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CLINICAL ARTICLE

Use of porcine small intestinal submucosa to reconstruct an ovarian defect ☆

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ABSTRACT

Objective: To investigate the feasibility of using porcine small intestinal submucosa (SIS) as a scaffold for repairing ovarian defects. *Method:* Fourteen female New Zealand rabbits undergoing ovarian resection were randomly allocated to 2 equal groups. The unilateral ovarian defects were repaired with SIS in group 1 animals and without SIS in group 2 animals (control). The volumes of the ovaries were calculated and the severity of adhesions was assessed in 1 animal from each group each month. The ovaries were removed and examined under a microscope. *Results:* The volumes of the SIS-grafted ovaries were larger than those of the operated ovaries of the control animals ($P<0.05$). The SIS-grafted ovaries had a lower adhesion score than the operated ovaries of the control group ($P<0.001$). SIS grafts showed hemorrhage and leukocyte infiltration until the 4th week after surgery, but the ovarian tissue appeared to be well organized from the 12th to the 16th week. At the 28th week, primordial follicles were scattered in the SIS graft. *Conclusion:* SIS graft could be used for repairing the ovary after surgery.

1. Introduction

In addition to genetic factors, chemotherapy, radiotherapy, pelvic infections, tubal disease, severe endometriosis, and heavy smoking are well-known causes of ovarian insufficiency [1,2]. Surgical excision of ovarian cysts can affect the follicular reserve and reduce the efficiency of ovarian stimulation [2]; ovarian failure can occur after both wedge resection and laparoscopic surgery [2].

Advances in tissue engineering have allowed the regeneration and repair of damaged tissues and organs [3]. It is essential that the biomaterial is biocompatible with the particular tissue that is to be repaired. Porcine small intestinal submucosa (SIS) is the preferred biomaterial because of its structural characteristics. Lantz et al. [4] implanted vascular grafts of feline, porcine, equine, ovine, caprine, bovine, and human jejunum SIS into canine femoral arteries to compare their strength and overall performance. Only feline and porcine SIS grafts were effective. Failure of SIS from other species was due to inadequate durability, inability of the graft to retain sutures, and thrombogenicity [4]. Porcine SIS has already gained approval from the US Food and Drug Administration (FDA) and is widely used as a biomaterial that has a minimum immune response [5]. Porcine SIS is constructive and often remodels itself toward a structure that resembles the injured native tissue [6]. It has already been used as a xenograft scaffold in dermal, cardiovascular, dura mater, urinary bladder, and orthopedic applications in various animal models [7,8], and encouraging results have been obtained for tissue regeneration and functional recovery without immunologic rejection [5].

The biocompatibility of porcine SIS with the ovary either in vitro or in vivo has not been reported. A recent study has indicated that oocyte-derived transforming growth factor-β (TGF-β) and fibroblast growth factor-2 (FGF-2) play key roles in folliculogenesis [9]. SIS is naturally impregnated with glycosaminoglycans, FGF-2, and TGF-β [10], which play important roles in the growth of the follicle. The aim of the present study was to examine the biocompatibility of porcine SIS for repairing the rabbit ovary, remodeling, and follicle development in an in-situ xenograft model.

2. Materials and methods

The preparation of SIS has been described previously [11]. Briefly, freshly harvested pig jejunum was gently cleaned and inverted, and the superficial layer of tunica mucosa was removed by scraping with a knife. The tissue was then reverted to its original orientation, and the

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serosa and tunica muscularis were removed. The remaining tunica submucosa with basilar layers, consisting, almost entirely, of extracellular matrix with a small number of intact cells was stored in phosphate-buffered saline containing 0.5% gentamycine sulfate at 4 °C until it was used.

The study was carried out in the Experimental Research Laboratory of Inonu University. It was approved by the Ethics Committee and complied with the guidelines for care and use of experimental animals. Fourteen female New Zealand white rabbits (body weight 2.5–3.0 kg; 12 to 15 weeks old) were purchased from the Animal Laboratory of Inonu University. The rabbits were housed in plastic cages and kept under standard conditions of 12-hour light and 12-hour dark periods, 20 °C constant temperature, and a humidity range of between 40% and 60%. The rabbits had free access to standard dry pellets and tap water during the study. The rabbits were arbitrarily assigned to two equal groups. Animals in group 1 were managed with SIS at wedge resection; animals in group 2 underwent wedge resection only. The contralateral ovary of each individual animal was asssigned as a local control for the treated ovary. After achieving an anesthetic plane appropriate for surgery, an incision was made approximately 5 cm superior to the pubic bone.

In group 1, an ovarian wedge resection was performed using electrosurgery (40 W, cutting) and approximately one-third of the ovary was removed. The site where the ovary was removed was filled with the SIS graft and sutured to the adjacent tissue from the operated ovary with 7-0 Vicryl (Ethicon, Somerville, NJ, USA). The 7 rabbits in group 2 underwent unilateral ovarian wedge resection without an SIS graft, and the site of the surgery was reapproximated using 7-0 Vicryl. Bleeding was controlled by needle-tip electrocautery for approximately 1 second.

After hemostasis was achieved, the rectus fascia was sutured with 2-0 silk suture, and the skin was closed with a 4-0 Prolene suture (Ethicon, USA). The rabbits were closely monitored after the surgery, and as they became stable they were placed into cages and monitored twice a day for their level of activity, oral intake, wound status, and fever. Each animal received both intraoperative and postoperative antibiotics for 4 days.

After surgery, 1 animal from each group was killed at 1-month intervals. First, their ovaries were examined to assess both the acute cellular response to the xenogeneic implant and the subacute and chronic responses of angiogenesis, neomatrix deposition, cellular proliferation and differentiation, and scaffold degradation. The extent and severity of adhesions at the site of the surgery were evaluated by using Mazuji's scoring system (Table 1) [12]. Second, both ovaries of each animal were surgically removed, gently rinsed in saline solution and their dimensions (length, width, and height) were measured using digital calipers; the volumes of the ovaries were calculated using ellipsoid formulae ($\pi/6 \times \text{length} \times \text{width} \times \text{height}$). Third, the graft site, along with the adjacent normal tissue was dissected and placed in 10% neutral buffered formalin for histologic examination. Later, the formalin-fixed tissues were embedded in paraffin wax, sectioned at 5-μm thickness, and stained with hematoxylin and eosin (H&E), Masson's trichrome, proliferating cell nuclear antigen (PCNA), inhibin-α subunit or c-Kit.

Histologic slides were evaluated for epithelization, inflammation, graft integration, tissue granulation, vascularity, stromal cells, and primordial follicles under light microscopy, and subjectively scored as shown in Table 2. For each ovary, follicles were evaluated according to Hutt et al. [13] at least 2 tissue sections were assessed in all follicles within 10 selected regions at a magnification of ×300.

SPSS version 11.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The significance of differences between the groups was obtained by Mann-Whitney U test ($P<0.05$), and the results are given as mean ± standard error of the mean (SEM).

Table 1
Mazuji's scoring system for the extent and severity of adhesions.

Grade	Observation
1	No adhesion
2	Very thin and pathological adhesion
3	Easily detectable moderate adhesion
4	Dense, continuous adhesion (adhesiolysis is not difficult)
5	Dense adhesion (adhesiolysis is difficult)

Table 2
A subjective grading system for scoring the degree of histologic change and integration of SIS graft into native ovary.

Scores	0	1	2	3
SIS organization	Totally disorganized	Slightly organized	Moderately organized	Well organized
Granulation tissue	None	Minimal	Moderate	Marked
SIS epithelization	None	25%	50%	75%–100%
PCNA+ cells	None	1–10	10–20	>20
PMNs	None	1–10	10–20	>20
Vascularity (blood vessels)	None	1–5	5–10	>10
Primordial follicles	None	1–5	5–10	>10

Abbreviations: SIS, small intestinal submucosa; PCNA, proliferating cell nuclear antigen; PMNs, polymorphonuclear leukocytes.

3. Results

The volumes of the SIS-grafted ovaries were larger than those of the operated ovaries of the control animals (140.25 ± 8.78 mm^3 vs 109.14 ± 8.15 mm^3, respectively, $P<0.05$). The operated ovaries were smaller than the contralateral ovaries in the control animals (109.14 ± 8.15 mm^3 vs 138.30 ± 8.97 mm^3, $P<0.05$), but the SIS-grafted ovaries were comparable in size to the contralateral ovaries of the group 1 animals (140.25 ± 8.78 mm^3 vs 151.21 ± 9.96 mm^3, $P>0.05$). The SIS-grafted ovaries had a lower adhesion score than the operated ovaries of the control group (1.14 ± 0.14 vs 3.85 ± 0.26, $P<0.001$). All contralateral intact ovaries had normal volumes, appearance, and were free of adhesions.

Examination of the ovaries under a microscope showed that the SIS-grafted ovaries and operated ovaries of the control animals differed qualitatively in the pattern of healing. The tissue surrounding the SIS graft had an appearance similar to that of the contralateral normal ovary, and except for a minor inflammatory response to the suture material, the native ovarian tissue was not affected by the SIS graft.

As shown in Fig. 1, at the 4th week after surgery, the response of host tissue to the SIS material was hemorrhage and cellular infiltration that included a mixture of polymorphonuclear leukocytes (PMNs); the PMN response in the SIS-grafted group was greater than that in the control group (2.28 ± 0.28 vs 1.14 ± 0.14; $P<0.008$) and mononuclear cells, but this was rapidly diminished to a negligible level by the 8th week. At the 12th week, deposition of neomatrix consisting of amorphous collagenous connective tissues and trichrome- and inhibin-stained cells were observed. Most of these cells resembled pregranulosa cells in morphology but were not associated with any of the associated structures. Although connective tissue deposition was similar in both groups, by the third month the SIS-grafted site showed a larger organization of neoconnective tissue (1.71 ± 0.42 vs 0.28 ± 0.18; $P<0.015$). The average score of vascularity of the remodelled tissue at the SIS site was not significantly higher than that of the control group (1.71 ± 0.18 vs 1.57 ± 0.20; $P=0.59$). At the 16th week after surgery, the SIS graft and adjacent ovarian tissue were well organized and the primordial and growing primary follicles had accumulated around the SIS–ovary boundary (Fig. 2). At the 20th–24th week, some primordial follicles were concentrated at the boundary of the SIS graft (Fig. 3). From the 28th week, primordial and growing primary follicles were well organized on the SIS boundary as well as in the SIS material, so that the SIS material could not be distinguished from ovarian tissue

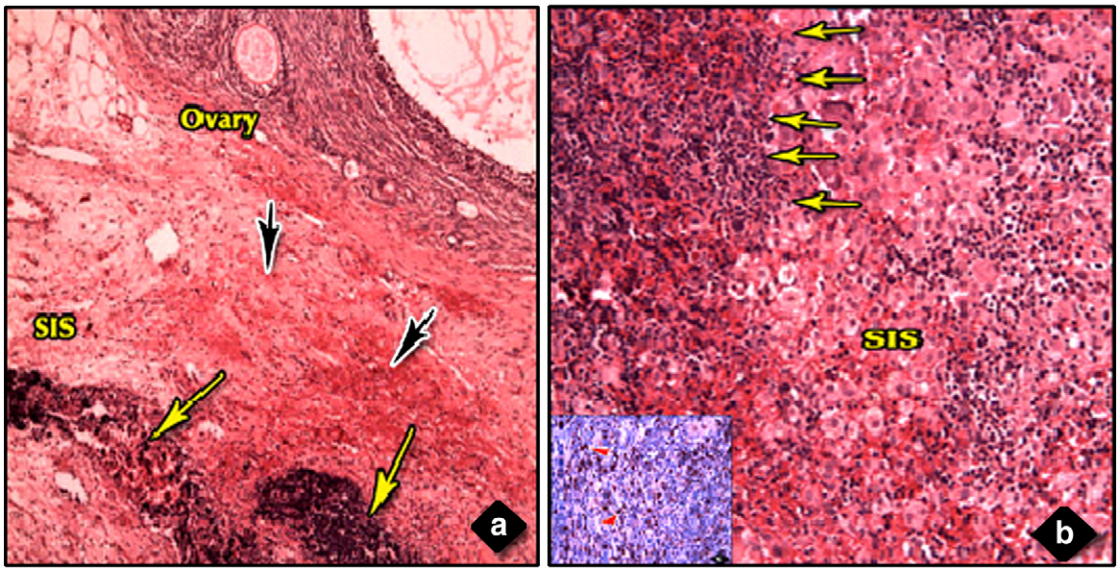

Fig. 1. Histologic response of host rabbit ovarian tissue to the porcine small intestinal submucosa (SIS) scaffold 4 weeks after repair. At this stage: (a) except for minor hemorrhages (black arrows) and lymphoid tissue (yellow arrows), the SIS-grafted layer was well organized (hematoxylin and eosin [HE] ×100); (b) inflammatory cells (some of which are indicated by yellow arrows) were abundant throughout the SIS–ovary boundary (HE ×200), and some cells among these inflammatory cells were stained with proliferating cell nuclear antigen (inset, red arrows, ×40).

(Fig. 4). However, no preantral or antral follicles were observed in the SIS material.

Immunohistochemistry studies of the tissue sections stained with PCNA, inhibin-α or c-Kit were used to confirm whether follicle-like structures found around and in the SIS graft were primordial follicles. At the 4th week after the surgery, stromal cells stained with PCNA, and at 24th week granulosa cells, primordial and primary follicles stained with PCNA. Oocyte PCNA staining was first seen in oocytes of primordial follicles and later in oocytes of other follicles. At the 28th week, the average score of the PCNA-stained stromal cells in the SIS-grafted group was higher than that in the control group (2.4 ± 0.42 vs 1.28 ± 0.28; $P < 0.028$). The ovarian surface epithelial cells surrounding the SIS graft, and normal ovarian tissue as well as most of the primordial and early primary follicles showed a marked level of PCNA staining, including those at the SIS–ovary boundary (Fig. 2). The primordial follicles together with some cells that might be stromal cells in the SIS graft also showed a good level of inhibin-α staining (Fig. 2). From the 8th to the 28th week c-Kit protein was observed in all specimens, but it was noticeably spread throughout the cytoplasm of the follicles from the 24th week. The surface epithelium, ovarian stroma, and pregranulosa cells of primordial follicles were not stained with c-Kit antibody. At the 8th week after surgery, some unidentified cells (distinct from fibroblasts) in the SIS graft stained with c-Kit. At the initial stage of follicle formation, the granulosa cells of primordial follicles did not show any staining, but as the follicle developed, c-Kit staining was detected in granulosa cells of primary and secondary

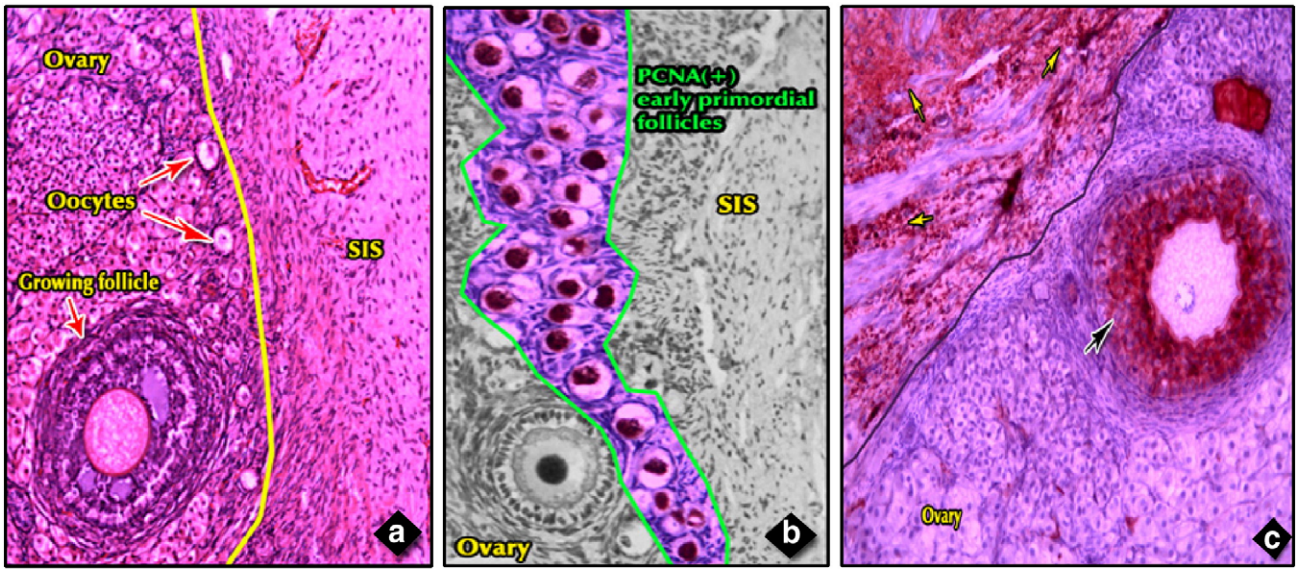

Fig. 2. Histoarchitecture of the rabbit ovary 16 weeks after the repair. At this stage, (a) a group of oocytes (red arrows) were present at the boundary of the cortex and porcine small intestinal submucosa (SIS) graft (yellow line) (hematoxylin and eosin ×100); (b) all of the oocytes that were present at the SIS–ovary boundary were stained with proliferating cell nuclear antigen (bounded by a green line, ×100); (c) both granulosa cells in the follicle wall (black arrow) and stromal cells at the SIS site (yellow arrows) were heavily stained with inhibin (×100). The boundary of SIS–ovary is shown with a blue line.

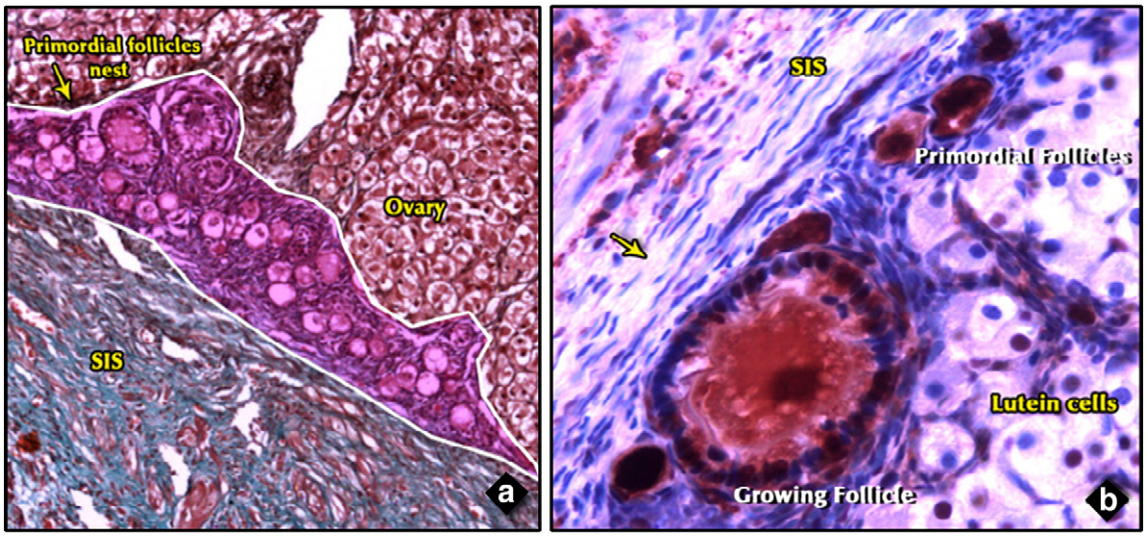

Fig. 3. Histoarchitecture of a porcine small intestinal submucosa (SIS)-grafted rabbit ovary 24 weeks after surgery. At this stage: (a) the SIS-grafted ovary comprised primarily primordial and growing follicles located very close to the SIS graft (bounded by a white line, Trichrome, ×100); (b) oocytes and granulosa cells of these follicles were stained with proliferating cell nuclear antigen, and were surrounded by lutein cells and SIS matrix (arrow, ×400).

follicles including small antral follicles. In the control group, however, none of the cells of the fibrovascular tissue stained with PCNA, inhibin-α, or c-Kit. Signs of follicle formation in fibrovascular tissue were not observed except in those in the adjacent normal ovarian tissue covering the fibrovascular tissue.

Epithelization in the SIS graft was partial until the 16th week after surgery, but it increased to 75% by the 20th week, and was almost complete by the 28th week. Interestingly, at the 28th week, the operated ovaries of the control animals revealed incomplete epithelization (only 25%). A similar amount of granulation tissue was detected in the SIS-grafted and control groups until the 8th week (1.0 ± 0.43 vs 2.14 ± 0.26; $P > 0.05$). From the 16th week, it had resolved in the SIS-grafted group but not in the control group. No signs of graft rejection were found in the tissue samples of the SIS-grafted ovary stained by H&E.

4. Discussion

Previous studies have shown that partial destruction of ovarian tissue, generally due to ovarian surgery, results in a reduction of the ovarian reserve leading to an inferior response in stimulated ovaries compared with the response of normal ovaries [14,15]. Even though an ideal material for ovarian defect repair has not yet been determined, SIS is a suitable material for surface epithelial cell attachment and follicle development because it possesses exclusive biological characteristics and functional growth factors that are essential for site-specific remodeling [10,11].

Porcine SIS was not recognized as a foreign protein by native ovarian tissue in the present study. This could be attributable to its restricted immunologic response to the Th2 pathway, which brings

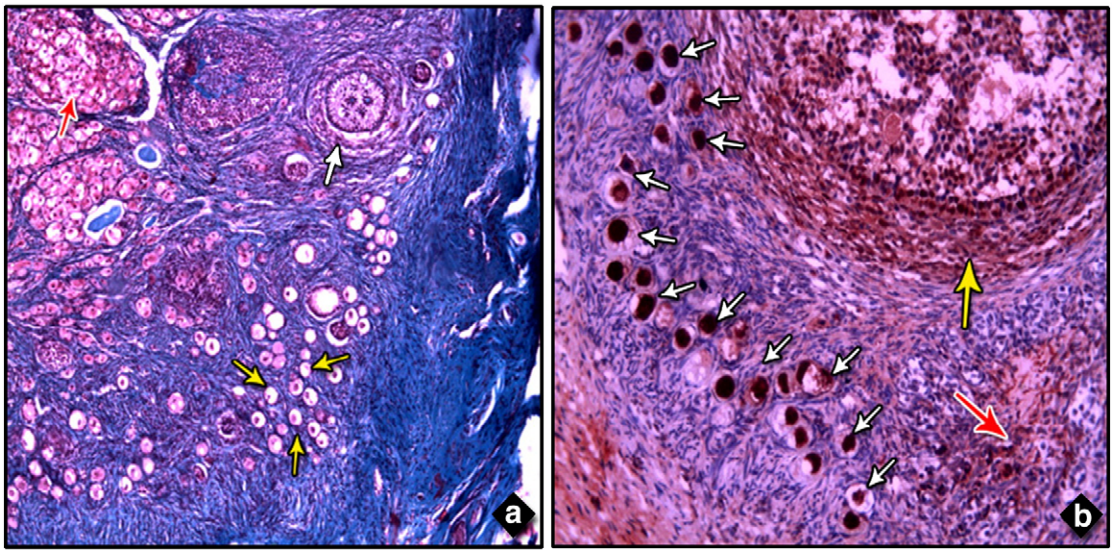

Fig. 4. Histoarchitecture of porcine small intestinal submucosa (SIS)-grafted rabbit ovary 28 weeks after surgery. At this stage: (a) the SIS graft has almost adapted itself to the ovarian tissue, and despite being irregularly ordered, numerous primordial follicles (yellow arrows), primary follicles (white arrow), and luteal cells (red arrow) can be observed (Trichrome ×100); (b) both granulosa and techa cells in the follicle wall (yellow arrow) and stromal cells at the SIS site (red arrow) as well as in the oocytes (white arrows) were heavily stained with proliferating cell nuclear antigen (×200).

about remodeling rather than rejection [5]. The moderate inflammation about the SIS graft, which subsided at 8 weeks after surgery, was thought to be part of the normal healing procedure, as described by Allman et al. [5]. The organization of primordial follicles required 24 weeks and complete epithelialization of the SIS graft required at least 28 weeks.

Adhesions that occur after ovarian surgery are also a crucial risk factor in the surgical procedure [16]. The present study clearly showed that the advantage of using a SIS graft is adequate support for the healing process without signs of adhesion compared with ovarian excision without the use of SIS graft in rabbit ovaries.

The present study demonstrated the success of porcine SIS in developing ovarian cells and possibly follicular structures using molecular markers. From the earliest to the final stages of follicular development, noticeable PCNA staining was found in oocytes. Normally an oocyte is meiotically arrested; the reason for staining with PCNA could be that the oocyte begins to synthesize nuclear RNA as it moves from the primordial to the growing phase [17]. Despite the ambiguity of PCNA expression in the primordial follicle, there is evidence that PCNA is a reliable indicator of cell proliferation [18], possibly accelerated by cytokines and other growth factors found in SIS material [10,19].

The low level of PCNA-stained granulosa cells in a large proportion of primordial follicles in the SIS graft in the present study suggests that granulosa cells do not undergo significant proliferation initially; this could be because of their slow growing process [20], but later it improves in the preantral and antral follicles. The ovarian surface epithelium surrounding the SIS graft showed PCNA immunoreaction, whereas stromal cells in the SIS-grafted site showed inhibin-α immunoreaction, which is in line with previous reports that have suggested that the initial increase in pregranulosa cells in the SIS site could arise from mitosis of surface epithelium or stromal cells [21], but are not in agreement with the proposals revealed by Lintern-Moore et al. [22].

Oocytes in the SIS graft, not yet being primordial follicles, were either unstained or exhibited faint cytoplasmic c-Kit staining, whereas oocytes in growing primary follicles showed distinct cell membrane staining. This is may be because of interactions between c-Kit and growth factors that were reinforced by SIS material playing a decisive role during germ-cell migration [23] and creating a suitable microenvironment to enhance ovarian tissue regeneration and primordial follicle development. However, the origin of the primordial follicles that were observed in the SIS material remains unclear. They might be follicles from the SIS–ovary boundary that diffused into the SIS, the ovarian tissue itself that may have diffused into the SIS graft, or possibly newly generated follicles. In the ovaries of the control animals, no follicles were observed in the surgically treated site.

It is possible that pregranulosa cells migrate into the SIS graft (as shown by inhibin-α staining) and then coalesce around oogonia that arise from the regenerated surface epithelium or stroma, or that SIS contributes to this process with multiple cytokines and growth factors such as FGF-2 and TGF-β, which play important roles in follicle growth [10], or that SIS, as has already been demonstrated in restoring partial tissue defects [6,24], temporarily supports the regenerative process of native tissues [25]. Once tissue regeneration occurs, the SIS metabolizes and gives rise to a functional tissue resembling native ovarian tissue (by the 210th day after surgery) confirming the results of Lantz et al. [4].

Information on the use of SIS for repairing the ovary and perhaps regeneration of reproductive tissue is limited in the present study because the number of animals involved was small. In addition, the histologic sections were not stained with markers that are specific to oocytes, and the origin of the primordial follicles detected in the SIS was not clearly identified and requires further investigation. However, histology of the remodeled SIS graft showed many qualities that are required in ovarian tissue regeneration, and porcine SIS might be considered for use in laparoscopic ovarian cystectomy, ovarian wedge resection, and laparoscopic ovarian drilling for remodeling ovarian tissue, and possibly even for regeneration of cell types such as granulosa cells or for primordial follicles.

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