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In Vitro Development of Ovine Embryos Following Maturation Under Limited CO_2

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An experiment was conducted to examine the influence of CO_2 during *in vitro* oocyte maturation on the *in vitro* ovine embryo development. Three treatments of CO_2 were subjected to the oocyte development. Those were 2h gasses prior to maturation in incubator (T1); without CO_2 either prior to or over maturation (T2) and CO_2 exposure both prior to and over 22h maturation (T3). A total of 324 oocytes were used. Putative zygotes were cultured for seven days and evaluated for their developmental stage. Presence of CO_2 (T3) increased the proportion of oocytes reaching Metaphase II (66.50 $\pm$ 3.5%; $p<0.05$). Whereas T1 and T2 resulted in lower number of Metaphase II oocytes, i.e., 46.00 $\pm$ 2.5% and 42.50 $\pm$ 2.0%, respectively. Gassed oocytes over 22h maturation (T3) cleaved higher (72.22 $\pm$ 3.36%) than ungassed oocytes in T1 and T2, i.e., 62.12 $\pm$ 3.38% and 60.00 $\pm$ 3.00%, respectively ($p<0.05$). Limitation of CO_2 during IVM did not affect the ability of oocytes to develop to blastocysts following *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (34.48 $\pm$ 2.9% vs. 33.00 $\pm$ 2.5% vs. 36.50 $\pm$ 3.0%, respectively for T1, T2, and T3; $P>0.05$). This study suggests that it is possible to mature ovine oocytes in the absence of CO_2 without loss its potensial development. It may therefore be an effective method of maturing ovine oocytes during transportation to IVP (*in vitro* production) laboratory.

INTRODUCTION

The quality of oocytes is one of the important factor for *in vitro* embryo production (IVEP). The number of high quality oocytes recovered from an ovary is also an important consideration for IVEP. In cattle, the capacity to produce IVEP has progressed remarkably over the last decade (Leibo & Loskutoff 1993; Hasler *et al*. 1995). In the past study reported, it was stated that a significant improvement in embryo yield could be achieved when *in vivo*-matured and *in vivo*-fertilized oocytes are cultured *in vitro* (Eyestone & First 1989). The *in vitro* production (IVP) in bovine has lead in progress, however, only a limited number of offspring have been produced in sheep IVP by using *in vitro* maturation/*in vitro* fertilization (IVM/IVF) techniques (Wani *et al*. 2000). More over a demand exists for basic research on zygote development and on the production of transgenic offspring in small ruminants. Previous studies of *in vivo* matured oocytes either by surgical or laparoscopic methods were reported by Baldassare *et al*. (1994) as well as *in vitro* embryo production by Baldassare *et al*. (1996). However, these are expensive methods and produce limitation number of recovered oocytes per ovary. Ovaries of slaughtered animals provide the cheapest and is the most abundant source of primary oocytes for a large scale production of embryos using a technique of IVM/IVF (Agrawal *et al*. 1995).

A selection of gametes is required for *in vitro* embryo production in which it presumably could possible to develop to blastocysts (Rodriguez *et al*. 2003). Competence oocytes of larger follicles (> 4, 6, or 8 mm) gave higher percentages of blastocysts than from smaller follicles (Lonergan *et al*. 1994). The smaller follicles (2 mm) were partly considered of oocytes that had not completed the RNA growth and synthesis, hence those cells were not competent. Hendriksen *et al*. (2000) stated that developmental competence of cumulus oocyte competences (COCs) might depend on changes both in the oocyte and cumulus cells.

In Indonesia, local regulations prohibit the slaughtering of productive cattle. The availability of bovine ovaries for IVP technologies is scattered in small numbers throughout several areas of West Java. Therefore, it would be advantageous for oocytes to be recovered at slaughterhouses and to undergo maturation while being transported to the central IVP laboratory. Suzuki *et al*. (1995) created a simple portable incubator using effervescent granules (EG) and Suzuki *et al*. (1999) have developed this simple portable incubator in their laboratory and which can be used successfully for production of bovine IVF embryo at the farm level.

As stated in above local problem, this study was designed to determine whether the presence of CO_2 during IVM was a critical factor affecting *in vitro* development of embryos. Availability of ovine ovaries from slaughter houses still enables to embark on this idea of the study in terms of the number of collected oocytes. Therefore, this study was conducted by using ovine oocytes as a model for farm animal *in vitro* embryo production.

MATERIALS AND METHODS

Collection of Ovaries and Oocytes. Ovaries were collected at local abattoirs of Bogor surroundings from unidentified ewes immediately after slaughtered and transported in saline held at 30 ^{o}C in a flask thermos within 2-3 hours to the laboratory. Oocytes were aspirated from follicles with diameter between 3

to 6 mm by an 18-gauge needle. Aspiration medium was H199 supplemented with 50 µg/ml Heparin and 0.4% bovine serum albumin (BSA). Only oocytes with compact cumulus cells or cumulus oocyte complexes (COC) were selected for *in vitro* maturation (IVM).

***In Vitro* Maturation.** The selected oocytes were collected in handling medium (H199 + 10% Fetal Calf Serum/FCS) then washed twice in bicarbonate buffered (25 mM $NaHCO_3$) 199 (B199) supplemented with 10% FCS and washed once in maturation medium. Maturation medium was B199 supplemented with 10% FCS, 10 µg/ml Folligon (PMSG-Intervet, Holland), 10 µg/ml Chorullon (hCG-Intervet, Holland), and 1 µg/ml estradiol (Sigma). Before culturing COC, IVM drops were prepared in 50 µl drops, each drop was contained of 10 oocytes. The maturation medium had pH 7.4 before treatments with or without gassing. The IVM treatments were performed in a humidified anaerobic gas-pack (Oxoid) which was placed in an incubator at 39 °C while CO_2 gas was flowed into the gas-pack for a few seconds to make approximately 5% CO_2. The CO_2 treatments were 2h prior to mature oocytes then matured in the incubator (T1); without exposure of CO_2 either prior to maturation or over maturation (T2) and CO_2 exposure both prior to maturation and over 22h maturation (T3). A total of 324 oocytes were used in this experiment.

Lacmoid Staining of Oocytes. The procedure was adapted from the Embryo laboratory of AgResearch, Hamilton, New Zealand (Margawati 1995). Twenty-two hours after maturation, part of oocytes were used for oocyte development examination. Adhered cumulus cells were removed by mechanical pipetting. The denuded oocytes were washed in 20 mM Hepes synthetic oviduct fluid (SOF) and every 10 oocyte were placed in a very small volume (10 µl) on a glass slide and covered by a coverslip and pressed down gently to make slightly flattened oocytes. Fixative solution was allowed to flow under the cover-slip then rimmed with rubber cement and immersed in the fixative solution for 2 days. These oocytes were stained in 1% lacmoid solution for about 1 up to 2 minutes then washed in 45% acetic acid. The chamber between slide and coverslip was then sealed with nail paint to prevent from drying out. These oocytes were examined under a microscope for maturation stages.

***In Vitro* Fertilization and *In Vitro* Culture.** Some of the left over COCs were transferred into IVF or BO medium (10 µl/COC). Fresh ovine sperm from a ram was collected and processed in the laboratory. A final sperm concentration was 2×10^6 sperm/ml in BO medium (Brackett & Oliphant 1975) and the oocytes were fertilized for 6h under 5% CO_2 in air. Putative zygotes were cultured for seven days as previously described by Thompson *et al*. (1995). The IVC medium contained of SOF/AA/BSA (SOF/Antibiotic/Bovine Serum Albumin) in a ratio concentration of SOF : Antibiotic was 97:3 then added 800 mg of BSA into 100 ml SOF/AA medium. The medium was then held in a humidified gas-pack incubator at 39 °C in atmosphere of 5% CO_2.

Statistical Analyses. The study was designed in a randomized block design with three treatments and each treatment was repeated 5 to 6 times. All data were analyzed using the general linear models (GLM) implemented in SAS v. 6.3.1 package to calculate means and its standard errors. Differences between treatments for the means were tested by Duncan's tests at a significance level of 5% ($p<0.05$).

RESULTS

***In Vitro* Matured Ovine Oocytes.** Photomicrograph of the oocytes development to Telophase I and Metaphae II is presented in Figure 1a and 1b. This figure showed only the third and fourth phase of oocyte development, i.e., Telophase I and Metaphase II. There was a significant difference ($p<0.05$) of the effect of CO_2 exposure on the percentages of oocytes developed to Anaphase I and matured to Metaphase II (Table 1). CO_2 exposure prior to oocyte maturation subsequently matured for 22 h (T3) produced the highest (66.50 ± 3.5%) matured oocyte numbers (Metaphase II). Those that subjected to only CO_2 exposure for two hours prior to maturation (T1) or even without exposure CO_2 (T2) showed low results, namely 46.00 ± 2.5% and 42.50 ± 2.0%, respectively.

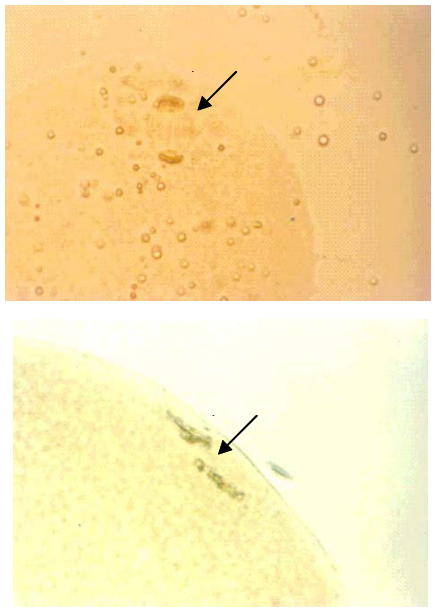

Figure 1. a. Telophase I, 300x and b. Metaphase II, x300.

Table 1. The effect of CO_2 exposure during *in vitro* maturation on the percentage of matured oocytes (Metaphase II), (Mean ± SEM)

Treatments	Number of oocytes	Oocytes development (%)			
		Metaphase I	Anaphase I	Telophase I	Metaphase II
T1	60	15.00 ± 1.50	28.00 ± 2.08ab	5.00 ± 0.08	46.00 ± 2.49d
T2	60	16.00 ± 1.50	33.00 ± 1.98b	4.00 ± 0.08	42.50 ± 2.00d
T3	60	10.00 ± 1.00	17.00 ± 1.88c	5.00 ± 0.08	66.50 ± 3.45e

Means with different superscripts in the same column differ significantly ($p<0.05$)

Table 2. *In vitro* ovine embryo development derived from oocytes matured in different CO_2 exposure during IVM (Mean ± SEM)

Treatments	Number of oocytes	% Embryo development (Mean ± SEM)		
		Cleavage (of oocytes)	Blastocyst (of oocytes)	Blastocyst rate (of cleaved)
T1	48	62.12 ± 3.38a	22.24 ± 2.24	34.48 ± 2.93
T2	50	60.00 ± 3.00a	20.00 ± 2.00	33.00 ± 2.50
T3	46	72.22 ± 3.36b	26.36 ± 2.49	36.50 ± 3.04

Means with different superscripts in the same column differ significantly ($p<0.05$)

***In Vitro* Ovine Embryo Development.** In this study, ovine embryo developed *in vitro* was derived from treatments of limited CO_2. Randomly, some of different stages of oocyte development underwent embryo development forming blastocyst stage (Table 2). It shows that CO_2 limitation during *in vitro* maturation did not affect the oocyte development in both blastocyst or blastocyst rate. However, it was reflected in different percentages of cleaved oocytes.

DISCUSSION

***In Vitro* Matured Ovine Oocytes.** After 22h maturation, pH of maturation medium was likely increased in ungassed oocytes. However, it tended to decrease and/or to be constant in gassed oocytes. The figure of oocyte development could be considered due to limitation of CO_2 exposure during maturation period. Development of oocytes at the condition of limitation of CO_2 (T1) and none exposure of CO_2 (T2) was not different in the percentage of occytes developed to Metaphase II. It might be that the oocytes matured in the medium containing HCO_3^- could manage the pH of medium or micro environment. In female reproductive duct, pH of oviduct and uterine fluid is regulated by concentration of HCO_3^- (Hunter 1988). HCO_3^- is likely to be necessary for many biochemical pathways. Therefore, it should always be supplemented in culture medium (Thompson 1995).

However, as stated by Wani *et al*. (2000), the addition of hormones is still controversial. Several researchers found no significant differences in the oocyte number reaching Metaphase II and oocytes forming blastocysts in the presence or absence of exogenous gonadotrophic hormones (Wahid *et al*. 1992; O-Brien *et al*. 1994). Basically, developmental competence of the oocyte is achieved coinciding by the ability of the follicle *in vivo* to respond the rapid growth to FSH stimulation (Hendriksen *et al*. 2000). However, previous studies reported that several exogenous hormones can influence IVM of ovine oocytes (Table 3). FSH will regulate the growth of oocytes in an ovary to achieve the stage of tertiary follicle. When the follicle has fully grown that termed as a competent oocyte, this oocyte responds to preovulatory surge of LH then ovulation occurs (Parrish & First 1993). This mechanism of reproductive hormones works at the appropriate stage of follicles. However, this study used the oocytes from unpredicted ewes collected from ovine ovaries in some slaughterhouses which some of the oocytes were predicted at the matured oocyte stage. Therefore, supplementation of exogenous hormones in this study might not affect much to the whole *in vitro* maturation of ovine oocytes.

Table 3. Hormones used for IVM of ovine oocytes

Source of exogenous hormones	References
FSH and LH	Staigmiller and Moor 1984
	Cognie *et al*. 1991
	Pugh *et al*. 1991
hMG	Galli and Moor 1991
	Wani *et al*. 2000
PMSG	Murzamadiev *et al*. 1983
hCG and estradiol	Shorgan *et al*. 1990

hMG= human menopausal gonadotrophin

***In Vitro* Ovine Embryo Development.** As stated by Walker *et al.* (1989) dependence on CO_2 is not absolute for embryonic development in ovine and in bovine (unpublished observations of M. Donnison and JG. Thompson, AgResearch-Hamilton, New Zealand), the embryo will develop to blastocyst stage when buffered with zwitterionic buffers (Good *et al*. 1966) in a CO_2-free atmosphere (Thompson 1995). In this study, however the findings show that the matured oocytes derived from limited CO_2 reflected in average of low development of ovine embryos. This may be due to inadequate culture conditions. Another reason, it might be that less (T1) or even without CO_2 exposure (T2) during *in vitro* maturation burdened oocytes underwent Metaphase II stage. However, those oocytes achieving the Metaphase II had passed the impairment of micro environment of *in vitro* maturation. This mechanism of oocyte development to Metaphase II proved statistically similar achievement of blastocyst rates in the three CO_2 exposure treatments (Table 2).

This finding is not different from a report of using a portable incubator in the absence of CO_2 for *in vitro* maturation subsequent to *in vitro* ovine embryo development (Byrd *et al.* 1995). In addition, Byrd *et al*. (1995) showed that their techniques of using a portable incubator in the absence of CO_2 allowed great numbers of oocytes to be transported to areas where an abattoir is not close proximity. This technique is also promising for the transport of oocytes from exotic animals or wildlife in remote locations.

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