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Effect of OPU Session Periods on the Efficiency of *In Vitro* Embryo Production in Elite Korean Native Cow

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Abstract

Up-to-date artificial insemination (AI) using frozen sperm consider as the most widely using technology for improvement of Korean Native Cow (Hanwoo) embryo production. However, it is time consuming, required at least 15~20 years to make more than 6 generations, and their offspring number is limited. To overcome such limitations, superovulation and *in vitro* fertilization have been developed. For superovulation, the number of produced embryos are not enough for commercialization and donor cows need rest period. This led to use of slaughterhouse ovary for *in vitro* fertilization, but it is impossible to repeat the collection from the same individual and it only can improve the genetic merits of offspring for one generation. Production of embryos using Ovum Pick-Up (OPU) technique, where oocytes can be repeatedly collected from living elite donor, might overcome these limitations. In this study, we investigated the possibility of using OPU technique from donors at different age and different session periods for mass-embryo-production. Oocytes were collected from 26 donor cows twice per week, 3 - 4 months per year, between 2013 and 2016. Results showed that, the average number of embryo produced in first year used donor was significantly higher than that in second year used donor (3.89 ± 2.85 vs 3.29 ± 2.70), however, there was no significant difference between third year used donor (3.51 ± 3.32) and other groups. Taken together, our data showed that repeated using of donor up to three years is possible for *in vitro* embryo mass-production. Moreover, OPU can be used as suitable embryo producing technique for livestock breed improvement.

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INTRODUCTION

Embryo transfer (ET) is able to produce the numerous embryos from superior genetic animal and can effectively produce offspring using surrogate mother of poor genetic traits (Kim et al., 1998, 2002). In 1951, first ET for Korean Nativer Cow (Hanwoo) was conducted through laparotomy and live offspring has been produced. In Korea, ET technique has been dramatically improved. In recent decades, hormones have been used for superovulation, non-surgical embryo recovery and transfer techniques have been developed. Repeated oocyte collection by OPU has become alternative and competitive to superovulation for embryo production in cattle (Boni 2012). Recently, *In vitro* production (IVP) system using Ovum Pick-Up (OPU) derived oocytes, vitrification and sperm sorting has used in industry of dairy and beef cattle (Brackett et al., 1982, 1989; Xu et al., 1987; Cran, 1993; Yanget al., 1993; 1993; Kubota, 1998;Yang et al., 1993; Kubota et al., 1998; Pierson, 1999, Pierson and Adams, 1999). Since the end of 1980, Hanwoo OPU followed by *in vitro* production become the most widespread embryos production technology in the research and commercial areas. The success and efficiency of production system of OPU embryos depends on quality and quantity of aspirated Cumulus-Oocyte Complexes (COCs). Moreover, OPU technician, OPU assistance, interval of OPU, hormone treatment and synchronization can dramatically affect the efficiency and success of OUP technique (Merton et al., 2003).

After the introduction of OPU-IVP, many efforts have been made to improve the efficiency of embryo production but all of these have focused on non-genetic factors (Van Wagtendonk-de Leeuw et al., 2000; Merton et al., 2003; Lonegan et al., 2006)). Currently, the embryo production system through the OPU technique and the development rate to blastocyst stage have been improved but it is insufficient to supply the blastocyst as needed. Several researchers have reported on the effect of the donor age (Su et al., 2012) and hormonal treatment (Blondin et al., 1997) on development rate of blastocyst according to *in vitro* and *in vivo* maturation (Hendriksen et al., 2000; Rizos et al., 2002) but there is no report on the period of use. In order to commercialize the production system of *in vitro* fertilization embryo using OPU derived oocytes, effective utilization system of donors should be established. In this study, we have suggested a newly developed method to reuse the OPU donors for producing the embryo and to use them efficiently.

MATERIALS AND METHODS

1. Chemicals

Unless otherwise indicated, all of chemicals and media used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Ethics statement

All of the methods and experimental procedures were conducted according to the approved (Approval ID: GAR-110502-X0017) guidelines and regulations by the institutional animal care and use committee (IACUC) of the division of applied life sciences, department of animal science at Gyeongsang National University, Republic of Korea.

3. Experimental design

The experiment has been conducted from April 2013 ~ December 2016. Total 27 elite Hanwoo, which belonged to the Institute of Agriculture and Life Science, Gyeongsang National University, were used for OPU.

4. Recovery of oocytes by OPU technique

OPU technique has been conducted during the first wave of follicular development, as described (Deb et al., 2011). Hanwoo (body weight, approximately 600 ~ 800 kg) were used for two OPU sessions, 4 days apart. Cows were restrained in a chute and given 3 to 4 mL of epidural anesthesia (2% lidocaine). The COCs were retrieved by an ultrasonic instrument equipped with a 5-MHz convex transducer connected to a vacuum operating at a negative suction pressure of 85 to 90 mmHg. The follicular content of each cow was aspirated individually into TL-HEPES buffer (114 mM sodium chloride, 3.2 mM potassium chloride, 2 mM sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2 mM calcium chloride, 10 mM HEPES, 1 μL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) and imaged with a stereomicroscope.

5. Oocyte collection and *in vitro* maturation (IVM)

OPU derive oocyte were cultured in IVM medium as described by (*Mesalam et al., 2017a*). In brief, COCs were collected and washed in (TCM-199) supplemented with 10% (v/v) FBS (Gibco BRL, Life Technologies, Grand Island, NY, USA), 1 μg/mLestradiol-17ß, 10 μg/mL follicle-stimulating hormone, 10 ng/mL epidermal

growth factor, 0.6 mM cysteine, and 0.2 mM sodium pyruvate. Thereafter, groups of up to 50 COCs were transferred to a 4-well dish (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 µL IVM medium and incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22 ~ 24 h.

6. *In vitro* fertilization (IVF) and IVC

COCs were fertilized with frozen thawed sperm of a single selected bull previously described (Mesalam et al., 2017a). Semen was thawed at 39°C for 1 min, sperm were washed and pelleted in D-PBS by centrifugation at 750 × g for 5 min at room temperature, and motile sperm were recovered. The pellet was resuspended in 500 µL heparin (20 µg/mL) prepared in IVF medium [Tyrode's lactate solution supplemented with 6 mg/mL bovine serum albumin (BSA), 22 mg/mL sodium pyruvate, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin] and incubated at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 15 min (to facilitate capacitation). Thereafter, sperm were diluted in IVF medium (final density of 1 - 2 × 10^6 sperm/mL). Matured oocytes were transferred to 600 µL IVF medium containing sperm for 18 ~ 20 h. After IVF, cumulus cells were removed by repeated pipetting and denuded zygotes were placed in a well of a four-well plate containing 700 mL CR1-aa medium (Mesalam et al., 2017a) supplemented with 44 mg/mL $C_3H_3NaO_3$, 14.6 mg/mL glutamine, 10 mL/mL penicillin/streptomycin, 3 mg/mL BSA, and 310 mg/mL glutathione for 3 days (IVC-I). The presumed zygotes were then cultured until day 8 of embryonic development (day 0, day of IVF) in a medium of the same composition (IVC-I), except that BSA was replaced with 10% (v/v) fetal bovine serum (IVC-II). Day 8 expanded blastocysts were ET for recipients.

7. Statistical analyses

Data are presented as mean values standard deviation, except for data reporting changes during embryo development, which are presented as percentages. Each experiment was performed at least three times, and results were analyzed with SPSS (ver. 18.0, SPSS Inc., Chicago, IL, USA). Differences between means were considered significant at $P < 0.05$.

RESULTS

1. Follicle and oocyte dynamics for session

The recovered cumulus-oocyte complexes were morphologically classified into four grades based on the number of cumulus cells layers and homogeneity of the cytoplasm (Table 1). The average number of aspirated oocytes per donor was significantly ($P < 0.05$) higher in third year used cows (14.03 ± 5.92) comparing to than those in first and second year session groups (9.76 ± 4.58, 10.68 ± 4.50, respectively, Table 1). Moreover, the average number of grade 1 and grade 2 per donor were significantly ($P < 0.05$) greater in first (1.46 ± 1.71 and 2.28 ± 1.85, respectively) and third year groups (1.52 ± 1.13 and 2.54 ± 1.42, respectively) than that in second year group (1.05 ± 1.28 and 1.85 ± 1.60, respectively) (Table 1).

2. Embryo development rate and the number of blastocysts

The percentage of cleaved embryos was determined on day 3 of culture. The cleavage rate was significantly ($P < 0.05$) higher in third year session (52.9 ± 5.00%) than those in first (48.7 ± 3.11%) and second year session (49.3 ± 3.16%) (Table 2). However, blastocyst developmental rate in first (39.3 ± 2.85%) and third year groups (25.0 ± 3.32%) were significantly higher ($P < 0.05$) than those in second year group (30.7 ± 2.70%).

Table 1. Effect of OPU session periods on the quality of collected oocytes

Donors (#)	No. of formed follicles (Mean ± SD)	No. of aspirated oocyte (Mean ± SD)	Grade 1 (%, Mean ± SD)	Grade 2 (%, Mean ± SD)	Grade 3 (%, Mean ± SD)	Grade 4 (%, Mean ± SD)
1st year (26)	13835 (18.98 ± 6.45)^c	7112 (9.76 ± 4.58)^c	1061 (14.9, 1.46 ± 1.71)^a	1659 (23.3, 2.28 ± 1.85)^b	3380 (47.5, 4.64 ± 3.23)^c	1012 (14.2, 1.39 ± 1.85)^a
2nd year (26)	13784 (20.06 ± 4.76)^b	7336 (10.68 ± 4.50)^b	720 (9.8, 1.05 ± 1.28)^b	1274 (17.4, 1.85 ± 1.60)^c	4448 (60.6, 6.47 ± 3.56)^b	894 (12.2,1.30 ± 1.94)^a
3rd year (7)	5009 (23.85 ± 6.51)^a	2947 (14.03 ± 5.92)^a	319 (10.8, 1.52 ± 1.13)^a	534 (18.1, 2.54 ± 1.42)^a	1843 (62.5, 8.78 ± 4.41)^a	251 (8.5, 1.20 ± 1.22)^a

$^{a\sim c}$ Values with different superscripts were significantly different ($P < 0.05$).

Table 2. Effects of OPU session periods on the in vitro developmental competency

Donors (#)	No. of oocytes collected (Mean ± SD)	No. cleaved embryos (%, Mean ± SD)	No. of blastocyst developed (%, Mean ± SD)
1st year (26)	7112 (9.76 ± 4.58)[c]	3464 (48.7, 4.77 ± 3.11)[c]	2796 (39.3, 3.89 ± 2.85)[a]
2nd year (26)	7336 (10.68 ± 4.50)[b]	3617 (49.3, 5.27 ± 3.16)[b]	2254 (30.7, 3.29 ± 2.70)[b]
3rd year (7)	2947 (14.03 ± 5.92)[a]	1561 (52.9, 7.43 ± 5.00)[a]	738 (25.0, 3.51 ± 3.32)[a,b]

[a~c] Values with different superscripts were significantly different (P < 0.05).

DISCUSSION

Production efficiency of OPU-derived embryo is very important for commercialization of ET and several studies have been conducted to increase its production efficiency (Kim et al., 2014; Jin et al., 2011, 2014; Choi et al., 2011). Roth et al (2008) reported that, recovery the 13.2, 17.2 and 16.0 embryos, respectively after met-estrus (Day 4, 8, 11 and 15) of Holstein. Su et al (2012) recovered 7.3, 6.1, 4.7 embryos on average using 12 months, 7 to 8 years and 15 years or elder. Galli et al (2001) collected 8.4, 8.2, 10.3 and 9.4 embryos on average from 1997 to 2000 for four years. Galli et al (2014) reported that recovery the 12.8, 7.3, 10.4 and 10.9 embryos in Holstein of stage of suckler, pregnant by rearing calf, non-pregnant by rearing calf and previous rearing. Jin et al (2014) collected 7.5, 7.6, 9.6 and 7.3 embryos on average from 2010 to 2013 in Hanwoo. Jin et al (2015) obtained an average of 10.0 in six Hanwoo and this study collected 9.8, 11.9 and 14.0 embryos. The production of implantable embryos per year increased annually with 1.7, 2.1, 2.9 and 3.0. Galli et al (2014) collected 2.49, 1.43, 1.86 and 1.73 embryos according to donor. Jin et al (2014) produced an average 3.0, 2.2, 3.2, 3.0 and 4.1 embryos annually, these data agree with our results reported that the average embryo production per donor were 3.9, 3.3 and 3.5 embryos at first, second and third year sessions respectively.

Another aspect to consider for the optimization of OPU is the number and quality of collected oocytes. Production of embryos using OPU-derived oocytes can be used for mass embryo production, but average of number is different from study to another. We investigated the development rate of blastocysts, grade of oocytes, recovery of oocytes and number of follicles according to reuse the selected superior donor for OPU-derived embryo production. Our results suggest that the number of collected oocytes and development rate to blastocysts can affected by condition of embryo collection, generative function according to age, technique and production area of embryos. We reported that, second year used donors derived grade 1 and grade 2 oocyte lower than first year used donor, but third year used donors were same as first year used donor. Moreover, efficiency of embryo production also shown similar results as follicle number data, suggesting an individual different among different donors. Jin et al (2015) reported that the period for reusing of donor was longer than half year. However, in order to exactly determine the reuse of donor, we have to consider not only efficiency of reusing but also production rate depends on the rest periods. We need to confirm the efficiency of production depends on rest period after OPU. In conclusion, our data showed that repeated using of donor up to three years is possible for *in vitro* embryo mass-production.

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