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Relationship between time post-ovulation and progesterone on oocyte maturation and pregnancy in canine cloning

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ABSTRACT

Canine oocytes ovulated at prophase complete meiosis and continue to develop in presence of a high progesterone concentration in the oviduct. Considering that meiotic competence of canine oocyte is accomplished in the oviductal environment, we postulate that hormonal milieu resulting from the circulating progesterone concentration may affect oocyte maturation and early development of embryos. From 237 oocyte donors, 2620 oocytes were collected and their meiotic status and morphology were determined. To determine optimal characteristics of the mature oocytes subjected to nuclear transfer, a proportion of the meiotic status of the oocytes were classified in reference to time post-ovulation as well as progesterone (P4) level. A high proportion of matured oocytes were collected from > 126 h (55.5%) post-ovulation or 40–50 ng mL^{-1} (46.4%) group compared to the other groups. Of the oocyte donors that provided mature oocytes *in vivo*, there was no correlation between serum progesterone of donors and time post ovulation, however, time post-ovulation were significantly shorter for < 30 ng/mL group ($P < 0.05$). Using mature oocytes, 1161 cloned embryos were reconstructed and transferred into 77 surrogates. In order to determine the relationship between pregnancy performance and serum progesterone level, embryos were transferred into surrogates showing various P4 serum levels. The highest pregnancy (31.8%) and live birth cloning efficacy (2.2%) rates were observed when the embryos were transferred into surrogates with circulating P4 levels were from 40 to 50 ng mL^{-1}. In conclusion, measurement of circulating progesterone of female dog could be a suitable an indicator of the optimal time to collect quality oocyte and to select surrogates for cloning.

1. Introduction

The critical importance of the oviductal environment for proper reproduction has been well-demonstrated by studies on sperm transport and storage, capacitation of spermatozoa, fertilization, and embryonic development in various species (Verhage et al., 1973; Steinhauer et al., 2004; Rodriguez-Martinez, 2007). In comparison to the information resulting from investigations in other species, in canines there is scarcity of data describing oviductal and follicular environment. Considering the critical importance of P4 in oocyte development in lower vertebrates (Hammes, 2004), we may postulate that the progesterone (P4) milieu may contribute to the acquisition of meiotic competence or the resumption of meiosis in canine oocyte and the development of preimplantation

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embryos, and that the concentration of circulating P4 can be used as an indicator for the optimal time to recover oocytes, or transfer embryos. Unlike other mammalian oocytes, canine oocytes are ovulated at the germinal vesicle stage and complete maturation in the presence of increasing P4 oviduct milieu, due to preovulatory luteinization (Concannon et al., 1988). In particular, MII oocytes, the most suitable oocyte for nuclear transfer as recipients were observed at from 54 to 127 h post ovulation in the oviduct. Furthermore, canine embryos remain in the oviduct until they reach the 8-cell stage of preimplantation (Reynaud et al., 2005), however those derived from nuclear transfer take longer, developing up to the morula stage (Jeong et al., 2016).

P4 is a pivotal hormone in oogenesis that plays a role in follicular growth, ovulation, and luteinization. P4 is also unequivocally required for maternal support of conceptus survival and development during pregnancies in mammals (Kim and Greenwald, 1987; Peluso, 2006). Willingham-Rocky et al. (2003) have reported lower P4 concentration in serum compared to that in preovulatory follicular fluid and bursal fluid collected before and after ovulation, respectively. It is proposed that the difference in P4 concentration between follicular fluid and the oviduct after ovulation facilitates meiotic competence of oocytes during meiotic arrest, and triggers meiosis resumption (Willingham-Rocky et al., 2003). In our previous result, the optimal time post ovulation to collect for MII oocytes varied with the initial rise of P4 of oocyte donors (Hossein et al., 2008). Recently, treatment of P4 receptor antagonist aglepristone at the end of proestrous stage prolonged the meiotic maturation of oocyte with normal morphology up to 335 h post ovulation. The underlying mechanism of P4 on oocyte has not yet been elucidated for dog species (Reynaud et al., 2015).

Oocyte cytoplasmic and nuclear maturation are key determining factors necessary for ensuring the progress of early embryo development at the embryonic genome activation and the subsequent embryogenesis in the preimplantation stage (Meirelles et al., 2004; Ferreira et al., 2009). In cattle, sheep and pig, follicular oocyte *in vitro* maturation protocols are well-established, allowing molecular studies of underlying mechanisms and applications in reproductive technologies such as *in vitro* fertilization, intra-cytoplasmic sperm injection, and cloning through somatic cell nuclear transfer (SCNT). However, the same is not true for canines. Despite numerous studies conducted to optimize canine *in vitro* maturation system by mimicking *in vivo* follicular and oviduct environment (Hewitt et al., 1998) through investigation of culture media (Rota and Cabianca, 2004), hormones (Hewitt and England, 1999; Hatoya et al., 2009), proteins (Rodrigues and Rodrigues, 2003), energy substrates (Songsasen et al., 2002; Sturmey et al., 2009), antioxidants (Hossein et al., 2007) and other compounds, canine IVM protocols is limited and yet to be optimized. To date, *in vivo* matured oocytes are the only sources of oocytes for canine SCNT (Jeong et al., 2012; Jeong et al., 2014; Lee et al., 2014).

Selection of suitable oocyte donors and surrogates is crucial for SCNT. Considering P4 is responsible for the early development of embryos and the maintenance of pregnancy, it could be an important factor for selecting oocyte donors and surrogates for canine SCNT. It has been reported that increased concentrations of serum P4 in the early developmental period have been implicated in embryo viability and maintenance of pregnancy in cattle (Henricks et al., 1971; Garrett et al., 1988; Mann and Lamming, 2001) and sheep (Ashworth et al., 1989; Satterfield et al., 2006). However, studies are yet to determine whether difference in serum P4 concentrations of canine surrogates at the time of embryo transfer is associated with changes in pregnancy rate.

Current cultivation protocols to mature oocyte and develop canine embryos are underdeveloped and *in vivo* maturation and development of oocytes and embryos are at present required for the production of cloned pups. Serum P4 is known to increase with time post-ovulation, following a LH surge, this makes it a candidate for gauging the maturation or developmental status of oocytes and embryos. In the present study, factors known to influence canine oocyte maturation as well as to determine optimal levels for embryo transfer were investigated. Serum progesterone of oocyte donors and recipients were measured to establish the relationship between oocyte maturation, serum P4 levels and time post ovulation.

2. Materials and methods

2.1. Care and use of animals

In this study that was conducted from February to April 2016. The female mixed breed dogs with the ages between 1–7 years (body weight 20–25 kg), were housed in indoor kennels, fed standard commercial dog food once a day, and given water *ad libitum*. All animal procedures were conducted in accordance with the animal study guidelines and approved by the committee at the Sooam Biotech Research Foundation, Korea (permit no. C-12-01).

2.2. Ovulation determination

Unless otherwise indicated, all other reagents were obtained from Sigma-Aldrich. All donors and recipients employed in the study were showing spontaneous estrous. The estrous stage was examined weekly by observing for vulval bleeding to detect the onset of the heat period. During heat, 2 mL of blood sample was collected daily by cephalic venipuncture and serum P4 levels of blood samples were measured by electrochemiluminescence immunoassay (ECLIA; Cobas e411, Roche Diagnostics, Mannheim, Germany; intra- and inter-assay coefficients of variation $< 4\%$). Ovarian ultrasonographies were periodically performed twice a day when serum P4 levels were found to rise more than 2 ng/mL. Time of ovulation was designated when ovaries become difficult to find for an apparent decrease in the number or contour of anechoic follicles, or for their disappearance anechogenicity by transabdominal ultrasonography and as the proportion of cornified cells were greater than or equal to 90% of epithelial cells from vaginal swabs, stained following Diff Quik (Sysmex Co., Kobe, Japan) standard protocols (Johnston et al., 2001).