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Original Research

Impact of Equine and Bovine Oocyte Maturation in Follicular Fluid From Young and Old Mares on Embryo Production in Vitro

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ABSTRACT

Equine follicular fluid (FF) provides autocrine and paracrine factors from theca, granulosa, and cumulus cells, both reflecting and impacting oocyte and follicle maturation. We hypothesized that maturation of oocytes in FF from old versus young mares has a deleterious effect on oocyte maturation and their subsequent developmental potential. Follicular fluid was collected from the large, dominant follicle from young mares (4–13 years) or old mares (21–26 years) and classified as: (1) Noninduced follicular fluid (NFF), FF from noninduced follicle 33 ± 3 mm, or (2) Induced follicular fluid (IFF), FF collected ~24 hours after administration of ovulation-inducing drugs when a follicle 33 ± 3 mm was observed. In experiment 1, immature equine oocytes were collected, matured in vitro for 30 ± 2 hours in 100% IFF, collected from young or old mares, with the addition of follicle stimulating hormone (5 mU/mL), then fertilized by intracytoplasmic sperm injection. In experiment 2, immature bovine oocytes were collected, matured in 100% IFF or NFF, collected from young mares or old mares, then fertilized via in vitro fertilization. In experiment 1, more blastocysts tended ($P = .08$) to be produced from equine oocytes that were matured in old versus young mare FF. In experiment 2, when IFF and NFF groups were combined, cleavage rates were higher ($P = .001$) when bovine oocytes were matured in FF from young than old mares. In contrast to our hypothesis, we observed no conclusive evidence that FF from old mares has a deleterious impact on oocytes and their early developmental potential.

1. Introduction

Reproductive decline due to advanced age is evident in many species [1–4], and a pronounced reduction in fertility is associated with aging in the mare [5–7]. In the old mare, age-associated changes in the oocyte have been determined to be an important cause of reproductive failure [8]. Mare age is associated with altered oocyte mitochondrial function [9] and morphology [5,6] and presumed to be the cause of decreased pregnancy rates after oocyte transfer [8,10] and intracytoplamic sperm injection [10,11]. In addition to intrinsic oocyte defects associated with aging, the extent that the follicular environment impacts oocyte viability and subsequent embryo production has not been determined.

Before ovulation, the follicle in light-horse mares reaches approximately 45 mm in diameter [12]. The ovarian follicle is composed of a thecal cell layer, basement membrane, granulosa cell layer, and finally the cumulus oocyte complex. The actions of each layer are of major importance to the production of a healthy oocyte that is capable of being fertilized, progressing through embryogenesis, and producing a live offspring [13]. The dynamics by which follicular fluid (FF) is manufactured in the equine follicle involves aquaporins and osmotic gradients, as well as cellular communications between the thecal and granulosa cell layers [14]. Follicular fluid impacts the microenvironment in which the equine oocyte grows and matures. Follicular fluid is a transudate from the thecal cell layer vasculature [14]. Initially, FF presents as small pockets of fluid in the developing follicle, and as the follicle grows, the fluid pockets combine to form an antrum [15]. Fluid movement into the

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follicle is dictated by osmotic gradients [16] with hyaluronan, versican, and inter-alpha-trypsin inhibitor being the main contributors to antrum osmotic pressure [14,16]. Follicular fluid components are comparable to blood serum, as validated by a study in which biomarkers were similar in FF and serum [17]. The osmolarity and pH of FF are similar to that of blood serum, although FF is slightly more alkaline [17,18]. Follicular fluid is a dynamic mixture of proteins, lipids, metabolites, hormones, enzymes, steroids, DNA, and large molecular weight molecules whose content changes with changing phases of the estrous cycle [17,19–23]. Therefore, systemic and follicular age-associated changes are likely to cause alterations in FF.

Efforts have been made to find correlations between the contents of FF and fertility in women, mares, and cows [24–29]. In a study where human FF was used in a bovine in vitro maturation system, a positive effect was noted when FF was collected from productive oocyte retrieval sessions compared to nonproductive oocyte retrieval sessions [30]. Although associations have been made between FF and fertility, the downstream effects of FF on embryonic development and the interaction with maternal age have yet to be clearly elucidated in the mare.

We hypothesized that FF from old mares has a deleterious effect on oocyte maturation and subsequent embryo development. The aim of the study was to assess the effect of culture in FF, collected from the follicles of old and young mares, on in vitro maturation (IVM) of equine and bovine oocytes and their subsequent embryo development.

2. Materials and Methods

2.1. Animals and Supplies

All procedures were done in accordance with Colorado State University's Institutional Animal Care and Use Committee. Unless stated otherwise, all chemicals and media were obtained from Sigma (St. Louis, MO, USA). Chemically defined media (CDM) was prepared at the Colorado State University's Animal Reproduction and Biotechnology Laboratory [31]. Media used for oocyte holding during sperm preparation and intracytoplasmic sperm injection (ICSI) (H-CDM-M, CDM buffered with HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, for use at room atmosphere), sperm preparation before ICSI and in vitro fertilization (IVF) (F-CDM, CDM for fertilization), bovine oocyte handling (H-CDM-M), bovine IVM medium, bovine embryo handling (H-CDM-1 and H-CDM-2, HEPES-buffered sequential embryo culture media), and bovine embryo culture (CDM-1 and CDM-2) were made as published [32], with the incorporation of later modifications [33].

2.2. Follicular Fluid Collections

Follicular fluid was collected from young mares (4–13 years) and old mares (21–26 years) that weighed 450 to 600 kg and were housed in dry lot paddocks or in a barn and fed grass or grass-alfalfa hay; mineral blocks and water were provided ad libitum. Follicular fluid was collected between March and September. Reproductive tracts of the mares were examined using transrectal ultrasound imaging with 7.5 MHz linear probe. When a dominant follicle reached 33 ± 3 mm in diameter, and endometrial edema consistent with estrus was imaged, FF was collected as: (1) Noninduced follicular fluid (NFF) from a dominant follicle with no induction of follicular maturation in the donor mare or (2) Induced follicular fluid (IFF) collected 24 ± 4 hours after induction of follicular maturation by administration of human chorionic gonadotropin (2000 IU, intravenous, Chorulon, Merck Animal Health, Madison, NJ) and deslorelin acetate (0.9 mg, intramuscular, SucroMate Equine, Thorn BioScience LLC, Louisville, Kentucky) to donor mares.

During FF collections, mares were restrained in stocks with their tails wrapped and tied, and the peritoneum was prepared aseptically. Just before the procedure, mares were administered xylazine (200–300 mg, intravenous, AnaSed LA, Boise, ID) and butorphanol (10 mg, intravenous, Dolorex, Merck Animal Health, Madison, NJ) for sedative and analgesic properties and N-butylscopollammonium bromide (120 mg, intravenous, Buscopan Injectable Solution, Boehringer Ingelheim, St. Joseph, MO) was given for relaxation of the rectum. Dosage was altered for individual mares as needed. Aspirations were performed as described [34,35]. To collect FF, a 12-ga, double-lumen needle was advanced into the center of the follicle lumen, and approximately 10 mL of FF was suctioned (−150 mm Hg) into a 50 mL conical tube (Corning CentriStar, Corning, NY). The FF was collected before the total collapse of the follicle around the needle and gross blood contamination. After FF was obtained, the collection vial was changed, and the aspiration was continued to collect the oocyte and follicular cells that were evaluated to confirm the stage of follicle maturation. Compact granulosa and cumulus cells were observed for NFF collections, and mucoid, expanded cells were observed for IFF collections [35]. Follicular fluid was aliquoted directly, without centrifugation or filtering. The fluid was stored at −80°C in 1 to 1.5 mL aliquots in vials (Nalgene Cryoware Cryogenic vials, Thermo Scientific, Waltham, MA) within 15 minutes of collection.

2.3. Experiment 1: Equine Oocytes

Oocytes were collected from mares (4–15 years, 450–600 kg, n = 12) that were housed in dry lot paddocks with access to covered shelters and fed grass-alfalfa hay; mineral blocks and water were provided ad libitum. At 10- to 14 day intervals, oocytes were collected from all follicles ≥5 mm in diameter using ultrasound-guided, transvaginal, follicle aspirations. Oocyte aspirations were performed as previously described [36] with the exception that a different lavage medium (ViGRO Complete Flush, Bioniche, Athens, GA) was used to which heparin (4000 IU/L) was added. Aspirates were collected into 500 mL bottles (Nalgene Sterile PETG media bottles, Thermo Scientific, Waltham, MA) and kept warm during the procedure by surrounding the bottle with ~37°C bags containing saline. When the oocyte aspiration was completed, aspirates were immediately filtered (EmCon Filter, AgTech, Manhattan, Kansas) and searched.

For each group of oocytes, IFF from a single mare's follicle (young, n = 15 and old, n = 18) was used for oocyte maturation and for media additives. No IFF from an individual donor was used for the donor mare's own oocytes. If more than one oocyte collection cycle occurred for a donor mare, the oocytes were alternated between young and old FF. Only two donor mares were aspirated once. Follicular fluid from the same follicle as used for oocyte maturation was also used for media additives.

Recovered oocytes were rinsed and held in holding medium (M199 with Hank's salts [Life Technologies, Waltham, MA] with 10% fetal calf serum). Searches were completed in <30 minutes, and oocytes (n = 2–9) were placed into a 1.1 mL glass vial (VWR Shell Vials 66015-702, Radnor, PA) containing room temperature medium (RTM) consisting of 40% M199 with Hank's salts, 40% M199 with Earle's salts, gentamicin (10 mg/mL) or penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin/mL) as previously described [36] with the exception that RTM included 20% IFF rather than FBS and was equilibrated in 5% CO_2 and air before use. Before use, a vial containing frozen IFF was thawed in warm water at roughly 35°C. After the addition of oocytes, the glass vial with RTM was immediately capped, and the lid was wrapped