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Culture of domestic cat ovarian tissue *in vitro* and in the chick embryo chorioallantoic membrane

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ABSTRACT

In vitro culture and transplantation procedures are essential protocols employed in the evaluation of ovarian follicle survival and development. Culture in the chorioallantoic membrane (CAM) of chick embryos is an intermediate method that provides important follicle development information and has not been tested for cat ovaries to date. The aim of this study was to investigate if *in vitro* and CAM culture could be used as short-term systems to study cat ovarian tissue development. The ovaries of eight cats were dissected into 3-mm^3 cubes, cultured *in vitro* and in CAM for up to 5 days, and stained with hematoxylin-eosin and Gomori trichrome. Cell proliferation was analyzed using anti-Ki67. Possible differences among groups were investigated by analysis of variance or the Kruskal–Wallis test followed by Bonferroni correction. The T-test or Wilcoxon test was used to verify differences between the CAM and IVC. Results revealed that 87.5% of all follicles were primordial during culture. The percentage of primordial follicles in the morphologically normal follicles (MNF) pool was always higher than 80%, with the exception of Day 3 of CAM culture, but the number of MNF reduced significantly from Day 0 (600 out of 777 follicles) to Day 5 in the CAM (91 out of 171) and IVC (296 out of 686). The number of primordial follicles in 1 mm^3 in Days 2, 3, and 5 in the CAM was significantly lower than that in the control (Day 0). No cellular proliferation was observed in culture. Vascularization occurred in the CAM culture, but with no association to follicular viability. In addition, both methods showed an increase in connective tissue during culture. Although no significant differences were observed in the percentage of MNF, there was a reduction in the total number of follicles, both for IVC and CAM-cultured ovarian tissue. Furthermore, anti-Ki67 did not stain any follicle after Day 0 in IVC or in CAM culture. Neither system was capable of promoting follicle growth and/or development. The results show that the CAM is not a suitable system for feline ovarian tissue and highlight the necessity to improve IVC systems in cats.

1. Introduction

Assisted reproductive technology is considered a significant tool for the conservation of threatened animal species [1,2]. With the exception of domestic cats, all other

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felids are present on the International Union for the Conservation of Nature (IUCN) Red list of endangered species [3], primarily because of human impacts such as hunting, habitat destruction, and diseases [4]. Owing to phylogenetic proximity between species, the domestic cat is an excellent model for feline reproduction studies, contributing to the understanding of felid reproductive physiology despite considerable species differences [5]. Ovarian tissue utilization has been studied for several decades with the expectation of improving preantral follicle use.

Despite significant efforts, IVC systems are not yet fully developed, at least not for most animal species. There are various difficulties in the development of an IVC system that foster preantral follicle development, such as a naturally long growth period required for such structures to reach the antral follicle stage, a period involving several growth factors and hormones [6–8]. Ovarian tissue transplantation is probably the most effective tool to evaluate follicle survival and development as it is the form closest to the physiological condition. However, grafting involves the use of immunosuppressed or immunodeficient animals (xenografts), or the availability of the same animal from which the tissue was removed (autograft) [9,10]. In contrast, IVC is easily performed in the laboratory without host animals although it does not mimic the physiological environment of the tissue. Indeed, cells can only rely on the nutrients and growth factors available in the culture medium, with all necessary exchanges occurring by diffusion as there are no blood vessels [11].

Culture in the chorioallantoic membrane (CAM) of chick embryos can be considered as an intermediate method for tissue evaluation as it allows the re-establishment of blood supply and affords the grafted tissue access to growth factors available for chick embryo development. Despite being a less controlled system than IVC, it is not as invasive as transplantation and involves simpler implementation. Moreover, the lack of a functional immune system before Day 17 of chick embryo development prevents graft rejection [12].

Although there have been satisfactory results of ovarian tissue IVC in some species [11,13–18], only a few studies in cats have been reported [19–24] and the system is not yet fully developed. Culture in the CAM has been performed in mice [25], bovine [25,26], and human [11,13,27,28] ovarian tissue. In cats, it has been used as model for studying the growth of the feline vaccine–associated fibrosarcoma cell line [29], but it has not been tested for ovarian tissue development to date. Therefore, the aim of this study was to investigate if *in vitro* and CAM culture could be used as short-term systems to study cat ovarian tissue development.

2. Methods

2.1. Ovary collection

The ovaries of eight healthy 1- to 5-year-old adult queens in interestrus were collected at a veterinary clinic following elective ovariohysterectomies and transported to the laboratory in physiological solution (0.9% saline) at 36 °C within 1 hour. Adipose tissue and ligaments were removed. The ovaries were washed with a 70% ethanol and saline solution. Each pair of ovaries was dissected into 3 mm^3 cubes with 21 fragments used.

2.2. Experimental design

If not specified, the reagents were obtained from Sigma–Aldrich.

This study was conducted in strict accordance with the applicable national and international guidelines. The protocol was approved by the Animal Experiments Ethics Committee of the University of Brasilia (permit number 30115/2013).

The ovarian pieces of each animal were distributed into the following treatment groups:

(1) Control: One piece was immediately fixed in 4% paraformaldehyde for histologic and immunohistochemical analyses.

(2) *In vitro* culture (IVC): Ten pieces were *in vitro* cultured in an incubator (Te-399, Tecnal, Brazil) at 37.5 °C and 5% CO_2 (in air) in a 96-well plate. Each well contained 350 μL of M-199 medium supplemented with 0.23-mM sodium pyruvate, 2 mM of L-Glutamine, 10% fetal calf serum (Gibco, the Netherlands), insulin-transferrin-selenium (6.25 μg/mL, 6.25 μg/mL, and 6.25 ng/mL), 100-U/mL penicillin, 0.1-mg/mL streptomycin, and 2.3-μg/mL FSH (Folltropin V, Vetrepharm, Canada). Every other day, 175 μL of the medium was replaced with fresh medium.

(3) CAM-culture: Ten pieces were cultured in the CAM of chick embryos as described by Martinez–Madrid et al. [27]. In summary, fertilized eggs were incubated for 3 days at 37 °C in 60% relative air humidity and rotated hourly. On Day 3 of incubation, a rectangular window (1 × 1.5 cm) was made in the eggshell. Two milliliters of albumen were withdrawn using a 21-gauge needle through the large blunt end of the egg. The window was covered with a piece of tape to prevent dehydration, and the eggs replaced in the incubator without rotation. On Day 10 of incubation, a small area of the CAM was gently traumatized by laying a 1 cm^2 strip of sterile silk paper on the surface of the epithelium and removing it immediately. The ovarian tissue graft was subsequently placed onto the traumatized CAM with sterile forceps and the eggshell re-covered.

For both culture systems, ovarian grafts were cultured for up to 5 days, with two pieces removed each day and fixed in 4% paraformaldehyde for histologic and immunohistochemical analyses.

2.3. Histologic processing

All fixed grafts were dehydrated in ethanol, clarified in xylene, and embedded in Paraplast Plus, 4 μm-thick semi-serial cuttings were performed. Every fourth section was