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Ovulation, fertilization and preimplantation embryonic development in raccoon dogs (*Nyctereutes procyonoides*)☆

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ABSTRACT

A study involving 32 sexual mature females was conducted to characterize ovulation, fertilization and early embryonic development *in vivo* in raccoon dogs. Oocytes and embryos were collected from the oviducts and uteri, evaluated by stereomicroscopy. Ovulation occurred 25–32 h after a female first accepted mounting, regardless of copulation, when the females were paired with a male in the same cage. Ovulated oocytes were at the primary stage. The number of ovulated eggs in females with or without mating was 9.96 ± 2.65 and 9.00 ± 1.92, respectively. Embryos at 2–4 cell, 8–16 cell, morula, blastocyst, and hatched blastocyst stage were observed at 29–73, 48–100, 98–126, 169–198 and 217–268 h after first mating, respectively. Embryos were located in the oviduct prior to 4-cell stage and moved into the uterus after 16-cell stage. Embryos at different stages were often obtained from the same female. During the zygote underwent a series of cleavage divisions, the diameter of the embryo cell mass continuously increased through the 2-cell and 4-cell stage, then started to decrease and was the minimum size at the morula stage. At the blastocyst stage, embryos increased in volume, and finally developed into a hatching blastocyst with a thinner zona pellucida. This is the first full report of preimplantation embryonic development in the raccoon dog, which will facilitate the application of advanced assisted reproductive technology in canine species.

1. Introduction

During past decades, assisted reproductive technology (ART) in mammals has played a very important role in enhancing animal reproduction, thus improving the quality of meat, eggs and milk, and saving endangered species. However, development of ART in the canids has resisted progress for decades due to their unique reproductive physiology. Canids experience obligatory prolonged ovarian inactivity or anestrus and may ovulate once or twice annually (Concannon, 2011). *In vivo*, circulating and local P4 levels begin to rise prior to the LH surge and subsequent ovulation (Olson et al., 1982). In addition, canine oocytes are ovulated at an immature stage compared with other mammalian species, and require 48–72 and 96–120 h postovulation in the oviduct to complete unclear maturation and cytoplasm maturation, respectively (Reynaud et al., 2005; Nagashima et al., 2015). There is great and growing interest in better utilizing the canid as

☆ This article is to cherish the memory of Professor P. C. Qin (1923–2008).

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a biomedical research model since over 350 heritable disorders/traits in canine species are homologous with human conditions, almost twice the number of any other species (Nicholas, 2003). The new gene-editing technology, such as CRISPR/CAS9 system, now enables writing the genome at will in a way that has never been simpler, more affordable, and widespread. To improve understanding of the genetic basis of potential human homologues of inherited disorders and traits in canine species by using CRISPR/CAS9, we must be able to precisely control canine reproduction and enable access to the germ line and embryo. In many mammals, such as dog (Tsutsui, 1989; Renton et al., 1991; Reynaud et al., 2005) and blue fox (Farstad et al., 1993), early embryonic developmental physiology has been systematically studied. However, there are only preliminary reports on early embryonic development in the raccoon dog (Feng et al., 1992).

The raccoon dog (*Nyctereutes procyonoides*) is an omnivorous canine with a high reproductive rate, short generation times and high population turnover (Al-Sabi et al., 2013). The breeding season is from early February to April. The female has only one heat period per season. In the wild the raccoon dog is often monogamous and lives in pairs from early autumn even after the pups are born. In captivity, however, it has adapted to polygamous mating.

Proestrus can be fairly long from 2 to 14 days, and oestrus lasts for 3–6 days. The gestation period is 59–64 days while little size varies from 1 to 10, and sometimes even more. The average is 6–7 pups per litter among domesticated raccoon dogs. The young reach maturity in 8–10 months (Norodd et al., 1988). While it is a native to Eastern Asia, about 9000 individuals were purposely introduced to different parts of European Russia and Western Asia between 1927 and 1957. Today, the raccoon dog is widespread in Asia, Northern and Eastern Europe and still spreading in Central Europe (Norodd et al., 1988; Drygala et al., 2016; Herfindal et al., 2016).

Farmed raccoon dog is an important species in regard to fur farming, which has acquired a great economic importance especially in the Nordic countries and Northeast area of China. The efficiency of its reproduction has always been the main issue for the fur farming. Although several publications on reproduction in the raccoon dog have been reported (Helle and Kauhhala, 1995; Xiao et al., 1995; Kauhala, 1996; Weng et al., 2006), many aspects of reproductive physiology in the raccoon dog remain unclear. The aim of this project was to investigate ovulation, fertilization and early embryonic development in the raccoon dog to further understand its reproductive biology thus laying the foundation for a biomedical research model and for improved reproductive outcomes for the farmed animal.

2. Materials and methods

2.1. Animals

The raccoon dogs (*Nyctereutes procyonoides*) were bred and raised at the fur animal farm of the Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences in Zuojia, Jilin province, China. To keep these animals healthy, they were vaccinated with canine distemper vaccine and enteritis vaccine twice a year. Farm workers identified the animals were healthy mainly by behavioural and physiological indicators which allowed sick animals to be quarantined. Such raccoon dogs were kept separated in outdoor cages and fed with commercial standard wet diet, consisting of fish, chicken eggs, beef liver, extrusion corn, mineral and vitamin additives with water available *ad libitum*. All animals were exposed to natural condition of cold ambient temperatures around −25 °C and seasonal daylight during the winter. Estrus occurred between the middle of February and the beginning of March. Thirty two healthy and mature female raccoon dogs, aged 1–3 years old and weighting 5–7 kg, were randomly divided into non-mated (n = 8) and mating (n = 24) groups. This sample size was determined based on the expense of animal and the need to have sufficient statistical power so that the main conclusions were drawn by testing at least 30 follicles or embryos. All animal experiments were conducted in accordance with the Guide to Care and Use of Experimental animal issued by the Animal Ethics Committees of Northeast Agricultural University and Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences.

2.2. Estrus monitoring, timing of ovulation and early embryonic development

Female raccoon dogs were tested for sexual acceptance twice daily (morning and evening), when the females were paired with a male in the same cage. In the non-mated group, females were exposed to a male, but were not allowed to breed. The time of first acceptance of mounting was assumed to be the beginning of ovulation. For the mating group, females were mated 2–3 times daily to increase the number of embryos produced *in vivo* since the multiple follicles ovulate over a period of time. The first mating was assumed to represent the commencement of embryonic development.

2.3. Oocyte/embryo collections

In the non-mated group, the oviducts from 8 female raccoon dogs were removed after the animals had been killed by electrical stunning either at 19–25 or 28–32 h following first acceptance of mounting. For the mating group, the oviducts or uteri from 24 female raccoon dogs were obtained after the animals had been sacrificed by electrical stunning at timed intervals following first coitus. Oocytes or embryos were collected from these excised oviducts or uteri by flushing with 3–5 ml pre-warmed phosphate buffered saline (PBS) plus 5 mg/ml BSA (Sigma, USA).

2.4. Embryo assessment

All the embryos were collected from the flushing fluids using stereomicroscopy. Their sizes were measured with a microscopic-micrometer while their characteristics were then noted under an inverted microscope.