Scaffold-based delivery of adipose tissue-derived stem cells in rat frozen-thawed ovarian autografts: preliminary studies in a rat model

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Received: 18 April 2015 / Accepted: 30 June 2015 / Published online: 24 July 2015
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Abstract

Purpose This study aimed to evaluate whether a gelatin-based Gelfoam sponge is feasible as a scaffold for adipose tissue-derived stem cell (ASC) therapy in rat frozen-thawed ovarian autografts.

Methods Two sets of studies were performed. The in vitro set evaluated ASCs' viability in the Gelfoam scaffold at different times of co-culturing (after 24, 48, 72, 96, and 120 h). The in vivo set used 20 12-week-old adult female Wistar rats. Frozen-thawed ovarian grafts were treated with ASCs delivered in Gelfoam scaffolds immediately after an autologous retroperitoneal transplant (ASCs-GS, $n=10$). The controls received Gelfoam with a culture medium (GS, $n=10$). Assessment of graft quality was conducted by vaginal smears (until euthanasia on the 30th postoperative day), histological

analyses, follicular density, and viability and fibrosis. Immunohistochemical staining for VEGF-A expression, vascular network (vWF), apoptosis (caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)), cell proliferation (Ki-67), and hormone receptors (estrogen and progesterone) were performed.

Results The cells remained viable in Gelfoam for up to 120 h of co-culturing. The graft morphology was similar among the groups. ASC therapy promoted the earlier resumption of the estrous phase (GS 16.6 ± 3 vs. ASCs-GS 12.8 ± 1.3 days) and enhanced estrogen receptors compared with the controls ($p<0.05$) without interfering with the quantity and viability of the ovarian follicles, fibrosis, endothelial cells, VEGF immunoexpression, apoptosis, or cell proliferation ($p>0.05$).

Capsule The Gelfoam scaffold could be a feasible and safe non-invasive technique for ASC delivery in the treatment of frozen-thawed ovarian autografts.

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Conclusion The Gelfoam scaffold could be a feasible and safe non-invasive technique for ASC delivery in the treatment of frozen-thawed ovarian autografts. Future studies should evaluate the real benefit of this treatment on the survival and endocrine activity of the graft.

Keywords Fertility preservation · Ovarian transplantation · Gelfoam scaffold · Cellular therapy · Stem cells · Ovary · Rat

Introduction

The cryopreservation of ovarian tissue for later transplantation is a fertility preservation alternative for patients who will undergo gonadotoxic treatments and, inevitably, face premature ovarian failure after treatment. Although it is effective in preserving fertility and endocrine function, there are still limitations such as the need to improve the adherence of the graft at the desired site and reduce the time in which it is vulnerable to ischemic and reperfusion damage [1–3]. Therefore, a strategy that should be better assessed is the use of an acellular scaffold for the attachment of grafts and the therapies employed therein.

Cell therapy with stem cells has emerged as one of the most promising treatments in the last decade, with clinical applicability in many specialties [4, 5]. In the field of regenerative medicine, the injection of stem cells into tissues under hypoxia has been much investigated for its ability to promote angiogenesis, secrete growth factors, and differentiate into multiple cell types such as endothelial cells [6]. Direct injection of adipose tissue-derived stem cells (ASCs) has been successfully tested in an experimental model of ovaries damaged by chemotherapy [7–9]. Our data show that cell therapy through the direct injection of rat ASCs transgenic for green fluorescent protein (GFP) in a fresh ovarian graft is feasible and safe. In addition, the ASCs remained in the ovarian stroma without inducing morphological distortions or functional impairment of grafts (unpublished observations, Damous et al.). However, the results were different in the frozen-thawed grafts, wherein the treatment induced atrophy and increased apoptosis [10]. From this scenario, we assume that the direct injection in the ovarian parenchyma after cryopreservation may have been aggressive, thereby overstimulating a local inflammatory response, which compromises the viability of the graft. Accordingly, it is now necessary to develop new strategies for the treatment of frozen-thawed grafts with cell therapy. One possibility is the use of an absorbable scaffold that allows the gradual release and attachment of the cells in a non-invasive manner.

Another crucial point is to ensure that the cells used for cell therapy are fixed at the desired location. In animal models, it has been increasingly common to transplant ovarian tissue installed in acellular scaffolds of materials such as fibrin [11–13], alginate [14], Matrigel [15], and collagen [16]. However, there is still no consensus on the ideal material. In clinical

practice, the gelatin-based Gelfoam sponge is a hemostatic sponge made from pig gelatin that is widely used in surgery. More recently, it has been investigated, both *in vivo* and *in vitro*, in experimental models of angiogenesis [17], immune response [18], bone healing [19], growth and regeneration of nerves [20], and vessel remodeling [21] to enhance the attachment of cells to the desired site. Therefore, this study aimed to evaluate whether the absorbable gelatin-based Gelfoam can be a feasible material as a scaffold in co-culture with ASCs for the treatment of rat ovarian frozen-thawed grafts.

Materials and methods

The study was performed at the Laboratory of Structural and Molecular Gynecology (LIM-58), Gynecology Discipline, Department of Obstetrics and Gynecology, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (FMUSP), in cooperation with the Laboratory of Genetics and Molecular Cardiology/Heart Institute (Incor)/FMUSP. The Institutional Ethics Committee approved the experimental procedures, which followed the institutional guidelines for the care and use of laboratory animals (protocol 190/10).

Two major sets of studies were performed, *in vitro* and *in vivo*. In both assays, an absorbable gelatin-based Gelfoam sponge (Gelfoam®, Pfizer Inc, New York, NY, USA) was used as an acellular scaffold for the ASC delivery.

In vitro assay

This assay aimed to confirm the ASCs' viability and integration with a gelatin-based Gelfoam scaffold (GS) at different co-culture times (24, 48, 72, 96, and 120 h). The experiment was performed in Petri dishes of 96 wells in triplicate, and only one batch of ASCs was used in one time-course experiment.

Cell isolation, *ex vivo* expansion, and ASC characterization

Inguinal subcutaneous adipose tissue was collected under sterile conditions from one 10-week-old male Wistar rat and rinsed with phosphate-buffered saline (PBS). Rat adipose-derived stem cells (rASCs) were isolated, characterized, and maintained in culture as previously described [22, 23].

The harvested tissue was dissociated by digestion with 0.075 % type IA collagenase (Sigma-Aldrich, Inc.) for 45 min. Enzyme activity was stopped, and the cell suspension was centrifuged at 300g for 15 min. Pelleted cells were recovered and plated onto 10-cm culture plates (NUNC, Rochester, NY). At 24-h intervals, cultures were washed with PBS to remove contaminating erythrocytes and other unattached cells and then reseeded with fresh medium. The plating and expansion

medium consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin antibiotics (Invitrogen Corporation, Carlsbad, CA).

Cells were maintained at 37 °C with 5 % CO₂ in tissue culture dishes and fed twice a week until they reached 80 % of confluence—usually within 5 to 7 days after the initial plating. Once 80 % confluence was reached (passage 0), adherent cells were detached with 0.25 % trypsin-EDTA (Vitrocell Embriolife, Campinas, SP, Brazil) and were either replated at 1×10^4 cells/cm² or used for experiments. Cultures were passaged every 3 to 5 days and used for experimental procedures until passage 3.

The morphological and replicative characteristics as well as the immunophenotype (CD90⁺, CD29⁺, CD44⁺, CD73⁺, CD31[−], CD45[−]) of the ASCs have been previously described in our laboratory [24]. Although the characterization has been performed extensively in mouse adipose-derived stem cells (mASCs), our group conducted some immunophenotyping assays in the rat adipose-derived stem cells (rASCs). The percentage of CD90- and CD29-positive cells, the primary markers of a variety of various adult stem cells, was found to be approximately 90 % (92.65 and 98.89 %, respectively) in rASCs at the third culture passage (unpublished data from Nakamuta et al.).

ASC co-culture in a Gelfoam scaffold

The ASCs were seeded on 2-mm² Gelfoam fragments immersed in 15 µl of low-glucose DMEM as a culture medium with 10 % fetal bovine serum at a concentration of 5×10^4 cells/cm² in 96-well plates and cultivated in this condition for 5 days, exchanging the culture medium every 2 days. Twenty-four hours before testing, the culture medium was replaced with FBS-free medium without phenol to avoid false positives and interpretation bias.

Evaluation of cell viability

The proliferation and viability of ASCs were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 24, 48, 72, 96, and 120 h after plating in GS. The MTT assay is based on the reduction of the tetrazolium salt to form formazan crystals by viable cells. Approximately 20 µl of MTT solution (5 mg/ml) was added to each well containing 100 µl of culture medium. After 3 h at 37 °C, the cell morphology was examined using an inverted optical microscope, and the culture medium was completely removed from each well. The formed crystals were dissolved in 200 µl of dimethyl sulfoxide (DMSO), and the plate was shaken for complete solubilization. The reading of the optical density was performed at 560 nm with a 670-nm background. The optical density is directly related to the number of cells.

In vivo assay

Experimental design

The study sample consisted of 20 12-week-old adult female Wistar (*Rattus norvegicus albinus*) rats. The animals had access to a breed-specific food formula and water ad libitum throughout the experiment and were maintained under adequate sanitary, lighting (12/12 h), and temperature conditions in the animal laboratory. The sample was distributed in two groups of study, with treatment with ASCs or not, as follows: Gelfoam scaffold group (GS, $n=10$) and ASCs in Gelfoam scaffold (ASCs-GS, $n=10$).

Vaginal smear collection

Vaginal smears were obtained daily pre-operatively from each rat between 8:00 a.m. and 10:00 a.m. Only the animals that exhibited at least two consecutive normal 4-day vaginal estrous cycles were included in the experiment. Ovarian transplantation was performed during the diestrus phase. Beginning on day 4 postoperatively (PO), vaginal smears were performed daily until euthanasia, which was performed between days 30 and 35, always when the rats were in the diestrus phase.

Vaginal smears were obtained with a swab soaked in physiological solution, placed on a standard slide, and immediately fixed in absolute alcohol for staining using the Shorr-Harris technique. The smears were analyzed under a light microscope at $\times 10$ and $\times 40$ magnification [25].

Operative procedures

Oophorectomy The animals were anesthetized with an intraperitoneal injection of xylazine and ketamine at doses of 15 and 60 mg kg^{−1} of body weight, respectively. The abdominopelvic cavity was opened, and the ovaries were identified. The ovarian pedicles were clamped and immediately ligated with 4–0 nylon suture. The fallopian tubes were resected with periovarian adipose tissue fragments. The wall closure was performed using a 5–0 nylon monofilament thread on two planes, including the peritoneum-aponeurotic muscle and the skin.

Ovarian cryopreservation and thawing After bilateral oophorectomy, the fresh whole ovaries were immediately frozen in a slow-cooling freezer (CL-8800, Cryogenesis software, Freezer Control) as previously described [26]. The whole ovaries were placed in 1.2-ml cryovials (Sigma-Aldrich®, Inc.) in M2 medium plus HEPES without penicillin and streptomycin (M2, Sigma-Aldrich®, Inc.) and with 1.4 M dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, Inc.) as a cryoprotector and were held at room temperature for 5 min. The cryovials were

sealed by twisting their caps and were placed in a temperature-programmed freezer (CL-8800, Cryogenesis software, Freezer Control) and cooled from 25 to 10 °C at a rate of 1 °C/min and then to −7 °C at a rate of 0.5 °C/min and maintained at −7 °C for 5 min. Then, ice nucleation was manually induced using pre-cooled forceps, and the temperature was held at −7 °C for another 5 min to allow for the release of latent heat fusion. The tissue was then frozen at −55 °C at a rate of 0.5 °C/min, plunged into liquid nitrogen at −196 °C, and stored for 24 h.

Ovarian transplantation and cellular therapy Twenty-four hours after ovarian cryopreservation, the rats were anesthetized as previously described for a second laparotomy.

Each animal received both autologous ovary transplants. Intact whole ovaries were implanted in the retroperitoneum in the proximity aorta and vena cava using a simple stitch of 4–0 nylon suture, without vascular anastomosis, each on one side of the psoas muscle.

At this point, the animals were distributed according to the treatment, as follows ($n=10$ for each group): Gelfoam scaffold group (GS with 15 μ l of DMEM solution/ovary) or ASCs-GS (Gelfoam with ASCs at a concentration of 5×10^4 cells in 15 μ l of vehicle/ovary). Both grafts from each animal received the same treatment. The standard dose of 15 μ l was the lowest dose that allowed the Gelfoam to stay soaked without leakage of the medium. The ASCs were placed in the Petri dish 10 min before applying on the ovarian graft.

After the treatments, the wall closure was performed using a 5–0 nylon monofilament thread on two planes, including the peritoneum-aponeurotic muscle and the skin.

Graft retrieval and histological preparation

The animals underwent a third surgical procedure between PO days 30 and 35, always in the diestrus phase. The abdominal cavity was opened, and the ovaries were macroscopically identified and assessed. The muscle bed was also assessed for vascularization and surrounding adhesions. The grafts were subsequently removed whole. Following this procedure, the animals were euthanized with a lethal dose of the previously used anesthetics.

One ovarian graft was immediately fixed in 4 % paraformaldehyde for at least 24 h. Following fixation, the ovaries were dehydrated, paraffin-embedded, serially sectioned at 5 μ m, and mounted on glass microscope slides. Routine hematoxylin and eosin (HE) staining was performed for histological examination with light microscopy. The ovarian cortex was sectioned into two pieces of equal size, and five representative sections were selected. The other graft was used to assess follicular viability, as described in the following section.

Follicular viability assay

Using a modified protocol [27], the analysis of the follicle viability was carried out. The ovaries were washed with M2, sectioned in pieces with a scalpel, and transferred to a 50-ml tube containing 1 mg/ml (200 IU/ml) of type IA collagenase solution (Invitrogen Corporation) and 2 ml of Leibovitz L-15 medium supplemented with L-glutamine and penicillin/streptomycin antibiotics (Invitrogen Corporation, Carlsbad, CA) to further dissolve the tissue further and make the follicles easy to count. This material was incubated in an oven at 37 °C for 1 h and slightly agitated every 10 min. The tissue digestion was interrupted with the addition of 1 ml of fetal bovine serum (Gibco). The solution was then filtered using a 100- μ m nylon strainer (Cell Strainer 100- μ m nylon, BD Falcon, Belgium) and centrifuged at 1500 rpm for 5 min at 20 °C. The pellet was resuspended in 1 ml of fetal bovine serum, and the resulting suspension was placed in a 12-well plate (one ovary/well) to be stained with 20 μ l of 0.4 % trypan blue (Life Technologies) at room temperature for 10 min.

A threefold manual count of each well was performed under an inverted microscope (EVOS® XL Core Cell Imaging System, AMG) at $\times 200$ magnification to allow for individualization of the white (viable) and blue (atretic) follicles in absolute numbers as well as in percentages of viable follicles.

Morphological, morphometric, and fibrosis analyses

Morphological evaluation was achieved through descriptive analyses of the grafts. The assessment of follicular quality was based on the cell density, the presence or absence of pyknotic bodies, and the integrity of the basement membrane and oocyte. The follicles were classified as normal or degenerated according to these criteria, and only the former were characterized and quantified [28].

Viable follicles were classified into three groups: immature follicles (including primordial, primary, and preantral), antral, and corpus luteum [29]. Primordial follicle exhibited only an oocyte and a layer of squamous cells. Primary follicle exhibited an oocyte and one or more layers of cuboidal or prismatic cells but no antrum. Preantral follicle exhibited an oocyte and an antrum. Mature follicles contained an oocyte with antrum. The corpus luteum exhibited intact luteal cells that contained a nucleus and cells surrounded by capillary blood vessels [28, 29]. To avoid counting the same follicle more than once, only individual follicles having an oocyte containing a nucleus were evaluated. Counting was performed in eight fields per section.

We also measure the thickness of the granulosa layer in the antral follicles, four measures per follicle (μm^2), and counted the number of follicular cells (three areas of 1,000 μm^2 /animal).

For fibrosis evaluation, the slices were stained by Picrosirius Red and measures were performed in eight different fields per animal in the ovarian stroma, at $\times 400$ magnification, and the results are expressed as a percentage of the positive area (arbitrary unity/mm²).

All images of the sections were obtained using an image acquisition software system (Leica DM2500; LEICA, Wetzlar, Germany). Images of the sections were obtained using an image acquisition software system (Leica DM2500), and measurements were made using Leica QWin V3 software. Two independent investigators blind to the experimental treatments performed all of the analyses under a microscope (LEICA).

Immunohistochemistry assays

Sections containing ovarian tissue were immunostained to measure angiogenesis using tissue VEGF expression (SANT-SC-152, 1:100, Santa Cruz Biotechnology), von Willebrand factor for endothelial cells (AB6994, 1:100, Abcam Inc., Cambridge, MA, USA), proliferation using Ki-67 expression (M724001-2, 1:100, Dako North America Inc., Carpinteria, CA, USA), and apoptosis using cleaved caspase-3 expression (SANT-SC-1226, 1:100, Santa Cruz Biotechnology) and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. Hormonal receptors of estrogen (E1644, 1:50, Springe Bioscience Corporation, Pleasanton, CA, USA) and progesterone (AB51896, 1:30, Abcam Inc., Cambridge, MA, USA) were also assessed.

Cross sections embedded in paraffin were treated with antigenic exposure and blocked with 2 % casein in PBS. The tissue sections were incubated with the respective primary antibodies overnight at 4 °C. The sections were incubated with a biotinylated rabbit secondary antibody (universal polymer anti-mouse and rabbit Histofine® 1:400, Vector Laboratories, Burlingame, CA, USA) and streptavidin peroxidase followed by the peroxidase substrate diaminobenzidine tetrahydrochloride according to the manufacturer's instructions. The tissue slices for the TUNEL assay were stained using a commercially available kit (In Situ Cell Death Detection Kit, Fluorescein, Roche, Germany, 11684795910) following the manufacturer's instructions. Red-brown coloring of the cytoplasm/nucleus was considered positive staining (any other coloring was considered negative staining). Negative controls were used to avoid bias. For the negative controls, the primary antibody was omitted in each different immunohistochemistry staining.

Images of the sections were obtained using an image acquisition software system (Leica DM2500), and measurements were made using Leica QWin V3 software. A red-brown coloring of the cytoplasm/nucleus of the granulosa cells of antral follicles (for apoptosis, VEGF and Ki-67 expression) or stroma (for fibrosis evaluation and endothelial

cells expression) was specified as positive staining (otherwise as negative staining). Hormonal receptors were evaluated both in the stroma and follicular cells. A positive cell staining assessment was performed in eight different fields per animal at $\times 200$ magnification, and the results are expressed as a percentage of the positive area (arbitrary unity/mm²). Two independent investigators blinded to the experimental treatments performed all measurements.

Statistical analysis

The results were expressed as the mean $\pm$ standard deviation of the mean. The two-way ANOVA was utilized to analyze the in vitro assay and unpaired *t* test to analyze the in vivo assay. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc, CA, USA). *p* values lower than 0.05 were considered significant.

Results

ASCs' viability in vitro in a Gelfoam scaffold

The ASCs remained viable in the GS for 120 h of co-culture, although there was a significant drop in viability at 24 and 48 h ($p < 0.05$ 24 vs. 48 h). Compared with the control, the ASCs-GS group shows a lower number of viable cells at all observation times, except at 96 h, when the values were similar ($p > 0.05$) (Fig. 1).

Vaginal smear, morphology, and fibrosis in ovarian grafts

All animals resumed estrous cycle, and the animals treated with ASCs-GS show earlier estrous cycle resumption compared with the controls (GS 16.6 ± 3 vs. ASCs-GS 12.8 ± 1.34 days, $p < 0.05$).

Fig. 1 Evaluation of adipose tissue-derived stem cells (ASCs) viability in a Gelfoam scaffold, as measured by MTT, at different times of conventional culture (ASCs) or co-culture with Gelfoam scaffold (ASCs-GS). Data were shown in mean $\pm$ SD. ASCs vs. ASCs-GS: $p < 0.05$ 24, 48, 72, and 120 h. ASCs vs. Times: $p < 0.05$ 24 vs. 48 h; 72 vs. 96 h. ASCs-GS vs. Times: $p < 0.05$ 24 vs. 48 h. two-way ANOVA

The ovarian grafts were easily identified during euthanasia through the nonabsorbable sutures used for attachment. The Gelfoam was completely absorbed after 30 days. Macroscopically, there were no signs of fibrosis or adhesions.

The morphology was the same in both groups. The ovarian tissue showed ovarian follicles in various stages of maturation, from the primary pre-ovulatory luteinizing, sometimes with a wall, and the presence of occasional albicantia and corpora lutea. There were no thickening and hyalinization of the blood vessel walls, obliteration of the lumen or myointimal proliferation in arterioles. There was mild fibrosis, no foreign body reaction, or inflammation (absence of neutrophils, plasmocytes, macrophages, and capsular tissue indicative of inflammation reaction around the graft). The application of the Gelfoam did not induce any distortion in the morphology or an increase in fibrosis in both grafts (GS 4.22 ± 2.91 vs. ASCs-GS: 5.24 ± 2.3 , $p > 0.05$, Table 1, Fig. 2).

Viability and follicular density

The follicular viability, as measured by trypan blue, was similar between groups (GS $84.4 \% \pm 4.47$ vs. ASCs-GS $86 \% \pm 2.68$, $p > 0.05$). The analysis of the follicular density by HE showed a predominance of developing follicles (primordial and preantral) to mature follicles (antral and corpora lutea). However, there was no difference between the untreated animals (GS) and treated animals (ASCs-GS) with respect to the density of immature follicles (5.33 ± 5.77 vs. 9.33 ± 2.65 , respectively, $p > 0.05$), antrals (1.33 ± 1.15 vs. 3.66 ± 1.86 , respectively, $p > 0.05$), and corpora lutea (4.33 ± 5.13 vs. 4 ± 2.19 , respectively, $p > 0.05$) (Table 1, Fig. 2).

In antral follicles, further assessment was performed, measuring the granular layer thickness (GS 561.7 ± 23.73 vs. ASCs-GS $544.4 \pm 58.25 \mu\text{m}^2$) and counting the number of follicular cells (GS 25.75 ± 1.7 vs. ASCs-GS 31.75 ± 2.7). Both analyses were similar between groups ($p > 0.05$).

Angiogenesis, apoptosis, and proliferation assays

Thirty days after the application of ASCs in GS in the ovarian graft, positive cells were quantified for angiogenesis (VEGF

immunostaining and endothelial cells by von Willebrand factor), apoptosis (cleaved caspase-3 and TUNEL), and cellular proliferation (Ki-67). Endothelial cells were quantified in the ovarian stroma and the others in the granulosa cells. As shown in Table 2, treatment with ASCs in GS did not change any of the parameters ($p > 0.05$) (Table 2, Fig. 3).

Estrogen and progesterone receptors

Hormone receptors were quantified in the ovarian follicles and stroma. Treatment with ASCs in GS increased the estrogen receptors both in the follicles (GS $3:23 \pm 1.5$ vs. ASCs-GS $9:35 \pm 0.84$, $p < 0.05$) and stroma (GS 2.15 ± 0.61 vs. ASCs-GS 7.6 ± 1.58 , $p < 0.05$). Progesterone receptors in the stroma were similar between groups (GS 2.98 ± 2.1 vs. ASCs-GS 4.22 ± 2.5 , $p > 0.05$), and in the ovarian follicles, there was no immunostaining (Fig. 4).

Discussion

Cell therapy with ASCs has been widely studied in regenerative medicine because ASCs can differentiate into other cell types and exert a therapeutic effect via paracrine or trophic factors at the site of the damaged tissue [30]. Despite the various mechanisms available for cell implantation at the desired site, a considerable amount is lost in drainage organs, such as the liver, lungs, and spleen. Only a small fraction migrates to the damaged tissue, depending on the method of administration and the disease model investigated [31, 32]. In addition to the loss of cells, another aggravating factor is that direct injection into the target organ may be detrimental to this tissue, which is already vulnerable. In recent years, cell therapy has been associated with tissue engineering techniques to enhance the release and retention of cells in the target tissue. Among these techniques, the use of an acellular scaffold has attracted increasing interest [6, 33], and our results show that GS can be a feasible and safe technique.

Therapy through an acellular scaffold has several advantages over direct injection of cells into the target tissue, as it allows prolonged contact and greater retention of cells,

Table 1 Ovarian follicles density (mean) and viability (percentage of viable follicles) and fibrosis quantification (arbitrary unit/ mm^2) according to treatment with adipose tissue-derived stem cells (ASCs) in

	Follicular viability (TB)	Immature follicles (HE)	Antral follicles (HE)	Corpora lutea (HE)	Fibrosis (Picrosirius)
GS	84.41 ± 4.47	5.33 ± 5.77	1.33 ± 1.15	4.33 ± 5.13	4.22 ± 2.91
ASCs-GS	86.06 ± 2.68	9.33 ± 2.65	3.66 ± 1.86	4 ± 2.19	5.24 ± 2.3

Data were shown in mean $\pm$ SD. Student *t* test $p > 0.05$

TB trypan blue, HE hematoxylin-eosin

a Gelfoam scaffold (ASCs-GS) or Gelfoam scaffold alone (GS), in autologous frozen-thawed grafted ovary ($n = 10$ each group)

Gelfoam Scaffold**ASCs -
Gelfoam Scaffold**

Fig. 2 Morphology of frozen-thawed ovarian autografts according to treatment with adipose tissue-derived stem cells (ASCs) in a Gelfoam scaffold (ASCs-GS) or Gelfoam scaffold alone (GS). HE staining (**a** and **b**), scale bar 100 μ m. The morphology was the same in both groups. Square areas show where fibrosis evaluation was performed in

the ovarian stroma, by Picrosirius Red staining (**c** and **d**), scale bar 20 μ m. The application of the Gelfoam did not induce any distortion in the morphology or an increase in fibrosis in both grafts. Fo follicles, CL corpora lutea. Arrows indicate bright red specified as positive staining

thereby preventing them from migrating to other sites in which they may have deleterious effects. Additionally, the scaffold must maintain cell viability for a sufficient amount of time for the gradual and continuous release of pro-angiogenic factors to reach therapeutic levels [33, 34].

In the present study, we evaluated whether the gelatin-based Gelfoam sponge may be a viable material as an acellular scaffold in co-culture with ASCs for the treatment of frozen-thawed ovarian grafts in rats. We chose this material for the scaffold because it is widely used in clinical practice as a hemostatic and apparently is a safe material; however, this

material needs to be investigated in animal models for use as a scaffold to treat ovarian grafts. Made from pig gelatin, the gelatin-based Gelfoam sponge is soft, highly porous; has a strong texture; is perfectly adaptable in an applied surface; and has slow absorption (approximately 4 weeks) [35]. For these characteristics of flexibility, biocompatibility, and biodegradability [36], it may be of potential use as a scaffold for cell therapy in an experimental model of frozen-thawed ovarian transplants.

In vitro, the ASCs in co-culture with GS remained viable in amounts similar to those in conventional culture (without the scaffold) for up to 5 days, during which time the reperfusion of the transplanted ovarian tissue occurs [37, 38]. Although the number of cells in co-culture with GS was smaller, the time could be sufficient to allow them to attenuate the ischemic injury to the graft.

In the in vivo assay, the application of GS did not interfere in the engraftment process, as there were no changes in morphology, signs of inflammation, foreign body reaction, increased fibrosis, apoptosis, or cell proliferation. Thus, this ASC application technique to the ovarian surface through GS seems to be an effective alternative to direct injection in the parenchyma, as it acts externally and noninvasively, allowing the slow and gradual release of ASCs in the crucial stage of graft adherence to the new site.

In the initial study of this line of research in experimental ovarian transplantation, the direct injection of ASCs in a fresh

Table 2 Immunohistochemistry analysis in antral ovarian follicles for VEGF expression, cellular proliferation (Ki-67) and apoptosis (cleaved caspase-3 and TUNEL), and angiogenesis in the ovarian stroma (vWF), according to treatment with adipose tissue-derived stem cells (ASCs) in a Gelfoam scaffold (ASCs-GS) or Gelfoam scaffold alone (GS), in autologous frozen-thawed grafted ovary ($n=10$ each group)

	VEGF	vWF	Ki-67	Caspase-3	TUNEL
GS	2.18 $\pm$ 0.72	2.57 $\pm$ 0.12	4.48 $\pm$ 2.18	6.7 $\pm$ 2.86	0.33 $\pm$ 0.15
ASCs-GS	5.8 $\pm$ 1.8	2.36 $\pm$ 0.24	4.34 $\pm$ 0.97	6.04 $\pm$ 1.81	0.4 $\pm$ 0.24

Data are shown in mean $\pm$ SD as a percentage of the positive area (arbitrary unit/ mm^2). Student *t* test $p>0.05$

VEGF vascular endothelial growth factor, vWF von Willebrand factor, TUNEL terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end

Fig. 3 Photomicrographs of immunohistochemistry analysis in ovarian follicles (*VEGF*, *Ki-67*, *caspase-3*, and *TUNEL*) and stroma (*vWF*), according to treatment with adipose tissue-derived stem cells (*ASCs*) in a Gelfoam scaffold (*ASCs-GS*) or Gelfoam scaffold alone (*GS*), in autologous frozen-thawed grafted ovary. A red-brown coloring of the cytoplasm/nucleus of the cells was specified as positive staining. For

the negative controls, the primary antibody was omitted in each different immunohistochemistry staining. Treatment with *ASCs* in *GS* did not change any of the parameters. *Scale bar* 20 μ m. Negative controls, *scale bar* 50 μ m. *VEGF* vascular endothelial growth factor, *vWF* von Willebrand factor, *TUNEL* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end

graft proved to be feasible and safe, with encouraging results (unpublished observations, Damous et al.). However, in the

frozen-thawed grafts, the same beneficial effect was not observed as in the fresh graft, but rather impaired morphology

Fig. 4 Photomicrographs of immunohistochemistry analysis of estrogen and progesterone receptors in ovarian follicles and stroma, according to treatment with adipose tissue-derived stem cells (*ASCs*) in a Gelfoam scaffold (*ASCs-GS*) or Gelfoam scaffold alone (*GS*), in autologous frozen-thawed grafted ovary. A red-brown coloring of the cytoplasm/

nucleus of the cells was specified as positive staining. For the negative controls, the primary antibody was omitted in each different immunohistochemistry staining. *Scale bar* 20 μ m. There was no immunostaining of progesterone receptors in the ovarian follicles and in the stroma were similar between groups

(diffuse atrophy and the predominance of corpora albicans) and increased apoptosis were observed in all treated animals. These data were confirmed by vaginal smears, in which there was a return of ovarian function [10]. From these data, we created a non-invasive model of ASC application in an acellular scaffold to allow their slow and gradual release on the ovarian graft surface.

In the present study, the immunostaining of hormone receptors for estrogen (ER) and progesterone (PR) was also evaluated. Paradoxically, ER was more expressed in the animals treated with ASCs, although this action did not occur simultaneously with the increase in the thickness or number of follicular cells in the granulosa layer. PR showed no expression in the ovarian follicles, only in the stroma, which is consistent with previous reports that the vast majority of ovarian cells do not express PR [39].

Altogether, our results allow us to suppose that the use of the Gelfoam scaffold for ASCs may be a feasible and safe strategy to treat the frozen-thawed ovarian graft, as it did not promote a change in morphology, an inflammatory reaction, or increased fibrosis and apoptosis. This technique can also have a beneficial effect on the endocrine activity of the graft, as it promoted increased immunostaining of RE and an early functional return of the estrous cycle, which may be a result of stimulation by the local microenvironment rather than systemic factors. However, the biological significance of these findings is a limitation of this study and is beyond the proposed initial goal. Recent studies have proposed the creation of an artificial ovary through follicular cells cultivation in an acellular scaffold [40, 41]. The GS can be another step towards this artificial ovary.

Conclusion

The ASCs remain viable in co-culture with the Gelfoam scaffold for up to 120 h after plating. The use of ASCs in the Gelfoam scaffold could be a feasible and safe technique for the non-invasive treatment of frozen-thawed ovarian grafts. Future studies should evaluate the real benefit of this treatment on the survival and endocrine activity of the graft.

Acknowledgments We thank São Paulo Research Foundation (FAPESP, Pío XI St, 1500, Alto da Lapa, São Paulo/SP-Brazil, 05468-901) for grant support (process numbers: 2010/17897-5 and 2012/09469-9) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for post-doctoral scholarship; Esmeralda Miristene Eher, Angela Batista Santos, Maria Cristina Rodrigues Medeiros, and Sandra de Moraes Femeizian (Immunohistochemistry Laboratory/Pathology Department/FMUSP) for technical assistance with immunohistochemistry assay; Leonard Medeiros da Silva (pathologist) for the help in the analysis of morphological data; and American Expert Journal for the english editing service.

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