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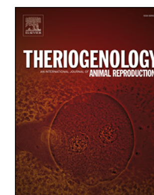

Morphometric assessment of *in vitro* matured dromedary camel oocytes determines the developmental competence after parthenogenetic activation

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The aim of the current study was to improve the selection method of camel oocytes after *in vitro* maturation by reducing exclusion criteria that were based only on the presence of the first polar body. A combined nuclear and morphometric assessment of camel oocytes after *in vitro* maturation was included to perform a judgment. The nuclear status of the oocytes, including the presence of the first polar body, meiosis I stage, and lack of nuclear materials, was investigated. The morphometric criteria that comprised the dimensions of each oocyte were as follows: diameter of the whole oocyte, including the zona pellucida (ZPO), zona pellucida thickness (ZPT), ooplasm diameter (OD), the perivitelline space (PVS) area, and PVS diameter. Among the oocytes with different nuclear status, there were no differences in ZPO and ZPT. However, oocytes with no nuclear material showed a significant reduction in OD (110.19 ± 1.4 μm) and a significant increase in PVS area (2139 ± 324.6 μm^2) and PVS diameter (13.9 ± 1.96 μm) when compared with oocytes in the meiosis I stage (117.41 ± 2.85 μm, 1287.4 ± 123.4 μm^2, and 8.56 ± 0.65 μm, respectively). To simplify the selection, the major difference between meiosis I and degenerated oocytes was the diameter of the PVS, which was greater than the ZPT in degenerated oocytes. Therefore, three groups were morphologically differentiated into oocytes with polar bodies (PB1), meiosis I (MI) oocytes, and degenerated oocytes. MI oocytes were able to extrude the polar body after activation but were not able to develop into blastocysts. In contrast, MI oocytes were able to develop into blastocysts after a biphasic activation protocol in which the oocytes were electrically activated and treated with ionomycin after 2 h. In conclusion, the results obtained by the morphometric assessment allowed us to develop a simple and objective classification system for *in vitro* matured dromedary camel oocytes, which will lead to accurate oocyte selection for the support of subsequent embryonic development.

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1. Introduction

There are several challenges in the *in vitro* production of camels embryos; for instance, a limited number of slaughtered camels is available, wherein the majority of the slaughtered animals are either old, culled for infertility, or very young and have not attained maturity [1]. In Saudi Arabia, the collection of ovaries from fertile camels is particularly challenging, because there are restrictions on fertile she-camel slaughtering, as given in the regulations of Ministry of Municipal and Rural Affairs in Kingdom of Saudi Arabia [2]. Additionally, the physiological complexity of follicle maturation during the follicular waves might be associated with the asynchronous maturation of oocytes retrieved from different follicular stages in the camel [3–5].

Therefore, the careful morphological selection of camel oocytes is a crucial step in the prediction of the subsequent developmental competence and to avoid the disposal of indispensable oocytes,

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especially when the available oocytes are limited. The morphometric selection of *in vitro* matured oocytes has been reported in humans and several animal species [6–16]. To our knowledge, no studies have previously utilized the morphometric classification for *in vitro* matured oocytes in camels.

Remarkably, a prerequisite to enable the developmental competence of the oocyte is the successful completion of meiosis. One main criterion in the judgment of oocyte maturation is the completion of meiosis I and the extrusion of the first polar body after asymmetric cell division with the vast majority of the cytoplasmic volume conserved for the oocyte [17]. After completion of the first meiotic division, the oocyte enters meiosis II and remains arrested in metaphase II [18]. Specifically, several reports revealed that approximately 50% of camel oocytes can extrude the first polar body after an *in vitro* maturation period [19–23]. Hence, approximately half of the cultured oocytes will be excluded from subsequent experiments, such as parthenogenesis, intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer, which use denuded oocytes. Additionally, the ooplasm diameter was associated with the developmental competency of the oocytes and considered as an essential factor for oocyte selection in humans and a variety of animal species [9,24,25].

Accordingly, the current study was carried out to determine several morphometric dimensions of camel oocytes, such as the diameter of the ooplasm and the perivitelline space in relation to the nuclear status after *in vitro* maturation. In addition, the study aimed to examine the effect of assisted activation on the oocytes that were morphometrically acceptable but did not extrude the polar body, to increase the yield of oocytes that could support early embryonic development.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals and hormones were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

2.2. Collection of ovaries, cumulus-oocyte complexes (COCs), and in vitro maturation (IVM)

Camel ovaries were obtained from a local abattoir in Riyadh and transported in 0.9% (v/v) NaCl solution at 30–33 °C to the laboratory within 4–6 h. The follicular contents from antral follicles (2–8 mm in diameter) were aspirated using an 18-ga needle attached to a 10 mL-disposable syringe. Cumulus-oocyte complexes (COCs) with evenly granulated cytoplasm that were enclosed by more than three layers of compact cumulus cells were selected and washed three times with HEPES-buffered tissue culture medium-199 (TCM-199) supplemented with 2 mM $NaHCO_3$, 5 mg/mL BSA, and 1 µL/mL gentamycin sulfate (Caisson Lab. Inc., Smithfield, UT, USA). The COCs were cultured in 4-well dishes that contained 20 to 25 oocytes in 500 µL of maturation medium at 38.5 °C in an atmosphere of 5% CO_2 in humidified air for 30 h. The maturation medium comprised bicarbonate-buffered TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS), 10 µg/mL FSH, 10 µg/mL LH, 1 µg/mL 17β–estradiol, 20 ng/mL epidermal growth factor (EGF), 1 µL/mL insulin-transferrin-selenium (ITS), 0.3 µM cysteamine, 0.15 mg/mL L-glutamine, and gentamycin sulfate 1 µL/mL, as previously described [26].

2.3. Morphometric classification of oocytes in relation to nuclear status after IVM

After IVM, the cumulus cells were stripped from the oocytes by trituration with 0.1% (v/v) hyaluronidase in HEPES-buffered TCM-199 and two washes in TCM-199 supplemented with FBS 10% (v/v). The oocytes were then stained with 5 µg/mL bisbenzimide (Hoechst 33342) for 10 min. The morphometric parameters of the oocytes were assessed according to previous studies, but with modifications [11,13]. The nuclei status and extrusion of the polar body were examined under an inverted microscope equipped with epifluorescence (Leica DMI4000 B, Leica Microsystems GMS GmbH, Wetzlar, Germany). Images were captured by the camera and software of the microscope (Leica Application Suite, Version 4.0) and the morphometric parameters of the captured images were analyzed by using ImageJ 1.50i software (NIH, USA) by use of the scale bar as an arbitrary scale for pixel analysis by the software. Each parameter was measured at least three times in the same oocyte image and all the results were recorded. The morphometric parameters described in Fig. 1 are as follows: oocyte outer diameter (ZPO), zona pellucida thickness (ZPT), inner oocyte diameter (ZPI), and ooplasm diameter (OD). Based on these parameters, other parameters were mathematically calculated: perivitelline space (PVS) diameter = ZPI − OD; inner oocyte area (IA) = 3.14 × $(ZPI/2)^2$; ooplasm area (OA) = 3.14 × $(OD/2)^2$, and PVS area = IA − OA. The diameters were measured in µm and the areas were measured in μm^2. The oocytes were classified into three groups depending on the nuclear status and morphometric analysis (the data are described in Table 1 and Fig. 3): 1) oocytes with extruded first polar body (PB1 group); 2) oocytes between metaphase I and anaphase I (MI group); and 3) oocytes with only a small amount of, or without, nuclear materials (degenerated group).

2.4. Morphometric assessment simplification

Based on the results of Table 1, the visual morphometric classification was simplified by using the stereomicroscope. The oocytes were classified according to a remarkable difference between the three groups: 1) PB1 group, which contained the first polar body; 2) MI group, which contained no polar body but was however characterized by a PVS diameter <10 µm, or the PVS diameter was less than the ZPT thickness of the same oocyte; and 3) the degenerated group in which the PVS diameter was greater than the ZPT of the same oocyte. This simple morphologic classification was used for this experiment and all further other experiments.

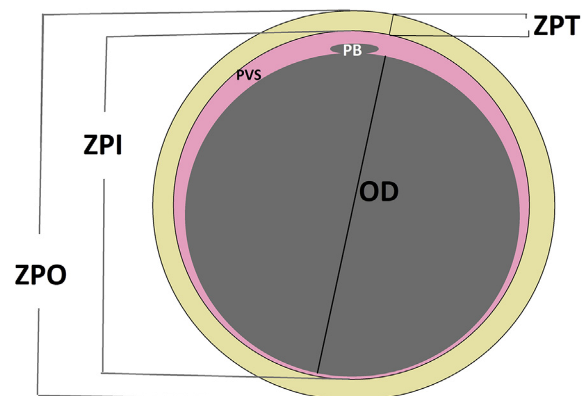

Fig. 1. The method of morphometric analysis of camel *in vitro* matured oocytes. Captured images were analyzed by using ImageJ software to measure the pixel distance. ZPO, outer diameter of zona pellucida included oocytes; ZPT, zona pellucida thickness; ZPI, inner diameter of the oocyte; OD, ooplasm diameter; PVS, perivitelline space; PB, polar body. All parameters were measured in µm.

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