第25卷 第6期 西北农业大学学报 Vol. 25 No. 6
1997年12月 Acta Univ. Agric. Boreali-occidentalis Dec. 1997

Fertilization of Bovine Oocytes by Spermatozoal Microinjection

Li Qingwang[1] Liboriussen T[2]

(1 *Department of Animal Science, Northwestern Agricultural University, Yangling, Shaanxi 712100, The People's Republic of China*)

(2 *Department of Cattle and Sheep, National Institute of Animal Science Research Centre, Foulum DK-8830 Tjele, Denmark*)

Abstract The objective of this study was to explore a method to increase the fertilization rate of bovine oocytes matured in vitro by the microinjection of a single immotile spermatozoon. The experiment was divided into three parts. In experiment 1, matured oocytes were activated for four treatments with 1 μmoL $\cdot$ L^{-1}, 1 μmoL $\cdot$ L^{-1} (cumulus cells were removed before activated), 5 μmoL $\cdot$ L^{-1} and 5 μmoL $\cdot$ L^{-1} (cumulus cells were removed before activated) calcium ionophore A23187, respectively. Spermatozoa were capacitated by 40 mg $\cdot$ L^{-1} heparin in four treatments. In experiments 2 and 3, matured oocytes were treated like experiment 1. Spermatozoa were capacitated by 50 mg $\cdot$ L^{-1} and 100 mg $\cdot$ L^{-1} heparin. After oocytes and spermatozoa were treated, a single immotile spermatozoon was injected into the ooplasm. The study results demonstrated that the fertilization rate of bovine oocytes matured in vitro increased with 5 μmoL $\cdot$ L^{-1} calcium ionophore A23187 by the immotile sperm injection compared with other treatments. The different concerntration heparin did not affect the fertilization rate of bovine oocytes for sperm capacitation. Cumulus cells were romoved before activated, which seriously reduced the fertilization rate of bovine oocytes by the microinjection of an immotile spermatozoon.

Key words bovine, oocyte, spermatozoa, microinjection

Microinjection techniques have been studied as another method of fertilization in vitro. Application of this technique in a clinical setting may provide therapeutic means to overcome certain type of infertility. Recently, some studies indicate that sperm cells as vectors may introduce foreign DNA into egg to conduct the expression of the foreign gene or genetic transformation of animals by means of sperm microinjection[1]. In the process of sexual sperm separated by the method of flow cytometer, one of the main problems existed was low sperm mortality and low separated speed[2], but the technical application of sperm microinjetion can resolve this problem efficiently. Meanwhile sperm microinjection is also a very useful means for examining the reliability of sexual sperm separated by PCR. From 1976 to 1992, lots of papers related to sperm microinjection had been published in human[3], mouse or rat[4], rabbit[5], hamster[6], sheep[7] and bovine[8~11]. Although few of fsprings had been achieved in mouse[4] and bovine[12] by

收稿日期 1997-05-26
作者简介 李青旺，男，1956年生，副教授，博士

means of sperm microinjection, generally speaking, the rate of fertilization and cleavage for oocytes was lower than that of fertilization in vitro. The main aim of this experiment was, as attempted, to explore different methods to improve the rate of fertilization for bovine oocytes matured in vitro by an immotile sperm microinjection into the the ooplasm.

1 Materials and Methods

1.1 Media preparation

Oocytes were aspirated and washed before cultured with TCM-Hepes Buffer supplemented with 2% heat-treated cow serum (c.s.) and 4.168 nmol $\cdot s^{-1} \cdot L^{-1}$ heparin (aspirated medium). Follicular oocytes were cultured to maturity in a TCM-Bicarbonate medium supplemented with 0.267 nmol $\cdot s^{-1} \cdot L^{-1}$ Suigonan-opl(hCG)(IVM medium). Spermatozoa was capacitated or eggs were activated in CR1-Bicarbonate medium supplemented with 40, 50, 100 mg $\cdot L^{-1}$ heparin for sperm capacitation (capacitated medium), and supplemented with 1 μmoL $\cdot L^{-1}$and 5 μmoL $\cdot L^{-1}$calcium ionophore A23187 for egg activation (activated medium), respectively. Activated and injected eggs were stored in TCM-Hepes Buffer supplemented with 15% c.s. and 5 mg $\cdot L^{-1}$ cytochalasin B (injected medium). Injected oocytes were washed in TCM-Hepes Buffer supplemented with 2% c.s. (washed medium), and were cultured in CR1-Bicarbonate Medium supplemented with 2% c.s. (IVC medium).

1.2 Culture of oocytes

Bovine ovaries were obtained at a local slaughterhouse in 10～20 min postslaughter. Oocytes were aspirated and washed twice in aspirated medium and one time in IVM medium, then oocytes were cultured in IVM medium (30～40 oocytes in 500 μL drops) under sterile paraffin oil at 39℃ in humidified 0.05 $m^3 \cdot m^{-3}$ CO_2 and 0.95 $m^3 \cdot m^{-3}$ N_2 for 24 to 25 h.

1.3 Oocyte activation

After maturation culture, oocytes were divided into four groups. Cumulus cells were removed with a stirring machine for 2 to 3 min in CR1-hepes for group 2 and 4. Oocytes were activated by the modified method of Younis et al. [8] using 1 μmoL $\cdot L^{-1}$ the calcium ionophore A23187 for group 1 and 2, and 5 μmoL $\cdot L^{-1}$calcium ionophore A23187 for group 3 and 4 in activated medium (in 200 μL drops) overlaid paraffin oil for 7 min at 39 C in humidified 0.05 $m^3 \cdot m^{-3}$ CO_2 and 0.95 $m^3 \cdot m^{-3}$ N_2.

1.4 Sperm preparation

Experiment 1 Frozen semen in 0.25 mL plastic straws was thawed in water at 37 C for 1 min and then washed twice with 2 mL CR1-Hepes medium by centrifugation at 700 g for 10 min. After discarding the supernatant, the sperm pellet was removed to 200 μL with heparin-containing (40 mg $\cdot L^{-1}$) IVF medium (in 200 μL drops) overlaid paraffin oil, and kept at 39℃ for 45 min in humidifield 0.05 $m^3 \cdot m^{-3}$ CO_2 and 0.95 $m^3 \cdot m^{-3}$ N_2 to induce capacitation. After incubating, sperm was sucked into a centrifuge tube containing 2 mL CR1-Hepes medium, and stored at −20℃ for sperm microinjection.

Experiment 2 and 3　Frozen semem was thawed and washed like Experiment 1, spermatozoa were capatitated by 50 mg • L^{-1} and 100 mg • L^{-1} heparin, respectively.

1.5 Sperm injection and subsequent culture

Cumulus cells were removed with stirring machine in CR1-Hepes medium for 2 min in group 1 and 3. After this proceeding, two drops (50 μL injected medium neared 1.5 cm away) were mounted in 100 mm petri dish covered with sterile paraffin oil. Three μL sperm dilution was placed on one drop, 10 oocytes was placed on another drop. The internal diameter of the tip of the injection pipette was 8～15 μmol • L^{-1}. Individual sperm was picked up and injected into the ooplasm of an ovum slowly. After injected, the oocytes were transferred into 1VC medium to be cultured for 6 d. The oocytes were examined under the fluoro-microscope with hoechst 33342 staining.

2 Statistical Analysis

The experiments were tested by Chi-square analysis.

3 Results

In experiment 1, when the injected oocytes had been cultured for 6 d in four treatments, and oocytes were stained with hoechest 33342 and examined under the flucro-microscope, the fertilization rate of oocyes was 17.5%, 10%, 46.3% and 20%, respectively (Table 1). In group 3, when oocytes with cumulus cells were activated with 5 μmoL • L^{-1} calcium ionophere A 23187, fertilization rate was higher ($P<0.05$) than that in group 1, 2 and 4. In group 4, matured oocytes removed cumulus cells before activated would considerably decrease the fertilazation rate compared with group 3. There were no significant differences ($P>0.05$) in group 1, 2 and 3 for fertilization rate of oocytes.

In experiment 2 and 3, when the injected oocytes had been cultured for 6 d, and spermatozoa were capacitated with 50 mg • L^{-1} and 100 mg • L^{-1} heparin, respectively, significantly higher fertilization rates were observed in group 3 than in other groups ($P<0.05$, Table 2 and 3). After cumulus cells were removed before activated, low fertilization rates were still observed in group 4.

The different concentration heparin for sperm capacitation among three experiments did not significantly affect fertilization rate of oocytes following sperm injection ($P>0.05$).

Table 1　The rate of fertilization and cleavage for injected bovine oocytes with 40 mg • L^{-1} heparin capacitation

Groups	Cumulus cells	A23187 (μmoL • L^{-1})	No. of oocytes injected(n)	Fertility (n)	8-16 cell (n)	Morula (n)
1	intact	1	40	7	4	0
2	remove	1	40	4	2	0
3	intact	5	41	19[a]	9	3
4	remove	5	40	8	3	0

a Proportion with superscript differ ($P<0.05$).

Table 2 The rate of fertilization and cleavage for injected bovine oocytes with 50 mg · L^{-1} heparin capacitation

Groups	Cumulus cells	A23187 (μmoL · L^{-1})	No. of oocytes injected(n)	Fertility (n)	8-16 cell (n)	Morula (n)
1	intact	1	40	8	3	0
2	remove	1	40	3	1	0
3	intact	5	41	17[a]	8	2
4	remove	5	40	7	4	1

a Proportion with superscript differ ($P<0.05$).

Table 3 The rate of fertilization and cleavage for injected bovine oocytes with 100 mg · L^{-1} heparin capacitation

Groups	Cumulus cells	A23187 (μmoL · L^{-1})	No. of oocytes injected(n)	Fertility (n)	8-16 cell (n)	Morula (n)
1	intact	1	40	6	2	0
2	remove	1	40	4	1	0
3	intact	5	40	16[a]	7	3
4	remove	5	41	8	4	0

a Proportion with superscript differ ($P<0.05$).

4 Discussion

In study of spermatozoal microinjection, most researches used a motile spermatozoon to inject into the ooplasm. In this experiment, killed bovine spermatozoa were microinjected into the ooplasm of bovine oocytes matured in vitro and cultured for 6 d in vitro. And the study indicated that bovine oocytes matured in vitro could be fertilized and developed into 8 cell to morula stage by the injection of an immotile sperm into the ooplasm, and our result was similar to the previous reports[10,11]. Goto et al[10] used 50 μmoL · L^{-1} calcium ionophore A 23187 to activate bovine oocytes by 10 min incubation, oocyte development to 2～4 cells, 6～12 cells, morula was 36.1%, 17.2% and 8.2%, respectively. Heuwieser et al[11] reported that bovine oocytes were activated in 10 μmoL · L^{-1} calcium ionophore A 23187 for 5 min incubation, the higher fertilization rate was observed (44%). In our study, when bovine oocytes were activated in 5 μmoL · L^{-1} calcium ionophore A23187 for 7 min incubation, after injected oocytes had been cultured for 6 days, development to 8～16 cells and morula was 21.95% and 7.3%, respectively (experiment 1). And the fertilization rate (46.3%) was significantly higher than that of oocyte activated with 1 μmoL · L^{-1} calcium ionophore A 23187 (17.5%). These results suggest that the calcium ionophore A 23187 can be uesd to stimulate oocyte activation and pronuclear development for in vitro matured bovine oocytes. The ability of calcium ionophore A 23187 to activate bovine oocyte depends on concentrations, 5 μmoL · L^{-1} calcium ionophore A 23187 may be effecient to bovine oocyte activation in vitro compared with other concentrations. In the present experiment, heparin had been used with three concentrations for sperm capacitation, but the fertilization rate of bovine oocytes among the three experiments makes no difference. In group 4, when cumulus cells were removed before activated, the fertilization rate of bovine oocytes was significantly de-

creased, which demonstrates that cumulus cells may play an important role in the process of oocyte activation or fertilization.

Acknowledgments: The author is grateful to Drs. Henrik Callesen and Steven Smith for their continuing guidance and strong supports, and also wishes to thank Mrs. Claus and Anne Mette for their much assistance.

References

1 Gandolfi F, Lavitrano M, Camaioni A et al. The use of sperm-mediated gene transfer for the generation of transgenic pigs. J Reprod Fertil. Abstr. Series. 1989, 4: 10
2 McEvoy J D. Alteration of the sex ratio. Animal Breeding Abstracts, 1992, 60(2): 97～111
3 Trounson A, Laws-King A, Sathananthan H et al. Fertilization of human oocytes by microinjection of a single spermatozoon under the zona pellucida. Fertility and Sterility, 1987, 48(4): 637～642
4 Mann J R. Full term development of mouse eggs fertilized by a spermatozoon microinjected under the zona pellucid. Biology of Reproduction, 1988, 38: 1077～1083
5 Keefer C L. Fertilization by sperm injection in the Rabbit. Gamete Research, 1989, 22: 59～69
6 Clarke R N, Johnson L A. Factors related to successful sperm microinjection of hamster eggs: The effect of sperm species, technical experience, needle dimension, and incubation medium on egg viability and sperm decondensation following microinjection. Theriogenology, 1988, 30(3): 447～460
7 Clarke R N, Rexroad C E J, Powell A M et al. Microinjection of ram spermatozoa into homologous and heterologous oocytes. Biology Of Reproduction, 1988, 38(1): 75
8 Younis A I, Keefer C L, Brackett B G. Fertilization of bovine oocytes by sperm injection. Theriogenology, 1989, 31: 276
9 Goto K, Kinoshita A, Takuma Y et al. Fertilisation of bovine oocytes by the injection of immobilised, killed spermatozoa. Veterinary Record, 1990, 127, 517～520
10 Goto K, Kinoshita A, Takuma Y et al. Fertilization by sperm injection in cattle. Theriogenology, 1991, 33(1): 238
11 Heuwieser W, Yang X, Jiang S et al. Fertilization of bovine oocytes after microsurgical injection of spermtozoa. Theriogenology, 1992, 38: 1～9
12 Goto K, Kinoshita A, Takuma Y et al. Birth of calves after the transfers of oocytes fertilized by sperm injection. Theriogenology, 1991, 35(1): 205

51-55　　(11)

牛体外成熟卵母细胞死精子微注射受精研究

李青旺[1]　Liboriussen T[2]　Libor., T

(1 西北农业大学动物科学系，陕西杨凌 712100)
(2 丹麦国家畜牧所牛羊系，DK-8830 Tjele, Denmark)

摘　要　本研究主要目的是应用死精子微注射法探索一种提高牛成熟卵母细胞体外受精的方法。试验划分为三个部分：试验 1 体外成熟卵母细胞分为 4 个处理组。1 组和 3 组成熟卵母细胞分别用 1 μmoL·L^{-1}和 5 μmoL·L^{-1}的钙离子 A23187 激活；2 组和 4 组卵母细胞在 1 μmoL·L^{-1}和 5 μmoL·L^{-1}的钙离子 A23187 激活前去除颗粒细胞。精子获能用 40 mg·L^{-1}肝素。在试验 2 和试验 3，成熟卵母细胞的激活方法和颗粒细胞的去除同试验 1，精子获能分别用 50 mg·L^{-1}和 100 mg·L^{-1}肝素。当卵和精子经上述处理后，一个获能后的死精子微注射到卵细胞质中。试验结果表明：体外成熟的牛卵母细胞，当用 5 μmoL·L^{-1}钙离子 A23187 激活后，经死精子显微注射，受精率明显地高于 1 μmoL·L^{-1}的钙离子激活组。当精子获能用不同剂量的肝素处理后，对牛体外成熟的卵母细胞受精率未产生明显的影响。在死精子显微注射受精过程中，当成熟牛卵细胞在钙离子激活前去除颗粒细胞将明显地降低其受精率。

关键词　牛，卵母细胞，精子，显微注射
中图分类号　S823.35，S814.4