STUDIES OF AUTOGRAFT OF CANINE OVARIES TO VARIOUS BODY SITES

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STUDIES OF AUTOGRAFT OF CANINE OVARIES TO VARIOUS BODY SITES

A Thesis

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ABSTRACT

Ovarian grafting may provide important insights into follicular development and oocyte maturation. Several reports have described the heterotopic transplantation of ovarian tissue but have not clearly identified the site that yields the best results after transplantation. To our knowledge, the transplantation of canine ovarian tissue under the skin of a bitch has never been reported. We investigated the influence of the graft site on the survival of canine follicles and oocytes and examined follicular development on the surface of canine ovarian tissue after autografting under muscle fascia and meiotic resumption of follicle-derived oocytes after the maturation culture period.

The first series of experiments was conducted to investigate the influence of the graft site on the survival of canine follicles and oocytes after autografting. Hemi-ovaries were grafted to 3 locations (quadriceps femoris muscle fascia; kidney capsule; and gastrosplenic ligament, the portion of dorsal mesentery between the spleen and the stomach) and maintained for 28–31 days, after which they were recovered from the animals under anesthesia. The autografted hemi-ovaries were bisected: 1/4 of the bisected portion of the ovary was used for histological assessment and the other, for evaluating oocyte viability. The remaining portions of the fresh hemi-ovaries were used as controls to assess the viability of follicles and oocytes in the non-transplanted ovaries. Most follicles in the histological sections of the grafts were classified as primordial or primary. Antral follicles were not observed in the grafts, regardless of the graft site. The percentages of viable follicles from the control ovaries and those grafted to the kidney capsule, quadriceps femoris muscle fascia, and gastrosplenic ligament were 17.4%, 22.9%, 18.3%, and 32.4%, respectively. Twelve oocytes were recovered from the 15

hemi-ovaries grafted in 5 bitches, of which 5 (41.7%) oocytes from autografts to the quadriceps femoris muscle fascia and the kidney capsule were cultured for the assessment of meiotic competence. Three oocytes were viable but remained in the germinal vesicle stage after a 72-h period of maturation culture. Results indicate that the near-surface quadriceps femoris muscle fascia and kidney capsule might be useful grafting sites, but follicle survival and meiotic competence of oocytes in the grafts must be improved.

The second series of experiments evaluated the follicular development on the surface of canine ovarian tissue after transplanting under the fascia near the surface of the thoracolumbar muscle and meiotic resumption of follicle-derived oocytes after the maturation culture period. After ovarian excision from a bitch, each ovary was cut approximately in half. The hemi-ovaries were transplanted into the original bitch at 3 different body sites (under the fascia of the quadriceps femoris muscle, in the thoracolumbar muscles, and in the deltoid muscle of the scapular region). All autografts were recovered from the bitch at 35 days after transplantation. A visible antral follicle was observed on the surface of the ovary grafted under the thoracolumbar fascia. Histological examination revealed viable follicles at different stages of development, regardless of graft site. Most granulosa cells in the follicles at different stages of development expressed proliferating cell nuclear antigen (PCNA). Three oocytes were collected from an ovary grafted under the fascia of the thoracolumbar muscle; 1 oocyte reached metaphase I after the maturation culture period. This report is the first of its kind to demonstrate follicular development and meiotic resumption of oocytes recovered from autografted canine ovarian tissues.

Our results indicate that the peripheral fascia and intramuscular sites may be suitable for grafting to monitor ovarian function and recover oocytes from the grafts. The advantages of grafting to the peripheral sites include grafting under local anesthesia, easy monitoring by ultrasonography, and easy access for graft or oocyte recovery. The use of both grafting and cryopreservation may prolong the lifespan of the female reproductive system.

GENERAL INTRODUCTION

Because of the particular physiology of the reproductive system in bitches, the development of reproduction technologies for dogs has lagged behind that of other species. It is known that bitches have a unique reproductive biology. For instance, the bitch is monoestrous, with a 2-month luteal phase and a prolonged but variable non-seasonal anestrus of 3–10 months at the end of both pregnant and non-pregnant cycles. Canine oocytes at ovulation are at the germinal vesicle stage and require at least 48 h to complete meiosis (Holst and Phemister, 1971; Tsutsui, 1989), a period considerably longer than the 18 h and 24 h required for murine and bovine oocytes, respectively (Randall *et al.*, 1990; Arlotto *et al.*, 1996). Before ovulation, basal levels of serum progesterone begin to rise as luteinizing hormone (LH) levels rise. Ovulation occurs approximately 2–3 days after the surge in LH (Wildt *et al.*, 1979). Information is available regarding the timing of ovulation relative to endocrine gland function but is limited regarding follicular growth during anestrus and proestrus. Although the development of canine ovarian follicles can be observed by real-time ultrasonography (Boyd *et al.*, 1993; England *et al.*, 1993), no established method to accurately determine or predict ovulation is currently available. Moreover, imaging is technically difficult because of the location and small size of the ovaries. The meiotic competence of oocytes collected from ovaries of bitches at estrus has been demonstrated to be significantly higher than that of oocytes during the anestrus and diestrus stages (Yamada *et al.*, 1993; Hewitt and England, 1997; Otoi *et al.*, 2001); however, the stages of ovaries obtained by ovariohysterectomy in veterinary clinics are mainly anestrus or diestrus, in which follicles are not visible on the surface of the ovary. An effective

procedure for in vitro maturation (IVM) of oocytes from the anestrous and diestrous stages is, therefore, needed for efficient in vitro embryo production.

For many species of mammals, including dogs, follicular dynamics such as the interval required for follicle growth and the hormonal responsiveness of ovarian follicles remain unclear. In vitro study of follicular dynamics of primordial and primary follicular stages is limited because in vitro culture systems for these follicles are lacking, both in domestic animals and in humans. Several studies suggest that follicles in the ovarian tissue can survive ovarian grafting, and they grow from early to advanced stages of development (Oktay *et al.*, 1998; Gosden *et al.*, 1994; Gunasena *et al.*, 1998; Weissman *et al.*, 1999); therefore, ovarian grafting can provide important insights into follicular development and oocyte maturation. Several grafting techniques have been reported, differing only in the location of the autografts, such as the bursal cavity, the kidney capsule, and subcutaneous sites. Subcutaneous ovarian grafting offers several advantages over intra-abdominal grafting (Oktay and Yih, 2002): the subcutaneous site is more easily accessible, and monitoring follicle growth is relatively simple because antral follicles in the graft can be studied by ultrasonography. In addition, subcutaneous grafting allows for antral follicle development (Weissman *et al.*, 1999); however, the kidney capsule and, to a lesser degree, the bursal cavity, became popular as transplantation sites because of the assumption that the ample blood supply in these locations favored revascularization and subsequently, graft survival (Gosden *et al.*, 1994). This is also the reason that recent research focuses on intramuscular grafting together with incisions in the transplanted ovaries to improve blood supply (Eimani *et al.*, 2009). Other authors believe that the transplantation site with respect to graft survival is not critical, as even cortical strips implanted subcutaneously achieve a rapid neovascularization (Weissman *et al.*, 1999). Yang *et al.* (2006) reported that the

transplantation site can have a direct influence on the number and quality of oocytes that can be harvested from grafted ovarian tissue.

Ovarian grafting to subcutaneous sites provides both a model to study canine ovarian function (Pris *et al.*, 2004) and ready access to the graft for oocyte collection (Oktay *et al.*, 2004; Paris *et al.*, 2004). The low meiotic competence of canine oocytes may be improved if oocytes can be collected from the antral follicles of the ovarian grafts for in vitro fertilization (IVF) (Farstad, 2000). It is difficult to prevent long-term rejection of ovarian allografts in bitches that are monoestrous with a 2-month luteal phase and have a prolonged non-seasonal anestrus of 3–10 months; therefore, autografts appear to be the most promising approach for studying follicular development. An important factor to consider in tissue survival and follicular development after transplantation is the graft site (Soleimani *et al.*, 2008). Hypoxic conditions could be the primary reason for initial follicle loss after transplantation (Aubard *et al.*, 1999); therefore, rapid revascularization after grafting is considered essential for maximum follicle survival. Because of the assumption that adequate blood supply favors revascularization and subsequent graft survival, the kidney capsule became popular as a transplantation site (Gosden *et al.*, 1994). Although several reports have described the transplantation of ovarian tissues to various heterotopic body locations (Gosden *et al.*, 1994; Weissman *et al.*, 1999; Oktay *et al.*, 2001; Schmidt *et al.*, 2005), the site that yields the best results after transplantation has not yet been clearly identified. In addition, subcutaneous transplantation of canine ovarian tissue has never been studied in bitches. Metcalfe *et al.* (2001) transplanted canine ovarian tissue to the kidney capsules of immunodeficient mice, but follicular development to the antral stage could not be confirmed.

In this study, we investigated whether the number of viable follicles after 1 month of ovarian tissue transplantation is influenced by the graft site. We also examined the quality and meiotic competence of the oocytes recovered from the ovarian tissues grafted to the quadriceps femoris muscle fascia, kidney capsule, and gastrosplenic ligament (the portion of dorsal mesentery between the spleen and the stomach) in dogs.

In the second study that involved ovarian grafting to subcutaneous sites, we identified follicular development on the surface of canine ovarian tissue 35 days after transplanting under the fascia of the left thoracolumbar muscle. We confirmed meiotic resumption of the follicle-derived oocyte after the maturation culture period.

Chapter 1

ASSESMENT OF CANINE OVARIES AUTOGRAFTED TO VARUOUS BODY SITES

ABSTRACT

The influence of graft site on the survival of canine follicles and oocytes after autografting was investigated. Hemi-ovaries were autografted to three locations (quadriceps femoris muscle fascia, kidney capsule, and gastrosplenic ligament), and grafted ovaries were recovered (under anesthesia) at 28-31 d after transplantation. The grafted hemi-ovaries were bisected: one-quarter ovary was used for histological assessment and another quarter for evaluation of oocyte viability. As controls, the remaining fresh hemi-ovaries were used to assess the viability of follicles and oocytes in non-transplanted ovaries. Most follicles in the histological sections of the grafts were classified as primordial or primary follicles. Antral follicles were not observed in the grafts, irrespective of the graft site. The percentages of viable follicles in the sections from control ovaries, and the ovaries grafted to the kidney capsule, the quadriceps femoris muscle fascia, and the gastrosplenic ligament were 17.4%, 22.9%, 18.3, and 32.4%, respectively. A total of 12 oocytes was recovered from the 15 hemi-ovaries grafted in five bitches, of which five (41.7%) oocytes from the ovaries grafted to the quadriceps femoris muscle fascia and the kidney capsule, were cultured for assessment of meiotic competence. Three oocytes were viable but remained in the germinal vesicle stage after 72 h of maturation culture. The quadriceps femoris muscle fascia might be useful for grafting like the kidney capsule, but improvement of follicle survival and meiotic competence of oocytes in the grafts is necessary.

INTRODUCTION

Several studies have reported that follicles in the ovarian tissue are able to survive ovarian grafting, and that they grow from early to advanced stages of follicular development (Oktay *et al.*, 1998; Gosden *et al.*, 1994; Gunasena *et al.*, 1998; Wissman *et al.*, 1999). Ovarian grafting should provide important insights into follicular development and oocyte maturation. For many mammalian species, including dogs, follicular dynamics such as the interval required for follicle growth and the hormonal responsiveness of ovarian follicles have not been elucidated. In bitches, because of the particular physiology of the female gamete, development of reproduction technologies has lagged behind that of other species. Most reports describe the maturation rates of canine oocytes cultured in vitro as extremely low (<20%) (Farstad, 2000), which is a major obstacle to the in vitro production of canine embryos. In fact, most follicles in the canine ovary remain below the ovarian surface, and they cannot be aspirated during the anestrous and diestrous stages of the estrous cycle. Therefore, a possible reason for the low maturation rate of oocytes is the use of oocytes with a small diameter (<110 μm), which were obtained from deeper cortical follicles (Otoi *et al.*, 2000). Ovarian grafts under the skin facilitate access to the graft for oocyte collection (Oktay and Yie, 2002; Oktay *et al.*, 2003), and completion of the meiotic maturation of oocytes may be increased if oocytes were collected from the antral follicles of the ovarian grafts for in vitro fertilization. Information about the timing of ovulation relative to endocrine gland function is available, but there is limited information about follicular growth during anestrus and proestrus. Although it is possible to observe the development of canine ovarian follicles by real-time ultrasonography (Boyd et al., 1993; England and Yeager, 1993), a method to accurately determine or predict ovulation has not been established.

Moreover, the location and small size of the ovaries make imaging technically difficult. Ovarian grafts under the skin facilitate monitoring of follicle growth (Oktay *et al.*, 2004). Ovarian grafting to the subcutaneous sites provides a model to study canine ovarian function (Paris *et al.*, 2004); however, no studies have been performed in bitches on the under-skin transplantation of canine ovarian tissue. Allografts and xenografts can survive indefinitely in immunodeficient or immunosuppressed host animals (Paris *et al.*, 2004; Soleimani *et al.*, 2008). Canine pre-pubertal ovarian allografts transplanted into the bursa of the recipient with immunosuppressive therapy have resulted in pregnancy (Pullium *et al.*, 2008); however, this implies that immunosuppressive therapy is required for the survival of ovarian allografts. In bitches, who are monestrous with a 2-mo luteal phase and a prolonged non-seasonal anestrus of 3–10 mo, it is difficult to prevent long-term rejection of ovarian allografts. Therefore, autografts appear as the most promising approach for studying follicular development. The graft site has been considered an important factor in tissue survival and follicular development after transplantation (Soleimani *et al.*, 2008). Several ovarian grafting techniques have been described; these basically differ only in the graft recipient site, which includes the bursal cavity, kidney capsule, and subcutaneous sites. Hypoxic conditions might be the main reason for initial follicle loss after transplantation (Aubard *et al.*, 1999); therefore, rapid revascularization after grafting is considered necessary for maximum follicle survival. The kidney capsule became popular as a transplantation site, because of the assumption that adequate blood supply favored revascularization and subsequent graft survival (Gosden *et al.*, 1994). To date, several reports have described the transplantation of ovarian tissues to various heterotopic body locations (Gosden *et al.*, 1994; Weissman *et al.*, 1999; Oktay et al., 2001; Schmidt et al., 2005), but have not clearly identified the site that yields the best results after transplantation. In this study,

we investigated whether the graft site influences the number of viable follicles after 1 mo of ovarian tissue transplantation, and examined the quality and meiotic competence of the oocytes recovered from the ovarian tissues grafted to the quadriceps femoris muscle fascia, kidney capsule, and gastrosplenic ligament in dogs.

MATERIALS AND METHODS

1. Experimental animals

Beagle bitches (age, 4–7 yr; mean weight, 10.3 ± 2.0 kg) in a closed breeding colony were used for this study. The dogs were housed individually in stainless steel cages, fed a diet of standard commercial dog food once a day, and given water ad libitum. All the animals involved in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council. All procedures were approved by the Animal Research Committee of Yamaguchi University.

2. Transplantation procedure

The dogs were fasted for 12 h before the surgical procedure. At 30 min before induction of anesthesia, 0.2 mg/kg meloxicam (Nippon Boehringer Ingelheim Co. Ltd., Hyogo, Japan) was administered subcutaneously for analgesia. General anesthesia was induced with an intravenous injection of 0.2 mg/kg midazolam hydrochloride (Astellas Pharma Inc., Tokyo, Japan) mixed with 0.2 mg/kg butorphanol tartrate (Meiji Seika Co. Ltd., Tokyo, Japan), followed by 4 mg/kg propofol (Fuji Pharmaceutical Co. Ltd., Toyama, Japan). After endotracheal intubation, the dogs were mechanically ventilated with isoflurane in pure oxygen and placed in dorsal recumbency. The carotid and femoral arteries and the jugular and femoral veins were unilaterally catheterized for monitoring the arterial and venous blood pressure. During the operation, 5% glucose in acetated Ringer's solution (Nikken Chemicals Co. Ltd., Tokyo) was infused at a rate of 10 mL/kg/h. Bilateral ovariectomy was performed using a ventral midline abdominal approach starting at the umbilicus and extending caudally (Janssens and Janssens,1991).

The ovarian pedicle was ligated using routine techniques and materials. The uterine artery and vein were then ligated and severed at the proper ligament (cranial tip of the uterine horn), and then, the ovary was removed. After excision, each ovary was bisected using a scalpel blade (Fig. 1-1a). The hemi-ovaries were stored in physiological saline and maintained at 38 °C. They were then transplanted to the animal of origin in three locations within 20 min of ovary excision. As a control, the remaining hemi-ovary was used to examine ovarian morphology and the viability of oocytes of fresh non-transplanted ovaries.

(1)Kidney capsule

The left kidney was exteriorized through ventral median celiotomy during ovariectomy. A hemi-ovary was inserted under the capsule of the kidney through a small incision (about 2 cm) made with a scalpel blade and iris scissors. After insertion of the ovary, the incision was closed with monofilament nylon (Dermalon 5-0; Davis & Geck Inc., NJ, USA).

(2)Gastrosplenic ligament

The gastrosplenic ligament near the splenic artery was exteriorized through ventral median celiotomy. A hemi-ovary was placed in an invagination in the gastrosplenic ligament, which was then closed with monofilament nylon in a purse-string suture pattern.

(3)Quadriceps femoris muscle fascia

A hemi-ovary was inserted under the fascia of the left quadriceps femoris muscle through a small incision (about 2 cm) made with a scalpel blade and iris scissors. After insertion of the ovary, the incision was closed with monofilament nylon.

Finally, the abdominal wall and the skin incisions were closed. All procedures were performed under aseptic conditions. All dogs recovered from anesthesia uneventfully and were allowed food and water 6 h after recovery. All grafted ovaries were recovered from animals at 28–31 d after transplantation under anesthesia, as previously described. After collection, each hemi-ovary was bisected using a scalpel blade, and one-quarter ovary was used for histological assessment and the other for evaluation of oocyte viability.

3. Histological assessment

The quarter ovaries of the recovered grafts and control tissues were fixed in 10% formaldehyde and manually processed for paraffin embedding. Histomorphological examination was conducted after sectioning 4-μm slices. Then, two serial sections with maximal area from each graft and control tissue were selected for hematoxylin–eosin (HE) (Fig. 1-2a) and proliferating cell nuclear antigen (PCNA) staining (Fig. 1-2b). For PCNA immunohistochemistry, according to the manufacturer's instructions of the Histofine SAB-PO kit (Nichirei Corp., Tokyo, Japan), tissue sections (4 μm) were dewaxed and rehydrated using ascending concentrations of alcohol before undergoing thermal antigen retrieval in citrate buffer (10 mM; pH 6.0). Slides were incubated at 25 °C for 90 min with anti-PCNA mouse monoclonal antibody (38 mg/mL; PCNA Clone PC10; Sigma, St. Louis, MO, USA), which was diluted 1:100 in PBS. Biotinylated rabbit anti-mouse IgG, IgA, and IgM antibody (Nichirei Corp.) was

applied as a secondary antibody for 30 min before treatment with peroxidase-conjugated streptavidin (Nichirei Corp., Tokyo, Japan). After treatment with a chromogenic substrate (3-amino-9-ethylcarbazol), the sections were counterstained with hematoxylin. Because PCNA is not expressed in the quiescent primordial follicles and marks the initiation of follicular growth (Oktay *et al.*, 1995), the follicles with granulosa cells expressing PCNA in the section were considered viable. In both HE- and PCNA-stained sections, follicles were classified as primordial (oocytes surrounded by one layer of flattened pre-granulosa cells), primary (surrounded by one layer of cuboidal granulosa cells), secondary (with two or more layers of granulosa cells without an antrum), or antral (with multiple layers of cuboidal granulosa cells and an antral cavity) (Oktay *et al.*, 1995). In PCNA-stained sections, the total number of follicles and the number of follicles with granulosa cells expressing PCNA were counted. The percentage of viable follicles was calculated by dividing the number of PCNA-positive follicles by the total number of follicles. To prevent counting errors, slides were assessed by two independent individuals, and the results were compared.

4. Oocyte viability

The quarter ovaries of recovered grafts and control ovaries were placed in modified phosphate-buffered saline (m-PBS; Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan) supplemented with 50 μg/mL gentamicin (Sigma) at 38 °C. They were then repeatedly sliced to release oocytes. All oocytes were collected and suspended in culture medium (TCM 199 with Earle's salts), buffered with 25 mM HEPES buffer (Invitrogen Corp., Carlsbad, CA, USA), and supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen Corp.) and 50 μg/mL gentamicin. The oocytes were microscopically examined for morphological quality and categorized using the following method by

Hewitt *et al.* (1998): Grade A oocytes were darkly pigmented and surrounded by one or more layers of cumulus cells. Grade B oocytes were lightly pigmented with incomplete layers of cumulus cells. Grade C oocytes were pale and irregularly shaped, with no cumulus cells. Only grade A and B oocytes were selected and transferred into 100-μL drops of culture medium, and covered with warm paraffin oil (3.5 mL, Sigma) in a polystyrene culture dish (35 × 10 mm; Falcon Becton Dickinson Labware, NJ, USA). Subsequently, the oocytes were cultured for 72 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

After incubation for 72 h, the oocytes were denuded using small glass pipettes, and fixed and permeabilized for 15 min at room temperature in Dulbecco's PBS (DPBS; Invitrogen Corp.) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma). Then, they were placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred into a small drop comprising PBS supplemented with 90% (v/v) glycerol (Sigma) and 1.9 μM bisbenzimide (Hoechst 33342; Sigma) on a slide. Subsequently, the oocytes were overlaid with a coverslip supported by four droplets of vaseline/paraffin and incubated overnight at 4 °C. The oocytes were examined using fluorescence microscopy with a 355-nm wavelength excitation filter. They were classified according to their chromatin configuration as "germinal vesicle," "condensed chromatin," "metaphase I," or "metaphase II." Oocytes with diffusely stained cytoplasmic characteristic of nonviable cells, and those in which chromatin was unidentifiable or not visible, were classified as "degenerated."

RESULTS

1. Histological assessment

One month after transplantation, all ovarian tissues were surrounded by a layer of fibrous tissue and blood vessels, irrespective of the graft site (Fig. 1-1b). In both HE- and PCNA-stained graft sections, most follicles were classified as primordial or primary follicles, and antral follicles were not observed in the grafts, irrespective of the graft location. The percentages of viable follicles in the sections from the control ovaries (total number of counted follicles, 409 follicles), and the ovaries grafted to the kidney capsule (170 follicles), the quadriceps femoris muscle fascia (224 follicles), and the gastrosplenic ligament (148 follicles) were 17.4%, 22.9%, 18.3%, and 32.4%, respectively.

2. Quality and viability of oocytes

A total of 12 oocytes was recovered from the quarter ovaries of the 15 hemi-ovaries grafted in five bitches, of which five (41.7%) oocytes, which were recovered from the ovaries grafted to the quadriceps femoris muscle fascia and the kidney capsule, were categorized as grade A or B (Table 1-1 and Fig. 1-2c). After in vitro maturation (IVM) culture, three oocytes were stained by bisbenzimide, but remained at the GV stage (Fig. 1-2d). No oocyte reached metaphase II (MII). In the non-grafted controls, 64 oocytes were recovered from the quarter ovaries of five hemi-ovaries, of which 33 (51.6%) oocytes were categorized as grade A or B. Only one out of 33 oocytes reached MII after IVM.

DISCUSSION

In this study, canine hemi-ovaries were autografted to three locations to compare the survival of the follicles and oocytes. After 1 mo of ovarian transplantation, growth of follicles in the ovarian tissues could be observed, irrespective of the graft site. Moreover, some viable oocytes were recovered from the ovaries grafted to the quadriceps femoris muscle fascia and to the kidney capsule, although the oocytes were unable to resume meiosis after IVM culture.

The graft site has been proposed to influence tissue survival and follicle development, as well as the quality of the oocytes obtained, after transplantation (Yang *et al.*, 2006). Cleary *et al.* (2003) reported increased follicle survival after grafting under the kidney capsule compared to that observed after grafting to the subcutaneous sites in xenografted ovarian tissues. Yang *et al.* (2006) demonstrated that a significantly higher number of oocytes were recovered from the ovaries grafted under the kidney capsule than from the ovaries grafted to the subcutaneous sites. Post-transplantation hypoxic ischemia has been suggested as the main cause of follicular loss in ovarian transplantation (Dissen *et al.*, 1994; Snow *et al.*, 2001). Therefore, rapid revascularization after grafting is considered necessary for maximum follicle survival. The duration of ischemia prior to revascularization of the transplanted ovarian tissue depends on several factors such as the graft site, tissue size, and quantity of angiogenic factors (Paris *et al.*, 2004; Bols *et al.*, 2010). The kidney has a rich blood supply and high concentrations of angiogenic growth factors, which facilitate rapid blood vessel ingrowth within 48 h of grafting (Dissen *et al.*, 1994). We transplanted the hemi-ovaries to the kidney capsule and gastrosplenic ligament tissue. The gastrosplenic ligament is the portion of dorsal mesentery between the spleen and the stomach. The vessels in the

ligament anastomose with the distal splenic vessels (Gurleyik *et al.*, 2000; Paulo *et al.*, 2006). Therefore, an abundant blood supply, similar to that of the kidney, was predicted when the hemi-ovaries were grafted to the tissue. In contrast, grafts in the less vascularized recipient sites such as the subcutaneous sites might take longer to revascularize, thereby prolonging graft ischemia and reducing follicle survival (Imthurn *et al.*, 2000). In this study, when the grafted ovaries were recovered, all ovarian tissues were surrounded by a layer of fibrous tissue and blood vessels, irrespective of the graft site. Moreover, the percentage of PCNA-positive follicles in the grafts under the quadriceps femoris muscle fascia was similar to those in the fresh control tissues and other grafts. The back muscle has been demonstrated to provide superior supportive neo-vascularization with specific blood supply to the graft site, thereby resulting in increased survival of the ovarian grafts than that at the kidney site (Soleimani *et al.*, 2008). Similarly, our results indicate that the quadriceps femoris muscle fascia may be as suitable as the kidney capsule as a graft recipient site.

Ovarian grafting generates follicle development, and provides a model to study ovarian function. The kidney capsule has become popular as a transplantation site, but it is difficult to access or monitor the tissues at this site. Peripheral graft sites such as the fascia lata or the antebrachium for ovarian tissue are more convenient and easily accessible for transplantation surgery, monitoring of graft function, and recovery of oocytes from the grafts. In this study, the length of the hemi-ovaries transplanted under the fascia of the quadriceps femoris muscle could be monitored by ultrasound, and the mean diameter of the grafted hemi-ovaries gradually decreased from 1.33 cm to 0.93 cm (decrease in diameter, 0.4 cm) (data not shown). Approximately 18−32% of the follicles counted in the graft sections was viable, but no antral follicles were observed in the grafts. The number of oocytes recovered from the grafted ovaries were much less than

those recovered from the non-grafted ovaries, and no oocytes from the grafts resumed meiosis. These results correlate with those of Metcalf *et al.* (2001), who transplanted canine ovarian tissue to the kidney capsules of immunodeficient mice, but could not confirm follicular development to the antral stage.

On the other hand, canine oocytes can resume meiosis in vitro, but the efficacy of the methods for IVM of oocytes is very low (Farstad, 2000). In this study, all oocytes from the grafts arrested at the GV stage after IVM culture, and only 3% of control oocytes could reach MII. The rate of maturation to MII of control oocytes was similar to that in our previous study, in which the maturation rate of the canine oocytes from the ovaries without visible follicles and pronounced luteal tissues was 2% (Otoi *et al.*, 2001). In a previous study, ovarian autotransplantation with vascular anastomosis yielded successful results with delivery of puppies (Paldi *et al.*, 1975). Pullium *et al.* (2008) demonstrated that bitches showed normal estrous cycles and pregnancy after mating following homologous ovarian transplantation into the bursa of the recipient. Moreover, Rimbach *et al.* (1993) assessed the survival of canine transplanted ovaries beyond 3 mo after grafting. In this study, all grafted ovaries were recovered from animals at 4 wk after transplantation, according to a previous study on xenotransplantation of canine ovarian tissues (Suzuki *et al.*, 2008; Ishijima *et al.*, 2006). However, folliculogenesis from the primordial to the preovulatory stage may take much longer in larger animals than in rodents (Van den Broecke *et al.*, 2001). Therefore, the low number of antral follicles and meiotic competence of oocytes recovered from grafts may indicate that a cohort of follicles had begun to grow but had had insufficient time to expand or that a proportion of follicles of all size groups had died, leaving a small number of each type. Moreover, transplantation of the ovarian tissue to the heterotopic

sites might affect not only the number but also the quality and developmental potential of oocytes (Yang *et al.*, 2006).

It has been suggested that the timing of onset of antrum formation and the progress of follicle development are advanced relative to that in the ovaries in situ (Mattiske *et al.*, 2002). In this study, when hemi-ovaries were autografted to each body site, no follicles on the surface of each ovary were observed. One ovary had a corpus luteum, and the concentration of progesterone in the donor's serum, which was measured by an enzyme-linked fluorescent assay using an autoanalyzer (SPOTCHEM VIDAS SV-5010; Arkray, Kyoto, Japan), was high (42.4 ng/mL). However, the progesterone concentration had decreased by the time of ovary recovery (0.28 ng/mL). In other bitches, no variations were observed in the concentrations of estradiol-17β (<11.4 pg/mL) and progesterone (<1.73 ng/mL) before and after grafting (data not shown). These results indicate that luteal activity in all the grafts was quiescent at the time of ovariectomy. The ovarian cycle of the bitch is unique among those of domestic animals, in that the bitch is monestrous with a 2-mo luteal phase and a prolonged but variable non-seasonal anestrus of 3–10 mo after both pregnant and non-pregnant cycles. Unlike most domestic species, the dog does not respond predictably to hormonal treatments that induce ovulation in other species (Inaba *et al.*, 1998; Vanderlip *et al.*, 1987). Therefore, it is not clear whether follicular development and oocyte survival are related to the endocrine status of donor or recipient at the time of transplantation. On the other hand, the young ovarian tissue contains more quiescent primordial and growing follicles than the adult ovarian tissue, and this tissue also forms more growing follicles than adult donor grafts. Therefore, it seems that antrum formation and the progress of follicle development may be related to the age of the animal (Vom Saal *et al.*, 1994).

However, the influence of donor age on follicular development and oocyte survival remains unclear from our results, because only adult bitches with previous whelping experience (4 to 7 yr of age) were used for the experiment. Therefore, further analysis of the role of endocrine status, age, and local factors in the control of follicle survival before and after grafting is necessary to improve the development of follicles and oocytes.

In conclusion, our results indicate that peripheral sites such as the fascia lata may be equivalent to the kidney capsule for transplantation. However, grafts to the quadriceps femoris muscle fascia did not support early follicle and oocyte development. The advantages of the peripheral recipient sites are that the grafts can be conveniently inserted using local anesthetic, easily monitored by ultrasound, and easily accessed for graft or oocyte recovery. Further investigation is necessary to improve the survival of the follicles in the ovaries grafted to the peripheral sites.

Chapter 2

FOLLICLE FORMATION IN THE CANINE OVARY AFTER AUTOGRAFTING TO A PERIPHERAL SITE

ABSTRACT

This study reports about follicular development on the surface of canine ovarian tissueafter autografting under the fascia of the thoracolumbar muscle and about meiotic resumption of follicle-derived oocyte after maturation culture. After ovarian excision from a bitch, each ovary of the pairs was cut approximately into half. The hemi-ovaries were transplanted into the bitch of origin at 3 different body sites (under the fascia of the quadriceps femoris muscle and the thoracolumbar muscle, and in the deltoid muscle in the scapular region). All grafted ovaries were recovered from the bitch at 35 days post-transplantation. A visible antral follicle was observed on the surface of the ovary grafted under the thoracolumbar fascia. Histological examination revealed viable follicles at different stages of development irrespective of graft site. Most granulosa cells in the follicles at different stages of development expressed proliferating cell nuclear antigen (PCNA). A total of 3 oocytes were collected from an ovary grafted under the fascia of the thoracolumbar muscle, wherein an oocyte reached metaphase I after maturation culture. This is the first report to demonstrate follicular development and meiotic resumption of oocytes recovered from autografted canine ovarian tissues.

INTRODUCTION

The reproductive biology of bitches has been known to be unique. For instance, ovulation occurs approximately 2–3 days after the surge in luteinizing hormone (LH) levels, and prior to ovulation, the serum progesterone levels begin to rise from basal levels coincident with the LH peak (Wildt *et al.*, 1979). Useful information about the time of ovulation relative to the endocrine glands is available, but there is limited information about follicular growth through the anestrus and proestrus. Although it is possible to observe the development of canine ovarian follicles by real-time ultrasonography (Boyd *et al.*, 1993; England and Yeager, 1993), a technique to accurately determine or predict ovulation has not yet been established. Moreover, the location and small size of ovaries makes imaging technically difficult. On the other hand, ovarian grafts under the skin allow easy monitoring of follicle growth and provide ready access to the graft for oocyte collection (Oktay *et al.*, 2004). Therefore, ovarian rafting to subcutaneous sites provides a model to study canine ovarian function (Paris *et al.*, 2004). If oocytes can be collected from the antral follicles of the ovarian grafts for in vitro fertlization, the low meiotic competence of canine oocytes may be improved (Farstad, 2000). However, transplantation of canine ovarian tissue under the skin has never been investigated in the bitch. Metcalfe *et al.* (2001) performed transplantation of canine ovarian tissue to the kidney capsules of immunodeficient mice, but they could not confirm follicular development to the antral stage.

In the present study, we report follicular development on the surface of canine ovarian tissue 35 days after autografting under the fascia of the left thoracolumbar muscle. We confirmed meiotic resumption of the follicle-derived oocyte after maturation culture.

MATERIALS AND METHODS

Bitch

A healthy beagle bitch (age, 5 years; weight, 10.3 kg) used in this study was reared in a 68 closed breeding colony and showed normal estrous cycles (interestrous interval, 241 days) and breeding (parity number, 6) until the start of experiment. At the time of ovariectomy, the concentrations of progesterone and estradiol-17β in the donor's serum, which were measured by an enzyme-linked fluorescent assay using an autoanalyzer (SPOTCHEM VIDAS SV-5010; Arkray, Kyoto, Japan), were 0.67 ng/mL and 9.6 pg/mL, respectively. The bitch was housed alone in a stainless steel cage (900 x 770 x 710 mm), and was given standard commercial dog food once a day, and water, ad libitum. She received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. All procedures were approved by the Animal Research Committee of Yamaguchi University.

Transplantation and recovery procedure

General anesthesia was induced using a mixture of isoflurane and oxygen; bilateral ovariectomy was then performed according to the method described by Janssens and Janssens (1991). Briefly, 0.2 mg/kg meloxicam (Nippon Boehringer Ingelheim Co. Ltd., Hyogo, Japan) was administered subcutaneously for analgesia before induction of anesthesia. General anesthesia was induced with an intravenous injection of 0.2 mg/kg midazolam hydrochloride (Astellas Pharma Inc., Tokyo, Japan) mixed with 0.2 mg/kg butorphanol tartrate (Meiji Seika Co. Ltd., Tokyo, Japan), followed by 4 mg/kg propofol (Fuji Pharmaceutical Co. Ltd., Toyama, Japan). After endotracheal intubation,

the dog was mechanically ventilated with isoflurane in pure oxygen and placed in dorsal recumbency. Bilateral ovariectomy was performed using a ventral midline abdominal approach starting at the umbilicus and extending caudally. The ovarian pedicle was ligated using routine techniques and materials. The uterine artery and vein were then ligated and severed at the proper ligament (cranial tip of the uterine horn), and then, the ovary was removed. After ovarian excision, each ovary of the pairs was removed from fat tissues and then cut longitudinally into half by using a scalpel blade (Fig. 2-1A). The hemi-ovaries were maintained in physiological saline at 38 °C, and then transplanted in the bitch of origin at 3 different 6 body sites within 20 min of ovary removal. As a control, the remaining hemi-ovary was used to examine the follicular morphology and oocyte viability of the fresh non-transplanted ovary.

The hemi-ovaries were inserted under the fascia of the left quadriceps femoris muscle and the left thoracolumbar muscle through a small hole (~2 cm) made using a scalpel blade and iris scissors. The third hemi-ovary was inserted into the deltoid muscle in the left scapular region through a hole (~5 cm) detached by an incision in the vertical direction along the muscle fiber. Finally, the hole and skin incision were closed.

The longest diameters of the hemi-ovaries transplanted into the fascia and intramuscular sites were measured every week until graft collection, with the Prosound α7 ultrasound scanner (ALOKA Co., Ltd., Tokyo, Japan) equipped with a 6.0–13.0-MHz linear-array transducer.

All grafted ovaries were recovered from the anesthetized bitch 35 days post-transplantation, according to a previous study on xenotransplantation of canine ovarian tissues (Ishijima *et al.*, 2006; Suzuki *et al.*, 2008). After collection, each ovary was cut approximately into half by using a scalpel blade. Histological assessment was carried

out on 1 half of each grafted hemi-ovary, and the other half was subjected to oocyte viability examination.

Histological assessment

Half of the recovered grafts and control tissues were fixed in 10% formaldehyde and manually embedded in paraffin. Histomorphological examination was carried out after serial sectioning to 4-μm thickness, and 4 sections with maximal area from each graft and control tissue were selected and stained with hematoxylin-eosin (HE) and proliferating cell nuclear antigen (PCNA). PCNA immunohistochemical technique was performed using a Histofine SAB-PO kit (Nichirei Corp., Tokyo, Japan), according to the manufacturer's instructions. Tissue sections were dewaxed and rehydrated using ascending concentrations of alcohol before undergoing thermal antigen retrieval in citrate buffer (10 mM; pH 6.0). Slides were incubated at 25°C for 90 min with anti-PCNA mouse monoclonal antibody (38 μg/ml; PCNA Clone PC10; Sigma, St. Louis, MO, USA), which was diluted 1:100 in PBS. Biotinylated rabbit anti-mouse IgG, IgA, and IgM antibody (Nichirei Corp.) was applied as a secondary antibody for 30 min before treatment with peroxidase-conjugated streptavidin (Nichirei Corp., Tokyo, Japan). After treatment with a chromogenic substrate (3-amino-9-ethylcarbazol), the sections were counterstained with hematoxylin. Mouse IgG (Dako Denmark, Glostrup, Denmark) as source of irrelevant primary antibody was used as negative control. Because PCNA is not expressed in the quiescent primordial follicles and marks the initiation of follicular growth (Oktay *et al.*, 1995), the follicles with granulosa cells expressing PCNA in the section were considered viable. In both HE- and PCNA-stained sections, follicles were classified as primordial (oocyte surrounded by 1 layer of flattened pre-granulosa cells), primary (surrounded by 1 layer of cuboidal granulosa

cells), secondary (with 2 or more layers of granulosa cells without an antrum), or antral (with multiple layers of cuboidal granulosa cells and an antral cavity) (Oktay *et al.*, 1995). In PCNA-stained sections, the total number of follicles and the number of follicles with granulosa cells expressing PCNA were counted. The percentage of viable follicles was calculated by dividing the number of PCNA-positive follicles by the total number of follicles.

Oocyte viability

Half of the recovered grafts and control ovaries were repeatedly sliced for oocyte recovery, according to the method described by Otoi et al. (2002) with minor modifications. All the oocytes were collected, and then, suspended in culture medium (TCM199 medium [Earle's salts] buffered with 25 mmol HEPES buffer [Invitrogen, Carlsbad, CA, USA] supplemented with 10% (v/v) fetal bovine serum [FBS; Invitrogen] and 50 μg/ml gentamicin). The oocytes were microscopically examined for morphological quality, and categorized according to the following classification based on the system described by Hewitt et al. (1998): Grade A oocytes were darkly pigmented and surrounded by 1 or more layers of cumulus cells; Grade B oocytes were lightly pigmented with incomplete layers of cumulus cells; Grade C oocytes were pale and irregularly shaped without any cumulus cells. Only Grade A and B oocytes were selected and transferred into 100-μL drops of culture medium covered with warm paraffin oil (3.5 mL; Sigma) in a polystyrene culture dish (35 × 10 mm; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA). The oocytes were subsequently cultured for 72 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

After 72-h incubation, the oocytes were denuded, fixed, and stained with 1.9 μmol bis-benzimide (Hoechst 33342; Sigma) on a slide (Otoi *et al.*, 2002). The oocytes were

examined using a fluorescence microscope with a 355-nm wavelength excitation filter, and classified according to chromatin configuration as "germinal vesicle," "condensed chromatin," "metaphase I," or "metaphase II." The oocytes with diffusely stained cytoplasmic characteristics of non-viable cells and those wherein the chromatin was unidentifiable or not visible were considered degenerated.

RESULTS

Histological and ultrasonographic assessment of autografts

After autografting, all the ovarian tissues were surrounded by a thin layer of fibrous tissue and blood vessels and they slightly diminished size irrespective of the grafting body site (Fig. 2-1C and D). A visible antral follicle was noted on the surface of the ovary grafted under the thoracolumbar fascia (Fig 2-1B). In HE stained sections signs of degeneration in blood vessels, such as nuclear pyknosis, were observed in part in the grafts. However, all grafts were well vascularized, had multiple growing follicles in the cortex, and were surrounded by a thin layer of fibrous tissue irrespective of the grafting body site (Fig. 2-2). In both HE- and PCNA- stained graft sections (Fig 2-3), most follicles (78.2% - 98.9%) in the control ovary (total number of counted follicles, 87 follicles), and the ovaries grafted to the quadriceps femoris muscle fascia (108 follicles) and the deltoid muscle (189 follicles) were classified as primordial or primary follicles, whereas 76.5% of follicles from an ovary grafted to the thoracolumbar fascia (34 follicles) were classified as secondary follicles. The percentages of PCNA-positive follicles in the sections from a control ovary, and the ovaries grafted to the quadriceps femoris muscle fascia, the deltoid muscle and the thoracolumbar fascia were 79.3%, 98.1%, 100%, and 100%, respectively. The viable follicles were prominent in the cortex of the grafted ovaries and the PCNA staining characteristics of preantral follicles were similar to those of a control ovary.

Ultrasonographic assessment during grafting revealed that the longest diameter of the grafts under the fascia of the quadriceps femoris muscle gradually decreased from 1.46 cm to 1.19 cm (decrease in diameter, 0.27 cm). In the thoracolumbar fascia and

deltoid muscle grafts, the decrease in the ovarian diameters were slightly lower (0.04 cm and 0.11 cm, respectively).

Quality and viability of oocytes from the autografts

A total of 3 oocytes that were classified as Grades A, B, and C, respectively, were collected from the ovary grafted under the fascia of the thoracolumbar muscle from which a Grade A oocyte (Fig. 2-1E) was recovered from the follicle formed on the ovarian surface (Fig. 2-1B). After in vitro maturation (IVM) culture, the oocyte derived from the follicle reached metaphase I (Fig. 2-1F). Another oocyte classified as Grade B remained in the germinal vesicle stage. A total of 5 oocytes were recovered from the ovary grafted under the fascia of the quadriceps femoris muscle, all of which were classified as Grade C. None of the oocytes was recovered from the deltoid muscle in the scapular region. In the non-grafted control, 8 oocytes were recovered from the fresh ovary, only 1 of which was categorized as Grade A, and it remained in the germinal vesicle stage after IVM culture.

DISCUSSION

In the present study, we observed follicular development on the surface of canine ovarian tissue grafted under the thoracolumbar fascia. Moreover, an oocyte from this follicle reached metaphase I after IVM culture. To our knowledge, although only one bitch was examined in this study, this is the first report to demonstrate follicular development and meiotic resumption of an oocyte recovered from a visible antral follicle on the surface of canine ovarian tissues that were grafted into the peripheral sites.

Ovarian grafting with follicular development provides a model for studying ovarian function. Although the kidney capsule is a popular transplantation site, it is difficult to access and monitor this site. Transplantation of the ovarian tissue to peripheral sites allows the tissue to be placed at a more convenient and easily accessible site for monitoring graft function and oocyte recovery. In the present study, we observed follicular development and some viable oocytes in the ovarian grafts. It has been suggested that the grafting site influences tissue survival and follicular development as well as the quality of the obtained oocytes after transplantation (Yang *et al.*, 2006). The main cause of follicular graft loss in the process of ovarian transplantation has been suggested to be post-transplantation hypoxic ischemia (Dissen *et al.*, 1994; Snow *et al.*, 2001). Therefore, rapid revascularization after grafting is considered essential for maximum follicular survival. The duration of ischemia prior to revascularization of the transplanted ovarian tissue depends on several factors, including graft site, tissue size, and the presence and amount of angiogenic factors (Paris *et al.*, 2004; Bols *et al.*, 2010). The kidney has rich blood supply and high concentrations of angiogenic growth factors, which allow rapid blood vessel in growth within 48 h of grafting (Dissen *et al.*, 1994).

We transplanted hemi-ovaries into the less vascularized sites (the fascia and intramuscular sites) that take longer to revascularize, thereby prolonging graft ischemia and reducing follicular survival (Imthurn *et al.*, 2000). Moreover, all ovaries had some corpora lutea at the time of ovariectomy, but the concentration of progesterone in the donor's serum was low (0.67 ng/mL). No variations were observed in the concentrations of progesterone (< 0.67 ng/mL) before and after grafting (data not shown), indicating that luteal activity in all the grafts was quiescent. However, we confirmed the presence of viable follicles at different stages of development 35 days of grafting irrespective of graft site. All the ovarian tissues were surrounded by a layer of fibrous tissue and blood vessels. Although the graft diameters gradually decreased after the grafting, the size of the ovarian tissue could be monitored by ultrasonography. It has been demonstrated that peripheral graft sites such as the back muscle or the antebrachium are suitable for ovarian allowgrafts (Oktay *et al.*, 2001; Lee *et al.*, 2004; Soleimani *et al.*, 2008). Therefore, our results indicate that the peripheral fascia and intramuscular sites may be one of suitable grafting sites for ovarian function monitoring and oocyte recovery from the grafts. The advantages of grafting to the peripheral sites include insertion under local anesthesia, easy monitoring by ultrasound, and easy access for graft or oocyte recovery. Use of grafting and cryopreservation in combination may prolong the female reproductive lifespan.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with people or organizations that could inappropriately influence or bias the content of this paper.

OVERALL DISCUSSION AND CONCLUSION

In this study, the following results were achieved: (1) the percentage of PCNA-positive follicles in the grafts under the quadriceps femoris muscle fascia was similar to that in the control tissues and other grafts, (2) the size and revascularization of the ovarian tissue could be easily monitored using ultrasonography after the grafting, (3) follicular development was observed on the surface of canine ovarian tissue grafted under the thoracolumbar fascia, and (4) an oocyte from this follicle could reach metaphase I after IVM culture. Although only 1 bitch was examined in this study, this is the first report, to our knowledge, of follicular development and meiotic resumption of an oocyte recovered from a visible antral follicle on the surface of canine autograft ovarian tissues that were transplanted to the peripheral sites. Taken together, our results indicate that the peripheral fascia and intramuscular sites might be suitable areas for monitoring ovarian function and recovering oocytes from the grafts.

Ovarian grafting results in follicle development (Cox *et al.*, 2000); therefore, it provides a model to study ovarian function. In many wildlife species, knowledge about follicular dynamics (e.g., the interval required for follicle growth and hormonal responsiveness of ovarian follicles) is unknown. This information is important if assisted reproductive technologies are to be employed to aid in the conservation of a species. Ovarian biopsies taken while animals undergo other surgeries or ovarian tissue collected from a recently deceased female can be used in grafting studies to provide key knowledge about follicular dynamics of a species when access to that species for research purposes is limited. If the objective of ovarian grafting is to restore long-term fertility or collect large numbers of mature oocytes from small pieces of ovarian grafts, maximizing the number of follicles that survive is paramount.

It has been proposed that graft site influences tissue survival and follicle development, as well as the quality of the oocytes obtained, after transplantation (Yang *et al.*, 2006). Cleary *et al.* (2003) reported that follicle survival after grafting under the kidney capsule was higher than that observed after grafting to the subcutaneous sites in xenografted ovarian tissues. Yang *et al.* (2006) demonstrated that a significantly higher number of oocytes were recovered from the ovaries grafted under the kidney capsule than from those grafted to the subcutaneous sites. Because hypoxic ischemia has been suggested as the main cause of follicular loss after ovarian transplantation (Dissen *et al.*, 1994; Snow *et al.*, 2001), rapid post-grafting revascularization is considered to be essential for maximizing follicle survival; the ischemic conditions cause follicles to succumb to apoptosis (Liu *et al.*, 2002). Revascularization of ovarian grafts was estimated to occur within 48 h of transplantation (Dissen *et al.*, 1994), when the majority of growing follicles are lost and as much as 50% of the primordial follicle population within an ovarian graft do not survive (Liu *et al.* 2002). This loss rate, in conjunction with cryopreservation, where about 9% of the follicle population is lost (Liu *et al.*, 2002), and the initial small size of the graft, severely limits the lifespan of the graft. However, ovarian grafts can maintain reproductive function for many months (Candy *et al.*, 2000). Several factors such as the graft site, tissue size, and quantity of angiogenic factors (Paris *et al.*, 2004; Bols *et al.*, 2010) directly affect the duration of ischemia before revascularization of the autograft ovarian tissue. The kidney receives a rich blood supply and has high concentrations of angiogenic growth factors, which facilitate rapid blood vessel ingrowth within 48 h of grafting (Dissen *et al.*, 1994). Promoting revascularization is the most obvious means for good graft establishment and maximum follicle survival. Vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-b) mRNA expression are upregulated during the

revascularization process of ovarian grafts (Dissen *et al.*, 1994). This upregulation of angiogenic stimulating factors may be mediated by pituitary gonadotropins (Dissen *et al.*, 1994), implying why ovarian grafts are more successful in bilaterally ovariectomized recipients than in intact recipients (Cox *et al.*, 2000). Treating the graft recipient with a combination of FSH and LH 2 days before and 2 days after grafting increased the number of follicles that survived within the ovarian graft (Imthurn *et al.*, 2000). Presumably, the effects of FSH and LH are mediated through VEGF; however, VEGF administration to ovarian graft recipients does not appear to improve graft establishment (Schnorr *et al.*, 2002). Ischemic reperfusion injury is also a problem with ovarian grafting. Treatment of ovarian graft recipients with an antioxidant protects follicles against such injury (Nugent *et al.*, 1998). Because of the inability of female germ cells to undergo mitosis during postnatal development, optimization of transplantation and cryopreservation protocols that minimize follicular depletion within ovarian grafts is essential if ovarian grafting is to be used as a long-term solution for some cases of infertility.

In the first series of experiments, the hemi-ovaries were transplanted to the kidney capsule and gastrosplenic ligament tissue. The vessels in the ligament anastomosed with the distal splenic vessels (Gurleyik *et al.*, 2000; Paulo *et al.*, 2006); therefore, an abundant blood supply, similar to that of the kidney, was anticipated when the hemi-ovaries were grafted to the tissue. In contrast, grafts in the less vascularized recipient sites, such as the subcutaneous sites, might take longer to revascularize, resulting in prolonged graft ischemia and reduced follicle survival (Inthurn *et al.*, 2000). When we transplanted hemi-ovaries into less vascularized sites (i.e., fascia and intramuscular areas), graft ischemia was prolonged and follicular survival was reduced

(Imthurn *et al.*, 2000); however, when the grafted ovaries were recovered, all ovarian tissues were surrounded by a layer of fibrous tissue and blood vessels, regardless of the graft site. In addition, the percentage of PCNA-positive follicles in the grafts under the quadriceps femoris muscle fascia was similar to that in the control tissues and other grafts. These results indicate that both the near-surface quadriceps femoris muscle fascia and the kidney capsule might be useful for grafting.

Ovarian grafting with follicular development provides a model by which ovarian function can be studied. Although the kidney capsule is a popular transplantation site, it is difficult to access and monitor. Transplantation of ovarian tissue to peripheral sites is more convenient and more easily accessible for monitoring graft function and oocyte recovery. In both series of experiments, the hemi-ovaries transplanted under the fascia of the quadriceps femoris muscle and the other fascia near the surface could be easily monitored by ultrasonography. In the second experiment, an antral follicle with follicular antrum formation and blood vessels from revascularization could also be monitored by ultrasonography. In addition, in the first series of experiments, the number of oocytes recovered from the grafted ovaries was far lower than that recovered from the non-grafted ovaries, and no oocytes from the grafts resumed meiosis. These results supported the report by Metcalf *et al.* (2001), wherein canine ovarian tissue was transplanted to the kidney capsules of immunodeficient mice but could not confirm follicular development to the antral stage. In contrast, in the second series of experiments, an oocyte from follicular development on the surface of canine ovarian tissue grafted under the thoracolumbar fascia reached metaphase I after IVM culture.

Gene banks have been established worldwide to store reproductive tissues and cells collected opportunistically from threatened wildlife species; however, to be of potential use, appropriate methods are required for optimum utilization of such materials.

Ovarian transplantation is a valuable tool that may enable the generation of mature oocytes from ovarian tissues salvaged from endangered wildlife species. Oocytes harvested from grafts could then be used in IVF or intracytoplasmic sperm injection to produce offspring. Of great advantage and in contrast to mature oocytes and embryos, ovarian tissues can be collected from females, irrespective of age and reproductive cycle, even after recent death. These factors make ovarian transplantation an attractive assisted reproductive technique for use in the conservation endangered wildlife species.

In recent years, non-human ovarian xenotransplantation studies have been extended and the feasibility of collecting oocytes from xenotransplants investigated, with oocytes successfully recovered from ovarian transplants in wallaby, cow, mouse, and marmoset (Snow *et al.*, 2002; Paris *et al.*, 2004). Fertilization and embryo cleavage have also been achieved with oocytes harvested from wallaby, marmoset, and mouse transplants to female recipients (Snow *et al.*, 2002; Paris *et al.*, 2004). Imthurn *et al.* (2000) reported the full potential of ovarian xenotransplantation, confirming (after much speculation) that live young can be produced from xenograft-matured oocytes. In this critical study, mouse ovarian tissue was xenotransplanted under the kidney capsule of bilaterally ovariectomized nude mice recipients. At graft collection, oocytes were released from the grafts, matured in vitro overnight, and fertilized in vitro. Resulting 2-cell stage embryos were transferred to foster mothers that subsequently produced healthy live young. This study suggested that oocytes that mature within ovarian grafts are normal and capable of producing normal, fertile young.

In conclusion, the advantages of grafting to peripheral sites include insertion under local anesthesia, easy monitoring by ultrasonography, and easy access for graft or oocyte recovery. By using a combination of grafting and cryopreservation, the lifespan of the female reproductive system may be prolonged.

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TABLE AND FIGURES

Table 1-1. Morphological quality and in vitro maturation of oocytes from hemi-ovaries grafted to each body site*

Grafting site	No. of oocytes collected	No. (%) of oocytes**			No. of oocytes examined	No. (%) of oocytes***				
		A	B	C		GV	D	MI	MII	Degenerated
Control	64	27 (42.2)	6 (9.4)	31 (48.4)	33	17 (54.8)	8 (25.8)	0 (0)	1 (3.2)	7 (21.2)
Kidney capsule	5	2 (40.0)	2 (40.0)	1 (20.0)	4	2 (50.0)	0 (0)	0 (0)	0 (0)	2 (50.0)
Gastrosplenic ligament	1	0 (0)	0 (0)	1 (100)	0	-	-	-	-	-
Quadriceps femoris fascia	6	0 (0)	1 (16.7)	5 (83.3)	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)

*Quartered ovaries obtained from five bitches were used for assessment of oocyte viability. Fresh quartered ovaries without transplantation were examined as controls.

**Oocyte grade: Grade A, darkly pigmented and completely surrounded by one or more layers of cumulus cells; Grade B, lightly pigmented with incomplete layers of cumulus cells; and Grade C, pale colored and irregularly shaped without any cumulus cells.

***Meiotic stage; GV: Germinal vesicle, D: Diakinesis, MI: Metaphase I, MII: Metaphase II.

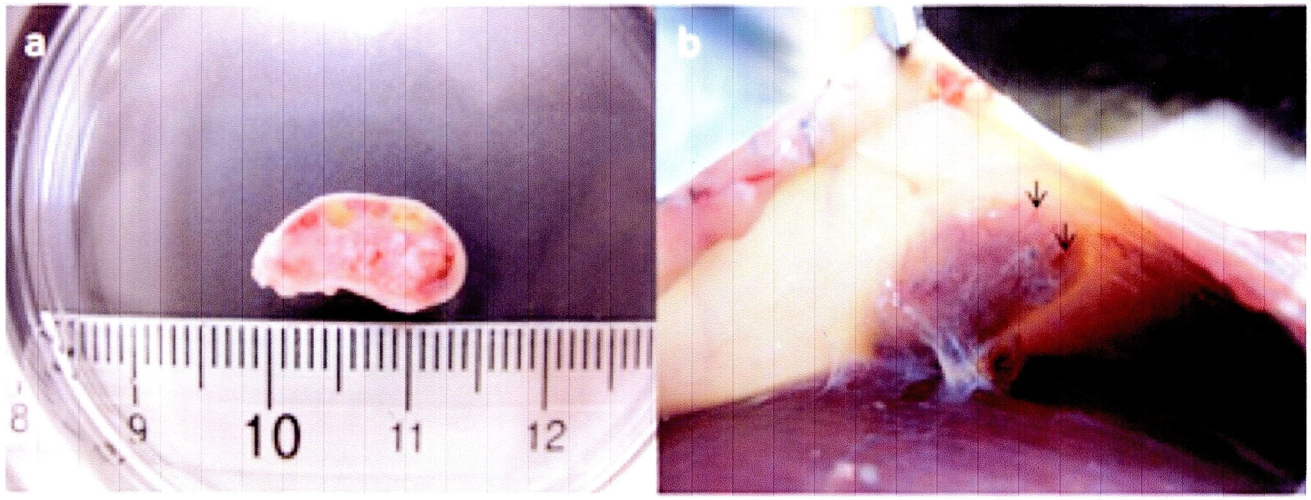

Fig. 1-1. Gross morphology of a hemi-ovary (1.4 × 0.7 × 0.4 cm) before (a) and after (b) grafting under the fascia of the quadriceps femoris muscle. The grafted ovary was surrounded by a layer of fibrous tissue and blood vessels

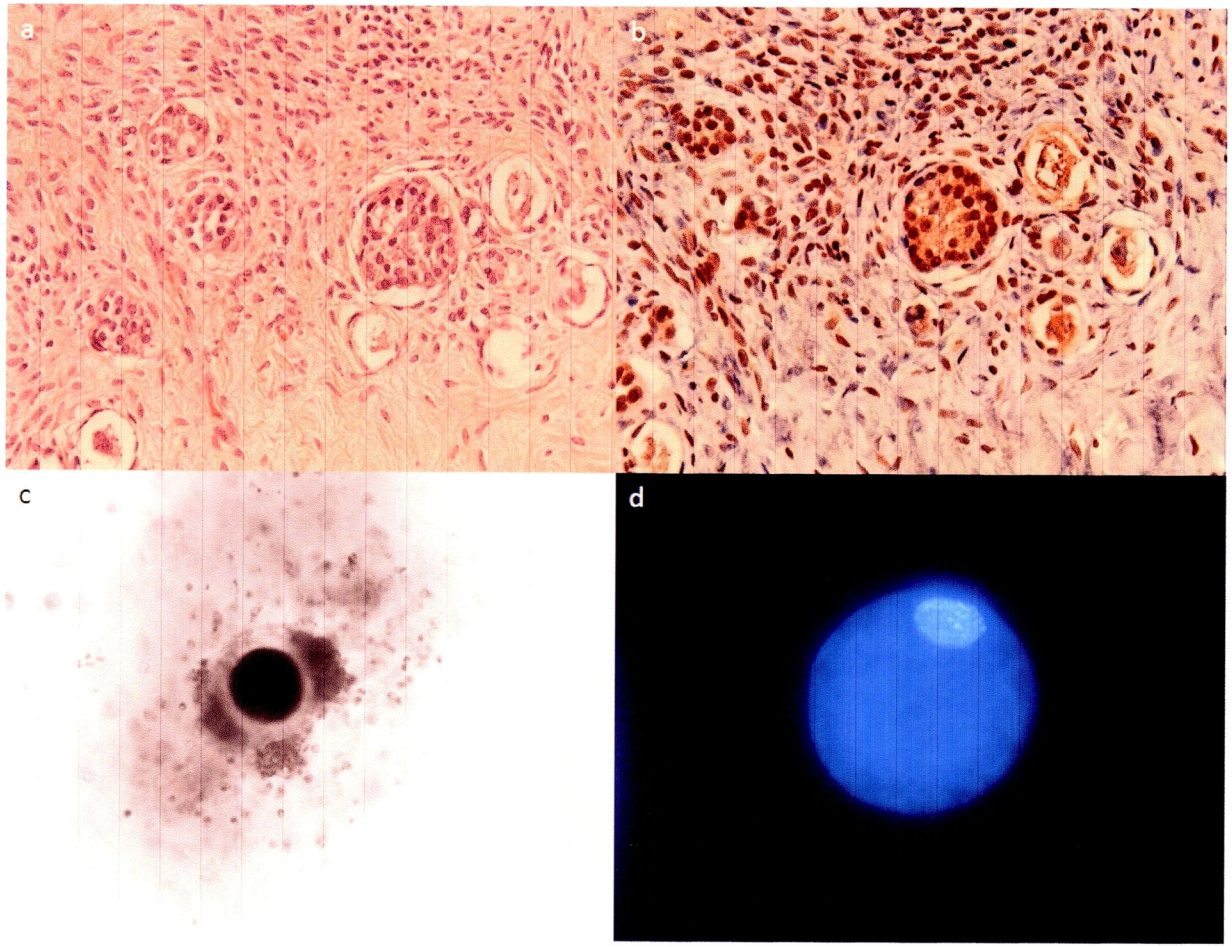

Fig. 1-2. Ovarian tissue and oocyte from a canine ovary grafted under the fascia of the quadriceps femoris muscle. Follicles in early stages of growth were present in ovarian tissue, which was stained with (A) hematoxylin-eosin, and (B) proliferating cell nuclear antigen (PCNA). In addition, note: (C) a grade B canine oocyte collected from a grafted ovary; and (D) the nuclear status of an oocyte (germinal vesicle stage) after 72 h of maturation culture (scale bars = 100μm)

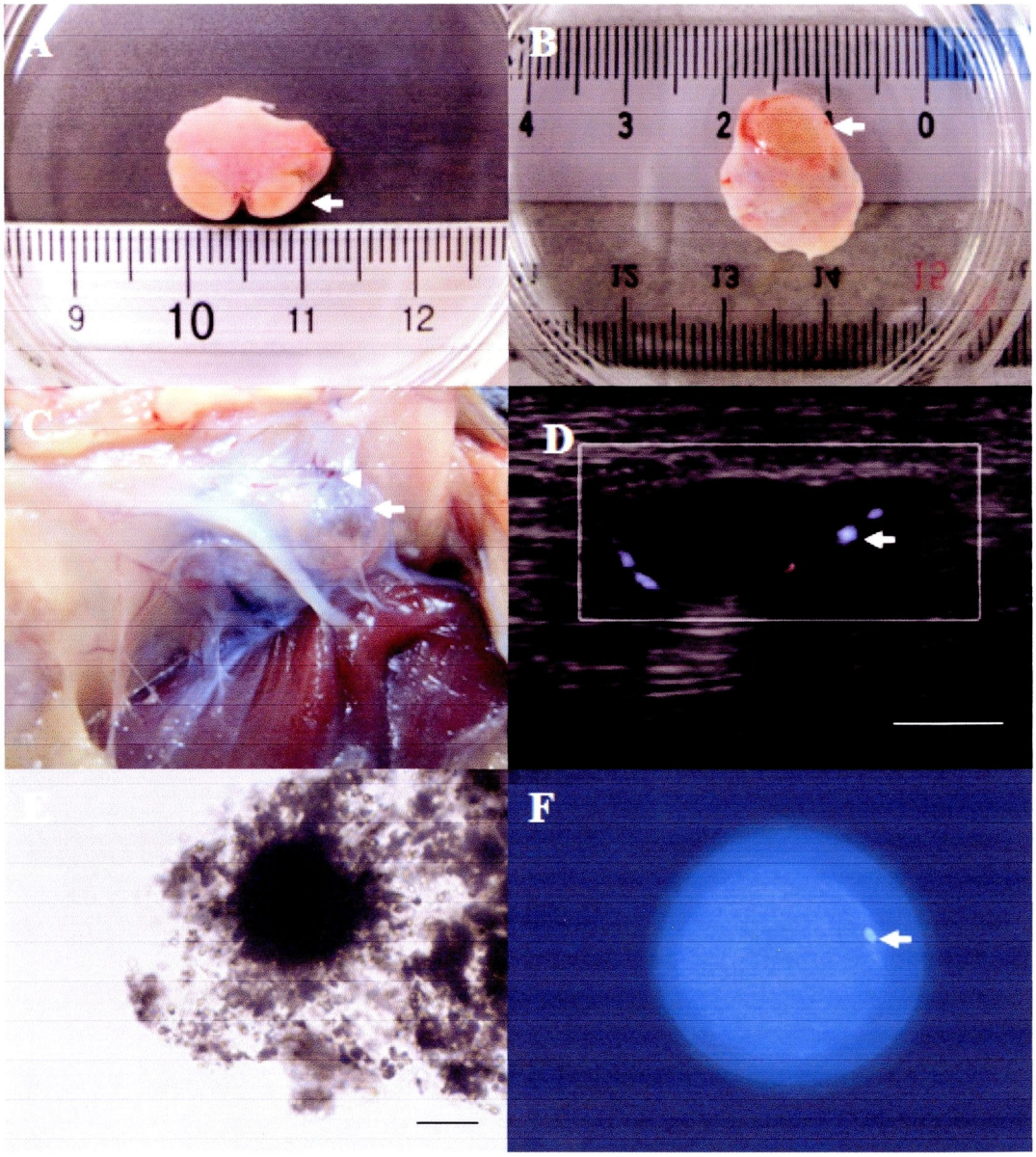

Fig. 2-1. Follicular development and oocyte recovery in an ovary grafted under the fascia of the thoracolumbar muscle. (A) Hemi-ovarian tissue prepared for transplantation. The arrow indicates the corpus luteum. (B) ovarian tissue with follicular development (arrow) 35 days after grafting. (C) Grafted ovarian tissue with follicular development (arrow) and blood vessels (arrowhead). (D) Cross section colour Doppler sonogram of a 9-mm growing antral follicle with surrounding blood flow (arrow). Bars = 100μm. (F) The nuclear oocyte reached metaphase I after 72 h of culture. The arrow indicates a metaphase chromosome.

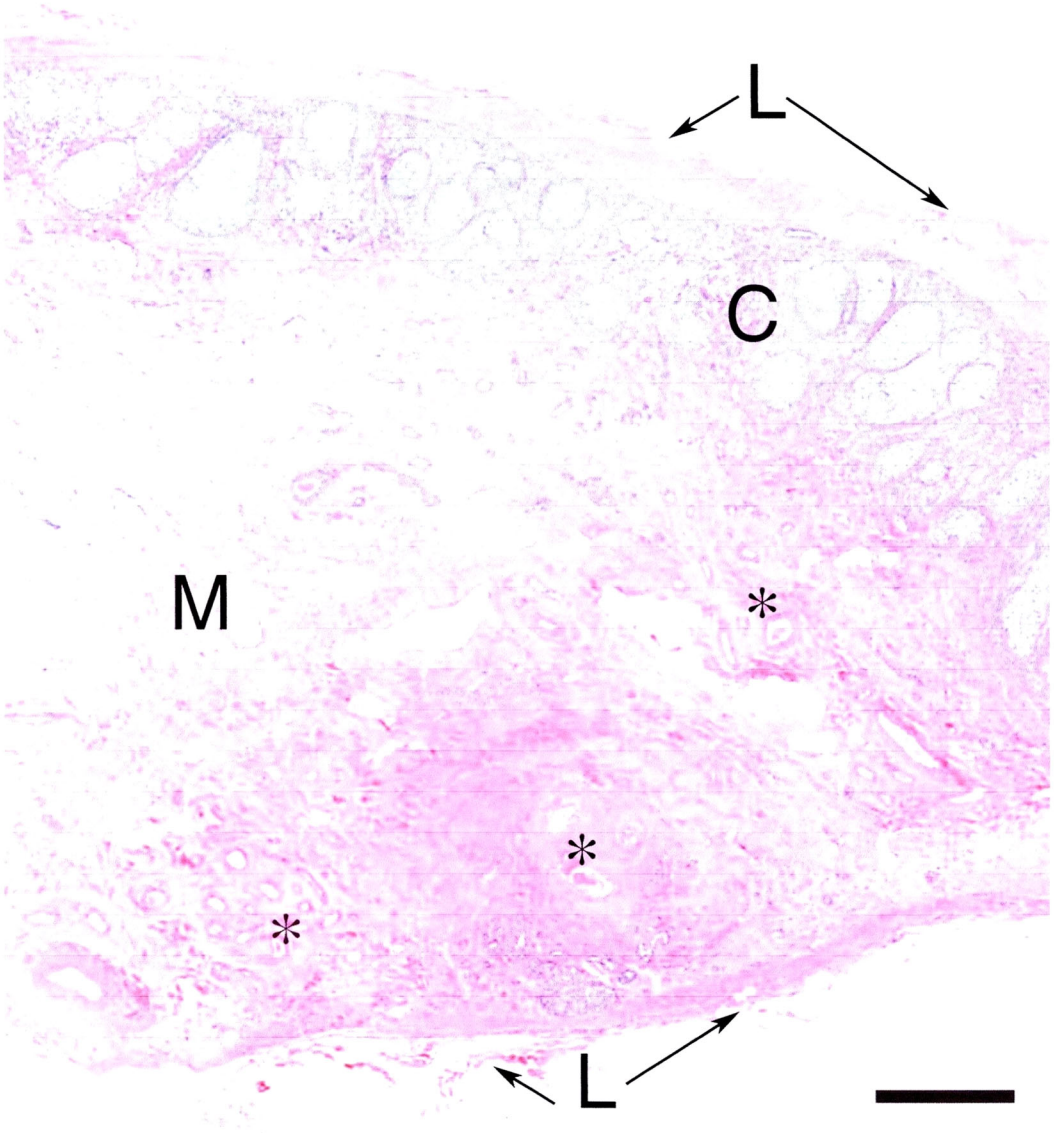

Fig. 2-2. Histological section of ovarian tissue recovered 35 days after grafting to the quadriceps femoris muscle fascia. Hematoxylin and eosin (HE) stained tissue, demonstrating a thin layer of fibrous tissue (L) surrounding the ovarian tissue, multiple follicles in the cortex, and blood vessels (*). Ovarian cortex (C) and medulla (M) are indicated in the image. Bar represents 500 μm.

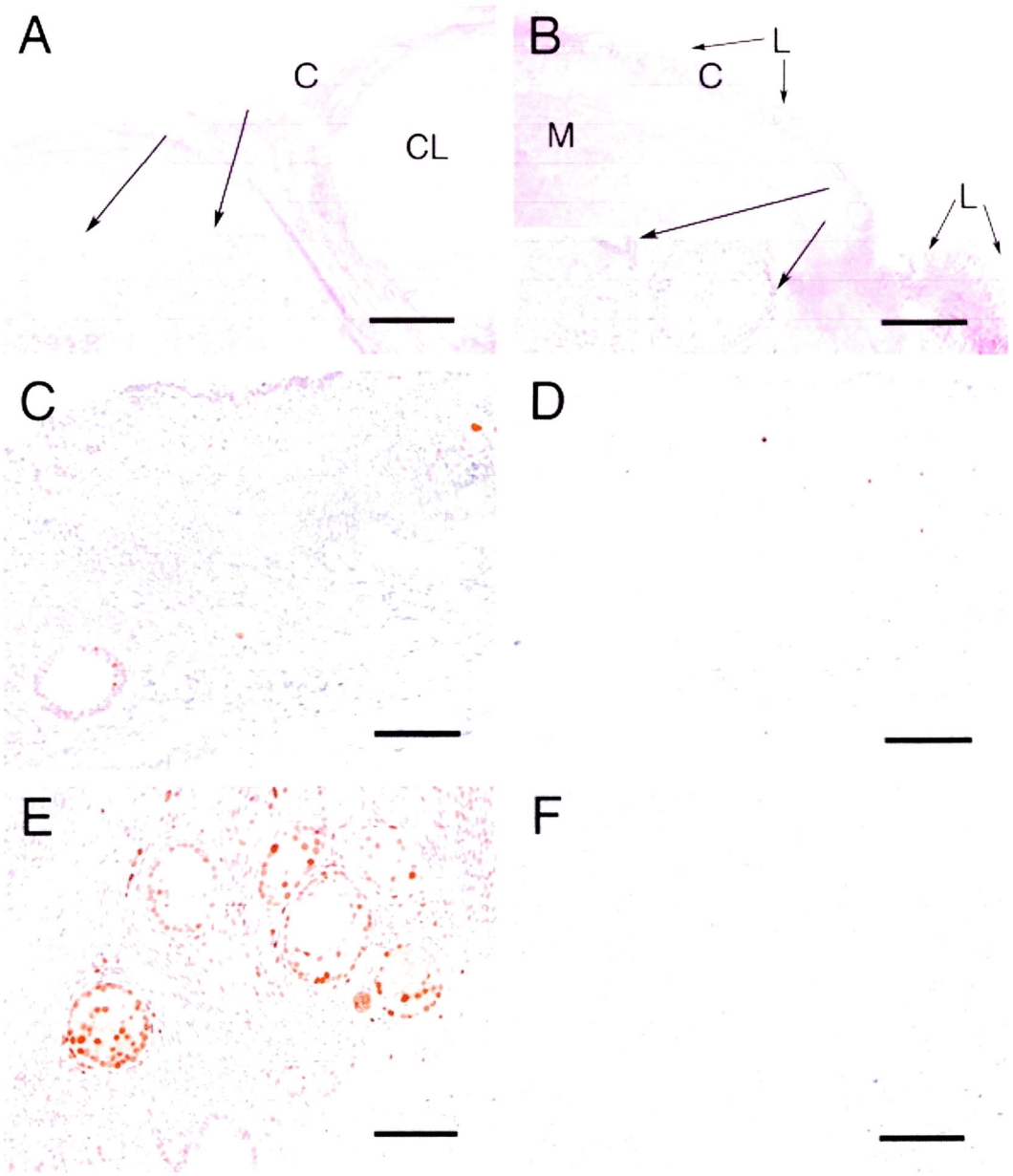

Fig.2-3. Histological appearance and expression of PCNA on ovarian images before and after grafting. Hematoxylin and eosin (HE) stained tissues from the control ovary (A) and the ovary grafted to the quadriceps femoris muscle fascia (B), demonstrating follicles in the early stages of growth. Insets represent magnified follicle cells. Ovarian cortex (C), medulla (M), corpus luteum (CL), and fibrous tissue layer (L) are indicated in A and B images. Expression of proliferating cell nuclear antigen (PCNA) in the control ovary (C) and the ovary grafted to the thoracolumbar muscle fascia (E), and negative control to C and E (D and F, respectively), showing that most follicles at different stages of development in grafted ovary displayed PCNA expression. Bars represent 500 μm in A and B or 100 μm in insets of A, B and C-F.

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