In Vitro Maturation and Fertilization of Ovine Oocytes in a System with Absence of 5% CO_2

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ABSTRACT. **In vitro maturation and fertilization of ovine oocytes in system with absence of CO_2 was studied. The cumulus oocyte complexes (COCs) were matured in four different maturation medium, namely: a). TCM-199 only, b). TCM-199 +10 mM Hepes, c). TCM-199 + 20 mM Hepes and d). TCM-199 + 30mM Hepes. The COCs were incubated in the incubator with 5% CO_2, incubator without 5% CO_2 and incubator with effervescent granule (EG) as the source of 5% CO_2 at 38.5°C for 24 hours. Four different time of incubation (20, 24, 28 and 32 hours) in system without 5% CO_2 was design to get the maturation rate. Treatments above were used to mature the oocytes for in vitro fertilization. The treatment of fertilization consists of the COCs fertilized for 18 hours in three incubator systems. The maturation rates of COCs in the incubator without 5% CO_2 or EG gradually increased by increasing of Hepes level from 0 mM to 20mM, whereas the maturation rates in the incubator with 5% CO_2 was decreased by increasing Hepes level in medium. However, they were not significantly different among the treatments, but there was the interaction between using of Hepes and incubator system. The level of 0 mM Hepes in the incubator without 5% CO_2 and 30 mM Hepes in the incubator with 5% CO_2 had the lowest maturation rate. The optimal maturation rate was obtained when COCs were incubated for more than 28 hours. The fertilization rate was not affected by incubator system. These results concluded that in vitro maturation and fertilization of ovine oocytes could be done in the incubator without 5% CO_2 by adding the 10-20 mM Hepes buffered into TCM-199 medium.**

Key words: IVF, ovine, 5% CO_2, Hepes, effervescent granule.

Introduction

The successful of embryo production is influenced by many factors such as the oocyte quality, medium and culture system. The optimum environment conditions are temperature 35–39°C and 5% CO_2 in air (Gordon, 1994). The CO_2 is required to maintain pH.

To increase the flexibility of embryos production in vitro some researcher tried to substitute the role or the source of CO_2. De Smed et al (1992), Le Gal (1995), Martino et al (1995) has successfully matured the goat oocytes in absence of 5% CO_2 in the incubator by using Hepes buffered medium. The production of cattle embryo in the incubator without 5% CO_2 has also reported by using effervescent granule (EG) as CO_2 sources (Suzuki et al, 1995; Khan et al, 1997; Suzuki et al, 1999)

The purpose of this research was to develop a method of in vitro maturation and fertilization of ovine oocytes in the absence of 5% CO_2 system, so that it will be possible to mature oocyte out side of laboratory. Moreover, it will increase the efficiency of utilization oocytes resources in the slaughterhouse or to be a model in application *dangerous animal* oocytes to yield the in vitro embryos.

Materials and Methods

Oocytes collection. Ovaries were collected from the slaughterhouse and brought to laboratory in physiological saline (NaCl 0.9%) supplemented with 100 mg/ml streptomycin and 100 IU/ml penicillin at 35°C within 5 hours. Ovaries were washed 3 times, and their oocytes were collected by slicing method in PBS solution (Nissui, Japan). Only oocytes with a homogenous cytoplasm and surrounded with one or more cumulus complete were selected for maturation.

In vitro maturation. After twice washing, the oocytes were matured in four different maturation media namely TCM-199 only (plus 0 mM Hepes), TCM-199 + 10 mM Hepes, TCM-199 + 20 mM Hepes and TCM-199 + 30mM Hepes. All media supplemented with 10% Calf Serum (CS, Sigma, St Louis USA), 10 mg/ml Follicle Stimulating Hormone (FSH, Sigma, St Louis USA) and 50 mg/ml

gentamycin sulphate. Groups of 15–20 oocytes were placed in 100 ml drop of those media and the drop covered with paraffin oil (Sigma, St Louis USA). Each of media was cultured at 38.5°C for 24 h in three types of incubator system (incubator with 5% CO_2, incubator with effervescent granule and incubator without 5% CO_2). In the incubator with EG, 5% CO_2 was created by adding 0.25 g effervescent granule to 5 ml deionized water (Suzuki et al, 1995).

Capacitation. In vitro capacitation and fertilization were carried out in two different fertilization media. Bracket-Oliphant (Bracket and Oliphant, 1975) was used for in vitro fertilization in the absence of 5% CO_2 and Defined Medium-Hepes (DM-H) for in vitro fertilization in absence 5% CO_2. The DM-H is a modified BO medium and containing 10 mM Hepes, 129.5 NaCl and 4.16 mM $NaHCO_3$ (pH 7.0). Fresh ejaculate of a proven fertility rams were collected by artificial vagina. The ejaculate was dilute with 2.5 mM caffein sulphate in medium BO or DM-H. After centrifugation at 500-G for 10 minutes, supernatant were discarded and 500 ml pellet was overlaid with 2 ml medium BO or DM-H enriched with 20% CS. The sperm were allowed to swim-up for 1 h at 38.5°C in an atmosphere of 5% CO_2 in humidified air. The upper layers of supernatant (approximately 0.5 ml) recovered and diluted with BO or DMHS to obtain a sperms concentration of 1 x 10^6/ml.

In vitro fertilization. Matured oocytes were washed twice to remove excess of cumulus cells by several pipetting. Groups of 5–15 oocytes were transferred to 50 ml medium BO or DM-H supplemented with 20% and 10 mg heparin/ml. The DM-HS fertilization medium was also supplemented with 7.75 mM calcium lactate (Sigma, St Louis USA). The sperms (50 ml) was added to into the fertilization medium and cultured for 18 hours at 38.5°C in the incubator with 5% CO_2, incubator with EG or incubator without 5% CO_2.

Evaluation. At the end of maturation and fertilization period, the oocytes were removed from cumulus cells by repeated pipetting in to PBS supplemented with 1% hyaluronidase and fixed in 3:1 ethanol: acetate acid for 48 h. They were stained with 1% aceto-orcein for 15 minutes and the nuclear status was observed under phase contrast microscope. The maturation was confirmed by the presence of metaphase plate II, and the fertilization by the presence of two or more pronuclei.

Results

The effect of different level of Hepes in TCM-199 medium and types of incubator on maturation rates of ovine oocytes was shown in Table 1. The maturation rates of oocytes in the incubator without 5% CO_2 or EG gradually increased by increasing of Hepes levels from 0 mM to 20mM, whereas the maturation rates in the incubator with 5% CO_2 was decreased by increasing Hepes level in medium. However, they were not significantly different ($P>0.05$) among the treatment. There was interaction between the using of Hepes and incubator system ($P<0.05$). The lowest maturation rate was obtained in the level of 0 mM Hepes in the incubator without 5% CO_2 and 30 mM Hepes in the incubator with 5% CO_2, respectively.

Table 1. Maturation rates of ovine oocytes mature in different levels of Hepes in TCM-199 medium and incubated in different incubation system

Incubation System	Hepes Levels			
	0 mM	10 mM	20 mM	30 mM
5% CO_2	69 (52/75)	56 (35/63)	53 (38/72)	47 (35/72)
Effervecent Granule	54 (38/70)	58 (41/71)	62 (41/66)	51 (35/65)
Without 5% CO_2	37 (31/74)	65 (50/71)	68 (51/74)	56 (41/73)

The optimal time of incubation of ovine oocytes in three incubator systems are shown in Table 2. The maturation rate of oocyte gradually increases by increasing of incubation time in three incubator systems. In the effervescent granule incubator system, maturation rate was higher ($P<0.05$) for 32 hours incubation period than incubation for 20 and 24 hours. However, they were not significantly different between the incubation period for 20 hours and 28 hours; and 24 and 28 hours in the maturation rate of the oocytes in different incubator condition. The pattern of maturation rate in effervescent granule system was similar with in 5% CO_2 incubator

Table 2. The maturation rate of ovine oocytes in different time of incubation and incubation systems

Incubation System	Time of incubation (hours)			
	20	24	28	32
5% CO_2	63 (22/35)^a	71 (26/36)^a	78 (27/37)^a	87 (36/41)^a
Effervecent Granule	62 (26/42)^a	68 (22/33)^a	76 (35/46)^a	83 (19/35)^a
Without 5% CO_2	62 (22/35)^a	69 (30/44)^a	75 (26/35)^a	69 (29/41)^a

Values within rows with different superscripts are significantly different ($P<0.05$)

system. However, in the system without 5% CO_2 incubator, the maturation rate of ovine oocytes was optimum after 28 hours of insemination.

The formation of 2PN, >2PN and fertilization rate of in vitro matured oocytes were not significant different among the three types of incubator system (P>0.05). On the other hand, the stage of 1PN oocytes in the system without 5% CO_2 incubator was higher (P<0.05) than that in 5% CO_2 incubator, as shown in Table 3.

Table 3. Pronuclear status of ovine oocytes after fertilization in different incubation systems

Incubation System	Pronuclear status			Total Fertilization
	1 PN	2 PN	>2 PN	
5% CO_2	6/40 (15)^a	21/40 (53)^a	1/40 (3)^a	22/40 (55)^a
Effervecent Granule	3/41 (7)^a	19/41 (46)^a	0/41 (0)^a	19/41 (46)^a
Without 5% CO_2	10/42 (24)^a	16/42 (38)^a	1/42 (2)^a	17/42 (40)^a

Values within column with different superscripts are significantly different (a-b, P<0.05)

Discussion

The result of this experiment showed that the use of Hepes in TCM medium could replace the role of CO_2 in maturation and fertilization process of ovine oocyte. These results are comparable to the results reported for goat oocytes that matured in 20 mM Hepes buffered TCM-199 medium (De Smed et al, 1992; Le Gal, 1996 and Martino et al, 1995). Hepes or phosphate buffered medium do not required a carbon dioxide controlled. Dependence of CO_2 is not absolute for embryonic development, embryo will develop to blastocyst stage when buffered with zwitterionic buffers in a 5% CO_2 free atmosphere (Good et al, 1966).

The lowest of maturation rate of oocyte in the maturation medium supplemented with 30 mM Hepes buffered medium in three incubator systems are possible caused by decreasing pH and higher osmotic pressure. At the end of culture period, pH medium tend to reach 7.0, while the optimum pH in maturation was 7.3 (Gordon, 1994). The increasing of osmotic pressure medium indicated with cytoplasm shrinkage of some oocytes. The use of Hepes in medium culture encourage to increase the osmotic pressure, so it was suggest to used 10 mM – 20 mM Hepes (Freshney, 1987).

The maturation rate of ovine oocytes after incubation in the incubator with EG showed that the 2.5 mg EG create 5% CO_2 needed for ideally condition of incubator environment in maturing oocytes. The amount of EG create 2.5% CO_2 in the air and maintain medium pH was around 7.4 ± 19 (Suzuki, 1999). The optimal gas atmosphere condition for in vitro maturation (IVM), in vitro fertilization (IVF) and culture are either 2.5 or 5% CO_2 and 20% O_2 (Pinyopummintr and Bavister et al, 1995).

The result of this experiment shows the highest of maturation rate with normal oocytes was attained for 28 hours incubation period. Although, the incubation period for 32 hours was higher than earlier incubation period, but we found some oocytes in this treatment with abnormal chromatin. No significant differ of maturation rate after 20 and 24 or 24 and 28 hours of incubation is comparable with earlier report (Shamsuddin et al, 1993).

Fertilization rate in the incubator with 5% CO_2, incubator with EG and incubator without 5% CO_2 were 40, 46, and 55% respectively. This result is lower than results reported on goat oocytes (De Smed et al, 1992; Le Gal, 1995 and Martino et al, 1995; Byrd et al, 1997). However, that result show that the fertilization rate of ovine oocytes in the incubator without 5% CO_2 or EG is nearly similar to that oocytes matured in standard 5% CO_2 incubator.

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