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Restoration of fresh cat ovarian tissue function by autografting to subcutaneous tissue: A pilot study

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ABSTRACT

Ovarian tissue transplantation could be a valuable technique for the preservation of endangered animals. The domestic cat affords an adequate experimental model for studies aimed at wild felids due to its phylogenetic similarity. Thus, this pilot study evaluated the efficacy of cat ovarian tissue autotransplantation to a peripheral site. Three adult queens were submitted to ovariohysterectomy. The ovaries were fragmented into eight pieces; two were fixed as a control and six were transplanted to subcutaneous tissue of the dorsal neck. Grafts were monitored weekly by ultrasound and fecal samples collected daily in order to monitor estradiol levels. Grafts were recovered on Days: 7, 14, 28, 49 and 63 post-transplantation for histological analyses. One graft was maintained in one animal for 8 months. A total of 2466 ovarian follicles were analyzed: 1406 primordial and 1060 growing follicles. All animals presented antral follicles in one or more of the grafts. The percentage of morphologically normal primordial follicles was always higher than 80%, except for Day 7 transplants. Although the proportion of growing follicles increased after transplantation, there was a general decrease in the percentage of morphologically normal growing follicles from Day 7 onwards. All animals demonstrated at least three estradiol peaks during the 63-day period, and one animal exhibited estrous behaviour on three occasions. Hormonal peaks directly correlated with the visualization of antral follicles (by ultrasound and/or histology) and the observation of estrous behaviour. Long-term results on one female showed the concentration of 37.8 pg/mL of serum estradiol on Day 233 post-grafting and the female exhibited estrous behaviour on several occasions. This graft showed one antral follicle, one luteinized follicle and two preantral follicles. In conclusion, cat ovary autotransplantation to the subcutaneous tissue restored ovarian function, with hormone production and antral follicle development, over both short and long term periods. This could be a valuable technique in the evaluation of ovarian cryopreservation methods in felids. Once the technique is shown successful, it may be applied in allografts or xenografts between different feline species.

1. Introduction

Ovarian tissue transplantation has been performed to study folliculogenesis and reproductive biology [1–4], restore fertility [5–7] and as a tool for evaluating ovarian tissue cryopreservation protocols [8–11]. Furthermore, ovarian tissue transplantation, whether associated with cryopreservation or not, could prove to be a valuable technique for the preservation of endangered animals.

Studies involving xenotransplantation of ovarian tissue from wild animals such as elephants [12], marsupials [13,14] and lions [15] to immunodeficient mice have demonstrated the promotion of follicle survival and development. Ovarian tissue from domestic animals such as sheep [9] and cats [10] has also restored its function under the same conditions. However, despite these positive results, embryo development following ovarian tissue xenotransplantation has only been reported after grafting to phylogenetically similar species, from mouse to rat [16], for example. This is probably due to certain species-specific particularities that limit the complete

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development of follicles in ovarian tissue xenotransplanted into phylogenetically distant species (e.g. estrous cycle length, hormone levels, etc.).

In cats, xenotransplantation of cryopreserved ovarian tissue to the immunodeficient mice kidney capsule enabled antral follicle development [10]. The best case scenario described in cats was germinal vesicle breakdown (GVBD) following *in vitro* maturation of oocytes aspirated from antral follicles developed in fresh ovarian tissue xenografted to immunodeficient mice [17]. In wild felids, lion ovarian cortexes have been xenotransplanted into immunodeficient mice, showing both follicle activation and development [15].

Autografting or allografting, on the other hand, have restored hormone secretion, ovulation and fertility in many species [4,18–24]. Live birth has been described after heterotopic allografting of ovarian tissue in mice [25]. Furthermore, autografting avoids grafted tissue rejection and, consequently, the need for immunosuppression in recipient animals. Recent studies have explored follicle development after ovarian tissue autotransplantation. More than 60 live births have been reported in women after autografting of cryopreserved ovarian tissue [5,26,27]. In sheep, the development of antral follicles occurred after heterotopic autotransplantation [28]. One sheep conceived after spontaneous intercourse and delivered a healthy lamb 545 days after orthotopic transplantation [29]. Antral follicle development was also described in dogs after heterotopic autotransplantation of ovarian tissue into the muscle fascia [2].

Most species from the Felidae family are in danger of extinction [30], with the domestic cat affording an adequate experimental model for studies aimed at wild felids due to its phylogenetic similarity [31]. However, there was only one study involving cat ovarian tissue autografting which demonstrated that although primordial and primary follicles survived 120 days of transplantation, they did not resume their growth [32]. Based on such limited data set results, the aim of this pilot study is to evaluate cat follicle development at different intervals following ovarian tissue autotransplantation. This technique, once proven successful, may be applied for allografting and xenografting in other felid species.

2. Materials and methods

2.1. Animals

This study was conducted in Brasília, Brazil (Latitude: −15.7801, Longitude: −47.9292 15° 46′ 48″ South, 47° 55′ 45″ West). Three healthy mixed breed adult cats (Felis catus), 1.5–3 years old and 2.5 to 4 Kg, were used in the experiment, each with a positive fertility history. The animals were vermifuged and verified as negative for feline immunodeficiency syndrome (FIV) and feline leukemia virus (FeLV) using a rapid test kit (Anigen Rapid FIV/FeLV Test Bioeasy, Alere, Ref. 34282). All of the test subjects were housed in individual cages (80 × 60 × 45 cm) with water and standard commercial cat food (Sabor & Vida, Guabi Pet Care, Brazil) *ad libitum*, allowed to adapt to the environment, and clinically observed for one month prior to the experiment. The cages were located in a cattery with a covered area and an open area, receiving natural daylight (11–13 h of sunlight). In tropical regions such as Brazil, where the seasons are not clearly defined, the cats may present as continuously polyestrous because in these places there is no significant change in day length throughout the year [33]. All procedures were approved by the Animal Ethics Committee of the Institute of Biological Sciences, University of Brasilia (protocol #76940/2012).

2.2. Ovarian tissue transplantation and recovery procedures

The queens were fasted for 12 h prior to the surgical procedure. The anaesthetic protocol used was based on those described by Cunha et al. [34] and Schiochet et al. [35]. Briefly, meperidine (Dolosal 50 mg/ml, Cristália, Brazil - 5 mg/kg) and acepromazine (Acepran 1%, Vetnil, Brazil - 0.2 mg/kg) were administered intramuscularly for analgesia and tranquilization. General anaesthesia was induced with intravenous injections of midazolam hydrochloride (Midazolam 1 mg/mL, Richmond VetPharma, Buenos Aires, Argentina - 0.5 mg/kg) and ketamine chloride (Cetamin 10%, Syntec, São Paulo, Brazil - 3 mg/kg). Anaesthesia was maintained by ventilation with isoflurane in pure oxygen.

Ovariohysterectomies were performed according to the method described by Fossum [36]. Both ovaries were harvested and cleaned with the removal of fat tissue and ligaments. Each ovary was dissected into four similar-sized pieces (10 × 3 × 3 mm), totaling eight pieces per animal. Two pieces were randomly chosen as controls and immediately fixed in Carnoy's solution (60% ethanol, 30% chloroform and 10% acetic acid). During the same surgical procedure, six small pouches (approximately 1 cm^3) were created in the subcutaneous tissue of the dorsal neck region by 1 cm incisions performed in the skin. After washing with 1% iodine and several times in sterile 0.9% saline solution, one piece of ovarian tissue was randomly placed inside each pouch and the skin incisions closed. Each queen received 5 mg/kg I.M. vitamin E (Monovin E – Bravet, Rio de Janeiro, Brazil). After surgery, each animal received an oral antibiotic (Enrofloxacin – Baytril 15 mg, Bayer, Brazil - 5 mg/kg) and an oral anti-inflammatory (Ketoprofen – Ketofen, Merial, Brazil - 2 mg/kg).

One ovarian graft was recovered on Days: 7, 14, 28 and 49 post-transplantation. The animals had all remaining grafts removed on Day 63, except for Animal 2, in which one fragment was maintained for long-term evaluations. All samples were fixed in Carnoy's solution and processed for histological analyses. The queens were sedated with intravenous ketamine (Cetamin 10%, Syntec, São Paulo, Brazil - 5 mg/kg) and xylazine (Calmium 2%, Agener União, Brasil - 0.5 mg/kg) during fragment extraction.

2.3. Graft evaluations

The animals were observed daily for any clinical signs and estrous behaviour. Relevant behavioural changes consisted of constant vocalizing, continuous rubbing of the head and neck against any object, rolling, lowering the forequarters, lordosis, and tail lateralization [37].

Grafts were monitored weekly by ultrasound evaluation (Mindray Digital Ultrasound Diagnostic Imaging System, model DP-6900Vet, B mode) in order to check their localization and evaluate the possible development of antral follicles.

2.4. Histological assessment

The ovarian tissue samples were dehydrated in ethanol, clarified in xylene and embedded in Paraplast Plus® (Sigma-Aldrich, Merck, Ref. P3683). The blocks were entirely sectioned (5 μm). All slides were stained with hematoxylin–eosin (HE) and every fourth section was evaluated under light microscopy. All follicles with visible oocyte nucleus were counted. Preantral follicles were classified according to their developmental stage as primordial or growing (primary and secondary) follicles [38]. Primordial follicles consist of an oocyte surrounded by a single layer of flattened granulosa cells, while growing follicles present one or more layers of cuboidal granulosa cells around the oocyte. Moreover, preantral follicles were classified as morphologically normal (MN) or atretic, based on the following criteria: integrity of the oocyte, presence or absence of pyknotic bodies, granulosa cells density and integrity of the basement membrane. Antral follicles were also counted and