Current status and future perspective of canine and feline ovarian tissue culture
Status atual e perspectiva futura de cultura de tecido ovariano canino e felino

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Abstract

The ability to maintain follicular structure in vitro while manipulating the surrounding biochemical and mechanical environment has improved our understanding of the mechanisms regulating the intimate relationship between the ovarian follicle and its maturing and differentiating oocyte (Eppig, 2001; Kreeger et al., 2005). Specifically, it has been established that bidirectional communication between the oocyte and surrounding granulosa cells is crucial for follicle and gamete development (Eppig, 2001). Beyond improving fundamental knowledge, in vitro folliculogenesis also has practical potential. Because the ovary contains thousands of primordial and primary follicles, in vitro culture could provide access to enormous numbers of oocytes that, if viable, could be matured and fertilized in vitro to produce embryos for offspring production. For the field in general, there are at two target groups, the most prominent being young women (or girls) whose germplasm is at risk from cancer treatment (Smitz et al., 2010). The second includes genetically valuable animals that have failed to reproduce naturally and then suddenly die or must undergo an ovariohysterectomy for medical reasons. For carnivores, there are three possible subgroups that could benefit: 1) companion dogs and cats; 2) special

Keywords: agarose gel, cats and dogs, ovarian tissue culture.

Resumo

A cultura de folículos ovarianos é uma ferramenta importante para o entendimento dos mecanismos que controlam o desenvolvimento de folículos e a diferenciação de oócitos. O benefício da recuperação de oócitos competentes em termos de desenvolvimento de folículos pequenos e imaturas (primordiais, primários, secundários e antral precoce) também seria significativo, variando do resgate de genoma de espécies em extinção à preservação da fertilidade em sobreviventes de câncer femininos. Até o momento, descendentes de folículos primordiais cultivados ocorreram somente em ratos. O progresso em espécies maiores e mais complexas tem sido limitado porque estes modelos de animais têm durações de foliculogênese mais longa, requerendo maior tempo de cultura para gerar folículos e oócitos completamente crescidos. Nesta apresentação ressaltamos o status atual deste tópico para carnívoros domésticos (i.e. cães e gatos), além de prioridades futuras de pesquisa.

Palavras-chave: gel agarose, cães e gatos, cultura de tecido ovariano.

Introduction

The first in vitro culture studies of intraovarian primordial follicles were published in the mouse (Eppig and O'Brien, 1996) and cow (Wandji et al., 1996) in the mid-1990s. The biological feasibility of the concept (i.e., the ability to nurture such premature follicles to the point of producing viable, fertilizable oocytes) was proven by O’Brien et al. (2003) with the production of 72 live mice pups. Since then, there has been progress through studies of other species, including observations of some sustained follicle viability and related oocyte growth in the baboon (Wandji et al., 1997), human (Telfer et al., 2008) and goat (Matos et al., 2011). In these species, primordial follicles have been able to advance to primary and secondary, and in the case of humans, to the antral stage. However, to-date, no offspring have been produced in species other than the mouse.

The ability to maintain follicular structure in vitro while manipulating the surrounding biochemical and mechanical environment has improved our understanding of the mechanisms regulating the intimate relationship between the ovarian follicle and its maturing and differentiating oocyte (Eppig, 2001; Kreeger et al., 2005). Specifically, it has been established that bidirectional communication between the oocyte and surrounding granulosa cells is crucial for follicle and gamete development (Eppig, 2001). Beyond improving fundamental knowledge, in vitro folliculogenesis also has practical potential. Because the ovary contains thousands of primordial and primary follicles, in vitro culture could provide access to enormous numbers of oocytes that, if viable, could be matured and fertilized in vitro to produce embryos for offspring production. For the field in general, there are at two target groups, the most prominent being young women (or girls) whose germplasm is at risk from cancer treatment (Smitz et al., 2010). The second includes genetically valuable animals that have failed to reproduce naturally and then suddenly die or must undergo an ovariohysterectomy for medical reasons. For carnivores, there are three possible subgroups that could benefit: 1) companion dogs and cats; 2) special
genotypes of these two species used as biomedical models for investigating human diseases; and 3) diverse wild carnivore species that are being managed to create sustainable populations to reduce extinction potential.

Our laboratory conducts folliculogenesis research in both the dog and cat for all of the above reasons. We are especially excited about the role of such studies for contributing to human reproductive health and wildlife propagation. For example, for the latter, five of the 36 extant canid and 16 of 37 felids are listed formally as threatened by extinction, mainly due to habitat loss, persecution and disease (International Union for Conservation of Nature – IUCN, 2014). This is one of the primary rationales for attempting to maintain viable populations of rare species ex situ, a complex process that can retain the necessary genetic diversity to ensure species integrity (Ballou, 1997). However, these intensive management programs require moving animals between institutions or, alternatively, germplasm. The latter approach has been incentive for adapting assisted reproductive technologies (so successful in humans and livestock; Comizzoli et al., 2010) to selected wildlife species.

But embryo-related strategies have not been used extensively in carnivores. Methods for estrous cycle stimulation and synchrony as well as gamete collection and culture that are so effective in cattle do not readily translate to canids and felids with their own unique (and more complex) reproductive anatomy and physiology. One of the most challenging issues is accessibility to mature oocytes capable of fertilization. For example, the canid ovary is encapsulated in an ovarian bursa that makes it difficult to recover oocytes from preovulatory follicles and the oviduct (Wildt et al., 1977; England and Allen, 1989). Combined with the challenge that both canids and felids resist (or are exquisitely sensitive) to exogenous gonadotropins given to provoke ovulation (Kutzler, 2005; Pelican et al., 2010), then it is not surprising that embryo technologies play a negligible role in carnivore reproductive management.

While conventional embryo strategies remain problematic, we believe it is timely to explore the feasibility of generating viable ova from the vast store of follicles within carnivore ovaries, most of which never ovulate. A prerequisite step to applying in vitro follicle culture to practical dog and cat reproduction is learning more about the basics of follicle (and corresponding oocyte) development. In this paper, we highlight recent advances in both the dog and cat in vitro follicle culture for the purpose of demonstrating not only potential, but the substantial challenges remaining.

Differences in culture medium requirement and sensitivity to in vitro culture between dogs and cats

We are interested in understanding the mechanisms regulating primordial follicle activation in the dog and cat, information crucial for formulating a consistently effective and long-term in vitro culture system. The first step has been to begin determining the basic requirements for maintaining living ovarian tissue and corresponding primordial follicles. Briefly, our protocol has involved the recovery of fresh ovaries within 6 h of excision followed by preparing cortical pieces that are cultured on an agarose gel for 14 days (Fujihara et al., 2014). In the presence of EGF, we can now maintain dog follicle viability for up to 7 days (Fig. 1C) compared to 3 days in the absence of this growth factor as reported in our previous study (Fujihara et al., 2012). Despite this improvement, it still remains challenging to maintain dog follicle viability in vitro. It is known that communication between the gamete and surrounding somatic cells is crucial for follicle growth and viability.
We suspect that the high incidence of apoptosis in the dog is related to an inability of the current system to sustain somatic cell survival that, in turn, diminishes nutrient support to the primordial follicle and oocyte. Furthermore, an inherent high susceptibility to oxidative stress of dog ovarian tissues may play some roles in the intolerance to in vitro culture. For this reason, future studies in this species should examine the value of a bioreactor culture system (Heise et al., 2009) for in vitro follicle development. This approach, which involves a suspension system consisting of orbiting test tubes and rotating-wall vessels, enhances nutrient and oxygen transport from the microenvironment into the ovarian tissues and has improved growth and viability of rat preantral follicles (Heise et al., 2009). Compared to a conventional static system (similar to what is now used in the dog and cat), the bioreactor approach has increased oxygen and nutrient supplies to rat follicles and allowed resident oocytes to more readily achieve meiosis (Heise et al., 2009). Finally, strategies for reducing oxidative stress (e.g., culture in low oxygen tension or supplementing antioxidants) should also be considered.

We also have had opportunities to culture ovarian cortical pieces from three maned wolf (Chrysocyon brachyurus) using the static incubation system routinely used in the domestic dog. We determined that the dog microenvironment retained viability for ~75% of the maned wolf primordial follicles (based on calcein-AM and ethidium homodimer staining) at 3 days of culture. However, follicle viability decreased significantly to 10-20% at 9 days of culture (M. Fujihara, 2014, Smithsonian Conservation Biology Institute; unpublished observations). Addition of EGF to the culture medium enhanced follicle viability as some (15%) follicles remained viable after 15 days in vitro culture in the presence of this growth factor compared to complete follicle degeneration in its absence (M. Fujihara, unpublished observations).

Figure 1. Histomicrographs of (A) cat primordial follicles within ovarian tissue after 14 days in vitro culture and dog follicles after (B) 14, (C) 3 and (D) 7 days in vitro culture. Bars represent 10 µm.

Understanding mechanisms controlling follicle growth in the cat

During the past few years, we have begun to utilize in vitro ovarian tissues culture to advance our understanding about mechanisms regulating cat folliculogenesis. Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) are known to play key roles in the remodeling of extracellular matrix during ovarian folliculogenesis, especially during the final stage of follicle development (Raymond et al., 2003). Specifically, during follicle development, follicles move from the rigid cortex toward the medulla to complete growth in the peri-medullary zone of less stromal density. Moderating rigidity is important for initiating primordial follicle growth and allowing responsiveness to paracrine factors. We recently examined the influences of retinoic acid, a vitamin A metabolite that has been shown to influence tissue rigidity by up- or down-regulation of MMPs on in vitro viability and activation of primordial follicles in cat ovarian tissue. Preliminary results from this study revealed that the addition of 5 µM retinoic acid decreased the percentages of primordial follicles (40.5 ± 4.5%) within cultured ovarian cortices compared to the fresh (61.6 ± 5.3%) and no-
retinoic acid supplemented tissues (67.8 ± 4.0%) or tissues supplemented with 1 µM RA (59.7 ± 4.0 %) (Fujihara et al., 2014b). Percentages of primary follicles also were higher (P < 0.05) after treatment with 5 µM RA (51.7 ± 3.3%) compared to other groups (fresh, 34.0 ± 3.8%; control, 29.7 ± 3.6%; 1µM RA, 37.3 ± 3.8%). Furthermore, MMP9 expression level increased 9.1-fold (P < 0.05) in follicles incubated in 5 µM RA compared to fresh tissue, whereas MMP2 and MM7 mRNA were similar (P > 0.05) among groups. Thus, our findings indicated that stimulating MMP9 expression by the addition of RA may help modifying ECM rigidity that in turn stimulating follicle growth.

Recently, we also examined the influence of kit ligand (KL) on cat follicle development. Studies in the mouse have shown that KL and its receptor, c-kit stimulate PI3K-Akt signaling pathway that regulated activation of primordial follicles (Hutt et al., 2006). Supplementing KL (50 ng/ml) to a culture medium increases the transition from primordial to primary follicles in the rat ovary (Nilsson and Skinner, 2004). Similar to the previous report, our preliminary research showed that addition of 100 ng/ml KL to a culture medium increased the number and size of secondary stage follicles in cultured cat ovarian cortices (P. Thuwanut, 2014. Smithsonian Conservation Biology Institute; unpublished data). Currently, we are exploring the mechanisms by which KL regulating primordial activation in the domestic cat model.

### Conclusion and priorities

Given the challenge of recovering large numbers of mature, fertilizable oocytes from canids, it makes sense to explore recovering the oocyte by growing intraovarian follicles in the laboratory. The production of mouse pups from oocytes recovered from cultured primordial follicles provides incentive. However, clearly carnivore reproductive physiology, morphology and function as well as the specific mechanisms driving follicle and oocyte development are more complex than in rodents. Each species also seems to vary significantly in their adaptability to culture methods as well as what regulates folliculogenesis. Nonetheless, early data demonstrates the potential of sustaining both live dog and cat ovarian tissue at least for days if not weeks in vitro. However, before becoming a practical propagative and management tool, far more basic research is required. For the dog, near term priorities include identifying an in vitro microenvironment that sustains oocyte viability. For the cat, there is a need to focus on determining the explicit paracrine elements that promote expansion of the antral cavity and oocyte growth, especially to the size of eggs normally recovered from preovulatory stage follicles. Finally, both species could benefit from exploring the potential of a dynamic, bioreactor system that permits enhanced exchange of nutrients and gas as well as removal of metabolic wastes.

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