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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

measured using ImageJ software. The smaller and the larger diameters of antral follicles were taken, using the basal membrane as the follicle limit, and the mean diameter was calculated.

2.5. Hormonal evaluation on fecal samples

Fecal samples were collected daily in plastic containers throughout the 63-day experiment, frozen and stored at −20 °C until assayed.

Fecal samples were dried using a SpeedVac Evaporator System (Forma Scientific Inc., Marietta, OH, USA), pulverized and fecal estrogen metabolites extracted with methanol using the protocol described by Grahamet al. [39]. A 1 mL aliquot of each fecal extract was dried and resuspended with assay buffer prior to analysis.

An enzyme immunoassay (EIA) protocol [40] was used to determine the concentration of fecal estrogen metabolites. The R0008 antibody (1:12000) for estradiol-17β was used with the respective HRP-conjugated hormone (1:120000, Clinical Endocrinology Laboratory, School of Veterinary Medicine, University of California, Davis, CA, USA). Both buffer and washing solutions were as previously described by Munroet al. [40], and the substrate solution as described by Grahamet al. [39]. Absorbance was read at 450 nm in a microplate reader. Antibody cross-reactivity was: 100% estradiol 17β, 0.73% estrone; while estrone sulfate, progesterone, testosterone, cortisol, and corticosterone were all <0.01%. The EIA sensitivity was 8.65 pg/well. The intra- and inter-assay coefficients of variation were less than 10.3% and the assay showed a parallel pattern between the serially-diluted pooled samples and the standard curve. Results were expressed as ng/g dry feces.

The EIA protocol used in this study was successfully used for nondomestic felids [41,42]. Additionally, it was validated biochemically for domestic cats by: (1) demonstrating parallelism between the serially-diluted pooled samples and the standard curve (Pearson's correlation coefficient, $r = 0.99$); (2) Addition of unlabeled estradiol standard to pooled fecal samples before extraction resulting in a significant hormonal recovery ($R2 = 0.9899$; $P > 0.01$).

Hormone data was evaluated separately for each animal. The results were analyzed in an iterative process of excluding values higher than the mean plus 2 standard deviations until basal values could be determined. Hormone values greater than the basal value were considered as peaks.

2.6. Long-term evaluations

One fragment of ovarian tissue was intentionally maintained in one animal (Animal 2) to examine the development of antral follicles and preantral follicle consumption several months after transplantation. The graft was recovered 233 days post-surgery, fixed and processed as previously described. Blood samples were taken at the time of removal. Estradiol and progesterone levels were determined in serum samples by Laborclin (SP – Brazil) using a chemiluminescence method (ADVIA – Centaur CP – Siemens).

2.7. Statistical analyses

The Chi-squared test was used to compare the percentages of follicles classified as primordial or growing, and as morphologically normal or degenerated, on different days after transplantation. Differences were considered as significant when $P < 0.05$.

3. Results

3.1. Ultrasound and behaviour evaluation

All animals were clinically healthy throughout the experiment. It was possible to manually identify the ovarian tissue grafts under the skin. Small hypoechoic circles were identified in eight of the grafts during the ultrasound evaluation, which is suggestive of antral follicles (Fig. 1). In addition, a dense capsule was observed around all fragments.

Only Animal 2 displayed estrous behaviour on Days 14, 47 and 63 post-transplantation, in the form of lowering the forequarters and lordosis. This behaviour was associated with the presence of hypoechoic circles in the graft ultrasound images of the same period, and subsequently with antral follicles observed in tissue histological analysis.

3.2. Histological evaluation of tissue grafts

All of the grafts were recovered. On histological evaluation, the grafts displayed a connective tissue capsule surrounding the ovarian tissue, with fatty tissue attached to the capsule. Blood vessels were observed through the capsule into the ovarian tissue.

A total of 2466 ovarian follicles were analyzed in control and transplanted pieces: 1406 primordial follicles and 1060 growing follicles. Prior to transplantation, the mean percentages of primordial and growing follicles were 90.5% and 9.5%, respectively (Table 1 – Control). Post-transplantation results demonstrated a significant decrease ($P < 0.05$) in the percentage of primordial follicles concomitant with a significant increase ($P < 0.05$) in the percentage of growing follicles (Table 1). The percentage of MN primordial follicles was always higher than 80%, except for Day 7, even though significantly lower rates ($P < 0.05$) were observed on Days 7, 14 and 63 post-transplantation in comparison with the control (Day 0). Although the percentage of growing follicles increased after transplantation, there was a significant ($P < 0.05$) decrease in the percentage of MN growing follicles from Day 7 to Day 49. Considering only MN follicles, primordial follicles always remained in the majority (84–98%).

On Days 7 and 14 post-transplantation, all animals presented numerous growing follicles with oocytes exhibiting an acidophilic cytoplasm and no nucleus (Fig. 2A). From Day 14, the most common degeneration observed was characterized as a follicle-like structure presenting juxtaposed cuboidal granulosa cells and no typical oocyte (Fig. 2B). In fact, these follicle-like structures comprised more than 80% of all of the degenerated follicles from Day 14 onwards which was significantly higher ($P < 0.05$) than on Days 0 (control) and 7 (Table 2). Other commonly observed signs of degeneration were oocytes with a pyknotic nucleus and detachment of follicles from the ovarian stroma.

Antral follicles were observed in all animals. Animal 1 showed one antral follicle (1.59 mm diameter) on Day 63 post-transplantation. Animal 2 showed one antral follicle (1.08 mm) on Day 28, a second (0.63 mm) on Day 49, and two others (1.3 and 0.8 mm) on Day 63 post-transplantation. Animal 3 exhibited luteinized follicles on Days 28 and 49 (2.12 mm and 0.73 mm diameter) post-transplantation and one antral follicle (1.1 mm) on Day 63 (Fig. 3).

3.3. Estradiol evaluation

Values higher than 292.0 ng/g dry feces for Animal 1, 235.0 ng/g dry feces for Animal 2 and 280.0 ng/g dry feces for Animal 3 were considered hormonal peaks. Animal 1 demonstrated three hormonal peaks on Days 20, 32 and 60 post-transplantation; Animal 2

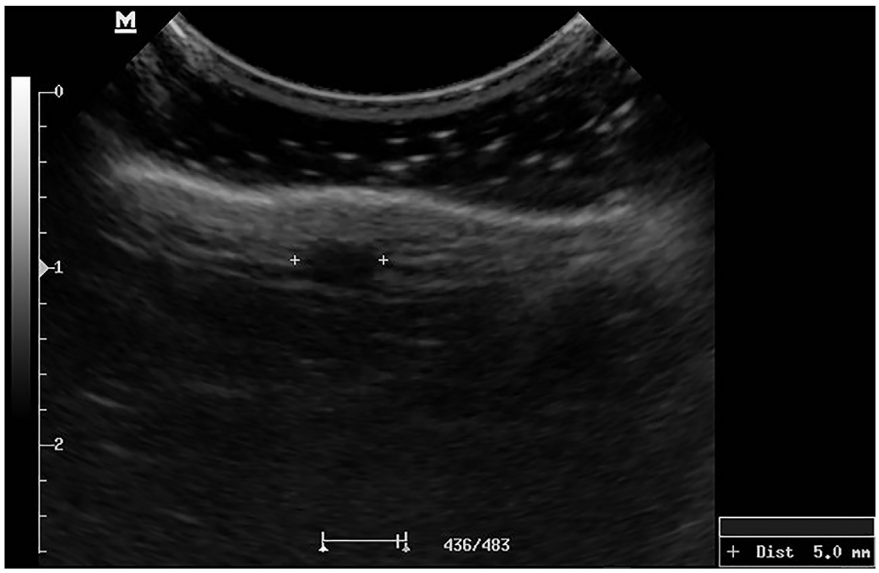

Fig. 1. Graft ultrasound image. Hypoechoic circle suggestive of an antral follicle 56 after transplantation.

showed peaks on Days 12, 50, 51 and 61 post-transplantation; and Animal 3 showed several peaks, with the highest on Day 60 (Fig. 4). The hormonal peaks were closely associated with the ultrasound observations of antral follicles, estrous behaviour observation and/or histological observation of antral follicles (Fig. 5).

3.4. Long-term results

During the 233-day post-transplantation period, Animal 2 demonstrated estrous behaviour on several occasions in the form of frequent vocalization and elevation of the hindquarters and tail. Day 233 post-grafting estradiol and progesterone serum concentrations were 37.8 pg/mL and 7.67 ng/mL, respectively. Histological evaluation of the graft showed one antral follicle (2.39 mm), one luteinized follicle and two preantral follicles (Fig. 6).

4. Discussion

This study shows, for the first time, that autografting of cat ovarian tissue to a peripheral site results in the resumption of ovarian function, together with the development of antral follicles, estradiol production and manifestation of estrous behaviour. The subcutaneous dorsal neck region proved to be a suitable site for grafting in cats, with revascularization of the tissue and ready access to the graft site, thereby facilitating the recovery of all grafts. In murine, subcutaneous tissue had the lowest rates of follicular development, and the kidney capsule is the most widely used site for ovarian tissue transplantation [43]. In humans, heterotopic transplantation has restored ovarian function, but the most promising option for follicular development is orthotopic transplantation which has resulted in more than 80 live births to date [44]. Grafting to peripheral sites is advantageous with regards to accessibility by local anaesthesia and facilitates aspiration for IVF [1]. Aubardet al. [28] autografted ovarian tissue to subcutaneous tissue of sheep abdomen and concluded that the technique was efficient in the restoration of ovarian activity. In cats, however, the abdominal wall subcutaneous tissue was inefficient because most grafts were non-recoverable [32]. According to the authors, this may have been caused by graft resorption or fat deposition at the grafting site, thereby making it difficult to localize the grafts. The dorsal region of the neck, used in this study as a grafting site, is not a common place for fat deposition in cats and is also difficult for the cat to scratch, which could eventually damage the grafted site. The fragments were involved in a dense capsule at the time of removal, probably because of an inflammatory response in the site of grafting [45].

Concerning the total number of follicles on different days after grafting (Table 1), it is important to remember that the data for each day is obtained from different grafts, and that a variation on the number of follicles between fragments of ovarian tissue is expected [46]. For this reason, we used the percentage of follicles on each day of grafting to compare the results. The proportion of growing

Table 1
Total number of follicles found in all three queens, mean percentage (±SD) of primordial and growing follicles, and morphologically normal (MN) primordial and growing follicles in cat ovarian tissue on the control (Day 0) and on Days 7, 14, 28, 49 and 63 post-transplantation.

Day	Total	Primordial follicles				Growing follicles			
	follicles	N	%	n MN	% MN	N	%	n MN	% MN
Control	472	426	90.5 ± 5.2^{a}	416	97.7 ± 0.7^{a}	46	9.5 ± 5.2^{a}	39	88.6 ± 9.9^{a}
Day 7	386	224	57.8 ± 16.6^{b}	164	68.7 ± 28.8^{b}	162	42.2 ± 16.6^{b}	9	7.2 ± 8.7^{bc}
Day 14	387	216	55.9 ± 1.4^{bd}	175	81.7 ± 24.6^{b}	171	44.1 ± 1.4^{bd}	4	2.3 ± 1.4^{b}
Day 28	342	165	48.6 ± 12.7^{c}	164	99.5 ± 0.7^{a}	177	51.4 ± 12.7^{c}	10	6.4 ± 5.1^{bc}
Day 49	296	143	50.5 ± 33.0^{cd}	142	99.5 ± 0.7^{a}	153	49.5 ± 33.0^{cd}	13	15.6 ± 18.4^{c}
Day 63	583	232	38.3 ± 32.1^{e}	205	90.7 ± 11.3^{c}	351	61.7 ± 32.1^{e}	21	8.0 ± 6.8^{bc}

N: total number; MN: morphologically normal.
a,b,c,d,e: Values with different letters in the same column are significantly different ($P < 0.05$).

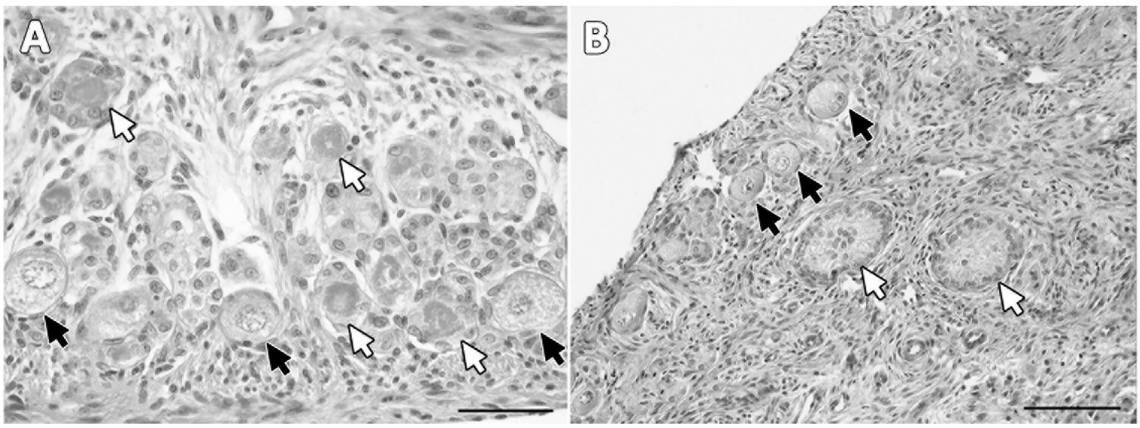

Fig. 2. Different degeneration signs observed in post-transplantation ovarian tissue. (A) Oocyte with acidophilic cytoplasm and absence of nucleus (white arrows), and morphologically normal follicles (black arrows) 7 days post-transplantation. Bar = 50 μm. (B) Follicle-like structure presenting juxtaposed cuboidal granulosa cells and no typical oocyte follicles (white arrows), and morphologically normal follicles (black arrows) 14 days post-transplantation. Bar = 100 μm.

follicles in relation to primordial follicles increased after grafting. However, there was a notable reduction in MN growing follicles from Day 7 onwards. In contrast, the percentage of MN primordial follicles continued to be high (over 80%), except on Day 7 (68%). These results suggest that there was primordial follicle activation, followed by degeneration of the newly growing follicles. This is probably due to the fact that growing follicles are less resistant to ischemia and poor nutritional conditions than primordial follicles [47]. In fact, it is well known that growing follicles are very susceptible to hypoxic conditions before reoxygenation of the transplanted tissue [48], which is established after 5 days in human ovarian tissue transplanted to nude mice [49]. Despite the revascularization, shown in the present experiment by the development of antral follicles, a high proportion of degenerated growing follicles were observed in longer periods of grafting. This may be a consequence of the death of existing growing follicles in the first days post-transplantation.

The most common degeneration observed was a follicle-like structure presenting juxtaposed cuboidal granulosa cells and no typical oocyte, especially from Day 14 onwards. These structures were also observed by Paynteret al. [50] after cryopreservation and *in vitro* culture of bovine ovarian tissue. Many authors have reported that oocyte degeneration is the most frequent sign of atresia in preantral follicles [51–54]. They report complete disappearance of the oocyte in preantral follicles while granulosa cells appear normal and continue to proliferate [53,54], showing that the oocyte is considerably more sensitive to adverse conditions than granulosa cells.

Table 2
Mean percentage (±SD) of follicle-like structures (FLS) with juxtaposed granulosa cells and no oocyte in relation to the degenerated follicles found (FLS/Degenerated Follicles) and to the total number of follicles counted (FLS/Total Follicles) in the four animals.

Day	% FLS/Degenerated Follicles		% FLS/Total Follicles	
Control	0 ± 0a	(0/17)	0 ± 0a	(0/472)
Day 7	13.0 ± 5.8^{a}	(31/213)	8.1 ± 7.2^{b}	(31/386)
Day 14	82.7 ± 22.7^{b}	(164/208)	42.4 ± 0.3^{c}	(164/387)
Day 28	99.2 ± 1.1^{b}	(167/168)	48.5 ± 14.5^{cd}	(167/342)
Day 49	98.1 ± 2.7^{b}	(140/141)	44.8 ± 37.0^{cd}	(140/296)
Day 63	89.2 ± 9.5^{b}	(325/357)	194.3 ± 33.5^{d}	(325/583)

FLS: follicle-like structures.
a,b,c,d: Values with different letters in the same column are significantly different ($P < 0.05$).

Although most growing follicles were degenerated, there was development of antral follicles in multiple grafts. The time necessary for follicle development in cats is not known, however, Songsasenet al. [55] described the presence of an antral cavity in 50% of preantral follicles cultured *in vitro* for 14 days. The timing of antral follicle observation in the present study (after 28 days of grafting) suggests that they were derived from primordial follicles activated post-transplantation, in addition to the other morphologically normal growing follicles observed. The antral follicles observed in the present study presented diameters smaller than natural preovulatory follicles in cyclic queens (reported as 3.6 mm on average, varying from 3.0 to 4.1 mm [56]), and this may be due to the ectopic site of the grafts.

Luteinized follicles were identified in the present study. These structures have been described in bovine ovaries after superovulation protocols [57]. According to the authors, a luteinized follicle is differentiated from a *corpus luteum* by the presence of an oocyte among the luteinic cells. From a physiological perspective, these structures may surge after spontaneous LH release at inappropriate moments, and were described after gonadotropin administration in goats [58]. In the present study, Animal 2 probably had an LH peak induced by increased estradiol secretion. Although the preovulatory LH surge in domestic cats usually occurs after the mechanical stimulus of coitus [59], there are reports of spontaneous ovulation in cats [60]. As the grafts were located at an ectopic site, it would be impossible for actual ovulation to occur, even in fully mature preovulatory follicles, so an LH peak led to the development of a luteinized follicle instead of a corpus luteum.

The use of hormonal assay to quantify estrogen secretion is a well-established technique to evaluate transplant efficacy. Despite estradiol secretion levels remaining at low concentrations over the first few days post-transplantation, several peaks were subsequently evidenced. Similar results were reported by Khoramet al. [61] following ovarian tissue autotransplantation in dogs. The noninvasive method to measure estradiol metabolites in feces proved effective in determining ovarian activity after transplantation in domestic cats, thus preventing the stressful alternative of daily vein puncture. Estradiol values observed in this study were similar to those reported for non-castrated female cats [62,63]. Estradiol peaks were observed on numerous occasions, which were concomitant to: estrous behaviour, visualization of antral follicles in histological section, and hypoechoic circles suggestive of antral follicles observed in ultrasound analysis. Only one animal presented estrous behaviour, with an interestrous interval

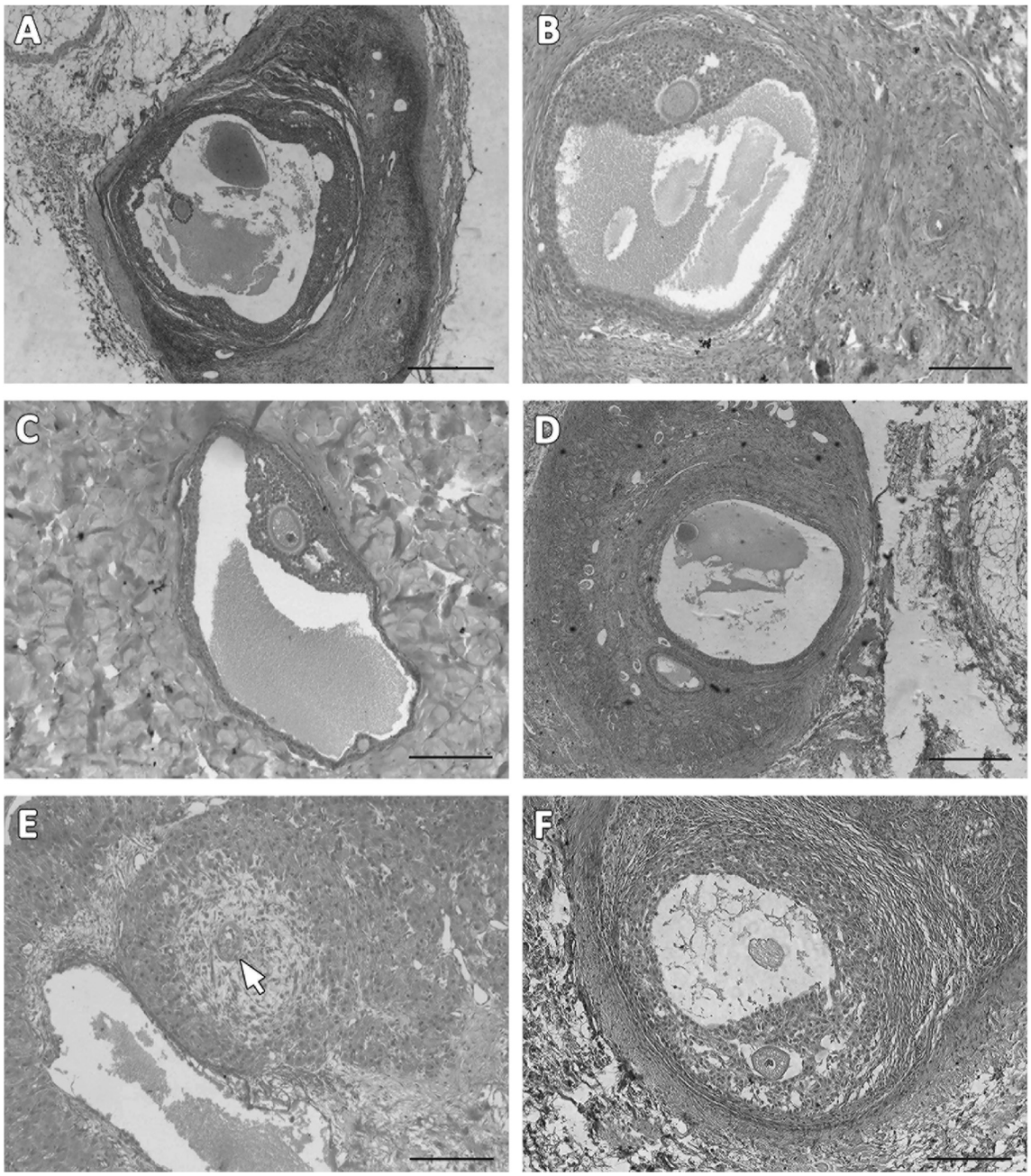

Fig. 3. Follicle structures found in ovarian tissue after transplantation. (A) Antral follicle in the Animal 1 graft on Day 63 post-transplantation. Bar = 500 μm. (B–D) Antral follicles in the graft from Animal 2 on Days 28 (B - Bar = 200 μm), 49 (C - Bar = 200 μm) and 63 (D - Bar = 500 μm) post-transplantation. (E–F) Follicles in grafts from Animal 3: (E) Luteinized follicle showing oocyte (white arrow) on Day 49 post-transplantation. Bar = 200 μm; (F) Antral follicle on Day 63 post-transplantation. Bar = 200 μm.

of 2–3 weeks, which is in accordance with the physiological interestrous interval, that may vary from 2 to 19 days [64]. It might be intriguing that sometimes the results of US examination, estradiol detection and histology did not coincide. However, it is noteworthy that US examination was performed weekly, fecal estradiol detection was performed daily and histology only on predetermined days. When follicles were seen by US but no estradiol was detected, it is important to keep in mind that the hormones detected on the feces are estradiol metabolites, and there is a delay of 1–3 days for them to be detected in the feces of domestic cats [59]. When estradiol was detected but antral follicles were not seen at the histology, it is relevant to remember that only one graft was removed in each day, so the estradiol production may be coming from a graft other than the one removed.

Because luteinized follicles were observed in animal 3 (on Days 28 and 49) progesterone was also measured in fecal samples (data not shown). All animals showed low fecal progesterone metabolite levels during all experimental period (0.16 ± 0.10 μg/g dry feces, range 0.02–0.89 μg/g dry feces). These results are close to basal levels in non-castrated female cats and to those reported for ovarectomized cats [62,65–67]. However, it is important to note that luteinized follicles do not always produce high levels of progesterone [68,69], which justifies our findings.

In long-term evaluations, we observed that ovarian activity

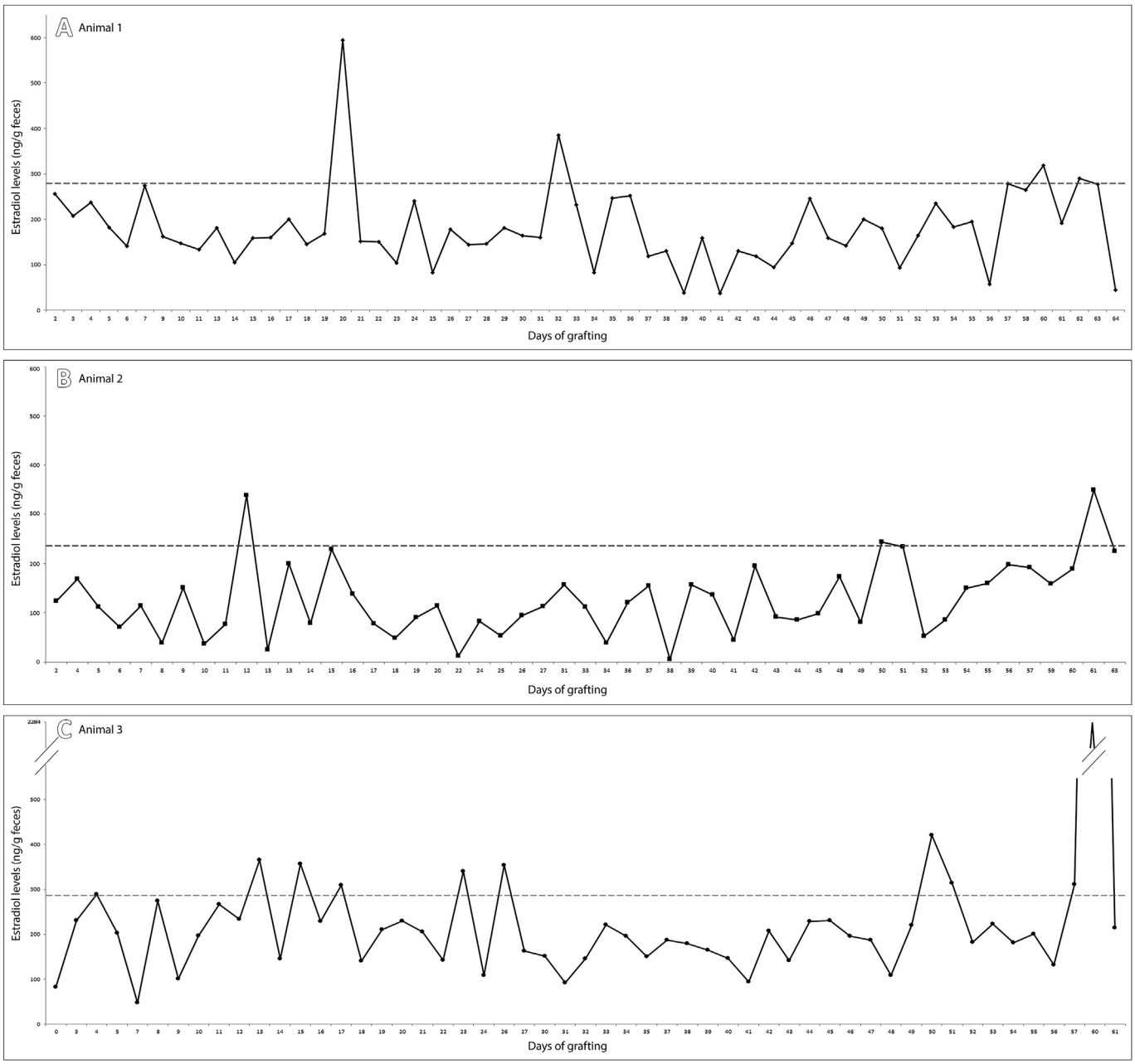

Fig. 4. Estrogen metabolite concentrations in the feces of animals 1 (A), 2 (B) and 3 (C) over the 63-day post-transplantation period. Dashed lines indicate the basal level for each animal. Values above the basal level are hormonal peaks.

remained until at least 8 months post-transplantation. Cat ovarian grafts also survived for long durations (up to 9 months) when xenotransplanted into the renal capsule of SCID mice, with small antral follicles and a few scattered primordial follicles in an abundant stromal tissue [9], similar to our findings. A plasma estradiol concentration of 37.8 pg/mL, compatible with the follicular phase in cats [59], was observed concomitant to estrous behaviour and antral follicle presence in histological sections of the graft on Day 233 post-transplantation. The serum levels of progesterone observed in this case corroborate with progesterone levels during luteal phase in domestic cats (>1.0 ng/mL) [70,71] indicating steroidogenic activity of the luteinic cells.

This was a pilot study conducted to test the viability of ovarian tissue autotransplantation to a peripheral site in domestic cats. Although it was a relatively small experiment, this type of study is important to gather information prior to conducting a more complex experiment as it enables protocol optimisation before investing time and/or significant financial resources in a large-scale study [72]. Ovarian tissue transplantation has been used to evaluate cryopreservation, and has restored ovarian function and follicle development in cows [73], goats [74] and rabbits [75]. Embryo development from cryopreserved ovarian tissue has been reported after grafting in rabbits [76] and sheep [28] and live birth in Rhesus monkeys [77], humans [5,26,27] and mice [78]. Our results showed that fresh feline ovarian tissue autotransplantation supports follicle development. Therefore, it could prove to be a valuable technique in the evaluation of ovarian tissue cryopreservation in this species.

In conclusion, the autotransplantation of fresh cat ovarian tissue

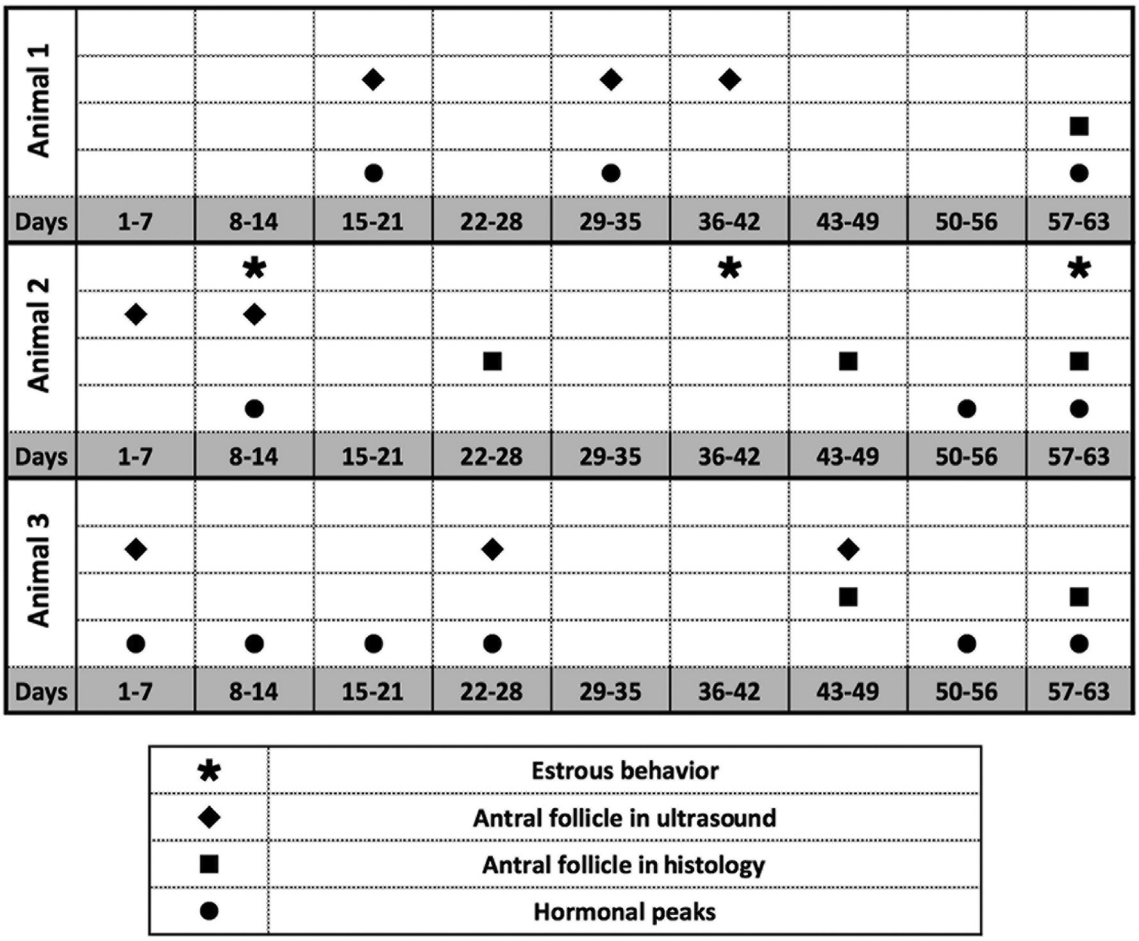

Fig. 5. Parameters observed over the 9-week experiment. Fecal estrogen metabolite peaks (●), observation of antral follicles in ultrasonography (◆) and histology (■), and estrous behaviour (✱) observed during the 9- week post-autografting period.

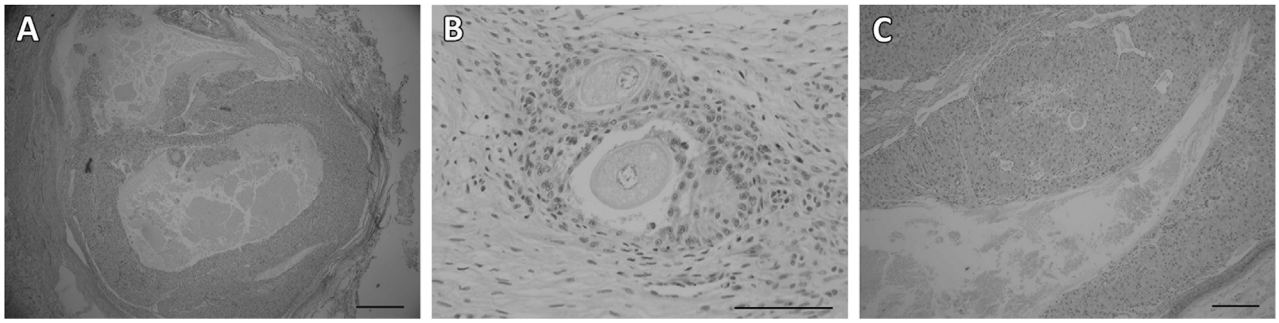

Fig. 6. Photomicrographs of the follicles developed after 233 days of grafting in Animal 2. Antral (A - Bar = 500 μm), preantral (B - Bar = 100 μm) and luteinized (C - Bar = 200 μm) follicles.

to subcutaneous tissue restored ovarian function and estrous cyclicity, with hormone production and antral follicle development, over both short and long terms. Although follicle development occurred, further evaluation of the oocytes in the growing follicles is necessary to confirm their viability. Due to the phylogenetic proximity of the domestic cat to wild felids, this technique could offer a more suitable alternative for wild felid ovary xenotransplantation experiments than laboratory rodents. Further studies evaluating the cryopreservation and harvesting of oocytes from transplanted ovarian tissue are necessary to contribute to advancing assisted reproduction technology for endangered species. After achieving success in the technique, it may be performed in allografts or xenografts between different feline species.

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