Viability of Canine Ovaries Autografted to Different Peripheral Sites

Maki Hirata[a]

Fuminori Tanihara[a]*

Masayasu Taniguchi[b]

Mitsuhiro Takagi[b]

Tsukasa Terazono[a]

Takeshige Otoi[a]

[a]Faculty of Bioscience and Bioindustry, Tokushima University, 2272-1 Ishii, Myozai-gun, Tokushima 779-3233, Japan

[b]Joint Faculty of Veterinary Science, Yamaguchi University, 1677-1, Yoshida, Yamaguchi-shi, Yamaguchi 753-8515, Japan

*All correspondence related to this paper should be addressed to:
F. Tanihara
E-mail: tanihara@tokushima-u.ac.jp
TEL: +81-88-635-0796

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ABSTRACT

Here, we investigated a suitable peripheral site for ovarian grafting that would facilitate monitoring of graft and oocyte recovery. Canine hemi-ovaries were autografted to three subcutaneous sites (under the fascia of the quadriceps femoris muscle and the thoracolumbar muscle and in the scapular deltoid muscle). All grafted ovaries were recovered from 10 bitches at 4 weeks post-transplantation and bisected for histological assessment and evaluation of oocyte viability. The longest ovarian diameters gradually decreased with the increase in the duration of grafting, irrespective of the grafting site. The mean number of total follicles was lower in the scapular deltoid muscle than in the quadriceps femoris fascia. Most follicles were classified as atretic follicles. Sixteen oocytes were recovered from 30 quartered ovaries; morphological analysis revealed three of these oocytes from the ovaries grafted under the fascia of quadriceps femoris and thoracolumbar muscles to be of good quality. Of these three oocytes, two reached metaphase I after 72 h of maturation culture. These results indicate that the environment in the fascia may be better suited for ovarian grafting than that in the intramuscular site. However, improvement of follicle survival and meiotic competence of oocytes in the grafts is necessary.

INTRODUCTION

Growing follicles are abundant within the ovaries of healthy female mammals. Although our understanding of follicular dynamics is based mainly on studies of normal ovaries in intact animals, techniques such as ovarian grafting have been expected to provide important insights into follicular development and oocyte maturation. For many mammalian species, including dogs,

information about follicular dynamics, e.g., the interval required for follicular growth and hormonal responsiveness of ovarian follicles, is not well known.

The reproductive biology of the bitches is known to be unique. For instance, ovulation occurs approximately 2-3 days after the luteinizing hormone (LH) surge, and prior to ovulation, the serum progesterone concentration begins to rise from the basal levels coincident with the LH peak (Wildt et al., 1979). There is useful endocrine information about the time of ovulation. However, there is limited information about the follicular growth through the anestrus and proestrus. Although observation of canine ovarian follicle development has been reported to be possible by real-time ultrasonography (Boyd et al., 1993; Hase et al., 2000), techniques for accurate determination or prediction of ovulation have not yet been established. Moreover, the position and small size of ovaries make imaging technically difficult. Therefore, transplantation of ovarian tissue to subcutaneous sites, such as the abdominal wall, allows the tissue to be placed at a more easily accessible site for monitoring of graft function (Paris et al., 2004).

Numerous studies have reported that follicles in the ovarian tissue are not only able to survive ovarian grafting, but also to grow from the early to advanced stages of follicular development (Gosden et al., 1994; Gunasena et al., 1998; Oktay et al., 1998; Weissman et al., 1999). The grafting site has been suggested to be very important for tissue survival and follicular development after transplantation (Soleimani et al., 2008). To date, several studies reported transplantation of ovarian tissues to different heterotopic sites (Gosden et al., 1994; Weissman et al., 1999; Oktay et al., 2001; Schmidt et al., 2005). The renal capsule became popular as a transplantation site because good blood supply is assumed to favor revascularization, and thus graft survival (Gosden et al., 1994).

However, it is difficult to access or monitor the transplanted tissue at this site. In a previous study, peripheral grafting sites such as the back muscle or the antebrachium have been demonstrated to be suitable for ovarian allografts (Oktay et al., 2001; Lee et al., 2004; Soleimani et al., 2008). The advantages of the peripheral sites are that the grafts can be conveniently inserted using local anesthetic, easily monitored by ultrasound, and easily accessed for graft or oocyte recovery. However, subcutaneous grafts have a poor blood supply and altered vascular integrity. Loss and damage of perivascular and endothelial cells were closely correlated with the loss of follicular and oocyte integrity. Thus, 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 objectives of the present study were to determine a suitable peripheral site of ovarian grafting for monitoring of the graft and to provide a method that allows the study of follicular dynamics and oocyte recovery in dogs.

MATERIALS AND METHODS
Bitches
Beagle bitches (age, 4–7 years; mean weight, 10 ± 2 kg) in a closed breeding colony were used for this study. The dogs were housed individually in stainless steel cages ($900 \times 770 \times 710$ mm), and were given standard commercial dog food once a day, and water, ad libitum. All 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.

Transplantation and Recovery Procedure
Bilateral ovariectomy was performed according to the method previously described by Terazono et al. (2012). 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. Bilateral ovariectomy was performed using routine techniques and materials. The uterine artery and vein were 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 halves using a scalpel blade. The hemi-ovaries were maintained in physiological saline at 38 °C, and then transplanted in the bitch of origin at three different 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.

Two 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 fibers. 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 using the Prosound α7 ultrasound scanner (ALOKA Co., Ltd., Tokyo, Japan) equipped with a 6–13-MHz linear-array transducer.

All grafted ovaries were recovered from the anesthetized bitches 4 weeks 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 halves using a scalpel blade. Histological assessment was carried out on one half of each grafted hemi-ovary, and the other half was used to examine the oocyte viability.

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 sections, and the four 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 deparaffinized 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 mouse anti-PCNA monoclonal antibody (38 μg/mL; PCNA Clone PC10; Sigma-Aldrich, St. Louis, MO, USA), which was diluted 1:100 in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA). Biotinylated rabbit anti-mouse IgG, IgA, and IgM (Nichirei Corp.) was applied as a secondary antibody for 30 min before treatment with peroxidase-conjugated streptavidin (Nichirei Corp.). 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 a 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 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 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 with an antral cavity) (Oktay et al., 1995). In PCNA-stained sections, the total number of follicles

Figure 1. *Oocyte from an ovary grafted into the thoracolumbar fascia. (A) Canine oocyte with Grade A was collected from the grafted ovary, and (B) the nuclear status of the oocyte reached metaphase I after 72 h of culture. The arrow indicates a metaphase chromosome. Bar = 100 μm.*
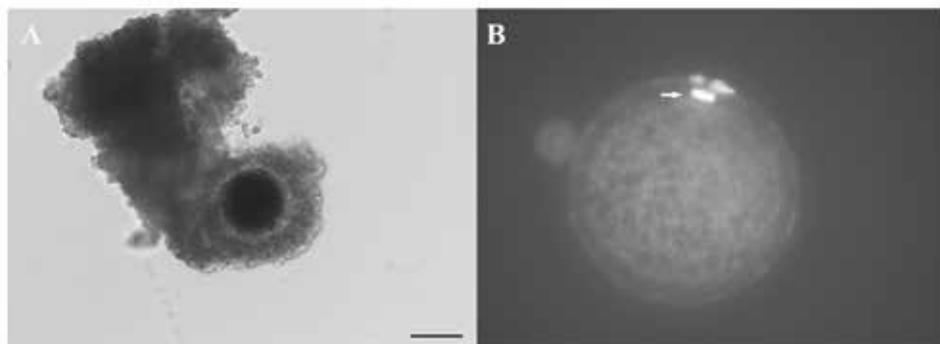
and the number of follicles with granulosa cells expressing PCNA were counted. The percentage of viable follicles was calculated by dividing the number of PCNA-positive follicles by the total number of follicles.

Oocyte Viability

Half of the recovered grafts and control ovaries were repeatedly sliced for oocyte recovery according to the method described by Terazono et al. (2012). All the oocytes were collected, and then suspended in culture medium (TCM199 medium [Earle's salts] buffered with 25 mmol HEPES buffer [Invitrogen] supplemented with 10% (v/v) fetal bovine serum [FBS; Invitrogen] and 50

μg/mL gentamicin [Sigma-Aldrich]). The oocytes were microscopically examined for morphological quality, and categorized based on the system described by Hewitt et al. (1998) as follows: Grade A oocytes were darkly pigmented and surrounded by one or more layers of cumulus cells (Fig. 1A). 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 of culture medium covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm; Falcon; Becton Dickinson Labware, Franklin Lakes, NJ,

Table 1. *Number of follicles in ovarian tissues after grafting to each body site**

Grafting site	Number of follicles per graft					Percentage of viable follicles**
	Total follicles	Primordal follicles	Primary follicles	Secondary follicles	Atretic follicles	
Control	162.6 ± 24.1[a]	20.1 ± 11.8[a]	6.6 ± 4.6[a]	3.8 ± 1.0[a]	132.1 ± 14.3[a]	13.2 ± 5.3[a]
Quadriceps femoris fascia	117.8 ± 21.7[a,b]	1.4 ± 0.7[b]	0.4 ± 0.3[a,b]	0.3 ± 0.2[b]	115.7 ± 21.0[a]	1.9 ± 0.9[b]
Thoracolumbar fascia	88.7 ± 16.1[b,c]	1.0 ± 0.8[b]	0[b]	0[b]	87.7 ± 15.6[a]	0.8 ± 0.5[b]
Scapular deltoid muscle	38.1 ± 10.4[c]	0.3 ± 0.3[b]	0[b]	0[b]	37.8 ± 10.3[b]	0.5 ± 0.5[b]

*The quartered ovaries obtained from 10 bitches were used for histological assessment. The fresh quartered ovaries without transplantation were examined as control. Data are expressed as means ± SEM.
**The percentage of viable follicles was calculated by dividing the number of PCNA-positive follicles by the total number of follicles.
a–cValues with different superscripts in the same column are significantly different (P < 0.05).

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 bisbenzimide (Hoechst 33342; Sigma-Aldrich) on a slide (Otoi et al., 2002). The oocytes were examined using a fluorescence microscope with a 355-nm excitation wavelength filter, and classified according to chromatin configuration as "germinal vesicle," "condensed chromatin," "metaphase I, (Fig. 1B)," or "metaphase II." The oocytes with diffusely stained cytoplasm, characteristics of non-viable cells, and those wherein the chromatin was unidentifiable or not visible were considered degenerated.

Statistical Analysis

Statistical significance was inferred from analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Percentage data were subjected to arcsine transformation before statistical analysis. Differences at a probability value (P) ≤ 0.05 were regarded significant.

RESULTS

Ovarian Diameter

The profiles of ovarian diameters during grafting at the proximal portion of the body surface are shown in Figure 2. The longest ovarian diameters gradually decreased with the increase in the duration of grafting, irrespective of the ovarian grafting site. In the ovaries grafted in the scapular deltoid muscle, the mean ovarian diameters at 3 and 4 weeks after transplantation were significantly shorter (P < 0.05) than that at the time of transplantation (0-week).

Histological Assessment of Autografts

As shown in Table 1, the mean number of

Figure 2. Profiles of the mean longest diameters in the hemi-ovaries after grafting into the quadriceps femoris fascia (-●-), the thoracolumbar fascia (-▲-), and the scapular deltoid muscle (-■-). The sizes of ovarian tissues were measured by ultrasound scanner every week until graft collection. The asterisks indicate significant differences compared with the ovarian size immediately after grafting to each body site (P < 0.05).
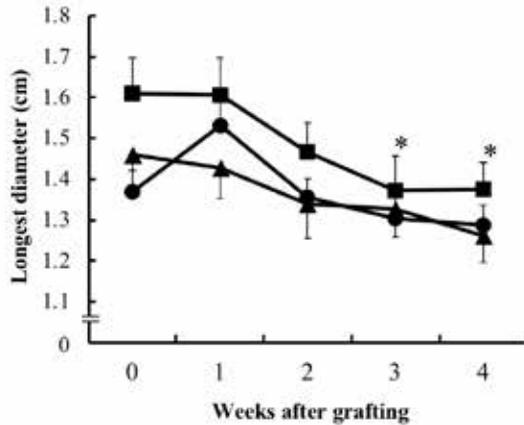
total follicles in the ovaries grafted in thoracolumbar fascia and the scapular deltoid muscle decreased (P < 0.05) as compared with that of the control non-grafted ovaries (88.7 and 38.1 vs. 162.6). Moreover, the mean number of total follicles was significantly lower (P < 0.05) in the scapular deltoid muscle than in the quadriceps femoris fascia. In both HE- and PCNA-stained graft sections, most follicles were classified as atretic follicles, and antral follicles were not observed in the grafted and control ovaries. The percentage of viable follicles in the sections from the grafted ovaries was significantly lower (P < 0.05) than that in the sections from the control ovaries, irrespective of the ovarian grafting site. There were no differences in the percentages of viable follicles among the grafted ovaries.

Quality and Meiotic Competence of Oocytes from Autografts

A total number of 16 oocytes were recovered from 30 quartered ovaries from 10 bitches (Table 2). Of which, three (18.8%) oocytes from the ovaries grafted under the fascia of quadriceps femoris muscle and thoracolum-

bar muscle were categorized as Grade A. Two oocytes reached metaphase I (MI) after in vitro maturation (IVM) culture, but none of the oocytes from the grafted ovaries reached metaphase II (MII). In the control hemi-ovaries, a total number of 183 oocytes were recovered from 10 quartered ovaries, of which 20.8% and 15.3% were categorized as Grades A and B, respectively. Only three (6.1%) out of 49 oocytes reached MII after IVM culture.

DISCUSSION

Transplantation of the ovarian tissue to a position other than its normal anatomical position allows the tissue to be placed at a more convenient and easily accessible site for surgery and monitoring of the graft function. In the present study, we found that the mean diameter of ovaries grafted in the proximal portion of the body surface gradually decreased with the increase in the duration of grafting, irrespective of the ovarian grafting site. Although surviving follicles could be observed in the ovarian tissues after 4 weeks of ovarian grafting, the percentage of the viable follicles and the total number of the recovered oocytes from the grafted ovaries decreased compared to the control non-grafted ovaries. 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). The duration of ischemia prior to revascularization of the transplanted ovarian tissue adversely affect the oocytes and growing follicles (Bols et al., 2010). In particular, damage to the perivascular and endothelial cells has an influence on the integrity of ovarian follicles and oocytes in ovarian grafts (Israely et al., 2003). Therefore, the grafts in the less vascularized recipient sites, such as the subcutaneous sites, might take longer time to revascular-

Table 2. 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	CC	MI	MII	Degenerated
Control	183	38 (20.8)	28 (15.3)	117 (63.9)	49	20 (40.8)	5 (10.2)	8 (16.3)	3 (6.1)	13 (26.5)
Quadriceps femoris fascia	5	1 (20.0)	0 (0)	4 (80.0)	1	0 (00)	0 (0)	1 (100)	0 (0)	0 (0)
Thoracolumbar fascia	7	2 (28.6)	0 (0)	5 (71.4)	2	1 (50.0)	0 (0)	1 (50.0)	0 (0)	0 (0)
Scapular deltoid muscle	4	0 (0)	0 (0)	4 (100)	0	-	-	-	-	-

*The quartered ovaries obtained from 10 bitches was used for assessment of oocyte viability; The fresh quartered ovaries without transplantation were examined as control.
**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, Grade C: pale colored and irregularly shaped without any cumulus cells.
***Meiotic stage; GV: Germinal vesicle, CC: Condensed chromatin, MI: Metaphase I, MII: Metaphase II.

ize, prolonging graft ischemia and reducing follicular survival (Imthurn et al., 2000).

The renal capsule graft site has been suggested to have a relatively good blood supply that facilitates the revascularization process, which is essential for graft establishment and support of follicular growth (Gosden et al., 1994). However, in a previous study, we demonstrated that peripheral sites, such as the fascia lata, were equivalent to the renal capsule for transplantation of canine ovarian tissues (Terazono et al., 2012). The transplantation site has been suggested to have direct consequences regarding the number and quality of oocytes that can be harvested from the grafted ovarian tissues (Yang et al., 2006). Therefore, in the present study, canine hemi-ovaries were autografted to three subcutaneous sites to determine a suitable peripheral site of ovarian grafting via assessment of the follicular and oocyte survival.

Our results showed that the percentage of viable follicles was similar among the grafted ovaries. However, the mean number of total follicles in the ovaries grafted in the scapular deltoid muscle was lower compared to that in the ovaries grafted in the quadriceps femoris fascia. No oocytes with good quality (Grades A and B) were recovered from the ovaries grafted in the scapular deltoid muscle. These results indicate that the scapular deltoid muscle is inferior to the fascia of the quadriceps femoris and thoracolumbar muscles for the grafting of canine ovaries. We transplanted the hemi-ovaries into the intermuscular space in the scapular deltoid muscle, whereas the other hemi-ovaries were inserted under the fascia of the quadriceps femoris and thoracolumbar muscles. Israely et al. (2003) reported that ovarian maintenance was better in the intramuscular transplants than in the subcutaneous region. They suggested that the rich blood supply within the muscle provided superior graft reception. Therefore, an abundant blood supply, similar to that of the renal capsule, was predicted when the hemi-ovaries were grafted into intermuscular

space. However, our results indicate that the fascia environment may meet the condition required for sufficient blood supply, whereas the intramuscular site does not.

It has been well known that canine oocytes can resume meiosis in vitro. However, the meiotic competence of oocytes is very low (Farstad, 2000). In this study, only 6.1% of the control oocytes with good quality could reach MII after IVM culture. On the other hand, oocytes recovered from the grafted ovaries remained at the MI stage. These results were similar to those of our previous study, in which the oocytes recovered from the ovaries grafted to peripheral sites could not reach MII (Terazono et al., 2012). It has been demonstrated that the estrous cycle stage of the donor influences the IVM rates of the canine oocytes collected from ovaries obtained by ovariohysterectomy (Yamada et al., 1993; Otoi et al., 2001).

In the present study, ovaries were collected from bitches whose estrous stages were classified to diestrus (five heads) and anestrus (five heads). When progesterone and estradiol concentrations were examined every 7 days, starting from transplantation to removal of ovaries (data not shown), there was no progressive rise in progesterone and estradiol concentrations, and the both concentrations remained relatively low during grafting (< 1.32 ng/mL and < 12.4 pg/mL, respectively). We confirmed the presence of viable follicles after 4 weeks of grafting in the proximal portion of the body surface. However, the viable follicles and the total number of recovered oocytes were very low. It has been suggested that folliculogenesis from the primordial to the preovulatory stage may take much longer in larger animals than in rodents (Van den Broecke et al., 2001). Moreover, the ovarian cycle of bitches is unique among those of domestic animals in that bitches are monoestrous with a 2-month luteal phase and a prolonged but variable non-seasonal anestrus of 3–10 months after both pregnant and nonpregnant cycles. Therefore, the small number of viable follicles and low meiotic competence of

oocytes recovered from grafts might indicate that a cohort of follicles had insufficient time to expand or that a proportion of follicles of all size-groups died, leaving a small number of each type. Moreover, transplantation of the ovarian tissue to heterotopic sites might affect the quality and developmental potential of oocytes (Yang et al., 2006).

Our results indicate that the grafting of canine ovaries under the fascia may be better than grafting into the intermuscular space with respect to follicular and oocyte survival. However, the viable follicles and quality and meiotic competence of the recovered oocytes from the grafted ovaries decreased after 4 weeks of transplantation, irrespective of the ovarian grafting site. Further investigation, including longer periods of grafting, is necessary to improve the survival of the follicles in the ovaries grafted to peripheral sites.

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