Disruption of Bovine Oocytes and Preimplantation Embryos by Urea and Acidic pH¹

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ABSTRACT

Feeding cattle diets high in degradable crude protein (CP) or in excess of requirements can reduce fertility and lower uterine pH. Objectives were to determine direct effects of urea and acidic pH during oocyte maturation and embryonic development. For experiment 1, oocytes were matured in medium containing 0, 5, 7.5, or 10 mM urea (0, 14, 21, or 28 mg/dl urea nitrogen, respectively). Cleavage rate was not reduced by any concentration of urea. However, the proportion of oocytes developing to the blastocyst stage at d 8 after insemination was reduced by 7.5 mM urea. In addition, the proportion of cleaved oocytes becoming blastocysts was decreased by 5 and 7.5 mM urea. For experiment 2, putative zygotes were collected ~9 h after insemination and cultured in modified Potassium Simplex Optimized Medium (KSOM). Urea did not reduce the proportion of oocytes developing to the blastocyst stage, although 10 mM urea reduced cleavage rate slightly. For experiment 3, dimethadione (DMD), a weak nonmetabolizable acid, was used to decrease culture medium pH. Putative zygotes were cultured in modified KSOM containing 0, 10, 15, or 20 mM DMD for 8 d. DMD reduced cleavage rate at 15 and 20 mM and development to the blastocyst stage at all concentrations. Results support the idea that feeding diets rich in highly degradable CP compromises fertility through direct actions of urea on the oocyte and through diet-induced alterations in uterine pH.

(Key words: urea, pH, preimplantation embryo, bovine)

Abbreviation key: COC = cumulus-oocyte complexes, DMD = dimethadione, KSOM = Potassium Simplex Optimized Medium.

INTRODUCTION

Feeding dairy cows large amounts of degradable protein in excess of requirements can reduce pregnancy rates per insemination (Canfield et al., 1990; Elrod and Butler, 1993). Diets high in protein content elevate urea nitrogen concentrations in plasma and uterine secretions (Jordan et al., 1983; Canfield et al., 1990; Elrod and Butler, 1993; Roseler et al., 1993), and elevations in blood or milk urea nitrogen concentrations have also been associated with low fertility (Butler et al., 1996; Larson et al., 1996; Rajala-Schultz et al., 2001).

The mechanism by which feeding large amounts of protein affects fertility is not completely known. One possibility is that high concentrations of urea associated with excess feeding of CP could disrupt oocyte growth or maturation, fertilization, or development. Feeding a diet generating large amounts of urea and ammonia increased growth of the second-wave dominant follicle, decreased capacity of oocytes from small (1 to 4 mm) and medium (>4 to 8 mm) follicles to cleave when fertilized in culture, and decreased ability of cleaved embryos formed from oocytes obtained from medium-sized follicles to develop in culture to the blastocyst stage (Sinclair et al., 2000). Moreover, exposure of oocytes to 6 mM of urea during maturation in vitro impaired meiosis and fertilization rate (De Wit et al., 2001).

Feeding large amounts of protein can also alter the uterine environment by reducing concentrations of magnesium, potassium, and phosphorus in uterine secretions (Jordan et al., 1983) and by reducing uterine pH (Elrod et al., 1993; Elrod and Butler, 1993). Effects of protein diet on potassium, phosphorus, and pH were only observed during the luteal phase. Although consequences of altered mineral concentrations on embryonic function are not known, a reduction in pH from 7.2 to between 6.9 and 7.1 has been reported to compromise embryonic development in mice (Edwards et al., 1998). In contrast, development of hamster preimplantation embryos was unaffected by a range of pH from 6.5 to 7.4 (Bavister et al., 1983; Carney and Bavister, 1987).

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Objectives of the present study were to 1) determine direct effects of urea on oocyte maturation and embryonic development and 2) evaluate whether low pH during the period of embryonic growth disrupts development. To test effect of pH, embryos were cultured in varying concentrations of dimethadione (**DMD**), a nonmetabolizable weak acid that lowers pH of the culture medium and has been used to study effects of pH on development of hamster and mouse embryos (Carney and Bavister, 1987; Edwards et al., 1998).

MATERIALS AND METHODS

Materials

Materials for in vitro maturation, fertilization, and embryo culture were obtained as described previously (Rivera and Hansen, 2001). Urea was purchased from Research Organics (Cleveland, OH), and hyaluronidase and dimethadione (5,5-dimethyl-2, 4-oxazol-idinedione) were from Sigma (St. Louis, MO). Frozen semen from bulls of several breeds (Angus, Holstein, and Brangus) was donated by Select Sires Inc. (Rocky Mount, VA) or was purchased from Southeastern Services Inc. (Wellborn, FL) or American Breeders Service (Madison, WI).

In Vitro Production of Embryos

In vitro production of embryos was performed as described by Rivera and Hansen (2001) using oocytes recovered from ovaries obtained at a local abattoir. Briefly, oocytes were collected by slashing the ovaries and washing in oocyte collection medium (Tissue Culture Medium 199 with Hank's Salts without phenol red, supplemented with 2% [vol/vol] bovine steer serum [containing 2 U/ml heparin], 1 mM glutamine, 100 U/ml penicillin-G, and 0.1 mg/ml streptomycin). Groups of 10 cumulus-oocyte complexes (**COC**) were matured by culture in 50- μ l microdrops of oocyte maturation medium (Tissue Culture Medium 199 with Earle's Salts supplemented with 10% [vol/vol] bovine steer serum, 50 μ g/ml gentamicin, 20 μ g/ml FSH, 2 μ g/ml estradiol-17 β , 22 μ g/ml sodium pyruvate, and 1 mM glutamine) for 21 to 24 h at 38.5°C and 5% (vol/vol) CO₂ in humidified air. Following maturation, COC were washed and groups of 30 oocytes were placed in four well plates of 600 μ l of IVF-TALP (Parrish et al., 1986) supplemented with 25 μ l of a mixture of 0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μ M epinephrine in 0.9% (wt/vol) NaCl. The COC were fertilized with $\sim 1 \times 10^6$ Percoll-purified sperm/well (Parrish et al., 1986). For each replicate, sperm were prepared from a pool of semen from three bulls. A different pool of bulls was sometimes but not always used for

each replicate. Following incubation for 8 to 10 h at 38.5°C and 5% (vol/vol) CO₂, putative zygotes were denuded of cumulus cells by vortexing and, if necessary, by incubation in 300 μ g/ml of hyaluronidase in Hepes-TALP. Embryos were then cultured in Potassium Simplex Optimized Medium (**KSOM**), modified by adding 3 mg/ml bovine serum albumin (essentially fatty-acid free), 50 μ g/ml gentamicin, Basal Medium Eagle essential AA solution, and Minimum Essential Medium nonessential AA solution. For embryo culture, putative zygotes were placed in drops of modified KSOM at 38.5°C and 5% CO₂ (vol/vol) in humidified air until d 8 after insemination. Cultures were performed in 25- μ l drops, and each contained 10 to 20 embryos per drop. Within a replicate, numbers of embryos per drop were constant for all treatments. For each replicate, one or more drops (typically two) of embryos were prepared for each treatment.

Experiments

Effect of urea during oocyte maturation on cleavage and subsequent development. Groups of ~ 10 COC were transferred to drops of oocyte maturation medium containing 0, 5, 7.5, or 10 mM urea. These concentrations are equivalent to 0, 14, 21, and 28 mg/dl urea nitrogen, respectively. Oocytes were matured for 21 to 24 h, washed in Hepes-TALP medium, and then subjected to fertilization in wells without the presence of urea. After fertilization, putative embryos were cultured in 25- μ l drops of modified KSOM. Cleavage rate was recorded on d 3 and the percentage of oocytes reaching the blastocyst stage recorded at d 8. The experiment was replicated seven times with a total of 204 to 229 oocytes per treatment group.

Effect of urea on early embryonic development. Putative zygotes were produced by in vitro maturation and fertilization. After fertilization, groups of 10 to 20 putative zygotes were transferred to 25- μ l drops of modified KSOM containing 0, 5, 7.5, or 10 mM urea for the duration of culture. Embryos were examined on d 3, 5, 7, and 8 for development. The experiment was replicated five times with a total of 167 to 180 putative zygotes per treatment group.

Effect of DMD on early embryonic development. A preliminary experiment was performed one time in which pH was measured in medium containing 0, 10, 15, or 20 mM DMD at various time points after incubation at 38.5°C and 5% CO₂ (vol/vol) in humidified air.

After fertilization, groups of 10 to 20 putative zygotes produced by in vitro maturation and fertilization were transferred to 25- μ l drops of modified KSOM containing 0, 10, 15, or 20 mM DMD. Embryos remained in these drops for the duration of culture.

Cleavage rate was recorded on d 3, and the percentage of oocytes reaching the blastocyst stage recorded at d 8. The experiment was replicated three times with a total of 102 to 124 putative zygotes per treatment group.

Statistical Analysis

The proportion of oocytes that cleaved and the proportion of oocytes and cleaved embryos that became blastocysts was calculated for each replicate (i.e., for embryos from all drops treated alike in a given replicate). Data were analyzed by least squares ANOVA using the general linear models procedure of the Statistical Analysis System (SAS, 1989). The mathematical model included effects of treatment and replicate; the error term represents treatment $\times$ replicate. When the main effect of treatment was significant, means separation procedures were carried out using least-squares means and the pdiff procedure of SAS to determine concentrations of urea that differed from untreated controls. Data were analyzed two ways—as untransformed data and after performing arcsin transformation. Probability values were similar for both analyses. Reported probability values are derived from the analysis of transformed data, whereas reported least-squares means and standard errors are derived from analysis of untransformed data.

RESULTS

Effect of Urea During Maturation

Cleavage rates were not reduced by urea at any concentration tested (Figure 1A). As compared with control oocytes, the proportion of oocytes that became blastocysts at d 8 after insemination was reduced ($P < 0.001$) by maturation in 7.5 mM urea but not by maturation in 5 or 10 mM urea (Figure 1B). The proportion of cleaved embryos developing to blastocysts was decreased by 5 ($P < 0.05$) and 7.5 mM urea ($P < 0.001$) but not by 10 mM urea (Figure 1C).

Effect of Urea During Embryonic Development

The highest urea concentration tested (10 mM) reduced ($P < 0.05$) cleavage rate slightly (Figure 2A). Nonetheless, in contrast to effects during maturation, urea did not reduce the proportion of oocytes (Figure 2B) or cleaved embryos (Figure 2C) developing to the blastocyst stage.

Effect of DMD During Embryonic Development

A preliminary study was conducted to describe the changes in the pH over time in media containing vari-

Figure 1. Development of bovine embryos treated with various concentrations of urea during in vitro maturation. The experiment was replicated seven times with a total of 204 to 229 oocytes per treatment group. Results are least-squares means $\pm$ SEM. Means significantly different from 0 mM urea are indicated by asterisks (* $P < 0.05$; *** $P < 0.001$).

ous concentrations of DMD (Figure 3). The initial pH values for modified KSOM with 0, 10, 15, and 20 mM DMD were 7.2, 6.6, 6.4, and 6.3, respectively. After 24 h in 5% CO₂, the respective pH values were 7.4, 7.1, 7.0, and 6.8.

As compared with controls, cleavage rate was not affected by 10 mM DMD but was reduced by 15 ($P =$

Figure 2. Development of bovine embryos treated with various concentrations of urea during culture. The experiment was replicated five times with a total of 167 to 180 putative zygotes per treatment group. Results are least-squares means $\pm$ SEM. Means significantly different from 0 mM of urea are indicated by an asterisk (* $P < 0.05$).

0.05) and 20 mM ($P < 0.01$) DMD (Figure 4A). Development to the blastocyst stage was significantly reduced by all concentrations of DMD regardless of whether data were expressed as percentage of oocytes developing to blastocysts (Figure 4B) or percentage of cleaved embryos developing to blastocysts (Figure 4C). The inhibition to development caused by DMD was nearly total; only 1.0% of oocytes cultured with 5 mM

Figure 3. Effects of various concentrations of DMD on culture medium pH at 38.5°C and 5% CO₂. Values are plotted for modified KSOM, modified KSOM containing vehicle for DMD (0 mM), and modified KSOM containing 10 to 20 mM DMD.

DMD became blastocysts, and no oocytes cultured in 15 or 20 mM DMD reached the blastocyst stage.

DISCUSSION

In this study, exposure of oocytes to physiologically relevant concentrations of urea during the process of maturation interfered with ability of the embryos formed after fertilization to develop to the blastocyst stage. Exposure of the embryo to urea after fertilization had no effects on development, indicating that the embryo itself was resistant to direct effects of urea. However, development was impaired by embryonic exposure to acidic pH similar to those of uterine secretions from cows fed excess CP (Elrod et al., 1993; Elrod and Butler, 1993). Taken together, the results implicate changes in urea concentrations and uterine pH as a cause of infertility associated with feeding diets containing excess CP.

As in the present study, De Wit et al. (2001) also observed an effect of urea exposure during maturation on the proportion of oocytes becoming blastocysts. However, the cause for the decline in development to the blastocyst stage was different than was the case here. In particular, De Wit et al. (2001) observed that the addition of 6 mM urea to maturation medium hastened completion of metaphase I, inhibited completion of metaphase II, reduced fertilization rate, and decreased the proportion of oocytes that became blastocysts. There was no effect of urea exposure during maturation on development of cleaved embryos to the blastocyst stage. Thus, urea reduced the proportion of

Figure 4. Effect of DMD during embryonic development. The experiment was replicated three times with a total of 102 to 124 putative zygotes per treatment group. Results are least-squares means $\pm$ SEM. Means significantly different from 0 mM DMD are indicated by symbols ($\dagger P = 0.05$; $*P < 0.05$; $**P < 0.01$).

oocytes becoming blastocysts because it reduced fertilization and cleavage rate, not because the embryos that were formed after fertilization were compromised. In the present study, in contrast, addition of urea during oocyte maturation did not inhibit cleavage rate but, rather, reduced the ability of cleaved embryos to become blastocysts. Thus, the overall reduction in the proportion of oocytes becoming blastocysts is attributed to decreased developmental competence of embryos formed from oocytes exposed to urea.

Differences in concentrations of urea between the two studies cannot account for the discrepancy between the current results and those of De Wit et al. (2001) because the reduction in development of cleaved embryos was observed in the present study at 5 and 7.5 mM urea. Perhaps differences in embryo culture media between the two studies are responsible for the discrepancy in effects of urea on embryo competence. The culture medium used in the study of De Wit et al. (2001) was complex and included serum, epidermal growth factor, insulin, and other constituents not present in the modified KSOM used in the present study. Future studies to determine whether specific nutrients or regulatory factors can overcome damage to the embryo caused by exposure of oocytes to urea or other stressors may lead to methods to improve embryonic survival under adverse conditions.

One surprising result of the current study was the observation that, in contrast to effects of 5 and 7.5 mM urea, exposure of oocytes to 10 mM urea during maturation did not affect cleavage or subsequent development. Conceivably, high concentrations of urea trigger some compensatory mechanism in the oocyte that increases resistance to urea toxicity. The nature of this phenomenon is unknown but is unlikely to involve transcription, because the oocyte is transcriptionally inactive during maturation and cannot respond to a cellular stress by increasing transcription of genes involved in cytoprotection. Heat shock, for example, can induce heat shock protein 70 synthesis at the two-cell stage but not in oocytes evaluated before or after completion of maturation (Edwards and Hansen, 1996). One possibility is that high concentrations (>7.5 mM) of urea trigger changes in membrane transport of urea to either inhibit movement of urea inside the cell or remove urea from the cytoplasm. Carrier-mediated exchange of urea occurs in other cells (Verkman et al., 1985; Hisanaga et al., 1991; Kato and Sands, 1998), but the system of urea transport in the oocyte and its regulation by urea concentration is not known.

The impairment of events during oocyte maturation by urea occurred at concentrations that are probably physiologically relevant. This is so because the concentrations affecting maturation (5 and 7.5 mM; equivalent to plasma urea nitrogen concentrations of 14 and 21 mg/dl) closely match the concentrations of urea in the plasma or milk of cows experiencing infertility due to excess feeding of CP (Canfield et al., 1990; Elrod and Butler, 1993; Butler et al., 1996; Larson et al., 1996; Rajala-Schultz et al., 2001). It is likely that feeding excess amounts of CP also elevates urea concentrations in the follicle, the site of oocyte maturation, al-

though intrafollicular concentrations of urea have not, to our knowledge, been monitored.

Urea concentrations are elevated in uterine secretions of cows fed large amounts of CP (Jordan et al., 1983) and in oviductal-uterine flushings of ewes fed supplemental urea (McEvoy et al., 1997). However, in contrast to effects of urea on the oocyte, present results suggest that exposure to urea has little effect on embryonic development when embryos are exposed continuously from the one-cell stage. The only effect seen was a slight reduction in cleavage rate when 10 mM urea was added after fertilization, suggesting that the highest concentration of urea tested inhibited ability of fertilized embryos to cleave. The reduction in cleavage rate was small, however, and addition of urea after fertilization did not reduce the proportion of embryos that developed to the blastocyst stage. Either the embryo may acquire some mechanism to counteract toxic effects of urea that the oocyte lacks, or urea is disrupting some process in the oocyte that does not occur during embryonic development.

In contrast to its resistance to urea, the embryo was very susceptible to disruption by reduction in pH, suggesting that the reduction in uterine fluid pH in the luteal phase of cows fed a high-protein diet (Elrod et al., 1993; Elrod and Butler, 1993) is inimical to embryonic survival. The approach taken to regulate extracellular pH was to add the acid DMD to culture medium. This acid is not likely to be toxic in and of itself because it is nonmetabolizable and had no effect on the growth of hamster embryos when pH was adjusted to a value similar to control embryos (Carney and Bavister, 1987). Rather, it is the decrease in extracellular pH that appears important. The addition of DMD resulted in a slightly acidified medium that gradually increased in pH as the medium equilibrated with the carbon dioxide in the gaseous phase. The degree of acidification produced by DMD resulted in a medium pH similar to that of uterine secretions of cows fed a diet high in CP (Elrod et al., 1993; Elrod and Butler, 1993). This condition was catastrophic for embryonic growth. Indeed, no embryo exposed to 15 or 20 mM DMD developed to blastocyst. This sensitivity to DMD makes the bovine embryo similar to the mouse, where culture in DMD from zygote stage reduces development to the blastocyst stage (Edwards et al., 1998).

CONCLUSIONS

Present results are consistent with the idea that detrimental effects of feeding protein in excess of requirements on fertility in dairy cows are mediated in part through the direct effect of urea on the process

of oocyte maturation and on diet-induced alterations in uterine pH. The observation that effects of urea on oocyte maturation were exerted in a narrow range of urea concentrations leads to the speculation that the oocyte may undergo some concentration-dependent compensatory mechanism to overcome actions of urea and that effects of urea on oocyte maturation may not be important under situations that result in very high circulating concentrations of urea.

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