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Estradiol benzoate treatment before ovum pick-up increases the number of good quality oocytes retrieved and improves the production of transferable embryos in Japanese Black cattle

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ABSTRACT

The aim of this study was to evaluate the efficacy of treatment with estradiol benzoate (EB) at luteal phase prior to the ovum pick-up (OPU) during in vitro production of transferable embryos in Japanese Black cattle. A total of 15 cows were used as oocyte donors for OPU. Of those, four donors were randomly allocated (three times) into each of two treatment groups as a crossover study, and OPU session was carried out six times per one donor. Another eleven donors were used in a paired difference test by one crossover trial. Donors in the control group received no hormonal treatment; whereas, donors in the EB group received 1 mg of EB as a single injection. First, we observed dynamics of ovarian follicles and emergence of follicular wave after EB injection using transrectal ultrasonography. The number and proportion of medium-sized follicles with 4 to 6 mm in diameter increased gradually and achieved a peak at 72 and 96 hours after EB injection. The OPU was performed 88 hours after EB injection. The EB-treated donors had a higher proportion of follicles with 4 to 6 mm in diameters at the time of OPU. The stimulation with EB significantly increased the numbers of follicles aspirated, and the good quality cumulus-oocyte complexes per OPU. Furthermore, in the EB group, the percentage of transferable blastocysts was significantly greater than that in the control group ($P < 0.05$). In conclusion, a single EB injection before OPU increases the number of medium-sized follicles and can produce more transferable embryos.

1. Introduction

Embryo transfer (ET) of *in vitro* production (IVP) embryos enables cattle industries to enhance and accelerate the diffusion of production traits (Kruip, Boni, Wurth, Roelofsen & Pieterse,1994; Numabe, Oikawa, Kikuchi, & Horiuchi, 2000; Numabe, Oikawa, Kikuchi, & Horiuchi, 2001; Stringfellow, Givens, 2010). The transfer of IVP embryos from Japanese Black donors to Holstein recipients increases the number of beef calves (Numabe *et al.* 2000; Numabe *et al.* 2001). Even if the cost of ET is twice more expensive than artificial insemination (AI), it is still profitable due to the increased revenue from beef calf sales in addition to the dairy production. Therefore, we are committed to the spread of ET. To achieve our aims, it was necessary to produce valuable embryos at a lower cost.

In the earliest years, bovine embryo production for ET was mainly *in vivo*-fertilized embryos from superovulated heifers or cows (Bousquet et al., 1990., Pontes et al., 2009). Currently, the retrieval of oocytes using ultrasound guided follicle puncture, or ovum pick-up (OPU), is linked to the procedures for IVP embryos, as it can exploit more Japanese Black cattle embryos (Numabe *et al.* 2000; Numabe *et al.* 2001). The OPU is a valuable technology that greatly enhances the potential of *in vitro* fertilization (IVF) systems in a variety of breeding conditions and species: Holstein (Ogata, Yu, Hidaka, Matzushige & Maeda, 2016, Vieira et al., 2014), Angus cross (Chaubal et al., 2007), Nelore (Pontes et al., 2009), Buffalo (Presicce et al., 2002, Presicce, 2007) and Japanese Black (Numabe *et al.* 2001) cattle.

For effective production of more IVP embryos by OPU-IVF, FSH stimulation treatment can promote the development of multiple follicles in the ovaries and improve embryos yield in non-lactating or lactating Holstein donors (De Roover, Genicot, Leonard, Bols & Dessy, 2005, 2008, Vieira et al., 2014). On the other hand, non-lactating Holstein cows can produce a higher blastocyst rate and a higher number of transferable embryos than lactating cows regardless of FSH stimulation treatment (Sendag, Cetin, Alan, Hadeler & Niemann, 2008, Vieira et al., 2014). In OPU without hormonal stimulation, ovum collection can be repeated every 1 to 3 weeks (Kruip et al., 1994). When

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comparing the number of embryos produced by one donor in 1 year, more than 2-fold embryos are produced *in vitro* than *in vivo* (Goodhand, Watt, Staines, Hutchinson & Broadbent, 1999, Kruip et al., 1994, Numabe *et al.* 2000, Pontes et al., 2009).

Breeding techniques which utilize gonadotropin-releasing hormone (GnRH) to control the follicular wave and ovulation synchronization methods, such as Ovsynch, have been developed (Pursley, Mee & Wiltbank, 1995). Previously, we reported that GnRH-stimulation before OPU improved the efficiency of embryo production in Holstein cows during early lactation (Ogata, Hidaka, Matzushige & Maeda,2015).

Ovarian follicular growth and development in bovines is characterized by two or three consecutive follicular waves per estrous cycle (Ginther, Knopf & Kastelic, 1989, Sirois, 1988). Each wave involves the recruitment of a cohort of follicles and the selection of a dominant follicle. The growth of follicular waves is initiated by a rise in circulating FSH. All follicles growing as a cohort contain specific receptors for FSH and depend on this gonadotropin when growing. Whereas, estradiol secretion is inversely related to FSH secretion and closely regulate the emergence and growth of follicular waves (Evans, Komar, Wandji & Fortune, 1997). Ovarian estradiol secretion is important for the final stages of dominant follicle growth (Evans et al., 1997). The dominant follicle is the major source of the fluctuations in circulating steroid concentrations and therefore is primarily responsible for the negative feedback effects of ovarian steroids during waves of follicular development (Araujo et al., 2009).

Administration of exogenous estradiol-17ß (E2) in progesterone-implanted cattle suppressed the dominant follicle and resulted in the consistent emergence of a new follicular wave, on average 4.3 days later, regardless of the stage of development of the dominant follicle (Araujo et al., 2009, Bo et al., 1995). Furthermore, the dynamics of ovarian follicular wave development during the estrous cycle can be manipulated by treating with estradiol benzoate (EB) to synchronize proestrous development of ovulatory follicle (Burke, Day, Bunt & Macmillan, 2000, Martinez, Kastelic, Bo, Caccia & Mapletoft, 2005). These data suggest that treatment with progesterone and E2, in combination, can be used to effectively control and synchronize follicular wave development. However, in OPU, there are few reports of treatment with EB alone, without the combination with progesterone source.

In this study, we hypothesized that the administration of EB prior to OPU would improve embryo production by OPU-IVF. We report here on the effectiveness of treatment with a single administration of EB prior to OPU for increasing the number of good quality embryos.

2. Materials and methods

2.1. Animals and Experimental design

Animal experiments in this study were approved by the Institutional Animal Experimental Committee of Hiroshima Prefectural Livestock Technology Research Center, where the experiments were performed. A total of four Japanese Black cows were used as donors for experiments. They were kept in stalls and fed grass silage and water.

Experiment 1 was designed to evaluate dynamics of ovarian follicles over time and emergence of follicular wave after one-shot EB (1 mg; estradiol benzoate, ASKA Animal Health Co., Ltd, Tokyo, Japan) intramuscular injection. In this experiment, four donors at luteal phase were stimulated with EB injection. At 0, 24, 48, 72, 96 and 120 hours after EB injection, ovarian follicles were visualized using a real-time ultrasound scanner (SSD-1000 type, Aloka Co. Ltd., Tokyo, Japan) equipped with a 7.5 MHz convex array transducer (UHT-9106 type, Aloka) and the number of follicles in ovaries were counted on ultrasound video images. All visible follicles were quantified and classified according to their diameters (small follicles: 2 to 3 mm, medium follicles: 4 and 6 mm and large follicles: more than 7 mm).

Experiment 2 was designed to evaluate effects of EB injection on the number and quality of oocytes aspirated by OPU. In this experiment,

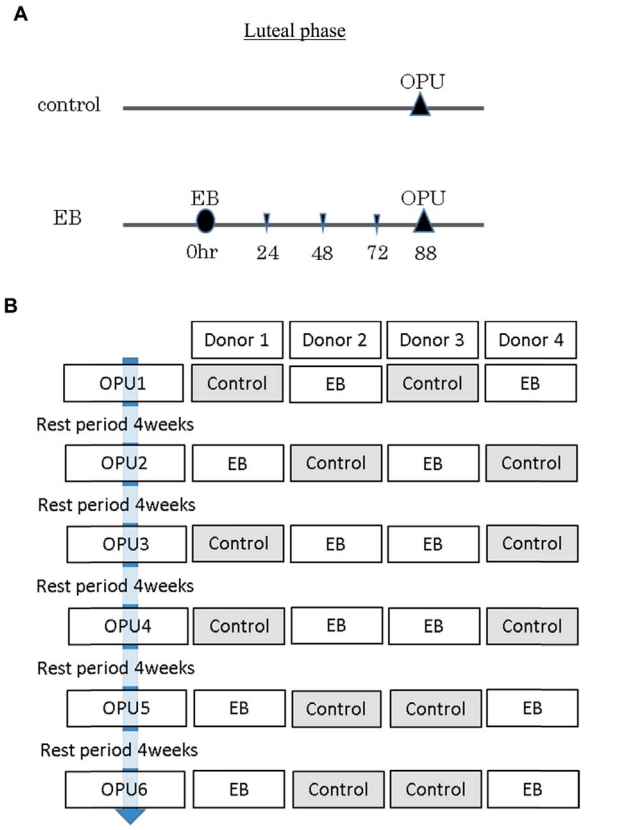

Fig. 1. Hormone treatment of four donors and ovum-pick up (OPU) schedule as the crossover design. Fig. 1A: control: The animals were not treated with hormone. OPU was performed on random days of estrous cycle. EB: Estradiol benzoate (EB) treatment at dosage of 1.0 mg IM was simultaneously administered. OPU was performed 88 hours after EB injection. Fig. 1B: Four donors were randomly allocated three times into each of two groups with/out the hormone (EB). OPU sessions was carried out total six times per one donor.

four donors were randomly allocated three times into each of two treatment groups (OPU without hormones as control group or with EB injection as experiment group). The experimental design was both group crossover study, and the OPU sessions were carried out total six times per one donor (Fig. 1A and B) The OPU was performed about 88 hours after EB injection according to the results of Experiment 1. Each OPU session was performed at more than four weeks intervals to avoid effects from repeated OPU and EB injection. In the preliminary study, we found that the number of COCs recovered decreased with the shorter intervals at less than 3-weeks.

Immediately before the OPU session, both ovaries were examined by transrectal ultrasonography. All visible follicles were quantified and classified according to the criteria shown as above in experiment 1 for control and EB groups. Furthermore, we examined the number of cumulus-oocyte complexes (COCs) recovered, classified the quality of COCs, and then cultured them.

Then, using *in vitro*-matured oocytes obtained from living cattle by OPU in control and EB groups, we examined the embryo production following IVF. Embryo development and transferable blastocysts were evaluated under an inverted microscope according to the International Embryo Transfer Society (IETS) manual (4th Edition IETS, IL, USA) (Stringfellow, 2010). Evaluation of the quality of the embryo was based on its morphological integrity. Embryos classified as transferable were all of code 1(excellent/good).

Experiment 3 was designed to evaluate effects of EB injection on the number and quality of oocytes aspirated by OPU as a paired difference test by one crossover trial using eleven donors. The OPU was performed about 88 hours after EB injection according to the results of Experiment