplasm (still associated with the cortex; Loos et al., 1992; Hyttel et al., 1997). At GVBD, mitochondria are distributed around lipid droplets and at Metaphase I (MI) the mitochondria (associated with increased number of lipid droplets) are distributed throughout the cytoplasm (Hyttel et al., 1997). At the completion of Metaphase II (MII), mitochondria are centrally distributed away from the periphery of the oocyte (Hyttel et al., 1997). Another organelle, the smooth endoplasmic reticulum is created from the nuclear envelope following GVBD and at MII is located at the oocyte periphery in large clusters (Hyttel et al., 1997). The Golgi complexes decrease in number throughout maturation and at MII are essentially absent (Hyttel et al., 1997). In the bovine, cortical granules distribute as the oocyte matures (Niimura and Hosoe, 1995). In early stages of maturation (first 6 hours) cortical granules are in large aggregates (Type I; Niimura and Hosoe, 1995). As the oocyte matures, cortical granules distribute throughout the cytoplasm in small aggregates and single particles (Typle II) and progressively become individual particles densely distributed along cortex of oocyte (Type III) after 6 hours and 18 hours of maturation, respectively (Niimura and Hosoe, 1995). Oocyte maturation is also dependent upon increased Ca^{2+} release. Incorporation of Ca^{2+} inhibitors can block or delay bovine oocyte maturation and may inhibit activation of MPF and MAP kinases (kinases required for resumption of meiosis; He et al., 1997). While Ca^{2+} levels seem to

be required for resumption in bovine oocytes, there is no evidence of spontaneous internal Ca^{2+} oscillations (He et al., 1997). Protein synthesis (Sirard et al., 1989) and protein phosphorylation (Kastrop et al., 1990) are needed to initiate GVBD. Upon completion of GVBD and during oocyte maturation, there are definite changes in protein synthesis (Kastrop et al., 1990) and declines in protein translation up to MII (Tomek et al., 2002). A reduction in cAMP levels is essential for GVBD (Sun et al., 1999). Increasing levels of cAMP blocks GVBD and phosphorylation of MAP kinases (Sun et al., 1999) necessary for oocyte maturation. Interestingly, increasing levels of cAMP post-GVBD did not affect progression of meiosis or MAP kinase phosphorylation (Sun et al., 1999). Within the cytoplasm of bovine oocytes, MPF and MAP kinases are simultaneously activated (from low levels) at 6 hours post-GVBD and then elevate their activities until 24 h of maturation (MII; Fissore et al., 1996). Specifically, activity of MPF is biphasic and begins prior to GVBD and increases until MI upon which activity decreases until Anaphase I (AI) and then increases through MII (Eppig, 1996).

Nuclear maturation

The nuclear changes that occur during oocyte maturation include a series of events that ultimately lead from a nucleus at germinal vesicle (GV)-stage to MII. Bovine oocytes with an intact nucleus are at GV-stage and breakdown of the nuclear membrane occurs at 6.6-8 hours after initiation of meiosis (Sirard et al., 1989). The timing of GVBD corresponds to chromatin condensation and segregation (van Soom et al., 1996). After GVBD, nuclear maturation progresses to MI within 10.3 hours, AI by 15.4 hours, Telophase I (TI) by 16.6 hours, and MII by 18 hours of maturation (Sirard et al., 1989).

Once at MII, one set of chromosomes is extruded from the oocyte (polar body) and the other retained within the oocyte (van Soom et al., 1996).

Summary: A thesis for describing the developmental effects of retinol on heat-stressed maturing oocytes

For this thesis, it was hypothesized that administration of retinol during heat-shock may protect oocytes from deleterious effects of elevated temperature on subsequent development. Several lines of evidence exist to support this model. First, heat-induced reductions in development of maturing oocytes are strongly attributed to cytoplasmic alterations with minimal effects on the nucleus (Braden and Austin, 1954; Komar, 1973; Baumgartner and Chrisman, 1981b; Hahnel et al., 1986; Hendrey and Kola, 1991; Edwards and Hansen, 1997; Kim et al., 2002; Payton et al., 2003b, 2003d). Second, heat-shock increases reactive oxygen species within other cell types which may be contributing to the negative effects on the maturing oocyte (Loven, 1988). Third, retinoids have been used to improve reproduction (Ascarelli et al., 1985; Brief and Chew, 1985; reviewed by Hurley and Doane, 1989; Coffey et al., 1993; Whaley et al., 1994; Shaw et al., 1995; Robertson et al., 1997; Whaley et al., 1997; Arechiga et la., 1998b; Eberhardt et al., 1999; Whaley et al., 2000; Duque et al., 2002a; Livingston et al., 2002). Fourth, retinoids have been described as antioxidants (Bendich and Olson, 1989; Mascio et al., 1991; Sies and Stahl, 1995; Chaudiere and Ferrari-Iliou, 1999; reviewed by Paiva and Russell, 1999; Palace et al., 1999) and may act to decrease reactive oxygen species produced by elevated temperature. Thus, the overall objective of this thesis was to

evaluate development of oocytes matured at an elevated temperature in the presence of retinol.

CHAPTER 3

RETINOL IMPROVES DEVELOPMENT OF BOVINE OOCYTES COMPROMISED BY HEAT STRESS DURING MATURATION

This chapter is the final version of a paper submitted for review for publication in the *Journal of Dairy Science* by JL Lawrence, RR Payton, JD Godkin, AM Saxton, FN Schrick and JL Edwards.

Abstract

The objectives of this study were three-fold: 1) to evaluate effects of a physiologically-relevant elevated temperature on in vitro development of maturing oocytes, 2) to evaluate effects of retinol on in vitro development of maturing oocytes, and 3) to evaluate effects of retinol for improving development of oocytes compromised by an elevated temperature. Bovine oocytes were matured for 24 h at 38.5 or 41.0°C for 12 h followed by 38.5°C for the remaining 12 h in 0 or 5 μM retinol. After insemination, cleavage and blastocyst development were assessed on days 3 and 8, respectively. Temperature, retinol, and the interaction were included in the statistical model. Culture of oocytes at 41.0°C decreased the proportion of 8-16 cell embryos and increased the proportion of 2-cell embryos. Also, 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 three of the seven experimental replicates, 41.0°C did not reduce blastocyst development. 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) whereby elevated temperature

reduced development to blastocyst by at least 20%. A significant temperature x retinol interaction was noted. Addition of retinol to the maturation medium prevented heat-induced reductions in development of oocytes to blastocyst. Results indicate that retinol may protect oocytes from the deleterious effects of heat stress.

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

Abbreviation key: FBS = fetal bovine serum, **hpi** = hours post insemination, **IVF** = in vitro fertilization, **KSOM** = potassium simplex optimized medium, **NEAA** = non-essential amino acids, **OMM** = oocyte maturation medium, **PZ** = presumptive zygotes.

Introduction

Heat stress is one of the most challenging problems facing the dairy industry. Seasonal periods of heat stress reduce milk yields (reviewed by West, 2003) and fertility in lactating dairy cows (reviewed by Hansen and Aréchiga, 1999 and Rensis and Scaramuzzi, 2003). Effects of heat stress for reducing fertility include reductions in the expression of estrus, decreases in first service conception rates, increases in the number of services per conception, and reductions in the overall pregnancy rates in dairy cows (Poston et al., 1962; Gwazdauskas et al., 1973; Rosenberg et al., 1977; Cavestany et al., 1985; reviewed by Hansen and Aréchiga, 1999 and Rensis and Scaramuzzi, 2003). As such, cows cycling during the summer remain nonpregnant for a longer time period than those bred during other seasons (Fuquay, 1981). Costs incurred by the dairy producer may reach up to $4.00/d per cow for every day a cow is not pregnant beyond 90 d after parturition (Britt, 1975).

Reduced fertility is of particular concern in lactating dairy cows when ambient temperatures increase from 20 to 35°C (Cavestany et al., 1985). Exposure of lactating cows to elevated temperatures, in conjunction with high humidity, impairs ability of the animal to balance heat loss with the heat gained from the environment and metabolic functions that are associated with lactation and maintenance (Fuquay, 1986). As a result, body temperatures of heat-stressed cows increase markedly. Ulberg and Burfening (1967) demonstrated that pregnancy rates decreased with each degree increase in rectal temperature. Rectal temperatures parallel those occurring in utero (Gwazdauskas et al., 1973) and thus provide an accurate assessment of the degree of hyperthermia that an embryo within the cow may experience. Rectal temperatures of dairy cows may reach or exceed 41.0°C (Gaalaas, 1945; Seath and Miller, 1946; Monty and Wolff, 1974; Roman-Ponce et al., 1977; Turner, 1982; Elvinger et al., 1991, 1992; Ealy et al., 1993). Heat-induced increases in rectal temperatures may be further elevated by administration of bovine somatotropin (Elvinger et al., 1992).

Exposure of Holstein heifers to elevated ambient temperatures during estrus increased the proportion of retarded and degenerate embryos recovered 7 d after superovulation and insemination (Putney et al., 1989) suggesting that the negative effects of heat stress for reducing reproduction occur while the oocyte is maturing in preparation for fertilization. Negative effects of heat stress for decreasing further development of the oocyte are in large part due to direct effects of elevated temperature. Edwards and Hansen (1996, 1997) cultured maturing bovine oocytes at 41.0°C and noted direct effects of elevated temperature for reducing 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., 2003d), and increased glutathione content (Payton et al., 2003b) have been reported, suggesting possible alterations in the cytoskeleton and increases in free radical production.

Vitamin A and other natural retinoids may act as antioxidants by scavenging free radicals and preventing lipid peroxidation (Ciaccio et al., 1993) and have been shown to improve reproduction in heat-stressed dairy cows and development of otherwise developmentally incompetent oocytes. For example, supplementation of postpartum lactating dairy cow diets with β-carotene during the summer increased pregnancy rates in the fall (Aréchiga et al., 1998a). Superovulation has been shown to reduce developmental competence of resulting embryos (reviewed by Merton et al., 2003). Injections of retinol palmitate (a metabolite of β-carotene) in combination with superovulation increased the mean number of quality grade 1 and 2 bovine embryos (Shaw et al., 1995). In addition, administration of all trans-retinol to ewes during superovulation followed by natural service increased ability of recovered embryos to develop in vitro (Eberhardt et al., 1999). Most recently, Livingston et al. (2002) reported that administration of all trans-retinol during oocyte maturation improved developmental competence of otherwise stressed oocytes. Based on these data, the working hypothesis of this study was that administration of retinol during maturation may improve developmental competence of oocytes compromised by heat stress. The objectives of this study were three-fold: 1) to evaluate effects of an elevated temperature, observed in heat-stressed dairy cows, on development of maturing oocytes, 2) to evaluate effects of retinol on development of maturing oocytes, and 3) to evaluate

the effects of retinol for improving development of oocytes compromised by an 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 (Harris; personal communication). Medium-199, gentamicin, penicillin-streptomycin, and non-essential 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 12-24 mM) was verified using a

UV-spectrometer and reading the absorbance in triplicate (wavelength 325 nm). Calculations for diluting 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 antral 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 a oocyte maturation medium (**OMM**) consisting of Medium-199 containing Earle's salts, 10% FBS, 50.0 μg/ml gentamicin, 5.0 μg/ml FSH, 0.3 μg/ml LH, 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 originating from the same two bulls was used for IVF in each experimental replicate. Approximately 13-17 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, 1X NEAA, 50 U/ml penicillin, and 50 μg/ml streptomycin at 38.5°C in 5.5% CO_2, 7.0% O_2, and 87.5% N_2 in humidified air for 8 d post-fertilization. Ability of PZ to cleave and develop to blastocyst was evaluated on d 3 and 8, respectively (d 0 = day of sperm addition). Cleavage was assessed by recording the number of 1-, 2-, 4-, and 8-16 cell embryos present on d 3 (70-75 hours post insemination; **hpi**). Total

number of nuclei was recorded in blastocysts (d 8; 187-195 hpi) after staining with Hoechst 33342 (5 μg/ml) for a minimum of 15 min and then exposing embryos to UV-light using a DAPI filter (330-380 nm).

Developmental competence of bovine oocytes matured at an elevated temperature in the presence of all trans-retinol

Previous efforts of Livingston et al. (2002) showed that the addition of 5 μM all trans-retinol to OMM improved the ability of bovine oocytes to develop to the blastocyst stage. For this study, oocytes were matured at 38.5°C for a total of 24 h or 41.0°C (first 12 h of culture) followed by 38.5°C for the remaining12 h in 0 or 5 μM all trans-retinol. Ethanol was used as a diluent for solubilizing all trans-retinol and was added to OMM at the same volume as that required to result in a concentration of 5 μM all trans-retinol (diluent control).

Variables of interest recorded while conducting this experiment included number of PZ recovered after denuding oocytes of cumulus and associated spermatozoa, number of PZ that were visibly lysed after denuding, number of PZ that cleaved and developed to blastocyst stage. In addition, number of nuclei in resulting blastocysts was also recorded. At least 2 independent evaluators that were uninformed of treatments participated in assessing variables of interest. To examine effects of elevated temperature and retinol during oocyte maturation for inducing parthenogenetic activation of the oocyte in the absence of sperm, two additional subsets of oocytes were cultured concurrent with the four treatment groups: 1) one group of oocytes (n=153) were matured at 38.5°C for 24 h in OMM, and 2) a second group of oocytes (n=145) were matured at 41.0°C (first 12 h of

culture) followed by 38.5°C for remaining 12 h in the presence of 5 μM all trans-retinol. After a total of 24 h in culture, oocytes were treated in a similar manner to treated oocytes with the exception that no sperm was added. Development to blastocyst was recorded.

Statistical Analysis

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 the interaction were evaluated. Experiment was replicated on seven different days (a total of 323-345 oocytes were included in each of the four treatment groups). Data were tested for normality (Shapiro-Wilk test; $W \geq 0.90$) and reported as least squares means ± standard error of the means. Protected least significant difference was used for means separation.

Results

Culture at 41.0°C did not affect the proportion 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, ability of oocytes to cleave after addition of sperm was not compromised (SEM=3.8; $P > 0.2$; Figure 1) when matured at 41.0°C for the first 12 h of culture. However, culture of oocytes at 41.0°C increased the proportion of 2-cell embryos (SEM=1.3; $P < 0.04$; Figure 1) and decreased proportion of 8-16 cell embryos (SEM=4.7; $P < 0.05$; Figure 1). Moreover, culture of oocytes at 41.0°C during the first 12 h of maturation reduced development to blastocyst (SEM=2.2;

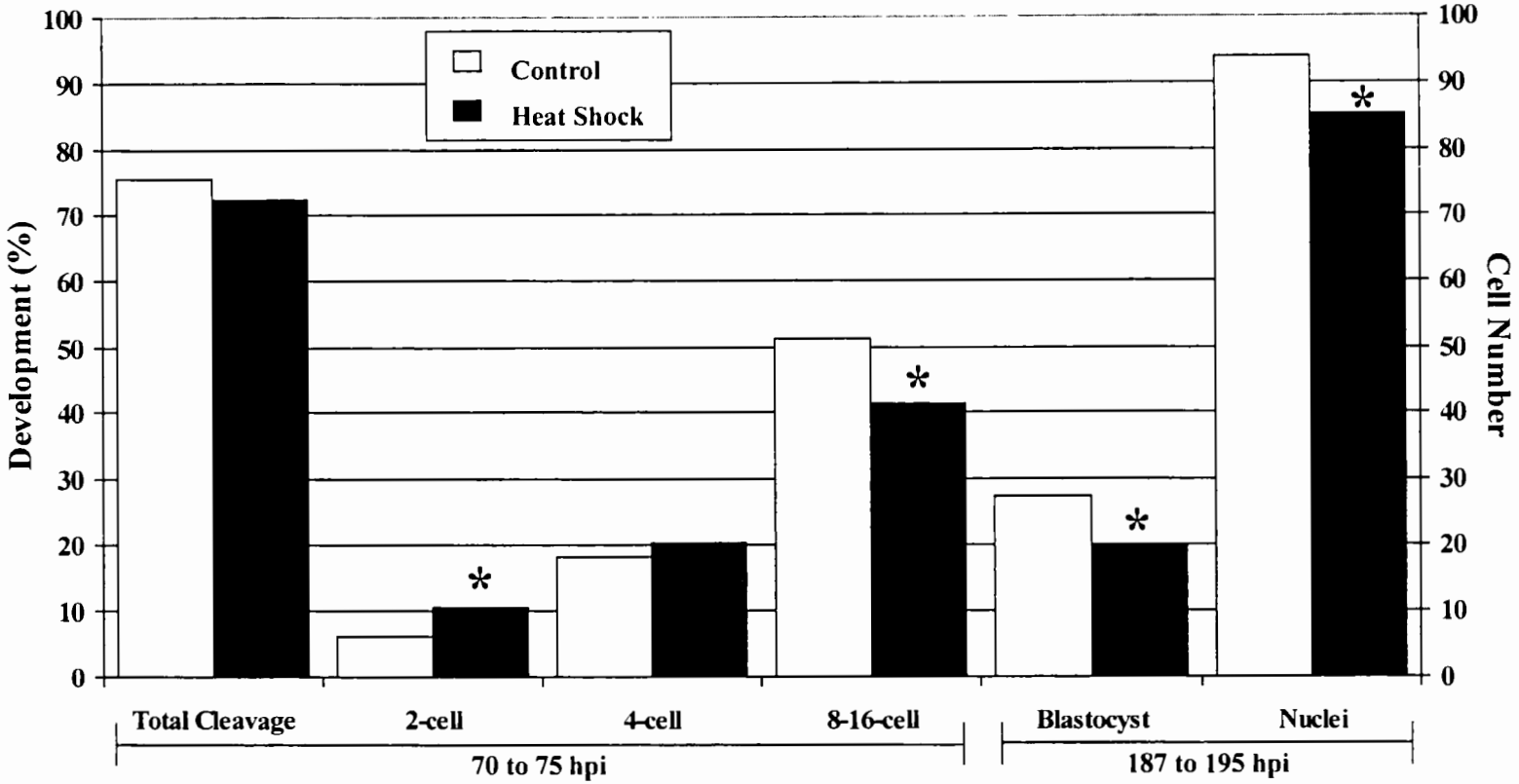

Figure 1. Development (%) of oocytes matured at 38.5°C (Control; □) or 41°C (Heat Shock; ■). After IVF, ability of oocytes to cleave was assessed between 70 to 75 hpi. The presence of more than one-cell was indicative of cleavage and the proportion of 2-, 4- and 8-16-cell embryos were also recorded. Beginning at 187 to 195 hpi, proportion of oocytes developing to blastocyst stage was recorded. Thereafter, total number of cells was estimated in individual blastocysts by counting the number of Hoechst-stained nuclei. *Heat shock differs from control; $P < 0.05$. SEM=6.7; P<0.005).

$P < 0.007$; Figure 1). Blastocysts derived from heat-stressed oocytes had fewer total nuclei (SEM=6.7; $P < 0.005$; Figure 1).

Addition of 5 μM all trans-retinol to 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; $P > 0.55$). Ability of PZ to cleave (SEM=3.8; $P > 0.9$; Figure 2), develop to 8-16 cell stage (SEM=4.7; $P > 0.6$; Figure 2) and blastocyst (SEM=2.2; $P > 0.5$; Figure 2) after addition of sperm was similar for control and retinol-treated oocytes. Also, 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). There were no significant temperature x retinol interactions observed ($P > 0.9$).

In three of the experimental replicates, 41.0°C did not compromise ability of oocytes to develop to the blastocyst stage. In order to have a more precise test of our hypothesis (administration of retinol during oocyte maturation may improve the developmental competence of oocytes compromised by heat stress), only those experimental replicates (n=4) whereby heat shock reduced development of oocytes by at least 20% compared to controls (Edwards and Hansen, 1996, 1997) were included in the statistical analysis. A significant temperature x retinol interaction was noted (n=4 reps; 189-197 oocytes/treatment) for blastocyst development (SEM=2.4; $P < 0.03$; Figure 3) and number of nuclei (SEM=5.0; $P < 0.04$; Figure 4).

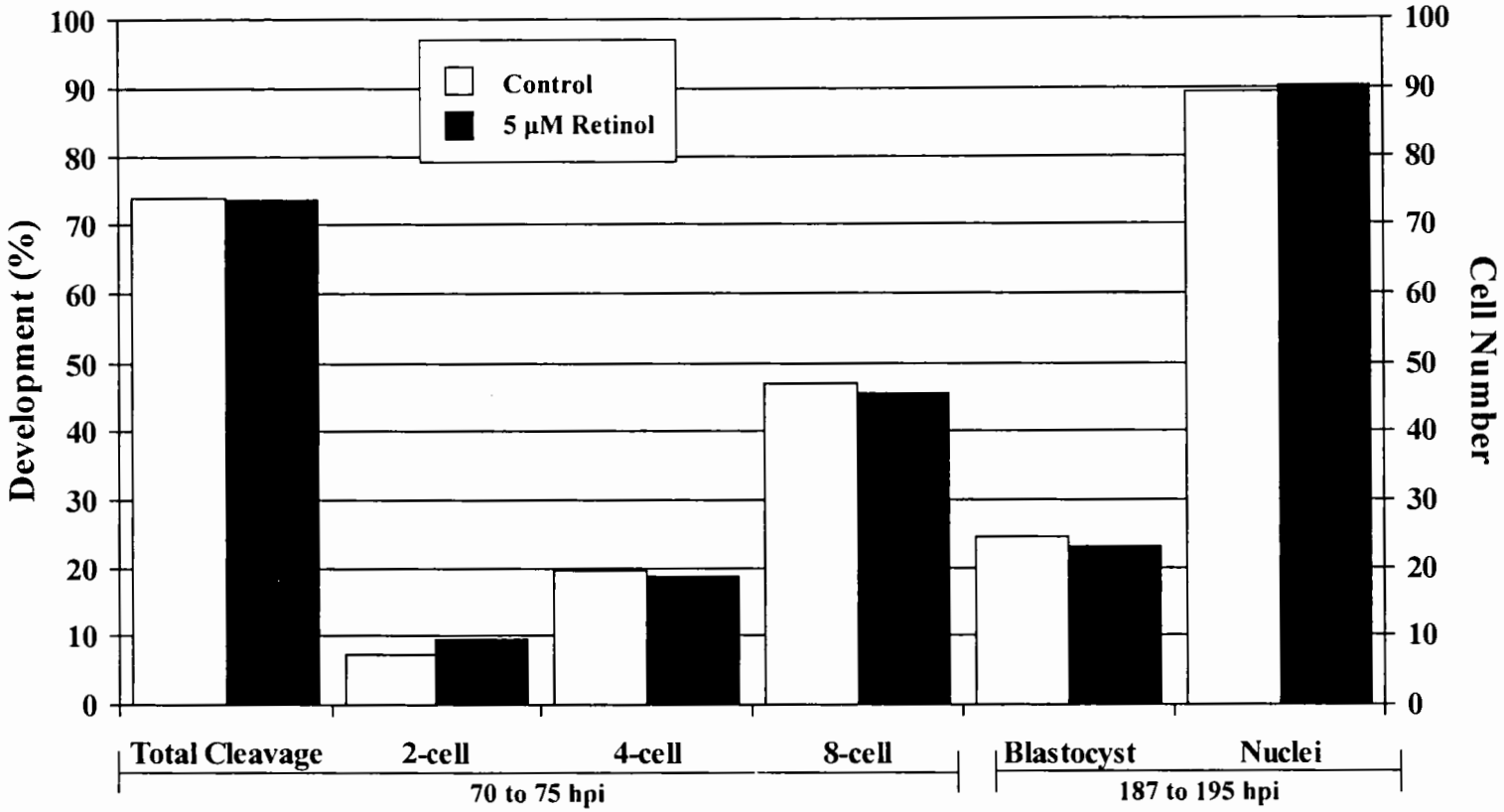

Figure 2. Development (%) of oocytes matured in 0 µM (Control; □) or 5 µM Retinol (■). After IVF, ability of oocytes to cleave was assessed between 70 to 75 hpi. The presence of more than one-cell was indicative of cleavage and the proportion of 2-, 4- and 8-16-cell embryos were also recorded. Beginning at 187 to 195 hpi, proportion of oocytes developing to blastocyst stage was recorded. Thereafter, total number of cells was estimated in individual blastocysts by counting the number of Hoechst-stained nuclei.

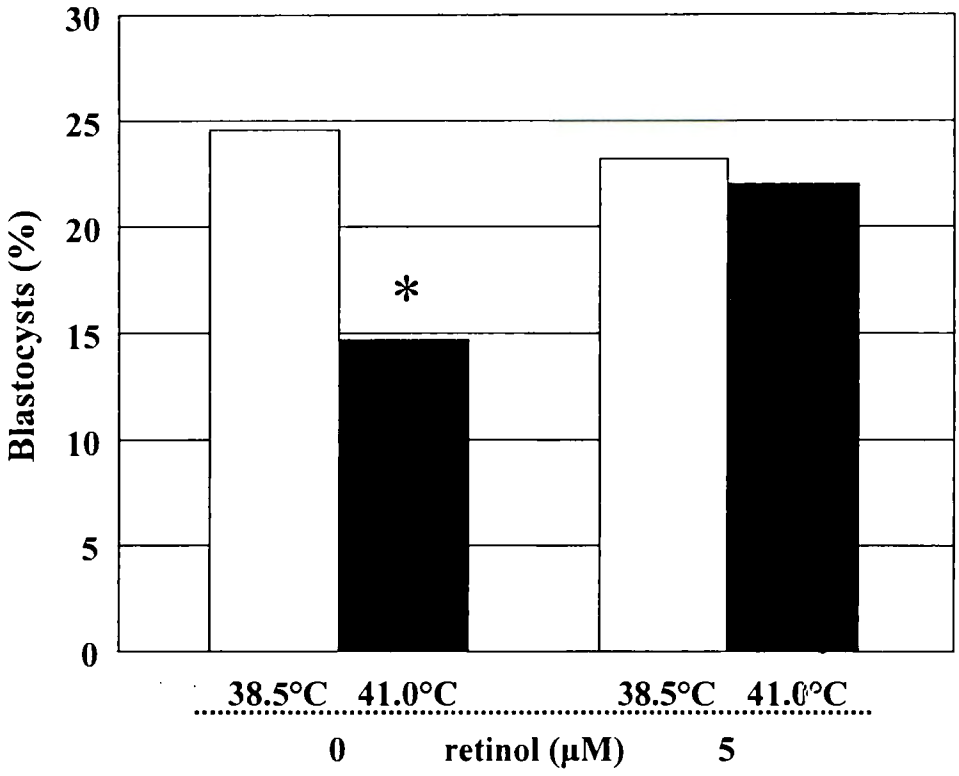

Figure 3. Proportion of oocytes developing to blastocyst after culture at 38.5°C or 41.0°C without (0) or with 5 μM all trans-retinol.
*Temperature x retinol interaction; $P < 0.03$.

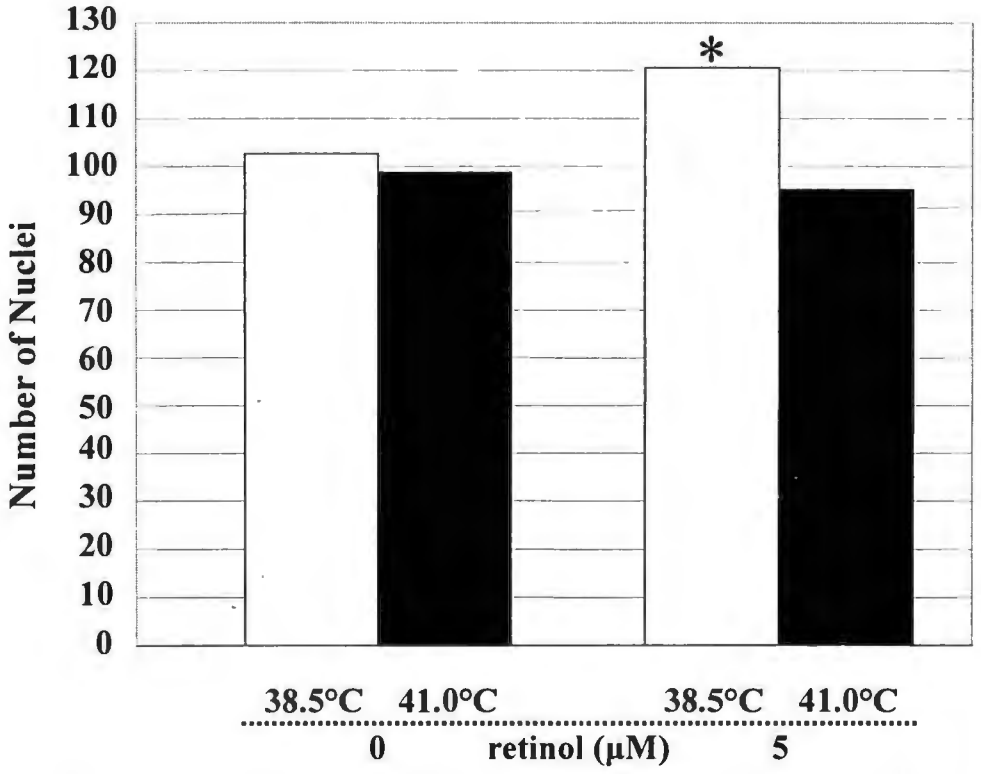

Figure 4. Number of nuclei contained within blastocysts derived from oocytes cultured at 38.5°C or 41.0°C without (0) or with 5 μM all trans-retinol. *Temperature x retinol interaction; $P < 0.04$.

Addition of 5 μM retinol to OMM and culture at 41.0°C for the first 12 h of culture did not increase parthenogenetic activation of oocytes as assessed by evaluating development to blastocyst (4.1 ± 1.6% and 2.6 ± 1.3% blastocyst development for oocytes cultured at 41.0°C for 12 h with 5 μM retinol or OMM for 24 h, respectively).

Discussion

Exposure of bovine oocytes to an elevated temperature during the first 12 h of maturation disrupted further development by decreasing the proportion of embryos developing to the 8-16 cell stage and increasing the proportion of embryos arresting at the 2-cell stage after addition of sperm. Moreover, heat shock reduced development of oocytes to blastocyst. Also blastocyst stage embryos derived from oocytes that were heat-stressed during maturation had fewer total nuclei. When added to the maturation medium, retinol ameliorated the negative effects of elevated temperature for reducing development of bovine oocytes compromised by a heat-stress of 41.0°C.

Heat-induced reductions in the development of maturing oocytes are in agreement with previous studies. Edwards and Hansen (1996, 1997) showed that culture of oocytes at 41.0°C during the first 12 h of maturation did not alter cleavage but reduced ability of oocytes to develop into blastocysts after fertilization. In the present study, a closer examination of cleaved embryos revealed that an elevated temperature of 41.0°C reduced the proportion of 8-16 cell embryos and the number of cells compromising blastocyst stage embryos. Similar observations have been made by others (Putney et al., 1989; Ju et al., 1998).

Specific mechanisms accounting for heat-induced reductions in development of maturing oocytes are poorly understood, but are likely the result of perturbations in critical events occurring during the first 12 h of oocyte maturation. Events known to occur during this time include posttranslational modifications of cyclin-dependent proteins (Wickramasinghe and Albertini, 1993), polyadenylation of mRNA coding for proteins necessary for spindle formation (Krischek and Meinecke, 2002), cytoskeletal modifications leading to germinal vesicle breakdown and progression to metaphase I (Sirard et al., 1989) and elevations in levels of maturation promoting factor ($p34^{cdc2}$/cyclin B kinase; Wu et al., 1997).

Examination of nuclear maturation (ability of the oocyte to progress from prophase I of meiosis to metaphase II and extrude the 1^{st} polar body) and components contained within the ooplasm altered by heat shock suggest that the majority of alterations are likely occurring within the cytoplasm of the maturing oocyte. Heat shock did not alter nuclear maturation as culture at 41.0°C for up to 24 h did not alter progression to metaphase II (Dorado et al., 2001; Payton et al., 2003d). Heat shock during maturation did, however, alter a number of components within the cytoplasm. Altered cortical granule types were observed by Payton et al. (2003d), suggesting possible alterations in the cytoskeleton (translocation of cortical granules to the oolemma is mediated by actin microfilaments in other species; Wessel et al., 2002). Glutathione content in matured oocytes was elevated after heat shock (Payton et al., 2003b), suggesting possible heat-induced increases in the production of free radicals. Effects of heat shock for altering components within the cytoplasm were perhaps most noted for altering de novo synthesis of intracellular proteins. Synthesis of intracellular proteins in

maturing oocytes was reduced by 30-50% depending on severity of the heat shock (Edwards and Hansen, 1996, 1997). Previously, Komar (1973) reported that exposure of murine oocytes to extreme elevations in temperature (44.0-45.0°C) after resumption of meiosis increased parthenogenesis (activation of the oocyte without sperm). In our study however, a physiologically-relevant elevated temperature of 41.0°C did not increase parthenogenetic development.

Heat-induced reductions in the development of oocytes were no longer evident when 5 μM all trans-retinol was added to 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 the control. Beneficial effects of adding retinol to maturation medium for increasing development of otherwise "stressed" oocytes have been observed previously (i.e. when development in controls was less than 20%; Livingston et al., 2002).

Specific mechanisms through which retinoids increase development of oocytes compromised by heat stress remain unclear. Retinoids may act as antioxidants by scavenging free radicals and preventing lipid peroxidation (Kartha and Krishnamurthy, 1977). Ciaccio et al. (1993) showed that administration of vitamin A to rats increased concentrations of all-trans retinol and all-trans 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 cells types, retinoids upregulate transcription of antioxidant genes such as superoxide dismutase (Ahlemeyer et al., 2001) and glutathione S-transferase (Lo and Ali-Osman, 1997) by binding to retinoic acid receptors.

Recently, Mohan et al. (2001; 2002) described the presence of 2 different types of retinoid receptors in the cumulus oocyte complex (retinoic acid receptor and retinoid X receptor). Additional analyses have shown both types of nuclear receptors as well as retinol binding proteins are localized within the cumulus cells surrounding the oocyte (Malayer; personal communication). Cumulus cells 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). Cumulus cells are transcriptionally active after resumption of meiosis (Fülöp et al., 1997; Tirone et al., 1997; Ochsner et al., 2003). As such, some of the positive effects of retinol for improving development of oocytes compromised by heat stress may be mediated by effects of retinol binding to receptors found within the cumulus. Mechanistic effects thereafter are unclear but in other cell types, retinoic acid decreased apoptosis by suppressing c-Jun N-terminal kinase (JNK) and activator protein 1 (AP1; Moreno-Manzano et al., 1999). In embryonal carcinoma cells, retinoic acid upregulated midkine expression (Kadomatsu et al., 1988). Midkine mRNA has been localized to granulosa cells (Karino et al., 1995) and addition of the functional protein to OMM improved development of bovine oocytes to the blastocyst stage (Ikeda et al., 2000).

Conclusions

Administration of retinol during oocyte maturation may protect oocytes from the harmful effects of heat stress for reducing oocyte quality. Further study on the mechanisms of retinol for improving development of oocytes compromised by heat stress

is needed. Doing so could lead to the development of therapeutic strategies for dairy producers faced with problems of heat stress for decreasing oocyte quality.

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