韓國家畜繁殖學會誌 17(2) : 149~157(1993)
Korean J. Animal Reprod.

Maximization of The Numbers of Follicular Oocytes Recovered from The Bovine Ovaries

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소 난소로부터 회수난포란수의 극대화 방법

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적 요

소 초기배의 체외생산을 위한 소 난포란 회수를 극대화할 수 있는 방법을 확립하기 위해 여러 가지 방법에 의해 채취된 난자의 발생능력을 검토하였다. 전통적인 흡입법(대조구), 개발된 회수법(slicing) 및 이들을 결합한 방법(결합법)을 비교하였다. 총 245개의 난소로부터 1,641개의 난포란을 실험에 이용하였다. 회수된 난자는 TCM199과 소 태아혈청을 기초로 한 배양액에서 24시간 체외성숙시켜 급속염색법에 의해 핵성숙을 판별하고, 7% 에탄올에 의해 활성화된 처녀발생란의 전핵형성 유무에 의해 세포질 성숙을 평가하였다. 회수된 평균 난자수는 난소당 흡입법, slicing 및 결합법이 각각 1.87, 11.05 및 7.88개를 얻어 새로 개발된 slicing에 의해 회수율을 5.9배 (11.05/1.87) 증가시킬 수 있었다. 핵 성숙은 흡입법 92.9%, slicing 79.1%와 결합법 71.7% 였다. 비록 흡입법에 위해 회수된 난자의 핵 성숙율이 높았지만 난소당 얻을 수 있는 성숙 난자의 수는 slicing할 경우 5배 까지 증가시킬 수 있었다. 세포질 성숙의 지표인 전핵의 형성율은 대조구 75%, slicing 67%, 그리고 결합법 62.5%였다. 이같은 결과는 개발된 slicing 법에 의해 도살장 난소로부터 보다 많은 수의 난자의 회수가 가능하며 이들의 핵성숙 및 세포질 성숙도 정상적으로 일어나며 난소당 전핵 초기배 수를 증가시킬 수 있음을 보여준 것이다. 아울러 증가된 난자수로 인하여 초기배의 생화학적 분석 및 외래유전자의 미세주입을 위한 지속적으로 안정된 초기배의 공급체계가 확립되었다.

I. INTRODUCTION

The production of bovine embryos from the superovulated donor is possible for embryo transfer program as well as research purpose. However, the high cost has to be considered. With the accumulated knowledge of embryogenesis, it is now feasible to produce the cattle embryos through *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) using immature follicular oocytes of the ovaries recovered from the slaughter house (Gordon and Lu, 1990).

The most common method used to obtain follicular oocytes is aspiration of the follicular fluit containing the oocyte with a syringe (Motlik et al., 1978; Leibried and First, 1979; Katska, 1984; Sirad et al., 1989). There have been a few methods to recover follicular oocytes for different purposes. These include mincing the ovaries, filtering through the stainless steel mesh (Oikawa, 1978), and enzymatic or mehchanical isolation of the oocytes (Taha and

Schellander, 1992). Transvagical laparoscope was also generally used to collect the follicular oocytes *in vivo* (Sirad and Lambert, 1985; Parson et al., 1990; Pierterse et al., 1991; Tan et al., 1991; Armstrong, 1992).

In case of monotocous animals such as cattle, sheep and horse, numbers of the oocytes could be obtained from the ovary *in vivo* and *in vitro* were very few, and available ovaries are also limited in some places. Therefore, there should be a method to provide sufficient numbr of the oocytes for the *in vitro* production of embryos, which can be commercialized in the near future. As an effort to recover more number of the oocytes, Suss and Madisson (1983) applied slicing of the ovarian surface to maximize the numbers of the follicular oocytes recovered from the ovary. However, it has not been fully described, without being exploited as a stable method for the recovery of the bovine oocytes (Kay and Frylinck, 1992; Carolan et al., 1992; Takagi et al., 1992; Choi et al., Steyn et al., 1993).

In this study, we made an effort to develop a stable method in detail of maximizing the number of bovine follicular oocytes recovered from the ovary, and demonstrate the developmental capacity of the oocytes collected by the established method.

Ⅱ. MATERIALS AND METHODS

1. Ovary collection

The ovaries were collected from Holsteins at the local slaughter house and whose estrous cycle stage were not known. Recovered ovaries were transported to the laboratory within an hour in 0.85% sterile saline with antibiotics, streptomycin sulfate 100 mg /l (Sigma Chemical Co., St. Louis, MN. U.S.A.) and penicillin G 100 i.u. /l (BDH Chemical Co., Poole, U.K.) in Thermo bottle at 39℃. The ovaries were washed in sterile saline to remove the blood and dirts, trimmed and swabbed with 70% ethanol-soaked gauze to remove microorganisms. The cleared ovaries were soaked into plenty of fresh saline to eliminate remaining ethanol. After washing, the ovaries were thansferred into the fresh Dulbecco's phosphate buffered saline(D-PBS) and kept at 39℃.

2. Recovery of oocytes cummulus complexes (OCCs)

Three recoverying methods were used to compare the numbers of follicular oocytes recovered : aspiration (ASP), slicing (SLC), and aspiration combining slicing (ASP + SLC).

1) Aspiration

The oocytes were recoverd from 2~6 mm follicles by aspirating follicular fluid containing the oocytes with a 18 gauge needle attached to 10 ml disposable syringe (Precion Glid, Becton Dickinson Co., N.J., U.S.A.). Following the aspiration, fluid of the syringe were released into a 6 cm sterile watchglass. The supernatant was removed, and fresh M2 medium containing heat inactivated 4%(v /v) fetal calf serum (Biologie Industries Co., Kibbutz Beth Haemek, Israel) (M2+FCS) was added. The oocytes were collected under 20 × stereo microscope (Kyowa Optical Co., Tokyo, Japan).

2) Slicing

Washed ovaries were bisected, and the corpora lutea and large follicles were removed. Slicing devices were made at each experiments. Three to five sterile single-use stainless steel blade (No. 21, Feather Industries Ltd., Tokyo, Japan) were assembled together in parallel by 3~5 mm apart with paper adhesive tape. 30 ml of D-PBS was poured into a 12 cm watchglass,

and 3 to 5 ovaries were sliced with the device on a surface of the ovary without tearing the tissue. The tissues were discarded, and the released contents were settled in the watchglass. After removing the supernatant. 10 ml of M2+FCS were added to give protein sources and to obtain fine view. Collected OCCs were transferred into fresh M2+FCS.

3) Aspiration combining slicing

The ovaries were firstly aspirated, then bisected and sliced as described above.

3. Media preparation

Culture medium was TCM 199 (Gibco Life Technologies Inc. Grand Island, N.Y., U.S.A.), with Earle's salts, 25 mM HEPES buffer, L-glutamine, 2,200 mg/l sodium bicarbonate, supplemented with 15% FCS (TCM 199 + FCS). Pregnant mare's serum gonadotrophin (PMSG) (Folligon, Intervet Co., Boxmeer, Holland) and human chorionic gonadotrophin (hCG) (Chorolon, Intervet Co., Boxmeer, Holland) were added at the concentrations of 10 i.u. /ml (TCM 199+FCS+Gn), respectively, for IVM (Sanbuissho and Threlfall, 1990). Medium was sterilized by 0.22 μm membrane filter (Millex GV, Millipore Corp., Bedford, MA., S.A.). And equilibrated on previous day at 39℃ with 5% CO_2 in air.

4. Classification of the recovered OCCs

The collected OCCs were divided into 5 groups depending upon their appearance of the ooplasm and attachment of the cumulus cell layers. The oocyte enclosed several layer of compacted cumulus cells were designated 'good' type. Three to four layers of cumulus cell-enclosed oocyted and denuded oocytes were designated 'fair' and 'poor', respectively. 'Atretic' type has degenerated cumulus cells and 'abnormal cytoplasmic (Abn)' has uneven ooplasm.

5. *In vitro* maturation

Collected OCCs were washed in M_2+FCS, and followed by TCM 199+FCS+Gn over 3 times, respectively. Washed OCCs were cultured in the medium under liquid paraffin oil (BDH Chemical Co., Poole, U.K.) in a disposable plastic dish (35 mm × 10 mm, Falcon 2001, Becton Dikinson Co., N.J., U.S.A.) at 39℃ with 5% CO_2 in air and high humidity water jacket CO_2 incubator (Sheldon Manufaturing Inc., Cornelius, ORG., U.S.A.) for 24 h (Sirare et al., 1989).

6. Granulosa cell monolyer (GCM)

Granulosa cells were collected into the 14 ml tube (Falcon 3001, Becton Dickinson Co. N.J., U.S.A.) containing M2+FCS and centrifuged at 3,000 rpm 5 min. The pellet was washed in M2+FCS and TCM 199+FCS by centrifugations three times, respectively. The pellet was dissociated mechanically by passing through 18 gauge needle with a 1 ml syringe. The cell concentration was adjusted to 2×10^6 cell/ml. The suspended cells were cultured under liquid paraffin oil at 39℃. On following day, the blood cells and dead cells were removed by a Pasteur pipette. Medium was replaced very 48 h.

7. Parthenogenic activation ethanol

At the end of IVM culture, the expanded OCCs were transferred into 0.5 ml of 3% (w/v) sodium citrate (Sigma Chemical Co., St. Louis, MO., U.S.A.) solution in a 1.5 ml Eppendorf tube to remove expanded cumulus cells (Kinis et al., 1989). Vigorous shaking and/or vortexing was applied prior to pouring into a 35 mm dish containing M2+FCS. Denuded oocytes

were searched under a dissecting microscope. The oocytes were washed in fresh M2+FCS 3 times. The oocytes were incubated in M2+FCS containing 7% ethanol (Merck, Darmstadt, Germany) for 7 min (Nagai, 1987, 1992). The ethanol-exposed oocytes were washed thoroughly in plenty of M2+FCS to remove remaining ethanol and transferred into GCM prepared previously.

8. Analyses

1) Nuclear maturation

Cumulus cells were completely removed with 3% sodium citrate after 24 h of maturation as described above. Nuclear configuration was analysed (Suss et al., 1988) by rapid staining method (Byun et al., 1991) and observed under thd 200× and 400× light microscope (BHS, Olympus Opticals Co. Ltd., Tokyo, Japan).

2) Cytoplasmic maturation

Cytoplasmic maturation was analysed by the presence of pronucleus in the activated oocytes at 18 h post activation by the rapid staining.

9. Photography

The nucleus stained by rapid staining was recorded on Konica GS negative film, ASA 100, with automatic exposure unit (PM-10ADS, Olympus Opticals Co. Ltd., Tokyo, Japan). GCM was recorded on Kodak Ektar negative film, ASA 25, with automatic camera (FE 2. Nikon Opticals Co. Ltd., Tokyo, Japan) under an inverted microscope (Diaphot TMD, Nikon Opticals Co. Ltd., Tokyo, Japan) using phase contrast filter (ELWD 0.3, Nikon Opticals Co. Ltd., Tokyo, Japan).

Ⅲ. RESULTS AND DISCUSSION

Bovine embryos produced *in vitro* using ovaries from slaughter house in one of the major sources for either production of cross-bred beef cattle or donor embryos in cloning of embryonic cells. Therefore, it is necessary to obtain larger number of follicular oocytes. Slicing method was chosen and further developed to evaluate number of the oocytes and their developmental potentiality. Preliminary experiments to establish the procedure were performed to minimize the time spent for recovery of oocytes and contamination from microorganisms on the surface of the ovary. The time required of the whole procedure to process 15 to 20 ovaries by 2 experiments was 1.5 h, compared with 2 h in the aspiration method. Wiping the ovaries with 70% ethanol-soaked gauze was sufficient to eliminate microbial contamination during subsequent maturation *in vitro*. From this prelimining experiment the slicing method was established and, presented in Fig. 1.

1. Number of follicular oocytes recovered with different procedures.

The ovaries were processed without selection, and allocated to 3 different groups. It was our intention not to select ovaries by their morphology to investigate the average number of the OCC obtained from the local slaughter house ovaries. The numbers of recovered oocytes from the 3 different methods, ASP, SLC and ASP+SLC were compared (Table 1). The number of OCCs per ovary was 1.87, 11.05 and 7.88 in ASP, SLC and ASP+SLC groups, respectively. In the bovine IVF system the number of follicular oocytes which are usually aspirated from visible follicles at the ovarian surface using a syringe and needle is approximately 5 OCCs per ovary (Motlik et al., 1978; Leibfried and First, 1979; Katska, 1984; Sirard et al., 1989). Yoon et al. (1989) reported that average 46.4 OCCs were obtained from a

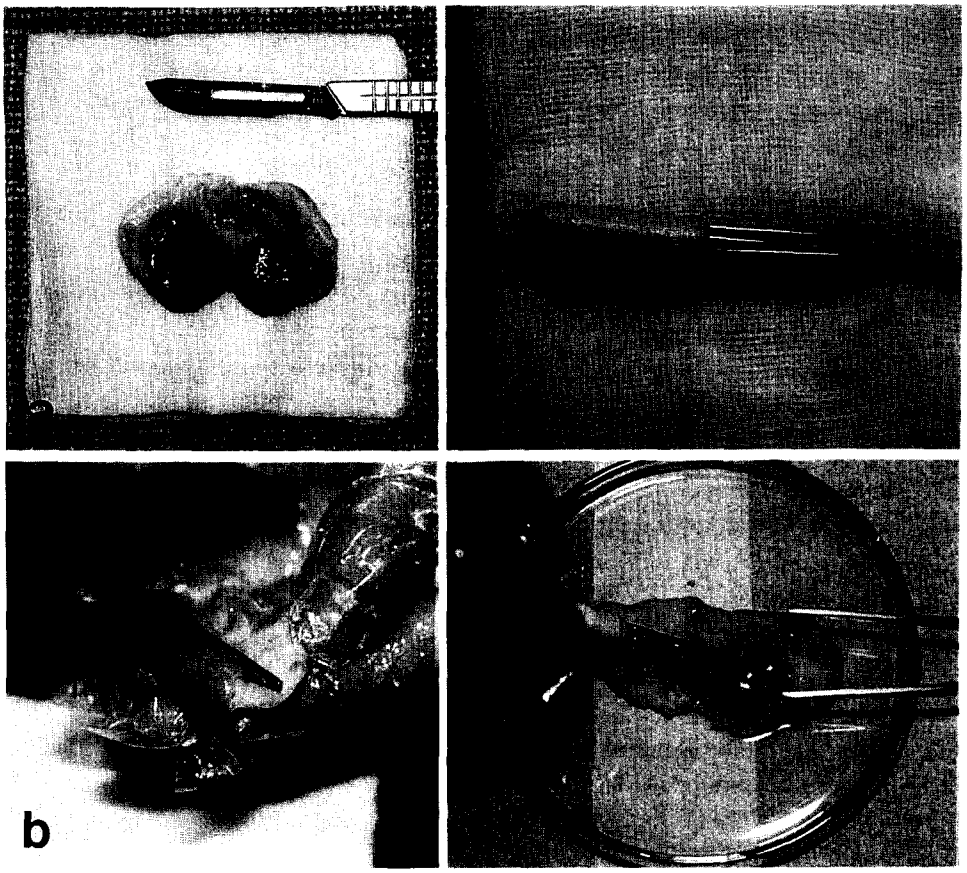

Fig. 1. The slicing method of the ovary. Ovaries trimmed and swabbed with a 70% ethanol-soaked gauze were cut and opened (a). Corpus lutea and large follicles were removed with a pair of sicssors (b). A slicing device (c) used for slicing the ovarian surface (d).

Table 1. Recovery of oocyte-cumulus complexes for culture with different procedures from bovine ovaries[1]

Collection procedure	No. of ovaries[2] processed	No. of the OCCs	No. of the OCCs per ovary (Range)	Recovery index[3] against control
ASP[5] (Control)	54	101	1.87 (1~10)	100
SLC[5]	35	387	11.05 (3~40)	591
ASP + SLC[4]	61	481	7.88 (2~20)	412

1. The values were obtained from 3 experiments.
2. Ovaries were not selected by their morphology.
3. Recorvery index was % of the number the OCCs in each procedure against the number of the OCCs obtained in control group.
4. Slicing was carried out in the aspirated ovaries.
5. ASP=aspiration, SLC=slicing

cow, i.e., 23.2 OCCs per ovary by ASP. Among them, 52.3% (12.2 OCCs per ovary) of the OCCs were good type althrough their further development to fertilization was not studied. Takagi et al. (1992) preseneted that 8.9±0.8 OCCs per ovary were recovered by ASP. Whereas Carolan et al., (1992) reported that 66.11±7.16 of bovine follicular OCCs were recovered by slicing the surface of the ovary with 3 blade-combined slice device. Among them 14.63±1.68 of OCCs were good type. SLC method could yield almost 6 times more than that of conventional ASP method. Similar result was also found, showing 3.47 fold (7.30 OCCs in SLC /2.10 OCCs in ASP) (Kay and Frylinck, 1992). When the two methods were consecutively used (ASL+SLC) the number of recovered oocytes did not increase although the two methods were combined. Thus the recovery index was 421(7.88 oocytes per ovary). This may be due to the loss of OCCs in the process of the combined method. The lower rate of the OCCs recovered in this study than those of others may be due to the source of ovaries.

2. Classification of the OCCs

Since SLC method ruptures some antral follicules in the cortex as well as the surface of the ovary, many types of the OCCs will appear. Among the 5 types of OCCs, good and fair types of the OCCs were used for subsequent experiments. The proportion of good and fair types of OCCs in ASP (85%) is higher than that of SLC (53%) as shown in Table 2. However the total number of the OCCs in SLC outnumbered that of the ASP, being nearly 5.1 times yield([242 /25 in SLC] /[46 /24 in ASP]).

Table 2. Recovery of oocyte-cumulus complexes (OCCs) for oocyte selection with different procedures from bovine ovaries.

Collection procedures	No. of ovaries	No. of the OCCs classified into the following categories[1](%)					
		Total	Good	Fair	Poor	Atr	Abn Cyt
ASP(Control)	24	54(100)	26(48)	20(37)	5(19)	1(1)	2(4)
SLC	25	458(100)	152(33)	90(20)	178(39)	23(5)	15(3)

Atr=atretic, Abn cyt=abnormal cytoplasm

Table 3. *In vitro* maturation of the OCCs recovered with different procedures and subsquently cultured for 24h.

Collection procedures	No. of ovary	No. of OCCs	No. of the oocytes at the follewing nuclear stages[1]					No. of M Ⅱ per ovary	Index[2]
			AV	M I	A I	T I	M Ⅱ	Abn	
ASP(Control)	45	84		4		2	78(92.9)		1.73	100
SLC	23	254		20	3	17	201(79.1)	13	8.74	505
ASP+SLC	27	209		23	18	8	150(71.7)	10	5.56	321

1. Nuclei were stained by rapid staining.
2. Rocovery index was % of the number the OCCs in each procedure against the number of the OCCs obtained in control group.
3. Abbreviations are GV, germinal vesicle; M I, metaphase I; A I, anaphase I; T I, telophase I; M Ⅱ, metaphase Ⅱ and Abn, abnormal nucleus.

Table 4. Pronuclei formation of parthenogenetically activated oocytes recovered by different procedures

Collecting Procedures	No. of the OCCs	Prounclei fiormation[1,2]		Total(%)
		Haploid	Diploid	
ASP(Control)	40	27	3	30(75)
SLC	45	28	2	30(67)
ASP+SLC	40	24	1	25 (62.5)

1. The OCCs co-cultured with GCM after parthenogenetic activation.
2. Pronuclei were stained by rapid staining at 18 h post-activation.

3. Nuclear maturation

The developmental potential of the recoverd oocytes with different methods were examined. The maturation rates of follicular oocytes were summarized (Table 3) according to the nuclear configuration. Metaphase Ⅱ stage oocytes were 78 (92.9%), 201 (79.1%) and 150(71.7%) in ASP, SLC and ASP+SLC, respectively. The proportion of numbers of matured oocytes was the highest in ASP among the different groups. But the maturation rate was higher (79.4% to 51.1%) when we compared with other experiment (Yoon et al, 1989). The total number of the OCCs in SLC was still 5.05 times greater than that of ASP. Similar maturation was obtained when the two methods were combined. Cumulus expansion was also found in all three groups, thus providing the evidence that the recovered OCCs from SLC underwent normal maturation process.

4. Cytoplasmic maturation

The morphological maturation was demonstrated both in cumulus expansion and nuclear configuration. It was interesting to know whether the cytoplasmic maturation accompanys in the oocytes obtained from SLC method. Pronucleus formation was analysed in the ethanol-activated oocytes. Formation of one or two pronuclei was found in successfully activiated oocytes. Pronucleus formation was similar among the 3 methods, being 75, 67 and 62.5% in ASP, SLC and ASP+SLC, respectively. Thus the oocytes demonstrated normal early development after parthenogenetic activation, which is one of indicators for cytoplasmic maturation. Although further development has to be shown, such a full development was previously obtained from activated oocytes in cattle (Lee et al., 1992). In conclusion, the developed slicing method is an alternative way of obtaining more number of the oocytes from slaughter house ovaries. Their developmental capacity was also demonstrated by nuclear and cytoplasmic maturation.

Ⅳ. SUMMARY

A new technique was established to maximize the numbers of follicular oocytes recovered from the ovaries obtained at the slaughter house. And their further developmental capacity was demonstrated. There recovery techniques were used; aspiration (ASP, control), slicing (SLC) and slicing combining aspiration (ASP+SLC). Recovered oocytes were cultured in TCM 199+15% FCS+gonadotrophins in an atmosphere of 5% CO_2 in air at 39℃ for 24 h. The nuclear maturation was detemined with chromo-

some configuration by rapid staining. And cytoplasmic maturation was examined by the formation of female pronuclei with parthenogenetic activation of the matured oocyte after 18 h of co-culture with granulosa cell monolayer.

Total 1,641 bovine follicular oocytes recovered from 245 ovaries. The number of oocytes per ovary was 1.87 in ASP, 11.05 in SLC and 7.88 in ASP+SLC, respectively. SLC would yield 5.9 folds increase, compared with ASP. The rate of maturation were 92.9% in ASP, 79.1% in SLC and 71.7% in ASP+SLC, respectively. Although the maturation rate in ASP was the highest, metaphase II oocytes per ovary in SLC was 5 times higher than that of ASP. The rates of pronuclei formation upon ethanol activation were 75% in ASP, 67% in SLC and 62.5% in ASP+SLC, respectively.

The results demonstrate that it should be possible to maximize the number of the follicular oocyte from the ovary for mass production of bovine embryos. Thus the established technique may provide efficient supply of bovine embryos for biochemical and molecular study of early bovine embryos.

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