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Short-term hypertonic exposure enhances in vitro follicle growth and meiotic competence of enclosed oocytes while modestly affecting mRNA expression of aquaporin and steroidogenic genes in the domestic cat model

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ABSTRACT

Using the domestic cat as a non-rodent, larger animal model, the objective was to determine the impact of a brief incubation in a hypertonic microenvironment on (1) ovarian follicle and oocyte growth in vitro, (2) developmental capacity of the resident oocyte, and (3) expression of aquaporin (AQP) genes in parallel with genes involved in regulation of folliculogenesis. In Study 1: Secondary or early antral follicles encapsulated in 0.5% alginate were allocated to one of three treatment groups: 1) culture in standard medium at 290 mOsm for 15 d (Control); 2) incubation in 350 mOsm medium for 1 h followed by culture in standard medium for 15 d (Hypertonic-1h); or 3) incubation in 350 mOsm medium for 24 h followed by incubation in standard medium for additional 14 d (Hypertonic-24h). After measuring follicle and oocyte diameters on Day 15, in vitro-grown oocytes were incubated for 24 h before assessing nuclear status. In Study 2: secondary or early antral follicles were subjected to one of the three treatments: 1) culture in standard medium at 290 mOsm for 48 h; 2) incubation in 350 mOsm medium for 1 h followed by culture in standard medium for additional 47 h; or 3) incubation in 350 mOsm medium for 24 h followed by culture in standard medium for additional 24 h. At the end of the culture period, all follicles were assessed for mRNA level of *Cyp17a1*, *Cyp19a1*, *Star*, *Aqp1, 3, 5, 7* and *8* as well as *Fshr* using qPCR. Freshly collected follicles also were subjected to gene expression analysis and served as the 'Non-cultured control'. Hypertonic-24h follicles grew larger ($P < 0.05$) than the control, whereas those in Hypertonic-1h group exhibited intermediate growth, especially when the culture started at the early antral stage. Oocytes in the Hypertonic-24h group were larger and resumed meiosis at a higher rate than in the other treatments. In vitro culture affected ($P < 0.05$) mRNA expression of *Cyp19a1*, *Star, Aqp1,* and *Aqp7* in both the secondary and early antral stage while *Fshr* was only affected in the former compared to the non-cultured control. Pre-incubating follicles in 350 mOsm medium for 24 h enhanced ($P < 0.05$) *Star* and *Aqp7* while decreasing ($P < 0.05$) *Aqp1* expression compared to the control in secondary follicles, but not in the early antral stage. In summary, short-term hypertonic exposure promoted cat follicle development in vitro (including the meiotic competence of the enclosed oocyte) possibly through a mechanism that does not involve water transport genes.

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1. Introduction

In addition to studying live animal models, much of our contemporary understanding about the mechanisms regulating ovarian folliculogenesis are partly derived from incubating follicles in vitro while manipulating the surrounding biochemical and mechanical environment [1–3]. Beyond improving fundamental knowledge, this research area has practical implications. Because the ovary contains thousands of small, immature follicles, the development of an effective culture system could provide access to enormous numbers of viable oocytes from a given donor that could be matured and fertilized in vitro to produce embryos. This

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emerging fertility preservation technology would benefit the genetic management of numerous animal genotypes (including those used to study human diseases [4,5] and diverse wildlife species [5]). Other beneficiaries are young female cancer patients who are at risk for permanent infertility due to their therapeutic treatments [6].

A three dimensional (3D) culture system involving alginate has been shown to be effective for growing mouse follicles to produce oocytes capable of fertilization and live births after embryo transfer [7]. This system has provided encouraging results for advancing follicle development in the human [8–10], rhesus macaque [11], baboon [12], goat [13], and dog [14,15], although with limited or no success at producing fertilizable oocytes. The lack of direct application from achievements in the mouse to other species likely is due to physical, anatomical and physiological differences [15,16]. For example, the size of a mature cow, sheep, or human follicle is five-to 20-fold larger than for the same stage mouse follicle [16]. Furthermore, the mouse oocyte becomes 'competent' upon follicular antrum development [16], whereas this capacity in larger species including for the cow [17], dog [18], human [19], and cat [20,21]) is more closely affiliated with the follicle and oocyte reaching a required size. For example, a human follicle must reach at least 5 mm in diameter for its resident oocyte to be able to complete nuclear maturation [19,22]. Such observations probably mean that follicles from larger species require an in vitro microenvironment that allows meeting some minimal size requirement, likely in the presence of an antral cavity.

To-date, mechanisms regulating antrum formation and expansion within the ovarian follicle have not been fully elucidated. It has been suggested that transport of water into the follicular antral cavity is facilitated by water channel proteins, or aquaporins (AQPs) [23–26] that are localized in granulosa cells. Thus far, at least 13 AQP subtypes (*AQP1-13*) have been found in mammalian cells [27]. *Aqp1* to *5* as well as *Aqp7* to *9* are present and participate in water transport and development of ovarian follicles of multiple mammalian species [23,26,28]. Specifically, mRNA and protein expressions of *Aqp1* and *5* are highest during the follicular period of cycling pigs, suggesting a role(s) in follicle advancement [28]. In the rat, *Aqp7*, *8*, and *9* facilitate water transport in antral stage follicles [23]. Finally, it has been shown that *Aqp1* and *9* may play roles in preovulatory follicle growth and ovulation in the human [26,29]. Based on these earlier observations, we speculated that AQPs may be exerting an influence by increasing water transport, that in turn, promotes the formation and expansion of the antral cavity.

Previous investigations explored the relationship between *Aqp* expression and osmolality of the culture medium. Specifically, it has been determined that subjecting pre-implantation stage mouse embryos (zygote-16 cell) to a short-term osmotic, hydrostatic, or oxidative challenge stimulates 'adaptive' and positive developmental responses [30]. More precisely, such embryos in a hypertonic environment (350 mOsm) are stimulated to produce more p38 mitogen-activated kinase that, in turn, increases *Aqp3* and *9* [31]. Furthermore, when pig zygotes are exposed to a slight hypertonicity for 48 h, more blastocysts form after transfer back into isotonic medium [32], and these embryos are more cryo-tolerant [28].

Using the domestic cat as a non-rodent, larger animal model, the objective of the present study was to assess the impact of a brief incubation in a hypertonic microenvironment on (1) follicle and oocyte growth in vitro, (2) developmental capacity of the resident oocyte, and (3) expression of AQP in parallel with genes involved in the regulation of folliculogenesis (follicle stimulating hormone receptor (*Fshr*) and steroidogenesis regulation, including *Cyp17a1*, *Cyp19a1* and *Star*). We hypothesized that short-term exposure of follicles to a hyperosmolar medium enhances follicle growth, and developmental competence of the resident oocyte by modulating expression of genes regulating water transport and folliculogenesis.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Collection of ovarian follicles

Ovaries from 60 domestic cats (6 mo-3 yr of age) were recovered after routine ovariohysterectomy at local veterinary and spay clinics and transported on ice in Leibovitz's L-15 medium supplemented with 10 IU/ml penicillin G sodium and 10 μg/ml streptomycin sulfate. Without predetermination, all ovaries used in the present study contained visible antral follicles on the gonadal surface and no evidence of corpora lutea or albicantia. Follicle isolation was performed within 6 h of the surgery. Briefly, the cortical sections (2–3 mm thick) were dissected from each ovary's surface and individual follicles physically-isolated using 23 g needles under stereomicroscopic viewing. The isolated follicles were placed in 'collection medium' comprised of Minimum Essential Medium (MEM) plus 3 mg/mL bovine serum albumin (BSA), 2 mM glutamine, 25 mM Hepes, and 10 IU/ml penicillin G sodium and 10 μg/mL streptomycin sulfate. Follicles were classified as secondary (mean ± standard error of mean [SEM] = 208 ± 7.9 μm diameter) or early antral (329.8 ± 5.4 μm) stage using the following criteria established earlier in our laboratory [21]. Specifically, follicles with multiple layers of granulosa cells and an apparent basement membrane, but lacking a clearly visible antral cavity (under stereomicroscopy), were considered to be in the secondary stage; those containing evidence of a small antral cavity were classified as in the early antral stage.

2.3. Follicle encapsulation and in vitro culture

Follicles were individually-encapsulated in 0.5% (w/v) alginate (FMC BioPolymers, Philadelphia, PA, USA) using methods adapted from Songsasen et al. [14]. In brief, alginate (50 μL) was pipetted onto a cover of a 65 mm Petri dish. A group of five to seven follicles was transferred into each alginate drop. Then each follicle was aspirated in ~5 μL of the surrounding alginate and transferred into a calcium chloride (5 mM $CaCl_2$/14 mM NaCl) solution. After allowing cross-linking for 2 min, each alginate-encapsulated follicle was washed twice in collection medium before being transferred into a 4-well culture plate (Nunc™, Fisher Scientific, Pittsburgh, PA, USA), each well containing 500 μL of pre-equilibrated growth medium. The latter was comprised of MEM containing 3 mg/mL BSA, 4.2 μg/mL insulin, 3.8 μg/mL transferrin, 5 ng/mL selenium), and 1 μg/mL FSH (Bioniche Animal Health, Belleville, ON, Canada). Individual follicles were cultured for a total of 15 d (or 360 h; Study 1) or 48 h (Study 2) at 38.5 °C in humidified 5% CO_2. For those incubated for 15 d, half of the culture medium (~250 μl) was aspirated from each culture well and this volume immediately replaced with fresh growth medium at every 72 h time-point.

2.4. Follicle and oocyte assessments

The diameter of each follicle was measured at the onset of in vitro culture (Day 0) as well as 7 and 15 d of in vitro incubation using an inverted microscope (Leitz DMIL, Leica Microsystem, Buffalo Grove, IL, USA) equipped with an ocular micrometer. Each follicle was sized from the outer layer of the somatic cells, with the