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Technical note

A simple and high-throughput method to assess maturation status of bovine oocytes: Comparison of anti-lamin A/C-DAPI with an aceto-orcein staining technique

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Abstract

A precise, accurate, nonambiguous and high-throughput method is required to assess nuclear maturation of mammalian oocytes. The objectives of this study were to compare the efficiency and ease of use of a simplified fluorescence imaging (anti-lamin A/C and 4′,6-diamidino-2-phenylindole [DAPI]) technique to the existing technique (aceto-orcein staining) for the evaluation of nuclear maturation of bovine oocytes, and to determine the kinetics of bovine oocyte maturation using an anti-lamin A/C-DAPI technique. In Experiment 1, oocytes were matured in vitro and stained with aceto-orcein and anti-lamin A/C-DAPI staining techniques. The proportions of oocytes lost during procedures and those that could not be classified (because of ambiguous morphology) during evaluation were lower ($P < 0.0001$) in oocytes stained with anti-lamin A/C-DAPI (9% and 2%) than those stained with aceto-orcein (31% and 13%), respectively. Anti-lamin A/C-DAPI was a quick procedure which could be completed within 7 h after completion of the maturation (compared with > 24 h for the aceto-orcein method). Furthermore, > 200 oocytes could be stained in one batch with anti-lamin A/C-DAPI technique. In Experiment 2, nuclear maturation kinetics of bovine oocytes at various time intervals (0, 6, 12, and 22 h) during in vitro maturation (IVM) was evaluated using the anti-lamin A/C-DAPI technique. Germinal vesicle, germinal vesicle breakdown, metaphase I, and metaphase II oocytes were predominant at 0, 6, 12, and 22 h of IVM, respectively. We concluded that the anti-lamin A/C-DAPI was an efficient and simple technique for nonambiguous evaluation of nuclear maturation status of large numbers of oocytes in a short interval.

Keywords: Cattle; Oocytes; Nuclear maturation; Aceto-orcein; Anti-lamin A/C; DAPI

1. Introduction

In the mammalian ovary, resting primordial follicles contain oocytes at the germinal vesicle (GV) stage. Germinal vesicle breakdown (GVBD) is a prerequisite for oocytes to come out of this arrest and for subsequent nuclear and cytoplasmic maturation to proceed [1]. In vitro maturation of GV stage oocytes is a critical step in IVF, intracytoplasmic sperm injection, and animal cloning. The assessment of nuclear maturation of mammalian oocytes at various intervals determines the efficiency of in vitro maturation procedure for healthy embryo production [2]. However, the ooplasm is filled with dark lipids which obscure the nuclear material [3]. Aceto-orcein staining is a common method for assessment of nuclear maturation of in vitro cultured oocytes in various species [4–7]; however, it is a laborious procedure. Recently, we experienced a substantial loss

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of oocytes during aceto-orcein staining and a high proportion of oocytes could not be classified because of their ambiguous morphology and/or staining. Alternative methods of assessing nuclear maturation of bovine oocytes became possible with the advent of DNA-specific fluorescent dyes, e.g., Hoechst 33342 [8,9] and 4',6-diamidino-2-phenylindole (DAPI) [10,11]. However, these fluorescence dyes cannot precisely differentiate between the GV and GVBD stages. Lamins, proteins present on the inner face of nuclear envelope, are responsible for the overall nuclear organization. Lamin A/C has been detected in the nuclear envelope of bovine and porcine GV stage oocytes [12–14]. In this study, we combined anti-lamin A/C and DAPI stain to clearly differentiate GV, GVBD, metaphase I (MI), and metaphase II (MII) stages of nuclear maturation in bovine oocytes. The objectives of this study were to compare the efficiency and ease of use of a simplified fluorescence imaging procedure (anti-lamin A/C-DAPI) to the existing technique (aceto-orcein staining) for evaluation of nuclear maturation of bovine oocytes, and to determine nuclear maturation kinetics in bovine oocytes using anti-lamin A/C-DAPI staining.

2. Materials and methods

2.1. Chemicals and supplies

All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

2.2. Oocyte collection

Bovine ovaries were collected from an abattoir and transported to the laboratory at approximately 25 °C within 6 h after collection. The cumulus-oocyte complexes (COCs) were manually aspirated from follicles < 8 mm in diameter using an 18-ga needle attached to a 5-mL syringe containing approximately 1.0 mL of wash medium consisting of Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Burlington, ON, Canada) supplemented with 5% calf serum (Invitrogen) (v:v). Cumulus-oocyte complexes were searched under a stereomicroscope and washed (three times) in wash medium. All COCs with more than three layers of cumulus cells and uniform cytoplasm (Grade 1) were selected for further processing.

2.3. In vitro maturation

Cumulus-oocyte complexes were washed (three times) in maturation media containing TCM-199 supplemented with 5% calf serum (v:v), 5 μg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 μg/mL FSH (Bioniche), and 0.05 μg/mL gentamicin. Groups of 20 COCs were placed in 100-μL droplets of maturation media under mineral oil and incubated for 22 h at 38.5 °C, 5% CO_2 in air and high humidity.

2.4. Experiment 1: comparison of aceto-orcein and anti-lamin A/C-DAPI staining techniques to assess nuclear maturation of bovine oocytes

After completion of 22 h of IVM, COCs were completely denuded by pipetting with 0.3% hyaluronidase in Ca^{+}- and Mg^{+}-free DPBS (Invitrogen) (wt/vol) and stained with aceto-orcein [15] or anti-lamin A/C-DAPI staining techniques.

2.4.1. Aceto-orcein staining

Oocytes were stained and evaluated as described [16]. Briefly, oocytes were mounted on glass slide (less than five oocytes per slide) under coverslip (supported with paraffin-vaseline corners) and fixed in ethanol: acetic acid (3:1, v:v) for 24 h. Then, oocytes were stained in 1% orcein (wt/vol) in 45% acetic acid (v:v) for 20 min and differentiated by gently running differentiation solution (20% glycerol [v:v] and 20% acetic acid [v:v] in distilled water), between the slide and coverslip. The oocytes were evaluated using phase-contrast microscopy for the stage of nuclear maturation as GV, GVBD, MI, and MII (Fig. 1).

2.4.2. Anti-lamin A/C-DAPI staining

Stock solutions of 4% paraformaldehyde (wt/vol) and 1% Tween 20 (v:v) were prepared in DPBS. All fixation, permeabilization, and immunostaining procedures were performed at 22 °C on a rocking platform in four-well plates and oocytes were washed (three times) in DPBS between steps. At the end of in vitro maturation, completely denuded oocytes were placed in four-well plates (5 to 20 oocytes per well) and fixed with 4% paraformaldehyde in DPBS (wt/vol) for 30 min. After fixation, oocytes were permeabilized with 0.5% Triton X-100 in DPBS (v:v) for 30 min, followed by 0.05% Tween 20 in DPBS (v:v) for 30 min. Oocytes were incubated with blocking buffer containing 2% bovine serum albumin in DPBS (wt/vol) for 1 h. Then, oocytes were incubated with mouse anti-lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:300 in blocking buffer for 1 h, washed (three times) in DPBS (5 min each) followed by incubation with secondary antibody Alexa Fluor 488 labeled anti-mouse IgG (Santa Cruz Biotechnology) 1:200 in blocking buffer for 1 h in the dark. Oocytes ($N = 5$ to 20) were transferred through