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Effect of Antioxidant on Development of Embryos in Korean Native Goats

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한국 재래산양의 체외수정란 생산에 대한 항산화제의 효과

최창용 · 손동수 · 김영근 · 한만희 · 권응기 · 최순호 · 최연호 · 최성복 · 조영묵 · 손삼규 · 노규진[1] · 최상용[1†]
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SUMMARY

본 연구는 우리나라 고유의 유전자원인 재래산양의 체내수정란 생산기술을 확립하고자 수행하였다. 흡입법(aspiration)과 세절법(slicing)에 의해 난소 한 개당 회수된 난자의 수는 3.9개와 4.1개를 나타내어 slicing 방법이 aspiration방법보다는 많은 숫자의 난자를 회수하였으나 유의적인 차이는 나타내지 않았다. 회수된 난자의 등급별 분포는 aspiration방법에서 Grade Ⅰ, Grade Ⅱ, Grade Ⅲ, Grade Ⅳ의 비율이 10.3%, 20.5%, 38.5%, 30.8%를 나타내었으며, slicing법에서는 9.8%, 22.0%, 39.0%, 29.3%를 나타내어 Grade Ⅲ과 Grade Ⅳ의 비율이 70% 이상을 차지하였다. 회수된 난자를 체외성숙시킨 결과 Grade Ⅰ과 Grade Ⅱ에서는 85% 이상이 metaphase Ⅱ (MⅡ)까지 도달하였으나, Grade Ⅲ과 Grade Ⅳ는 40% 이하의 체외성숙율을 나타내었다. 체외수정용 배양액으로 BO를 사용하였을 경우 Grade Ⅰ 및 Ⅱ에서 84.4%의 난분할율을 나타내어 TALP를 사용하였을 때의 58.8%보다 높은 난할율을 보였다. 또한 배양액의 종류별 체외발달율에 있어서는 상실배 및 배반포기배로의 발달은 mSOF를 배양액으로 이용하였을 경우 15.0%의 발달율을 나타내었으며, 체외배양 시 항산화물질인 glutathione (GSH)을 첨가함으로서 26.8%의 상실배 및 배반포배로의 발달율을 나타내었다.

(Key words : aspiration, slicing, glutathione (GSH), IVF, Goats)

INTRODUCTION

In vitro embryo production (IVP) in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and physiology and for commercial application of the emerging biotechnology such as nuclear transfer and transgenesis (Baldassare et al., 2002). In addition, the application of IVP and interspecific transfer has been proposed as a strategy for the rescue of some endangered species (Ptak et al., 2002). The method of IVP of embryos involves three main steps: maturation of primary oocytes collected from antral follicles, fertilization of the matured secondary oocytes with semen, and culture of the putative embryos for up to day 8 until formation of

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blastocysts that can be transferred to recipients or cryopreserved for future use.

Compared with the procedure in cattle and sheep, IVP in goats has been subjected relatively few reports, despite its usefulness for both basic research and in commercial applications. Since it was difficult to culture goat embryos to the 8 to 16 cell stage *in vitro*, a stage specific developmental block has been postulated in these animals (Wright and Bondioli, 1981). In an attempt to overcome the developmental block occurring *in vitro* and to approximate efficiency *in vivo*, various embryo culture conditions have been widely investigated. Generation of reactive oxygen species is an inevitable consequence of oxidative reactions. Thus cellular protective process has evolved to include antioxidants or free radical scavengers to prevent cellular damage caused by reactive oxygen species. The aim of the present study was to establish effective *in vitro* systems for oocyte maturation, fertilization and develop of Korean native goats.

MATERIALS AND METHODS

1. Oocytes Recovery

Goat ovaries were collected from a local slaughterhouse and transported to the laboratory within 2~3 h of harvest at 35℃ in PBS supplemented with 100 units/ml penicillin G (Sigma, USA) and 100 ug/ml streptomycin (Sigma, USA). Oocytes were collected either by aspiration or slicing methods. For aspiration, antral follicles were aspirated into 10 ml disposable plastic syringe fitted with an 18G needle. For slicing, ovaries were placed in a petri dish containing 5 ml Hepes-199 supplemented with 0.2% BSA and held with an artery forceps. The visible surface of follicles was carefully dissected, and finally the whole ovary was sliced with the help of a scalpel blade into thin pieces. Large pieces of ovarian tissues were removed by through washings. Oocytes were recovered under a stereomicroscope (×40).

The isolated oocytes were washed 3 times with Hepes-199 supplemented with 0.3% BSA and classfied into 4 categories (Grade I, II, III and IV) according to the character of the cumulus cells (Wiemer et al., 1991; Cox et al., 1994) oocytes with more than 4 layers of compact cumulus cell and uniform cytoplasm (Grade I), with 2~3 layers of cumulus cells (Grade II), with less than 2 layer or partialy naked cumulus cells (Grade III) and without cumulus cells and degenerated cytoplasm (Grade IV).

2. *In Vitro* Maturation

Sets of 15 oocytes were matured in 100 ul droplets of TCM-199 supplemented with 10% FBS, 10 ug/ml FSH (Sigma, USA), 10 ug/ml LH (Sigma, USA) and 10 ng/ml epidermal growth factor (EGF, Sigma, USA) at 38.5℃ in a humidified atmosphere of 5% CO_2 in air. After 24 h *in vitro* maturation (IVM), some of expanded cumulus cells were removed by vortexing for 2 min in Ham's F10 medium containing 0.5 mg/ml hyaluronidase, and fixed overnight in methanol:acetic acid (3:1, v/v) and stained with 1% aceto-orcein to reveal the chromosome status. Oocytes were classified as germinal vesicle (GV), metaphase I (MI) and metaphase II (MII).

3. *In Vitro* Fertilization

Sperm collected from cauda epididymis were treated with swim-up procedures. Briefly, sperm were washed once by centrifugation at 300 g for 10 min in 10 ml of sperm-Talp. From 0.5 ml of sperm pellet, five aliquots of 0.1 ml were transferred into five 2-ml round-bottom test tubes and then each was overlaid with 0.5 ml of IVF-Talp containing 0.3% BSA, 10 ug/ml heparin and 2.5 mM Caffeine. With the tubes tilted to a 45 angle, incubation at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 1 h allowed swim-up of motile

sperm. The top 0.4 ml of the supernatant was harvested and further centrifuged at 300 g for 10 min to yield a sperm pellet, and resuspended in 0.6 ml of IVF-TALP or BO (Brackett and Oliphant) medium.

For IVF, expanded cumulus cells were partially removed from oocytes after 24h IVM by vortexing them for 10 sec in Talp and divided to two IVF groups. Goup 1 oocytes were fertilized in BO medium and group 2 oocytes were fertilized in IVF-Talp. Sets of 15 oocytes were then inseminated with sperm which had been prepared by swim-up at a final concentration of 2×10^6 sperm/ml in 50 ul of each IVF media for 18 h at 38.5℃ in a humidified atmosphere of 5% CO_2 in air.

4. *In Vitro* Culture

After 18 h of co-incubation with sperm, set of 15 oocytes were removed from the fertilization drops. The cumulus was stripped off by repeated pipetting through a small-bore pipette and cultured in 100 ul TCM199 (co-cultured with 100~200 goat oviduct epithelial cells/100 ul) or modified synthetic oviduct fluid (mSOF) medium (Takahashi and First., 1992) supplemented with 1×MEM non essential amino acid, 1×BME amino acid and 0.3% BSA. At 48 h and 120 h post-insemination, the cultures were "fed" by adding 25 ul of fresh IVC medium to each drop, and were maintained for 192 h post-insemination. At that time, the rates of development into blastocysts were determined. To investigate the effect of GSH, embryos were cultured in mSOF supplemented with 1 mM GSH.

5. Statistical Analysis

Differences were analyzed among treatments using the General Linear Model (GLM) procedure in the Statistical Analysis System (SAS). A probability of $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

1. Effects of Different Collection Methods on the Number of Recovered Oocytes

In order to compare the efficiency of the number and quality of oocytes by different collection methods, oocytes were collected either by aspiration or slicing methods (Table 1). A total of 276 ovaries were aspirated and 176 ovaries were sliced. There were not differences on the grade and number of oocytes collected per ovary by slicing and aspiration. The total number and grade I of oocytes were 3.9 and 0.4 in aspiration, and 4.1 and 0.4 in slicing, respectively.

The number of high quality oocytes recovered per ovary has been an important consideration in the *in vitro* embryo production. Mammalian ovaries contain thousands of oocytes, but with the currently available methods only a relatively small proportion of the total vesicular follicle population can be utilized. A couple of methods were reported on the collection of follicular oocytes from slaughtered ovaries in domestic animals, including aspiration,

Table 1. Effect of collection methods on the number of recovered oocytes (Mean±SD)

Collection methods	No. of used ovaries	Number (%) of collected oocytes/ovary				
		Grade I	Grade II	Grade III	Grade IV	Total
Aspiration	276	0.4±0.1 (10.3)	0.9±0.3 (20.5)	1.8±0.7 (38.5)	1.3±0.6 (30.8)	3.9
Slicing	176	0.4±0.2 (9.8)	0.9±0.2 (22.0)	1.9±0.8 (39.0)	1.3±0.5 (29.3)	4.1

There were no significantly ($P<0.05$) difference between collection methods.

dissection and slicing with advantages and disadvantages. In general, goat ovaries were sliced or dissected for obtaining the follicular oocytes due to small size of ovary and follicle. Crozet et al. (1995) compared that the number of oocytes collected by aspiration and slicing are 2 and 6 per ovary, respectively. It has been suggested to do aspiration when follicle diameter is 2~8 mm, whereas slicing when the diameter is less than 2 mm. Out of 452 ovaries, the present study showed that ~4 oocytes were collected both by aspiration and slicing, and did not differ of the number of oocytes collected between the methods. Approximately 1.3 oocytes were categorized in grade I an II when the oocytes were evaluated by morphological assessments, such as the layers of cumulus cells. In cattle, follicular oocytes collected were ranged ~70% of good quality (Grade I and II) (Katska, 1984; Lu et al., 1987 Iwasaki et al., 1987; Hamano and Kuwayama, 1993). Whereas, in goat, only 30% of the oocytes remained Grade I and II (Pawshe et al., 1994; Park et al., 2000). This is similar to the present study and caused to retardation of the developmentof the field in goat *in vitro* embryo production.

2. Effects of Oocytes Quality on Nuclear Maturation of Oocytes

After 24 h of maturation, different grade oocytes removed their cumulus cells were evaluated nucleus status, as assessments of germinal vesicle (GV), metaphase I (MI) and metaphase II (MII). Table 2 shows that most oocytes (93%, 37/40) in grades I and II were in the metaphase II stage. However, 35% of the oocytes had developed to metaphase II in grades III and IV. The rates of oocytes had developed to metaphase II in groups I and II were significantly ($P<0.05$) higher than those in groups III and IV.

The present study has confirmed that a period of 24 h for IVM of goat oocytes is sufficient for nuclear maturation. This time is similar to that used in goats by Yonis et al (1991), but was slightly longer than that used by Martino et al (1994) and Rho et al (2001). The reason remains unclear, but might be different breeders that were used. In this study, ~93% oocytes in grade I and II developed to metaphase II. However, 35% of oocytes in grade III and IV reached to metaphase II. This may have been due to slicing method which probably leads to the collection of immatured and non-competent oocytes from small diameter follicles. De Smedt et al (1994) reported that ~24% of the oocytes collected from 1 to 2 mm follicles developed to metaphase II, indicating that oocytes from follicles larger than 2 mm in diameter are needed for the ability to achieve nucleus maturation.

Table 2. Effect of oocyte quality on nuclear maturation of oocytes matured *in vitro* for 24 h

Oocytes	Oocytes used	Number (%) of nuclear status*		
		GV	MI	MII
Grade I	20	-	-	20 (100)[a]
Grade II	20	3 (15)	-	17 (85)[a]
Grade III	20	4 (20)	8 (40)	8 (40)[b]
Grade IV	20	10 (50)	4 (20)	6 (30)[b]

* GV, germinal vesicle; MI, metaphase I; MII, metaphase II.

[a,b] The value with different superscripts within the same column were significantly different ($P<0.05$).

3. Effects of IVF Media and Oocytes Grade on the Cleavage Rates of Oocytes

Table 3 shows the cleavage rates of oocytes fertilized in different IVF media. At 24 h of maturation, the oocytes removed partially theircumulus cells were fertilized with sperm that treated with swim-up procedure. In groups I and II oocytes, the rates of cleavage were significantly ($P<0.05$) higher in BO medium than that in Talp medium (85% vs. 59%, respectively). However, the

Table 3. Effects of IVF media and oocytes grade on the cleavage rates of oocytes fertilized *in vitro*

IVF medium	Oocytes grade*	No. of oocytes	
		Inseminate	Cleaved (%)
BO	I ~ II	138	117 (84.8)[a]
	III ~ IV	322	110 (34.2)[c]
TALP	I ~ II	34	20 (58.8)[b]
	III ~ IV	120	27 (22.5)[c]

* Grade I, > 4 layers of cumulus cells and uniform cytoplasm; Grade II, with 2-3 layers of cumulus cells; Grade III, less than 2 layer or partialy naked cumulus cells; Grade IV, without any cumulus cells.

[a,b,c] The value with different superscripts within the same column were significantly different ($P<0.05$).

rates of oocytes in groups III and IV did not differ between BO and Talp medium. In both BO and Talp medium, the cleavage rates were significantly ($P<0.05$) different between grades I~II and III~IV.

There have been many reports on comparisons of cleavage and development into blastocysts between different IVF media using BO, Talp and SOF. In the present study, sperm preparation for IVF was swim-up which is mostly used on based of the selection of motile spermatozoa. The present study has shown that the proportions of cleavages of grade I and II oocytes fertilized in BO medium were significantly ($P<0.05$) higher than that in Talp medium (85% vs. 59%, respectively). This finding is consistent with the reports that, in BO medium, the rates of cleavage were 58% (De Smedt et al., 1992; Yadav et al., 1998) and 22% in Talp (Mogas et al., 1997). However, advanced cleavage rates were obtained in the resent study compared to previous reports (De Smedt et al., 1992; Mogas et al., 1997; Yadav et al., 1998).

4. Effects of Culture Medium and Antioxidant Supplement on Embryos Development

The cleaved embryos were compared the development into morula and blastoctst among different culture conditions as presented in Table 4. The embryo cultured in mSOF media without GSH was developed to 15% of morula and blastocyst stage. Addition of GSH in mSOF enhanced the development to morula and blastocyst stage as 27%, which did significantly ($P<0.05$) differ compared to mSOF. However, no morula and blastocyst developed in TCM199 medium co-cultured with GOEC.

Mammalian embryos undergo species-specific *in vitro* block to development. Reactive oxygen species, derived from oxygen metabolism, was suggested as a principal cause of this block because they are apparently involved in the two-cell block of mouse embryos. The period of *in vitro* development at which this block occurs in the mouse embryo is associated with a rise in reactive oxygen species such as H_2O_2 (Nasr-Esfahani et al., 1990;

Table 4. Effects of culture media and antioxidant supplement on embryo development

Culture media	Addition of GSH	Two cell embryos used	No. (%) of embryos developed to		
			Morula	Blastocyst	Mor+Blast
TCM199	-	104	0	0	0[a]
mSOF	-	80	3 (3.8)	9 (11.3)	12 (15.0)[b]
mSOF	+	82	6 (7.3)	16 (19.5)	22 (26.8)[c]

[a,b,c] The value with different superscripts within the same column were significantly different ($P<0.05$).

Nasr-Esfahani and Johnson, 1991, 1992). This suggestion is in agreement with the results of improved embryo development by lowering the oxygen concentration *in vitro* in mice (Quinn and Harlow, 1978; Pabon et al., 1989), sheep and cattle (Thompson, 2000) and goat (Batt et al., 1991). GSH, in general, is known to be an important cellular protecting component from oxygen stress. In bovine embryo, GSH could improve development to morula and blastocyst from cultured oocytes by about two-fold. Moreover, they showed that the GSH addition was effective specifically from the 8 to 16 cell stage, which is a known developmental block stage in bovine embryo. The present study shows that no embryos cultured in TCM199 developed to blastocyst, however 15% of cleaved embryos cultured in mSOF developed to morula and blastocysts, suggesting that the use of nutrient-rich medium is unable to attain the developmental competence. Similar to this, it has been reported that only 3% of embryos cultured in TCM199 developed to blastocyst (Mogas et al., 1997) and ~28% embryos cultured in mSOF developed to blastocysts (Cognie, 1999). Inadequate *in vitro* culture conditions enhancing oxidative damage by increasing ROS production caused to reduce the development of embryo, resulting in developmental blocking at 8- to 16- cell stage and in retarding the progress to develop to late stage embryos. From the present study, GSH supplementation into mSOF for embryos development *in vitro* plays important role on enhancing the development.

CONCLUSION

The present study clearly showed that BO medium for IVF enhanced fertilization and mSOF supplemented with GSH increased the developmental rates into blastocysts. However, *in vitro* embryo production in the Korean native goats still remains further research to be solved. The further study, in particular based on the studies of gene expression and regulation related to retardation on the development of embryos, will provide greatly enhancement of the goat IVF fields.

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