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Theriogenology 77 (2012) 347–355

Theriogenology

www.theriojournal.com

In vitro maturation of canine oocytes co-cultured with bovine and canine granulosa cell monolayers

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Received 27 October 2010; received in revised form 21 July 2011; accepted 3 August 2011

Abstract

The present study investigated the effects of bovine granulosa cell monolayers (BGML) and canine granulosa cell monolayers (CGML) on nuclear maturation of canine oocytes with and without cumulus cells. Cumulus-oocyte complexes (COCs) or cumulus-free oocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM, control group), DMEM with BGML (BGML group), or DMEM with CGML (CGML group) for 72 h at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. All media were supplemented with 10% of FCS, 50 ng/mL of EGF, 2 μg/mL of estradiol-17β, 0.1 IU/mL of hCG, 0.1 IU/mL of FSH, 0.25 mM of pyruvic acid, 100 μM of β-mercaptoethanol, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. In cumulus-enclosed oocytes retrieved from ovaries at estrus and/or diestrus, the highest percentage of M-II oocytes ($P < 0.05$) was present in the BGML group (27.0%) compared with the CGML group (7.9%) and the control group (3.5%). In cumulus-free oocytes collected from ovaries at estrus and/or diestrus, the proportions of M-II oocytes co-cultured with the CGML were low (3.0%) and similar ($P > 0.05$) to proportions achieved with control (3.0%). However, the presence of BGML improved ($P < 0.05$) the ability of denuded oocytes to develop into M-II (10.2%). The BGML group had the highest overall meiotic resumption ($P < 0.05$), and least oocyte degeneration ($P < 0.05$) among experimental groups. In conclusion, BGML had a positive impact on the *in vitro* maturation system, as well as meiotic resumption of canine oocytes.

Keywords: Granulosa cell monolayers; IVM; IVP; Canine oocytes; Dog

1. Introduction

Assisted reproductive techniques (ARTs) including IVM of oocytes are eminently desirable for rescuing genetic materials from individuals that fail to reproduce. These techniques are also valuable in improving the genetic management of rare populations maintained *ex situ* as an insurance for counterparts living in nature [1]. However, despite recent tremendous advances in the application of ARTs to other domestic animals, consistent and controlled reproduction either by natural or assisted breeding has remained elusive in canids. This is mostly due to their unique reproductive characteristics, including polyovulation, non-seasonal repro-

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0093-691X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2011.08.007

ductive cycle, ovulation of immature oocytes at the germinal vesicle stage, and a 48 to 72 h period of postovulatory oocyte maturation in the oviduct. Therefore, a combination of these factors make it difficult to develop ARTs for canids [2,3].

The major challenge in developing IVM systems is creation of environmental conditions that can support oocyte development and would resemble an *in vivo* situation. Many researchers have examined the feasibility of IVM of canine oocytes [4–8]. Other researchers have also attempted to add oviductal epithelial cells [9], synthetic oviductal fluid (SOF) [10], mouse embryonic fibroblasts (MEF), and canine embryonic fibroblasts (CEF) [11] to the oocyte culture medium to address the above problems. In other studies, concentrated materials of protein sources [8] including gonadotrophins [12] and growth factors [13,14] have also been used. Nevertheless, the rates of maturation of canine oocytes to metaphase-II (M-II) remain low ($< 25\%$), especially when compared with those of many other mammalian species. For example, maturation rates of 90% have been achieved in cattle [15], and an 87% rate was attained in sheep [16]. Other reported successful maturation rates included 88% for pigs [17], 93% for mice [18], and 70% for cats [19]. The acquisition of developmental competence of oocytes is a limiting step that determines the ability of the oocyte to undergo successful fertilization and embryonic development. Furthermore, the inability to develop a consistent and an effective IVM system has resulted in limited success rates in IVF and IVC [20].

The final differentiation of the oocyte is orchestrated by a complex network of growth factors and cytokines leading to proper nuclear and cytoplasmic maturation. There is evidence that supplementation of IVM medium with granulosa cells improves nuclear and cytoplasmic maturation of oocytes in cattle [21], sheep [22], goats [23], camels [24], and monkeys [25], as well as the incidence of normal fertilization [26]. Since granulosa cells are found *in vivo* within developing follicles and undoubtedly play an important role in the oocyte maturation process, the present study was designed to investigate the influence of granulosa cell monolayers on the nuclear maturation of canine oocytes with and without cumulus cells during IVM.

2. Materials and methods

2.1. Assessment of reproductive status of the donors

The reproductive status of the donors was categorized as follows [27]: (a) anestrus, when the ovaries had no follicles or pronounced luteal tissues; (b) estrus (follicular phase), when one or more visible follicles were present; and (c) diestrus (luteal phase), when one or more evident corpora lutea were present.

2.2. Collection and preparation of cumulus-oocyte complexes (COCs)

Ovaries were collected from healthy domestic bitches undergoing routine ovariohysterectomy in local veterinary clinics. The animals ($n = 42$) were of various breeds, ranging in age from 8 mo to 7 yr. Both ovaries from each bitch were transported to the laboratory within 1 h after collection, in a thermo flask containing physiological sterile saline supplemented with 100 IU/mL of penicillin (Calbiochem, Inc., La Jolla, CA, USA) at 37 °C. After transportation, the fat, ligaments, and medulla were carefully trimmed off and removed. The COCs were released by repeatedly slicing the ovarian cortex with a scalpel blade (Feather, Osaka, Japan) at 37 °C. These COCs were placed in 35 mm petri dish (Falcon # 3001; Becton Dickinson, Lincoln Park, NY, USA) containing PB1 medium [28] supplemented with 3 mg/mL of BSA (Sigma, St. Louis, MO, USA) and 100 μg/mL of streptomycin (MEIJI Co., Tokyo, Japan), and examined under a dissecting microscope (SMZ1500, Nikon Instech Co., Ltd. Tokyo, Japan). After three washes in the same medium, the COCs were selected by observation under an inverted microscope (DMIR/E, LEICA Co., Wetzlar, Germany) according to previously described criteria [29]. The parameters were those reported to favor meiotic competence, which included the uniformity of ooplasm, homogeneous dark cytoplasm with more than three layers of compact cumulus cells and oocytes > 110 μm in diameter. The vitelline diameter of all COCs was measured with a calibrated ocular micrometer.

2.3. Preparation of cumulus-free oocytes

Cumulus-free oocytes were prepared by exposing the selected COCs to 0.2% of hyaluronidase (Sigma) for 15 min with gentle pipetting to remove cumulus cells.

2.4. Preparation of bovine granulosa cell monolayers (BGML)

Bovine granulosa cells were prepared as described by Maeda et al [21]. Briefly, bovine ovaries were collected from a local abbatoir and transported to the laboratory in a thermo flask. The granulosa cells were obtained by aspiration of a small antral follicle (2 to 5 mm in diameter) with an 18 ga needle. The follicular