Lowered Maturation Rates of Bovine Follicular Oocytes Cultured In Vitro after One-day Storage

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Abstract

In vitro maturation of bovine follicular oocytes stored at various temperatures (37, 20-25: room temperature, 10, 4, 0 and −4°C) with or without nitrogen gas infusion was investigated. After one-day storage, maturation rates of oocytes cultured in a medium containing TCM 199, 4 mg/ml bovine serum albumin and antibiotics for 27-30 hours were extremely decreased. The highest rate of maturation of the stored oocytes was obtained at the storage of 4°C without nitrogen gas (36.1%), which was a significant difference ($P<0.025$) in comparison with that of oocytes cultured immediately after collecting from follicles (66.7%).

The present study shows that one-day, prolonged storage of bovine oocytes would be hurmful for their viability of meiotic division during the culture in vitro.

Introduction

A number of studies have been made for short-term storage of sheep and cattle embryos at 5−10°C, room temperature and 37°C[1-7]. However, it seems that studies on low temperature storage of follicular oocytes or unfertilized eggs have been limited in experimental animals such as rabbits and mice[8-10]. The report of CHANG[10] showed that some unfertilized rabbit eggs were still fertilizable following exposure at 0−10°C for 48−72 hours.

The present experiment was planned to investigated in vitro maturation of bovine follicular oocytes cultured after one-day storage at various temperatures.

Materials and Methods

Ovaries were removed from cows at a local slaughterhouse and brought to the laboratory in 0.9% saline at 30°C within one hour. Follicular oocytes were collected by puncturing the follicles of <5 mm in diameter with a 22 gauge needle. The oocytes surrounded by cumuls cells were only used for storage in the present experiment. The oocytes were randamly kept in 1.0ml of the Dulbecco's phosphate buffered saline (PBS) containing 4 mg/ml bovine serum albumin: BSA (Fraction V: Sigma Co., U. S. A.), 50 μg/ml streptomycin and 100 i. u./ml penicillin at the various temperatures (37, 20−25: room temperature, 10, 4, 0 and −4°C) with or without gassing of nitrogen (N_2) into a small test tube (60 mm

long, 10 mm in diameter). After 24 hours storage, 5–10 oocytes were placed into the other test tube containing 1.0 ml of a culture medium which consists of TCM 199, 4 mg/ml BSA and the antibiotics. The medium had been allowed to preincubate for 2–3 hours at 37–38°C after gassing with 5% CO_2 in air for 30 seconds. The pH of the medium was adjusted to 7.2. At the transfer of oocytes into the medium, gassing for one minute was again performed. The test tubes were tightly stoppered and kept in an incubator at 37–38°C for 27–30 hours. After the culture was terminated, the oocytes were denuded of cumulus cells in 0.2% sodium citrate[11]. The denuded oocytes was fixed in acetic alchohol (1: 3) for 24–48 hours and stained with 0.75% aceto-orcein. The stage of the meiotic division was examined for each oocyte by a phase contrast microscopy. Classification of the stage of oocyte maturation was followed by the report of FUKUI and SAKUMA[12]. As a control, oocytes were cultured in the medium immediately after collecting from follicles without storage. All procedures were repeated in duplicates.

The factorial design (2×6) was as follows: with or without N_2 gas infusion, storage temperatures; 37, 20–25 (room temperature), 10, 4, 9 and −4°C. The proportions of oocytes reached to the metaphase stage of the second meiotic division were subjected to least square method for analysis of variance after angular transformation[13]. The analysis for the proportion of matured oocytes between each two temperatures was also attempted by the Duncan's new multiple range test[13].

Results

The results of the experiment are summarized in Table 1. The maturation rate of oocytes cultured immediately after collecting was 66.7% which was higher than those of oocytes stored

Table 1. Maturation of bovine follicular oocytes cultured in vitro after one-day storage at various temperatures

With or without N_2 gas	Temperatures (°C)	No. of oocytes examined	Stages of maturation*						Degenerative
			GV	P-I	M-I	A-I	T-I	M-II	
	No storage	33	0	3	4	2	1	22 (66.7)	1
With	37	37	9	18	3	0	0	0 (0.0)	7
	20–25	36	17	12	2	2	0	2 (6.1)	1
	10	40	10	15	4	0	0	5 (12.2)	6
	4	33	22	3	1	0	0	5 (15.2)	2
	0	33	18	2	4	0	0	2 (6.7)	7
	−4	36	17	4	3	0	0	2 (5.5)	10
	Total	215	93	54	17	2	0	16 (7.4)	33
Without	37	33	4	13	3	1	0	0 (0.0)	12
	20–25	48	31	7	1	0	0	5 (10.4)	4
	10	45	21	9	6	0	0	7 (15.6)	2
	4	36	13	7	0	0	1	13 (36.1)	2
	0	33	17	5	1	0	1	5 (15.2)	4
	−4	38	17	6	4	0	0	3 (7.9)	8
	Total	233	103	47	15	1	2	33 (14.2)	32

* GV, germinal vesicle; P-I, prometaphase I (germinal vesicle breakdown); M-I, metaphase I; A-I, anaphase I; T-I, telophase I; M-II, metaphase II.

Table 2. Analysis of variance for the data of Table 1.

Source of variation	DF	MS	F
With or without N_2 gas	1	84.06	3.77
Temperatures	5	195.82	8.78***
Interaction	5	12.50	0.56
Error		22.30	

*** $P<0.001$

Table 3. Results by the Duncan's new multiple-range test between each two temperatures

Temperatures (°C)	37	20—25	10	4	0
— 4	4.47**	0.50	2.16	4.60**	1.13
0	5.46**	5.10**	0.92	3.36	
4	8.93**	4.24**	2.64		
10	6.75**	1.71			
20—25	5.10**				

** $P<0.01$

and cultured. For the stored oocytes, the highest rate of oocyte maturation was obtained at the storage of 4°C without N_2 gas infusion (36.1%), which was a significant difference ($P<0.025$) as compared with that of oocytes cultured immediately after collecting.

The analysis of variance has shown in Table 2. There was a highly significant difference among the storage temperatures ($P<0.001$), but it was failed to find a significant difference between with and without N_2 gas infusing into the test tubes before storage. Among the 6 different temperatures, the storage at 4°C seemed to be more appropriate for the subsequent oocyte maturation than the other temperatures employed in the present study. However, there was no significant difference between 0°C and 10°C analysed by the Duncan's new multiple-range test (Table 3). The storage at 37°C resulted in the lowest rate of maturation after the culture.

Discussion

Maturation rates of follicular oocytes were extremely lowered after one-day storage at the every temperatures examined in comparison with that of oocytes cultured immediately after collecting from follicles. The use of N_2 gas to arrest the viability of follicular oocytes during the storage was failed to find the effect on the subsequent oocyte maturation. CHANG[8] reported that some unfertilized rabbit eggs were still fertilizable following exposure at 0–10°C for 2–3 days. SHERMAN and LIN[10] also reported that unfertilized mouse eggs survived up to 6 hours at 0°C. In the present study, the bovine oocytes aspirated from follicles showed low maturation rates after one-day storage at 0, 4 and 10°C. It has been considered that induction of aneuphoidy caused by the disruption of the second spindle during cooling since microtubules became disassembled at temperatures around 4°C and below, and the meiotic chromosomes are the free in the unfertilized egg cytoplasm[14].

DAVID et al[15]. described that resistance of cattle embryos to cooling to 0°C may not develop until the blastocyst stage. However, the early stage of embryos of cattle[3] and sheep[16-18] have been successful for 1 day and 2–10 days storages, respectively. Cleavage of cattle embryos is arrected at 10°C and the embryos stored at this temperature for one day appears to resume the normal development when transferred to the rabbit oviduct[3, 19]. For short-storage of embryos, there are lacking of data to suggest whether storage should be kept at 37°C, at room temperature of about 20°C or at an intermediate temperature of 30°C[20]. However, it has been agreed that sharp fluctuation in temperature should be avoided. From the results that the maturation rate was decreased after the one-day storage at 10°C in the present study, bovine oocytes

may not have resistance to cooling at this temperature or the below temperatures. In rabbit eggs, the storage at 0°C causes swelling of the cells and darkening or roughness of the cell membrane[21]. At 10°C, prolonged storage may produce granulation, loss of spheroid shape, and marked indentation in the cytoplasmic membrane of the egg. In the bovine oocytes stored in the present conditions, these abnormal evidences were observed. No cryoprotective agents were used for the storage at 0°C and −4°C in the present study. Sherman and Lin[9] reported that unfertilized mouse eggs survive up to 3.5 hours in a modified Locke's solution containing 5% glycerol at −10°C. During the cooling procedures, the induction of ice crystallization in the freezing medium at −3 to −7°C is necessary to achieve the subsequent survival[22]. With this respect, a cryoprotective agent should be used for storage at below 0°C.

The storage at 37°C in this experiment gave the poorest results of oocyte maturation. The cumuls cell mass of the oocytes stored at 37°C and some of those stored at room temperature had been disappeared after the one-day storage. As cumulus cells surrounding oocytes would be one of the most important factors influencing in vitro maturation of isolated oocytes [12, 23-25], this may be a major cause of the low rate of oocyte maturation after storage at high tempratures. Smith and Tenney[26] showed that mouse oocytes resulted in no difference in the maturation and degeneration rates of oocytes from chilled ovaries for 4 hours and of those from the contralateral ovaries dissected at 37°C. Whereas, Smith et al.[27] placed rabbit ovaries into cold media until the oocytes were recovered, anf found that the number of the oocytes with the first polar body decreased with duration of the exposure in the cold culture media. Fukui et al.[28] also placed bovine ovaries at 4°C or room temperature (15–20°C) in the same stored medium (Dulbecco's PBS) for one-day and cultured those isolated oocytes in vitro. The maturation rates were 18% and 33% for storage at 4°C and room temperature, respectively. As indicated by Smith et al.[27], the one-day storage of ovaries also decreased the maturation rate, which was similar to the isolated oocytes stored and cultured in vitro in the present study.

It was concluded that in vitro maturation of bovine oocytes collected from follicles was extremely depressed after one-day storage at various temperatures examined. More shortened storage should be investigated in future works as combined with determination of an appropriate temperature and components of a stored solution for the subsequent maturation in vitro of bovine extra-follicular oocytes.

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References

1) Averill, R. L. W. (1956): The transfer and storage of sheep ova. Proc. 3rd Int. Congr. Anim. Reprod., Cambridge **3**: 7–9.

2) Kardymowicz, M., O. Kardymowicz, W. Kohl and A. Lada (1963): Storage of fertilized sheep ova at low temprature. Acta. Biol. Cracov. Ser. Zool. **6**: 31–37.

3) Sreenan, J. M., P. Scanlon and I. Gordon (1970): Storage of fertilized cattle ova in vitro. J. Agric. Sci., Cambridge **74**: 593–594.

4) Moore, N. W. and R. J. Bilton (1973): The storage of fertilized sheep ova at 5°C. Aust. J. Biol. Sci. **26**: 1421–1427.

5) Trounson, A. O., S. M. Willadsen, L. E. A. Rowson and R. Newcomb (1976): The storage of cow eggs at room temprature and at low temperatures. J. Reprod. Fert.

46: 173–178.

6) Nelson, L. P., R. P. Elsden and L. G. Case (1979): Pregnancy rates after storage of bovine embryos at 37°C or room temperatures. J. Anim. Sci. **49**, Suppl. 1: 321 (Abstr.).

7) Kanagawa, H. (1980): One to two day preservations of bovine embryos. Jpn. J. Vet. Res. **28**: 1–6.

8) Chang, M. C. (1952): Fertilizability of rabbit ova and the effects of temperatures in vitro on their subsequent fertilization and activation in vivo. J. Exp. Zool. **121**: 351–381.

9) Sherman, J. K. and T. P. Lin (1958): Survival of unfertilized mouse eggs during freezing and thawing. Proc. Soc Exp. Biol. Med. **98**: 902–905.

10) Shbrman, J. K. and T. P. Lin (1959): Temperature shock and cold storage of unfertilized mouse eggs. Fertil. Ster. **10**: 384–396.

11) Hirschel, M. D. and A. G. Hunter (1979): Protease inhibitors in the bovine cystic follicle. J. Anim. Sci. **49**, Suppl. 1: 304 (Abstr.).

12) Fukui, Y. and Y. Sakuma (1980): Maturation of bovine oocytes cultured in vitro: Relation to ovarian activity, follicular size and the presence or absence of cumulus cells. Biol. Reprod. **22**: 669–673.

13) Steel, R. G. D. and J. H. Torrie (1960): Principles and Procedures of Statistics. McGraw-Hill Book Co., New York.

14) Whittngham, D. (1980): Preservation of embryos of the laboratory animals. Proc. 9th Int. Congr. Anim. Reprod. and A. I., Spain **2**: 237–243.

15) David, J. D. E., W. A. Jones, R. Newcomb, G. F. Smith and D. F. Whishart (1976): Embryo transfer with particular reference to cattle. Soc. Study of Animal Breeding, Published by the British Vet. Assoc., London.

16) Averill, R. L. W. and L. E. A. Rowson (1959): Attempts at storage of sheep ova at low temperatures. J. Agric. Sci., Cambridge **52**: 392–395.

17) Buttle, H. R. L. and J. L. Hancock (1964): Birth of the lambs after storage of sheep eggs in vitro. J. Reprod. Fert. **7**: 417–418.

18) Kardymowicz, O. (1972): Successful in vitro storage of fertilized sheep ova for ten days. Proc. 7th Int. Congr. Anim. Reprod. and A. I., Munich **1**: 499–502.

19) Sreenan, J. M., P. Scanlon and I. Gordon (1968): Culture of fertilized cattle eggs. J. Agric. Sci., Cambridge **70**: 183–185.

20) Betteridge, K. J. (1980): Procedures and results obtainable in cattle. In Current Therapy in Theriogenology. Ed. by D. A. Morrow, p. 74–88, W. B. Saunders Co., Philadelphia.

21) Chang, M. C. (1948): Probability of normal development after transplantation of fertilized rabbit ova stored at different temperatures. Proc. Soc. Exp. Biol. Med. **68**: 680–683.

22) Moore, N. W. (1980): Long-term storage of frozen embryos. In Current Therapy in Theriogenology. Ed. by D. A. Morrow, p. 104–106, W. B. Saunders Co., Philadelphia.

23) Mcgaughey, R. W. (1977): The culture of pig oocyte in minimal media, and the influence of progesterone and estradiol-17β on meiotic maturation. Endocrinology **100**: 39–45.

24) Sato, E., A. Irtani and Y. Nishikawa (1977): Factors involved in maturation of pig and cattle follicular oocytes cultured in in vitro. Jap. J. Anim. Reprod. **23**: 12–18.

25) Sato, E., A. Iritani and Y. Nishikawa (1978): Rate of maturation division of pig follicular oocytes cultured in vitro. Jap. J. Zootech. **49**: 400–405.

26) Smith, D. M. and D. Y. Tenney (1978):

The effect of cold shock on mouse oocyte maturation. J. Reprod. Fert. **54**: 401–403.

27) SMITH, D. M., J. P. P. TYLER and G. F. ERICKSON (1978): Effects of medium composition and progesterone on maturation in vitro of rabbit oocytes from graafian follicles of different sizes. J. Reprod. Fert. **54**: 393–400.

28) FUKUI, Y., A. MIYAMOTO and H. ONO (1982): In vitro maturation of bovine follicular oocytes at various temperatures before culture. Jap. J. Fertil. Ster. **27**: 514–519.

1日保存後に体外培養されたウシ卵胞内卵子の低成熟率

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摘　　要

種々の温度（37，室温：20～25，10，4，0 そして −4°C）および窒素封入の有無において1日保存されたウシ卵胞内卵子の体外成熟について検討した。ウシ未成熟卵子は直径5mm以下の卵胞から採取され，ダルベッコのPBS液で1日保存後，TCM199，4mg/mlウシ血清アルブミンおよび抗生物質を含む培養液で27～30時間体外培養された。成績は，種々の条件下で保存された卵子の体外成熟率は採取後直ちに培養に供されたものより有意に低かった（$P<0.025$：0～36%，67%）。保存温度では4°Cで保存された卵子の成熟率は最高であった（36.1%）。また，窒素封入の効果は認められなかった。以上の成績から，1日保存後のウシ卵胞内卵子の生存性および体外成熟能は著しく低下することが示唆された。

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