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Interaction of donor age, parity and repeated recovery of cumulus–oocyte complexes by ovum pick-up on *in vitro* embryo production and viability after transfer

Jong-In Jin [a,1], Nasser Ghanem [a,c,1], Seong-Su Kim [a], Byung-Hyun Choi [a], A.-Na Ha [a], Kyeong-Lim Lee [a], Du-Won Sun [a], Hyun-Tae Lim [a], Jung-Gyu Lee [a,b], Il-Keun Kong [a,b,*]

[a] Department of Animal Science, Division of Applied Life Science (BK21 Plus), Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea
[c] Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt

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ABSTRACT

The objective of this study was to investigate the effect of age and number of parities on the potential of the Korean native cow (Hanwoo breed) as oocyte donor in ovum pick-up and subsequent *in vitro* embryo production. All animals were divided into two different groups based on age (3–6 and 7–9 years) and parity (2–3 and 4–7). Ovum pick-up (OPU) was performed to ten Korean native cows over a 4-months period at intervals of 3–4 d (twice weekly). The population of follicles (≥ 3 mm diameter) present in both ovaries and the grades of all cumulus-oocyte complexes (COCs) retrieved were recorded. All COCs were matured, fertilized and allowed to develop *in vitro* and the morphological quality of embryos was evaluated before transfer to recipients. There were 18.4 ± 5.7 (mean $\pm$ SEM) follicles available for aspiration throughout the experiments; the population of follicles decreased from 19.1 ± 6.3 in the first month to 16.9 ± 4.2 in the last month ($p \leq 0.05$). However, with advancement in age and parity of animals, there was an increase in number of ovarian follicles. Furthermore, the average number of COCs retrieved was 12.5 ± 5.6 in the first month and this number was reduced in the fourth month (10.4 ± 5.4). The total number of recovered COCs was increased in old cows with increased number of parities. Consequently, embryo production decreased from 4.8 ± 3.1 to 4.2 ± 2.8 on the first and last months, while, it was increased in old cows than young ones ($p \leq 0.05$). Furthermore, animals with high number of antral follicular count produced more COCs and embryos. Therefore, we concluded that repeated OPU of Korean native cows resulted in significant decrease in the *in vitro* production of embryos in young compared with old cows; this has negatively impacted embryo survival after transfer.

1. Introduction

Female reproductive technologies in cattle are important tools in hastening genetic gain in nucleus breeding herds and also for reducing the lag between the breeding population (the nucleus) and the commercial population (van Arendonk and Bijma, 2003). Retrieval of cumulus-oocyte complexes (COCs) via ovum pick-up followed by *in vitro* embryo production (OPU-IVP) are among the biotechnological tools that could be used to increase the number of offspring from elite females, accelerate genetic gain and reduce the generation interval, especially for a nucleus breeding scheme (Kruip et al., 1994; Merton et al., 2003).

Hanwoo is a Korean native breed of cattle known for their high marbling. The percentage of intramuscular fat is approximately 15–23% in Hanwoo cattle fattened for slaughter (Kim et al., 2005; Oh et al., 1970). In addition, the muscle fibres in Hanwoo cattle are thin with minimal connective tissues and have a characteristic flavour (Jo et al., 2012). With the increasing demand for Hanwoo meat and consequently more utilization as main beef animals in Korea, there is an increased emphasis on genetic improvement of Hanwoo cattle (Lee et al., 2013). With the bovine genome sequenced and more bovine genes for traits of economic interest being identified, OPU-IVP will be invaluable in rapidly multiplying rare genes or Quantitative Trait Loci (QTL) of high values (van Wagtendonk-de Leeuw, 2006).

In order to maximize oocyte recovery, OPU without hormonal stimulation could be routinely done twice a week (Galli et al., 2001; Hasler et al., 1995) for an extended period of time without

* Corresponding author at: Department of Animal Science, Division of Applied Life Science (BK21 Plus), Gyeongsang National University, Jinju 660-701, Republic of Korea.
E-mail address: ikong7900@gmail.com (I.-K. Kong).
[1] These authors contributed equally to this work.

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any long-term detrimental effects on the donor cow's fertility and well-being (Chastant-Maillard et al. 2003; van Wagtendonk-de Leeuw, 2006). A twice weekly OPU avoids the presence of the dominant follicle that could negatively affect COCs developmental competence (Machatkova et al. 2004). An early study (Kruip et al., 1994) has reported that repeated OPU does not affect the number of COCs that developed into blastocysts when it was done twice weekly for 5 months. The previous report did not find clear differences between dairy versus beef breeds, nor the extension of OPU (three *vs*. five months). However, large differences between individual cows and between cow/bull combinations were observed in the average population of follicles and oocytes recovered (Kruip et al., 1994) for non-stimulated and stimulated sessions in Belgian Blue donor cows (De Roover et al., 2008). In non-stimulated cows, some donors consistently showed a low population of follicles (n=4.5) and oocytes (n=1.9) per session compared with others that showed high-outcome sessions (n=18.5; n=7.7). In Brazilian donor cattle (Nelore), oocyte production rate was the key factor for the number of *in vitro* embryos produced (Thibier, 2004). In Nelore cows, individual variation in the population of follicles and oocytes have been reported (Pontes et al., 2009, 2011), with an average of 30 oocytes produced per procedure. In this regard, the effect of repeated OPU on the number and quality of COCs, in addition to subsequent embryo production, has not been reported so far in Hanwoo cows. Our research team reported the use of OPU-IVP on Hanwoo females several years ago (Deb et al., 2011). The objective of the present study was to determine the effect of long-term repeated OPU on the *in vitro* embryo production of Korean native cows with different ages and parities.

2. Materials and methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Hanwoo females were managed and used in accordance with Gyeongsang National University guidelines (approval no. GAR-110502-X0017).

2.1. Retrieval of cumulus-oocyte complexes by OPU

Ovum pick-up was performed during the first wave of follicular development, as described previously (Garcia and Salaheddine, 1998; Ghanem et al., 2007). Ten Hanwoo cows, with body weight ~400 to 600 kg and with apparently normal estrous cycles, were subjected to OPU twice weekly (follicle aspiration every 3–4 day) at growth phase (D 3–4 after estrus). Estrus and subsequent ovulation were synchronized by two administrations of prostaglandin F_2-alpha (500 μg Cloprostenol i.m, Estrumate®, Essex Tierarznei, Munich, Germany) at 11 d interval followed by GnRH injection (Receptal, 2500 IU i.m, Intervet, Unterschleissheim, Germany) on the day of estrus onset. Estrus was detected two days after the last prostaglandin F_2-alpha treatment. Cows were restrained in a chute and given caudal epidural anesthesia (5 mL of 2% lidocaine; Je-Il Pharmaceutical, Daegu, Republic of Korea). Ovarian follicles were visualized using an ultrasound scanner equipped with a 5 MHz convex probe (Scanner 100S; Esaote-Pie Medical, Maastricht, The Netherlands) fitted in a custom intra-vaginal OPU probe-holder. Follicles ≥3 mm in diameter were punctured with 18-gauge disposable hypodermic needle (Cook Veterinary Products, Queensland, Australia) connected to a 50-mL conical tube (BD Falcon, New Jersey, USA) by Teflon tubing (Cook Veterinary Products). Negative pressure of 85–90 mmHg was applied using a vacuum aspiration pump (GAST, Benton Harbor, Michigan, USA) and the aspiration vacuum was adjusted to a flow rate of 15 mL of water per minute. The follicular content of each cow was aspirated individually into 50 mL conical tube containing Tyrodes lactate (TL)-HEPES medium (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, 2.0 mM calcium chloride, 10 mM HEPES, 1 μL/mL phenol red, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin) supplemented with 2% (v/v) fetal bovine serum (FBS) and 5 IU/mL heparin. The collection tubes and aspiration medium were kept at 38 °C in a water bath.

2.2. In vitro maturation (IVM)

The retrieved COCs from each cow were morphologically evaluated based on the thickness and compactness of the cumulus investment and the homogeneity of the ooplasm (Gordon, 2003). Accordingly, grade 1+2 COCs were considered good quality while grade 3+4 were considered low quality ones. All COCs retrieved of each donor were washed twice in maturation medium (TCM-199) supplemented with 10% (v/v) FBS, 1 μg/mL of estradiol-17β, 10 μg/mL of FSH, 0.6 mM of cystein and 0.2 mM of sodium pyruvate, 50 μg/mL gentamycin sulphate and transferred in 700 μL of IVM medium at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 23–24 h, and cultured as described previously (Dey et al., 2012).

2.3. In vitro fertilization (IVF)

In vitro-matured COCs were fertilized with frozen-thawed sperm previously used in my laboratory (Dey et al., 2012). Semen was thawed (36 °C for 1 min), and sperm were then washed and pelleted in Dulbecco's PBS (D-PBS) by centrifugation at 750 × g at room temperature for 5 min. The pellet was diluted with 500 μL of heparin (20 μg/mL) in IVF medium [Tyrodes lactate solution supplemented with 6 mg/mL of BSA (A-6003), 22 μg/mL of sodium pyruvate, 100 IU/mL of penicillin, and 0.1 mg/mL of streptomycin] and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 15 min. Capacitated sperm were diluted in IVF medium (1×10^6 sperm/mL). Matured oocytes were transferred into IVF medium (700 μL) containing sperm and cultured at 38.5 °C for 18–20 h.

2.4. Embryo in vitro culture (IVC)

After IVF, cumulus cells were removed by pipetting and the denuded presumptive zygotes were placed in 700 μL of CR1-aa medium (Rosenkrans et al., 1993) supplemented with 44 μg/mL of sodium pyruvate, 14.6 μg/mL of glutamine, 10 μl/mL of penicillin/streptomycin, 3 mg/mL of BSA, and 310 μg/mL of glutathione for 3 d (IVC-I). Presumptive zygotes were then cultured until D 7 of embryonic development (D 0=day of IVF) in a medium of the same composition as IVC-I, except that the BSA was replaced with 10% (v/v) FBS (IVC-II). Cleavage rate was recorded on D 3 after IVF (72 hpi). On D 7, the stage of development and embryo quality were evaluated for each donor.

2.5. Embryo evaluation and transfer

Embryos were evaluated based on the guidelines of International Embryo Transfer Society (IETS) manual (Stringfellow and Givens, 2010). Accordingly, D 7 blastocyts with quality code 1 were used for embryo transfer. Estrus was synchronized as mentioned previously. A single fresh blastocyst was transferred into a recipient by non-surgical standard procedures at D 7 of the estrus cycle.

2.6. Statistical analyses

Mean ± SEM for follicular population, COCs quality, embryo