Animal Reproduction Science 122 (2010) 244–252

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Comparative ultrastructural analysis of diestrous and anestrous canine Grade 1 cumulus-oocyte complexes

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ARTICLE INFO

Article history:
Received 5 May 2010
Received in revised form 9 August 2010
Accepted 18 August 2010
Available online 26 August 2010

Keywords:
Canine
Diestrus
Anestrus
Cumulus-oocyte complexes
Ultrastructure

ABSTRACT

The objective of the present study was to characterize, by means of transmission electron microscopy, immature Grade 1 cumulus-oocyte complexes (COCs) obtained from ovaries collected from bitches at diestrus and anestrus, after routine ovariohysterectomy. Cumulus-oocyte complexes were recovered after slicing the ovarian cortex and Grade 1 COCs were selected and prepared for transmission electron microscopy. All oocytes were at germinal vesicle stage in two different configurations. In 29 out of 37 COCs, oocytes presented a central or eccentric located nucleus (GVc), frequently containing a reticulated nucleolus, with a predominance of profound follicular cell processes (FCP), abundant endoplasmic reticulum (ER) and a moderated number of lipid droplets. In eight out of 37 COCs, oocytes had a peripheral located nucleus (GVp) containing small compact nucleolus, a thin perivitelline space, both superficial and profound FCP, moderate ER content and abundant lipid droplets. A higher ($p < 0.05$) proportion of GVp was found in oocytes collected in diestrus (36.8% – 7 out of 19) than in anestrus (5.6% – 1 out of 18). In addition, distinct ultrastructure characteristics among GVc associated with estrous phase were noted. Furthermore, a number of structures, some of which had not been reported before, were present in canine prophase I oocytes at variable frequencies. The evident variation in the presence, quantity and distribution of cell organelles in canine immature Grade 1 oocytes is discussed in relation to the biological phase of the oocyte and the reproductive stage of the donor bitch.

1. Introduction

A very low efficiency of canine oocyte *in vitro* maturation and subsequent fertilization (IVM/F) have been reported by several authors, with less than 20% of the oocytes reaching metaphase II (MII), and with no progeny born from IVM/F (Songsasen and Wildt, 2007). The ability of canine oocytes to mature *in vitro* is related with several morphological characteristics of the cumulus-oocyte complexes (COCs) (for review see Luvoni et al., 2005). Good quality canine oocytes show a darkly pigmented and homogeneous cytoplasm, a diameter over 100 μm (excluding the zona pellucida) and are surrounded by two or more layers of cumulus cells (Luvoni et al., 2005). However, these criteria appear to be insufficient to select oocytes fully competent to mature *in vitro* (Songsasen and Wildt, 2007).

In other species, studies on the ultrastructure of the immature oocyte contributed to relate differences in ultrastructural characteristics with developmental competence (De Loos et al., 1989; O'Brien et al., 1996; Wassarman and Josefowicz, 1978). Until the recent work by Viaris de Lesegno et al. (2008a), studies on the ultrastructural characterization of dog oocytes were limited to the early events of the developing oocyte (Szabo, 1967; Tesoriero, 1981) and to immature COCs from pre-pubertal bitches (Haenish-

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doi:10.1016/j.anireprosci.2010.08.018

Woehl et al., 2003). Viaris de Lesegno et al. (2008a) studied the ultrastructure of canine oocytes from preovulatory follicles before the LH peak to 4 days after the onset of ovulation, providing a much needed standard for evaluation of the ultrastructural characteristics of canine oocyte maturation *in vivo*.

Canine IVM/F is usually performed after selection of Grade 1 COCs obtained from ovaries after routine ovariohysterectomy of bitches at diestrus or anestrus stages. However, some studies have reported that the number of usable oocytes collected from individual bitches and the frequency of maturation in oocytes cultured *in vitro* are affected by the reproductive stage of the donor bitch (Nickson et al., 1993; Otoi et al., 2001; Yamada et al., 1993). Furthermore, a recent study by Kim et al. (2007) demonstrated that the glutathione content of the oocyte, an important marker of cytoplasm maturation, was influenced by the reproductive stage of the donor animal. To our best knowledge, there are no studies investigating if differences between oocyte populations collected at different reproductive stages are matched by dissimilarities at the ultrastructural level. Therefore, the present study was conducted to compare the ultrastructural characteristics of immature Grade 1 COCs obtained from diestrous and anestrous bitches.

2. Materials and methods

Except where otherwise indicated all chemicals were obtained from Sigma Chemicals Company (Barcelone, Spain).

2.1. Animals

Reproductive tracts were obtained from 8 healthy pubertal bitches, aged 3–10 years old and of different breeds, undergoing routine ovariohysterectomy (OVH) at diestrus ($N = 4$) and anestrus ($N = 4$). After OVH, ovaries were immediately transported (less than 4 h until processing) to the laboratory at room temperature in 0.9% NaCl supplemented with 1% penicillin–streptomycin. Blood was collected from bitches before OVH, in EDTA-coated tubes, for determination of plasma progesterone concentrations. Blood samples were immediately centrifuged for 5 min at 5000 rpm and plasma was stored at −20 °C until assayed. Progesterone concentrations were measured using a chemiluminescence assay (Immulite®, Siemens Systems; the inter- and intra-assay coefficient of variation were <16% at concentrations below 2 ng/ml and <7% at concentration above 15 ng/ml). The reproductive status of the donors was confirmed by morphological examination of the reproductive organs and plasma progesterone concentration (Hewitt and England, 1998). Briefly, ovaries without follicles or pronounced luteal tissue associated with a progesterone concentration below 2 ng/ml were considered to be from animals in anestrus. Ovaries from diestrous animals had one or more pronounced corpora lutea associated with a plasma progesterone concentration above 15 ng/ml.

2.2. Cumulus-oocyte complexes collection and processing for light and transmission electron microscopy

The ovarian cortex was sliced and washed in phosphate buffer saline (PBS, Gibco-Invitrogen, Barcelona, Spain) supplemented with 0.1% bovine serum albumin (BSA), at 37 °C. At the stereomicroscope, COCs with a multilayered compact cumulus-oophorus and a dark, evenly granulated cytoplasm with a diameter over 100 μm (Grade 1) were selected for analysis (Luvoni et al., 2005). Selected COCs were fixed in Karnovsky (2 h, 4 °C), washed with 0.15 M sodium cacodylate buffer, pH 7.2 (overnight, 4 °C), post-fixed (2 h, 4 °C) in 1% OsO_4 in buffer containing 0.8% hexanocyanoferrate potassium [$K_3Fe_3 + (CN)_6$] and washed for 15 min in buffer. Specimens were then dehydrated through a graded series of ethanol (50, 70, 90, 2 × 100%; 30 min each) followed by propylene oxide (15 min plus 15 min), impregnated with propylene oxid:epon (3:1, 1 h; 1:1, 1 h; 1:3, 1 h) and embedded in epon (3 h at room temperature, 3 days at 60 °C). A complete sectioning of COCs, in semithin sections of 1 μm and in ultrathin sections taken at every 10 μm was performed with a diamond knife (Diatome). Semithin sections taken at every 10 μm were stained with methylene blue and studied in an optical microscope (Nikon Eclipse E600, 400× magnification). The oocyte diameter, excluding the zona pellucida, was calculated from the average of two perpendicular diameters measured at equatorial sections of the oocyte (Nikon software Digital Sight dS-L1). Ultrathin sections were collected on copper grids (Taab-200 mesh) and contrasted for 20 min in uranyl acetate followed by 10 min in Reynolds lead citrate. All grids were examined and photographed in a JEOL 100CXII transmission electron microscope operated at 60 kV. A total of 41 COCs (20 from anestrous bitches and 21 from diestrous bitches) were studied, with 5–6 COCs analysed per animal.

2.3. Analysis of cytoplasm structures

The morphological study of oocytes was focused on a semi-quantitative analysis of the cytoplasm structures preformed on the equatorial sections of the oocyte, as described by Fair et al. (1997). The location of the oocyte nucleus, determined at light microscopy, was described as (1) central, i.e. located at the center of the oocyte (2) eccentric, i.e. located between the zona pellucida and the center of the oocyte; (3) peripheral, i.e. located close to the zona pellucida. In addition, the criteria to classify oocytes followed the description of Viaris de Lesegno et al. (2008b) with a slight modification. Briefly, GVc oocytes presented a round, central or eccentric located nucleus, whereas GVp oocytes had a peripheral located, more or less flattened nucleus. Organelle location was described as (1) peripheral, i.e. located at the oocyte cortex; (2) in the inner cell, i.e. confined to the area between the peri-nuclear region and the cortex; (3) peri-nuclear, i.e. in the area surrounding the nucleus or (4) all over, i.e. disseminated throughout the oocyte. The amount of lipid droplets (LD) was estimated as (1) plentiful, i.e. so numerous that there were few areas of cytoplasm matrix; (2) moderate, i.e. many lipid droplets, but small areas of cytoplasm matrix were readily observed;