Maturation and Freezing of Bovine Oocytes

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Story in Brief

Immature bovine oocytes were aspirated from small to medium size follicles of bovine ovaries by needle and syringe. The ovaries were obtained from animals slaughtered in the Perkins or Oklahoma City slaughter houses. The oocytes were transported to the lab in a prewarmed thermos and used in *in vitro* studies to determine maturation and livability under various treatments. The oocytes were treated in three ways: (1) incubated in synthetic oviduct fluid (SOF) for 24 hrs, (2) frozen, thawed, then incubated in SOF for 24 hrs, or (3) incubated, frozen, thawed, then incubated again to test the effects of partial maturation. The maturation and freezability of these oocytes was checked by making chromosome spread preparations of the oocytes. These were examined, using both phase and standard light microscopes, for the stage of division. Incubation for 24-26 hours in SOF resulted in 92 percent of the oocytes maturing to the metaphase II stage. Attempts to freeze resting or partially matured oocytes are in progress.

Introduction

In vitro work has been done with oocytes and embryos in the areas of maturation, and maturation and long term storage, respectively. Maturation of immature bovine oocytes, aspirated from follicles has been quite successful (i.e., 80 percent or more of the oocytes completing maturation) when cultured in a modified SOF media. The maturation process takes the oocyte from the resting stage of prophase I to metaphase II of the meiotic division, at which time the egg is ready to be fertilized. Embryos, flushed from cows and incubated *in vitro*, have gone from the two-cell stage of development to the eight-cell or morula stage; from the eight-cell stage to the blastula stage; and from the 16-cell or morula to the expanded blastula stage. However, the percentage of embryos continuing development *in vitro* has been variable and generally not as high as oocyte maturation. Long term storage of embryos at -320°F has primarily been studied in small laboratory animals, such as the mouse and rabbit, and up to 70 percent of the stored embryos have resumed division post-freezing. One of the areas that has not been investigated is the livability of oocytes frozen at either the resting stage or some stage of maturity.

The objectives of this study were to characterize the effects of (1) incubation time on chromosomal development, and (2) various freezing procedures on viability of oocytes.

Experimental Procedure

Ovaries were removed from reproductive tracts of cows slaughtered at Oklahoma City or Perkins. Follicular contents were immediately aspirated from two to six mm follicles with a 12 ml syringe and an 18 gauge needle. The fluid containing the oocytes and surrounding cumulus cells was placed in test tubes or a thermos at 38°C for transport to the laboratory. Oocytes with several layers of cumulus cells were selected and rinsed in modified SOF.

For the maturation studies, 10 to 20 eggs were placed in 12 x 75 mm test tubes containing one ml of modified SOF media. The tubes were gassed with a mixture of gases containing 90 percent N_2, 5 percent O_2, and 5 percent CO_2, stoppered; and incubated in an oven at 38°C. Several eggs were removed every two to four hrs of the incubation period to determine the progression of the chromosomes through meiotic division to metaphase II.

Four different treatments were utilized to investigate the possibility of freezing unfertilized oocytes. In Treatments 1 and 2, oocytes were placed in 0.1 ml of culture media and placed in a water bath at 38°C. The cryoprotective agent, dimethylsulfoxide (DMSO), was added in two increments of 0.05 ml at 10 min. intervals resulting in a final concentration of 1.5 M DMSO. The tubes were transferred to an ice bath for 10 min, transferred to a seeding bath at -4.5°C, seeded, and then transferred to the cooling chamber. Treatment 1 tubes were cooled to -110°C at a rate of 0.33°C per min, and Treatment 2 tubes were cooled at a rate of 0.8°C per minute. Treatment 3 was the standard semen freezing technique, which was an 8 min freeze in liquid N_2 vapor to -180°C. In Treatment 4, the oocytes were incubated for 12 hr, as described for the maturation studies, then frozen at 0.8°C per min to -110°C. This approach was designed to test the effects of partially maturing the oocytes.

Oocytes frozen in Treatments 1, 2, and 3 were thawed after three to six days of storage, at a rate of 16°C per minute until they reached 0°C, and were then transferred to a 38°C water bath. The DMSO was diluted by addition of 0.2 ml, 0.2 ml, and 0.4 ml of culture media at two min intervals. Oocytes were then rinsed in culture media, transferred to culture tubes, and incubated at 38°C for 24 hrs.

Oocytes frozen in Treatment 4 were quick thawed in 35°C water, and then treated the same as described above for preparation for culturing.

Oocytes in Treatments 1, 2, and 3 were incubated for 24 hrs while those in Treatment 4 were incubated 12 hrs. Following incubation, follicular cells were removed from the oocytes by enzymatic digestion with hyaluronidase, treated with a hypotonic solution of 0.2 percent sodium citrate, fixed on slides with a

Table 1. Effects of incubation time on stage of division of bovine ooctyes

Hr of incubation	No. of oocytes	Percent of oocytes in				
		Prophase I		Metaphase I	Telophase I	Metaphase II
		Germ. ves.	Distinct. chrom.			
4	39	100	---	--	--	--
8	36	---	100	--	--	--
12	19	---	55	45	--	--
14	19	---	---	84	11	5
18	20	---	---	30	45	25
20-22	22	---	---	5	35	60
24-26	--	---	---	--	8	92

1:3 mixture of acetic and ethanol, and air dried. They were then fixed for at least 1 hr in the same fixative, and stained with Giemsa stain to visualize the stage of chromosome division.

Results and Discussion

The process of oocytes maturation begins with germinal vesicle, the resting stage of the immature oocyte, breakdown and the appearance of distinct chromosomes finishing prophase I of the meiotic division. The chromosomal division proceeds through metaphase I where they resemble cross-type structures, anaphase, and telophase. Then, after a short prophase, they go to the final maturation phase, metaphase II, where the divisional activity waits until fertilization occurs. Table 1 presents the summary of the several studies conducted to develop procedures, and characterize the effects of incubation time on oocyte maturation. These data show that germinal vesicle breakdown and appearance of distinct chromosomes occurred between four and eight hours after initiation of culturing. At 14 hours, 85 percent of the oocytes were at the metaphase I stage. Between 18 to 22 hrs of culture are necessary for the oocytes to reach the maximum percentage progressing to the telophase stage. It took 24-26 hr of culture for the maximum percentage of the oocytes to progress to the metaphase II stage. Table 1 presents the data on only those oocytes where the divisional stage was identifiable. The number of cultured oocytes not showing an identifiable stage was usually less than 5 percent. Therefore, we conclude that 85-90 percent of the immature oocytes secured from excised ovaries and matured *in vitro* will progress through meiotic division as they would in the intact cow. Such consistent techniques will prove to be quite valuable in studies on oocyte properties and utilization.

Table 2 presents a summary of the attempts to freeze oocytes. The freezing rates in Treatments 1, 2 or 3 did not yield viable oocytes post-thaw. This is likely due to the fact that the oocyte, and the surrounding follicle cells are greatly different in size and have differing optimum freezing rates. One or the other is likely killed in freezing. Reports have shown that the follicle cells supply nutrients to the oocyte as it matures, and disruption of these cells by freezing may be the reason frozen oocytes do not mature. Treatment 4 was an

Table 2. Summary of effects of freezing on oocytes

Treatment	No. of oocytes	No. maturing
1 (.33°C/min)	270	0
2 (.8°C/min)	96	0
3 (8 min to -180°C)	122	0
4 (12 hr incub + .8°C/min)	75	0

attempt to get around this problem with the theory that partially matured cells may be better able to withstand freezing. Although the first study (Table 2) indicates no cells resuming maturation, later studies have yielded low percentages of viable cells after partial maturation, freezing, and completion of the required incubation period. Studies are continuing on ways to increase the manipulation and utilization of oocyte populations.
