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FSH stimulation of anestrous cats improves oocyte quality and development of parthenogenetic embryos

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ABSTRACT

In the domestic cat, the efficiency of *in vitro* embryo production systems is negatively affected during the nonbreeding season. The objective of this research was to evaluate the effect of FSH stimulation in anestrous cats, on quality of cumulus-oocyte complexes (COCs) and *in vitro* developmental competence after parthenogenetic activation. To accomplish this purpose, anestrous cats were grouped into: (1) FSH treated (serial doses of 5 mg of porcine FSH each, every 24 hours, for 4 days) and (2) untreated control. The COCs were classified morphologically and a proportion of grade I and II COCs was used for expression analysis of *FSHR*, *LHCGR*, *EGFR*, *PTGS2*, *EGR1*, *GDF9*, and *GATM* by RT-qPCR. In addition, another proportion of grade I and II COCs was matured *in vitro* and used for parthenogenetic activation. After 8 days in culture, blastocyst and hatching blastocyst rates were assessed, and the expression of *OCT4*, *SOX2*, *NANOG*, *CDX2*, and *GATA6* was evaluated. The COCs in the FSH group had an enhanced quality, a higher expression of *LHCGR* and a lower expression of *GATM* than did COCs from the control group ($P < 0.05$). Furthermore, embryos in the FSH group had increased blastocyst and hatching blastocyst rates, and those embryos had a higher expression of *OCT4* and *GATA* than their counterparts from the control group ($P < 0.05$). In conclusion, ovarian stimulation of anestrous cats with FSH improved quality and increased the expression of *LHCGR* in COCs. The enhanced *in vitro* developmental competence, after parthenogenetic activation of oocytes from FSH-treated cats, coincided with an increased expression of *OCT4* and *GATA6* in blastocysts and hatching blastocysts.

1. Introduction

The domestic cat (*Felis silvestris catus*) is a valuable model for developing assisted reproductive techniques that might be used for the conservation of endangered wild felids [1,2]. At present, 17 species of felids are classified as threatened and eight as near threatened according to the Red List of Threatened Species of the International Union for Conservation of Nature [3]. The IVM of oocytes and *in vitro* embryo production systems are useful tools for preserving the genetic pool of endangered wild felids [4,5]. However, a reported problem of the *in vitro* embryo production systems in the domestic cat is the discontinuous availability of competent oocytes due to seasonal effects [6–8]. The domestic cat is a seasonally polyestrous long-day species, which cycles continually during spring and summer (breeding season), whereas the anestrus occurs between autumn and winter (nonbreeding season) [9,10]. The efficiency of producing *in vitro*-matured oocytes and *in vitro* blastocysts decreases during the nonbreeding season in the domestic cat [6–8]. It has been discussed that a decrease in circulating FSH levels or fewer FSH receptors in the granulosa cells might negatively affect cat oocyte quality during the nonbreeding season [6,7].

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FSH stimulates proliferation, prevents atresia, and induces the synthesis of LH receptor and cytochrome P-450 aromatase in granulosa cells [11,12]. This assures the induction of oocyte maturation and the acquisition of competence to support embryo development after fertilization. Indeed, the exposure of cat oocytes to high concentrations of FSH during IVM is required to enhance maturation, fertilization, and *in vitro* development during the nonbreeding season [7]. On the other hand, an absence of seasonal changes in the relative expression of FSH receptor (*FSHR*) in immature COCs of domestic cats has been described [13]. Furthermore, the relative expression of FSH-induced genes such as prostaglandin-endoperoxide synthase 2 (*PTGS2*), early growth response protein-1 (*EGR1*), and epidermal growth factor receptor (*EGFR*) does not experience seasonal changes in mature COCs of domestic cats [13]. As such, the latter results were not consistent with the decrease in developmental capacity of cat oocytes after fertilization during the nonbreeding season [13].

To our knowledge, there are no previous reports about the effect of ovarian stimulation with FSH during the nonbreeding season on cat oocyte competence. During follicular development, the acquisition of oocyte competence requires a permanent and intimate relationship between the oocyte and follicular environment. Particularly, the presence of gonadotrophins and growth factor receptors in the cumulus cells is critically important during oocyte development and maturation inside the follicle [14]. For these reasons, we hypothesize that the ovarian stimulation of anestrous cats with FSH improves oocyte competence by changing gene expression pattern in cumulus cells. To test our hypothesis, two experimental groups were created: (1) anestrous cats treated with porcine FSH (pFSH) and (2) untreated anestrous cats (control). We first evaluated the morphological quality of immature COCs and the expression pattern of specific genes in these COCs: gonadotrophin receptors (*FSHR* and *LHCGR*), FSH-induced genes (*EGFR*, *PTGS2*, and *EGR1*), and genes related to oocyte competence (*GDF9* and *GATM*). In addition, we used parthenogenetic activation as a method to evaluate the *in vitro* developmental competence of *in vitro*-matured oocytes from both the groups. Finally, blastocysts and hatching blastocysts from each group were analyzed to compare the expression level of pluripotency markers *OCT4*, *SOX2*, and *NANOG* and differentiation markers *CDX2* and *GATA6*.

2. Materials and methods

All chemical reagents were purchased from Sigma Aldrich Chemicals Company (St. Louis, MO, USA), except for those otherwise indicated.

2.1. Animals

The animals used in this research were clinically healthy female cats weighting 2 to 4 kg and aged between 6 months and 5 years. All animals came from different owners but had similar housing conditions. The animals were kept in tempered rooms without being caged and had water and food *ad libitum*.

2.2. Ethics statement

All animal experiments were approved by the Ethics Committee of the Faculty of Veterinary Sciences, University of Concepcion. The use of animals for this research was permitted by previous consent of their owners, according to the requirements of the Ethics Committee. For surgical ovariohysterectomy, anesthesia was induced and maintained with a dose of 0.5 mg/Kg of xylazine (Xilacina 2%, Centrovet Chile) and 5 mg/Kg of ketamine (Ketamina 100, Chemie Chile).

2.3. Experimental design

In this research, two experimental groups were created. The first group consisted of 11 anestrous cats that were treated with four serial doses of 5-mg pFSH, every 24 hours (FSH group), whereas the second group included 13 anestrous cats that did not receive any treatment (control group). The ovaries were obtained by ovariohysterectomy, and in the case of FSH group, ovariohysterectomy was done 24 hours after the last dose. In both groups, each cat corresponded to an individual biological replicate, whereby the COCs recovered from each cat were classified and processed separately. The COCs recovered from each pair of ovaries were classified according to their morphologic characteristics as either grade I (excellent), II (good), III (fair), or IV (poor) quality. A proportion of grade I and II COCs were used for IVM. The remaining grade I and II COCs were used in RT-qPCR relative expression analysis. Genes studied were: *FSHR*, *LHCGR*, *EGFR*, *PTGS2*, *EGR1*, *GDF9*, and *GATM*. After IVM, only mature oocytes were used for parthenogenetic activation. The activated oocytes were cultured *in vitro* for 8 days and blastocyst and hatching blastocyst rates were visually assessed. A proportion of blastocysts and hatching blastocysts from each group was used for total cell counting. In addition, another proportion of blastocysts and hatching blastocysts were used for RT-qPCR relative expressions analysis of pluripotency markers *OCT4*, *SOX2*, and *NANOG* and differentiation markers *CDX2* and *GATA6*.

2.4. Animal selection and reproductive status diagnosis

Only healthy anestrous cats were used in this research. For this reason, the study was done from April to July, which corresponds to the anestrus season of domestic cats in the Central South region of Chile in the southern hemisphere (36°59′47.00″S). In the FSH group, only cats without estrus behavior [9] and anestrus as confirmed by vaginal cytology were selected for treatment. In the control group, anestrus was confirmed by visual inspection of the morphologic characteristics of the ovaries after ovariohysterectomy. Only cats with inactive ovaries (follicle $<$ 2-mm diameter and without luteal tissue) [8,15] were included in the study.

2.5. Cumulus-oocytes complexes collection and in vitro maturation

The ovaries were obtained by ovariohysterectomy. In the FSH group, the cats were treated with a subcutaneous