J Assist Reprod Genet (2011) 28:761–769
DOI 10.1007/s10815-011-9605-x

FERTILITY PRESERVATION

Alginate scaffold for organ culture of cryopreserved-thawed human ovarian cortical follicles

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Received: 11 February 2011 / Accepted: 21 June 2011 / Published online: 23 July 2011

Abstract
Purpose To compare macroporous alginate scaffolds with Matrigel for culturing frozen-thawed human primordial follicles in organ culture.
Methods Twelve girls/women donated ovarian tissue. One tissue sample was fixed immediately after thawing (uncultured samples). Slices were cultured for 2 weeks on either Matrigel or on alginate scaffolds with a serum-free culture medium. Growth evaluation consisted of follicular counts and classification, immunohistochemistry and measurement of 17β-Estradiol (E_2) production.
Results The number of developing follicles was significantly higher in alginate scaffold-cultured samples than on Matrigel with a concomitant decrease in the number of primordial follicles in alginate scaffold-cultured samples than uncultured samples. The number of atretic follicles after 1 week was significantly higher in the Matrigel-cultured samples than in the alginate scaffold cultured samples. E_2 production was similar in both groups.
Conclusions Three dimensional alginate scaffolds are a promising putative in vitro technology for developing human primordial follicles.

Keywords Alginate scaffold · 17β-Estradiol (E_2) · Human primordial follicles · Matrigel · Proliferating cell nuclear antigen (PCNA)

Introduction

As cancer treatment improves, more young women of reproductive age survive [1]. However, many have ovarian failure and premature menopause as a consequence of the

Capsule Culturing human ovarian cortical tissue on a macroporous alginate scaffold seems to promote better follicular development than culturing on Matrigel

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radiation and chemotherapy [1]. Among the limited options currently available for fertility preservation in these patients is cryopreservation of ovarian cortical tissue containing immature primordial follicles followed by autotransplantation [2–5]. However, this procedure carries the risk of reintroducing hematological or other malignancies [6–9]. This drawback could be overcome by embryo transfer after *in vitro* fertilization of oocytes from primordial follicles matured *in vitro*. Researchers are, therefore, seeking to improve techniques of culturing primordial follicles [10, 11].

Two main approaches to culturing primary and primordial follicles have been attempted [10, 11]. One is to culture whole slices of ovarian tissue (organ culture) in order to retain the interactions between the follicles and the surrounding stroma cells. Using this method, primordial follicles from cows [12] and humans [13–18] have been grown to secondary stages [13]. Matrigel is often used as a supporting matrix for human primordial follicles because it was found to increase their survival [13, 19]. However, Telfer *et al*. [20, 21] succeeded to activate human and bovine primordial follicles without Matrigel.

The second approach involves the use of isolated primordial and primary follicles [22, 23]. By employing this method researchers can directly monitor follicular growth during the culture period, a particularly important consideration given the poorly populated state of human ovarian tissue in adults. Recent advances in biomaterials scaffolds have led to the development of three-dimensional (3D) culturing systems that mimic the physiological environment and imitate the internal architecture of the living tissue [24–32]. Several teams have applied hydrogel beads composed of alginate, a biocompatible polysaccharide widely utilized for cell transplantation [24, 25], to encapsulate and culture isolated ovarian secondary follicles from mice [26], rats [27], nonhuman primates [28] and humans [29]. The follicles grew to antral stages [26–30]. Moreover, the oocytes from mice were successfully fertilized and implanted to yield live births [26]. This culture system was also used for smaller human follicles, but they only survived in culture [31].

The aim of the present study was to investigate for the first time, the effectiveness of a 3D macroporous alginate scaffold for culturing human primordial follicles in organ culture. We chose alginate for its mechanical stability, biocompatibility, and hydrophilicity, in addition to its high porosity (>90%) and large pore size of 80–150 μm which allow for efficient mass transport [24, 25]. The porous structure can be easily manipulated by controlling various parameters during fabrication of the scaffold, such as cross-linker concentration, solution viscosity, and freezing regimen. The alginate scaffold has been successfully used to cultivate hepatocytes [24], cardiomyocytes [25] and human embryonic stem cells [32], while preserving their functional activities. Given these unique features we hypothesized that it would enhance the diffusion rate of the culture media to the tissue and generate an extracellular matrix-like environment adjacent to the tissue. The follicular growth findings were compared to tissue slices cultured on Matrigel.

Materials and methods

Sample sources and retrieval

The Ethics Committee of Rabin Medical Center approved the study protocol, and written consent was obtained from every adult patient or the parents of all minors. The study included 12 frozen-thawed ovarian samples from girls and women (aged 10–27 years, mean±standard deviation, SD = 16 ± 6 years). All these patients underwent Gynecological laparoscopies. All operations were performed for ovarian biopsy cryopreservation before chemotherapy [1–3, 5].

All samples were handled in our laboratory within 1 h of surgery. One slice from every patient (measuring 1–2 mm) was fixed in Bouin's solution (components purchased from BDH Chemicals Ltd., Poole, England, and Sigma, St. Louis, MO, USA) immediately after ovarian dissection (uncultured fresh control), and the remaining ovarian slices were frozen.

Cryopreservation and thawing of ovarian tissue

Tissue slices were frozen with a 1.5 M dimethylsulfoxide (DMSO) (Sigma) solution [19, 33–35]. Before freezing, the samples were kept on ice with DMSO. All samples were frozen slowly in a programmable freezer (Kryo 10; series 10/20, Planer Biomed, Sunbury on Thames, UK), and immediately placed in liquid nitrogen.

The tissue slices were thawed by three washouts with alpha minimal essential medium (αMEM) (Biological Industries, Beit Ha'emek, Israel), 5% human serum albumin (HSA) (Irvine Scientific, Santa Ana, CA, USA), followed by incubation at 37°C [34]. One slice of every thawed ovarian sample (similar in size to the uncultured fresh control) was fixed in Bouin's solution immediately after thawing (uncultured thawed control).

Alginate scaffold preparation

Alginate scaffolds, 10 mm in diameter and 2 mm thickness (Fig. 1) were prepared from alginate with a high guluronic (G) acid concentration (LVG; 65%G monomer content, molecular weight = ~ 100 kDa, NovaMatrix FMC

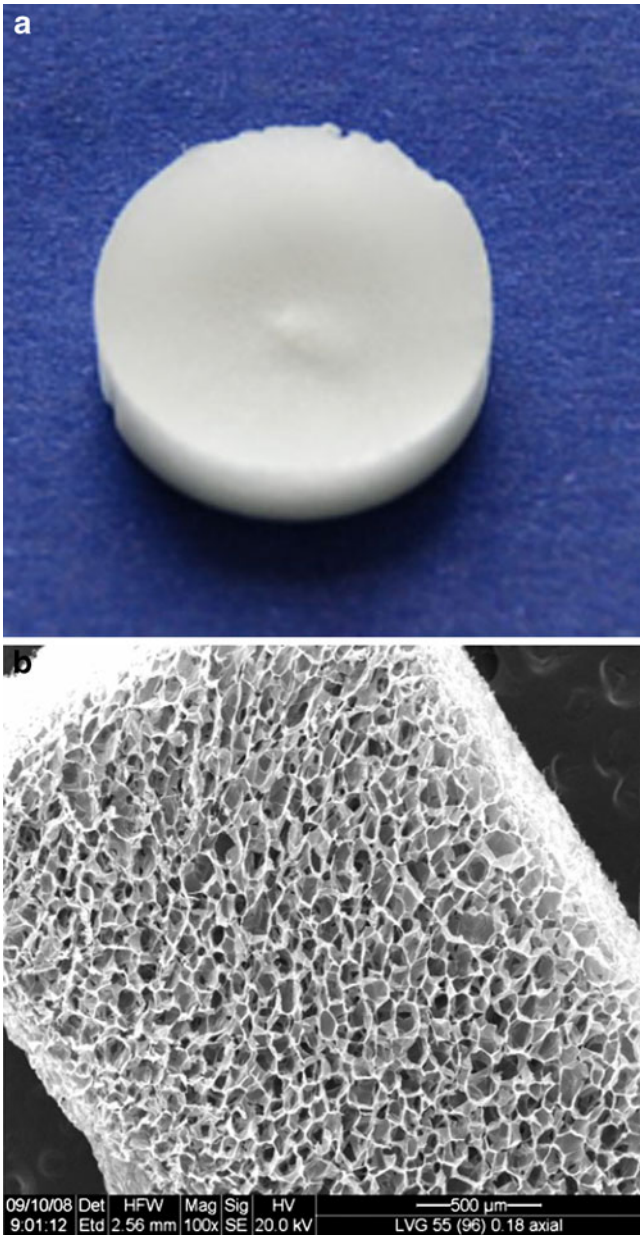


Fig. 1 Photographs of the macroporous alginate scaffolds used in the study. **a** An alginate scaffold with a diameter of 10 mm and thickness of 2 mm. **b** Scanning electron microscope micrograph of the alginate scaffold showing its porous structure and large pore size. Original magnification X100, scale bar = 500 µm

Biopolymers, Drammen, Norway) using a freeze-dry technique [36]. In brief, alginate was dissolved in double distilled water (1.2% w/v) and calcium cross-linked by adding a solution of D-gluconic acid (Sigma) and hemi-calcium salt (Sigma) (1.32% w/v); the solution was homogenized to obtain homogenous calcium ion distribution and cross-linking. The final component concentrations in the cross-linked solution were 1.0% for the polymer and 0.22% (w/v) for the cross-linker. The cross-linked solution was poured into 48-well plates (200 µl/well), frozen at −20°C for 24 h and lyophilized. The scaffolds were sterilized by exposure to ultraviolet light in a biological hood for 1 h. Scanning electron microscope (SEM) inspection revealed 90% scaffold porosity and a pore diameter of 80–150 µm.

Matrigel preparation

Millicell CM inserts (Millipore, Bedford, MA, USA) fitted into 24-well plates (Nalge Nunc International) were pre-coated with an extracellular matrix gel (Matrigel reduced in growth factors; BD Bioscience, Franklin Lakes, NJ, USA) [19, 35].

Culturing methods

Only frozen-thawed samples were used for incubation so that follicular density could be studied histologically prior to culture [19, 35]. This practice eliminated the risk of utilizing poorly populated ovarian tissue. Moreover, previous studies in which we participated showed no differences in *in vitro* development of fresh and frozen-thawed isolated human ovarian cortical follicles [23], and no morphological differences between follicles from frozen-thawed and fresh human ovarian cortical tissue [34]. Because the ultimate fertility preservation and restoration technique for cancer patients will require the use of frozen-thawed follicles [10], studies using frozen-thawed ovaries are crucial.

Each ovarian sample was cut to thin ovarian cortical slices (1–2 mm, width = ~1 mm, as uniform in size as possible and similar in dimensions to the uncultured controls) and divided into two study groups. Group 1 was placed on the alginate scaffolds (47 slices, at least three slices per patient) and group 2, on the Matrigel precoated inserts (52 slices, at least three slices per patient). The slices were placed in every well and were covered with medium. The basic culture medium consisted of αMEM (Biological Industries), human recombinant follicle stimulating hormone (FSH, 1 IU/ml) (Gonal-F, Serono, Aubonne, Switzerland), 10% HSA (Irvine Scientific), insulin, transferrin, and selenium (Sigma) [19, 35]. The samples were incubated for 2 weeks in a standard incubator (95% air, 5% CO_2), and the culture media were changed every second day. Once a week, at least one slice (on average two slices) was removed from culture and fixed immediately in Bouin's solution (BDH Chemicals and Sigma), and the spent medium samples were collected for 17β-estradiol (E_2) measurement [19, 35, 37, 38].

Histological preparation

The fixed specimens from both groups were prepared for paraffin embedding, sectioning, and staining with hema-

toxylin and eosin (H & E) [19, 35, 37, 38]. The number of follicles in the uncultured and cultured samples was counted with an image analyzer in two different section levels per sample (50 μm between sections, to avoid counting the same follicle twice) (analySIS Soft Imaging System, Digital Solutions for Imaging and Microscopy System, GmbH, Munster, Germany), and the follicles were classified according to Gougeon [39] as follows: *primordial* (with a single flat layer of granulosa cells-GCs surrounding the oocyte), *primary* (with a single cuboidal GC layer surrounding the oocyte), *secondary* (with at least two GC layers and a theca layer surrounding the oocyte) and antral (with a fluid filled cavity within the GCs). Developing follicles were defined as primary and secondary [19, 35]; atretic follicles were characterized by pyknotic cells, eosinophilia of the ooplasm, and clumping of the chromatin material [39]. Unstained sections were placed on OptiPlus positive charged microscope slides (BioGenex Laboratories, San Ramon, CA, USA) for immunohistochemistry assay.

Immunohistochemistry for proliferating cell nuclear antigen (PCNA)

PCNA is a nuclear protein that plays an essential role in cell-cycle regulation [19, 35, 37]. It serves as a useful marker of proliferating cells such as GCs. The sections were first microwaved with citrate buffer at pH 6.0 (Dako Corporation, Santa Barbara, CA, USA) to enhance antigen retrieval. They were then incubated with the primary antibody for PCNA, a monoclonal mouse anti-PCNA antibody (1: 70 and 1: 100, Dako). Sections were incubated with two negative control solutions that replaced the primary antibody. The first was prepared with a normal mouse IgGa2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to the same concentrations as the primary antibody and the second included phosphate buffered saline. Solutions from a Dako EnVision System, horseradish peroxidase–3-amino-9-ethylcarbazole (AEC) kit (Dako Corporation) were used for immunostaning: red-brown AEC (Zymed Laboratories Inc., Invitrogen, Carlasbad, CA, USA) staining indicated PCNA expression. We defined a follicle as being positively stained if at least one of its GCs expressed PCNA.

E_2 accumulation in the culture medium

E_2 concentrations were measured by a double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA), which has a detection limit of 1.4 pg/mL [19, 35, 37, 38].

Statistical analysis

Data were statistically analyzed by analysis of variance, chi-square test and Fisher's exact test, as required. P values of less then 0.05 were considered significant.

Results

Follicular counts and classification

The distribution of follicles by group is presented in Table 1. We preferred to refer to developing follicles rather than to primary and secondary follicles separately as very few secondary follicles were identified in the sections. Figure 2

Table 1 Follicular counts and classification in ovarian tissue slices cultures on alginate scaffold or Matrigel and uncultured controls for a two-week period

Follicular classes	Alginate scaffold		Matrigel		Uncultured samples
	Week 1	Week 2	Week 1	Week 2	
[a]Primordial	138 (36%)	121 (33%)	231 (46%)	120 (46%)	445 (78%)[1]
	12±9	15±8	38±15	15±11	31±13
Developing	190 (51%)[2]	205 (58%)[2]	94 (18%)	92 (34%)	135 (22%)
	13±9	17±9	11±5	11±9	12±5
Atretic	48 (13%)	31 (9%)	183 (36%)[3]	51 (20%)	0
	5±2	3±1	22±10	6±2	0

[a] Follicular numbers are given as sum of follicles per group and week (percents were calculated from total number of follicles in group per week) and as average±standard deviation

[1] Significantly higher in uncultured group than in alginate scaffold group ($p<0.003$ for week 1, $p<0.002$ for week 2)

[2] Significantly higher in alginate scaffold group than in Matrigel after one and 2 weeks ($p<0.03$ for both weeks)

[3] Significantly higher in Matrigel group than in alginate scaffold group for week 1 and for both weeks together ($p<0.005$ for week 1, $p<0.008$ for both weeks together)

presents photographs of ovarian sections containing follicles at various stages of the experiment. There were no morphological differences between follicles in the uncultured fresh (as presented in Fig. 2a) controls and the uncultured thawed controls (as presented in Fig. 2b).

The number of developing follicles was significantly higher ($p<0.03$) in samples cultured on macroporous alginate scaffolds than in samples cultured on Matrigel. After 1 week, there was a 51% developing follicles in the alginate group compared to 18% in the Matrigel group; corresponding rates at 2 weeks were 58% and 34% ($p<0.03$ for both weeks). In addition, the proportion of primordial follicles decreased significantly in the alginate scaffold group compared to the uncultured samples group to 36% after 1 week and 33% after 2 weeks compared to 78% ($p<0.003$ for week 1, $p<0.002$ for week 2). The number of atretic follicles was significantly higher in the Matrigel group than the alginate scaffold group accounting for 30% and 10% of all follicles, respectively for both weeks together ($p<0.008$); and 36% and 13%, respectively, after 1 week ($p<0.005$).

Immunohistochemistry for PCNA expression

An example of a cultured ovarian section after PCNA staining is shown in Fig. 2c. In most cases PCNA staining was weak. PCNA staining was identified in a portion of the GC and oocytes from primordial-primary and secondary follicles. After 1 week the number of PCNA-stained follicles was significantly higher in the alginate scaffold group (79.7% of all follicles) than in the uncultured controls (35.7%) ($p<0.0001$). In the second week, the rate of PCNA-stained follicles was significantly higher in the alginate scaffold group (82.2%) ($p<0.0001$) and the Matrigel group (66.1%) ($p<0.0004$) than in the uncultured controls. Moreover, in the second week the rate of PCNA-stained follicles was also significantly higher in the alginate group than in the Matrigel group ($p<0.04$). It is noteworthy that there were no follicles in any of PCNA-stained sections from the Matrigel group in the first week despite their presence in the corresponding H & E stained sections (because of different tissue sectioning levels). The negative controls did not stain positively (blue) (Fig. 2d).

E_2 accumulation in the medium

E_2 levels were detectable in spent media samples after culture on either alginate scaffold or Matrigel throughout the culture period. E_2 concentrations were significantly higher in week 1 (mean±SD = 152±208 pg/ml/slice for alginate; mean±SD = 81±147 for Matrigel) than week 2 (mean±SD = 264±326 for alginate; mean±SD = 33±47 for Matrigel) in both groups ($p<0.003$).

Discussion

The present study examined for the first time the feasibility of culturing human cortical ovarian slices on macroporous alginate scaffolds. The study suggests certain trends regarding improvements in follicular culture on alginate scaffolds than Matrigel. The proportion of developing follicles after culture on alginate scaffolds was significantly higher than after culturing on Matrigel and compared to uncultured slices, with a concomitant decline in primordial follicles. In parallel, there were more atretic follicles in the Matrigel group than the alginate scaffold group. Detectable E_2 levels were measured in spent media samples after culture in both systems, although these levels decreased during the second week.

A limitation of the present study, similar to other studies on human ovarian tissue [5, 10, 13–20, 22, 23, 29, 31, 34, 35, 40], is the heterogenecity in follicular numbers between samples. Young age is strongly correlated with high follicular density, and therefore, we chose samples from young patients. However, the availability of human ovaries for research is limited and therefore, by contrast to rodents [26, 27], selection of tissue from human subjects of uniform ages is unpractical. Moreover, as follicular distribution is uneven within individual human ovaries [5, 40], this might have led to our culturing ovarian slices with lower follicular numbers than in the uncultured controls.

The lower number of primordial follicles in the alginate scaffold group than the Matrigel group can be due to higher activation rates of primordial follicles or to more atretic follicles in the Matrigel group. This is in line also with more PCNA stained follicles, indicating better GC proliferation rates, in the alginate scaffold group than in the Matrigel group. Alternatively, similar to other studies with organ culture of human ovarian tissue [13–20], it is possible that we placed tissue slices that initially contained less primordial follicles on alginate scaffolds.

PCNA oocyte staining was observed not only in the current study but also in various previous studies [19, 35, 37, 41]. It cannot be attributed to cell division, since the oocyte is not engaged in mitotic activity [37, 42]. However, the mammalian oocyte is not quiescent. Although no new DNA is created, DNA polymerase delta might be activated to repair possible damage to the nuclear and mitochondrial DNA in the oocyte, and during its activity, it or possibly the proliferating mitochondrial DNA itself might incorporate PCNA [42]. This might account for the positive PCNA staining.

As E_2 is secreted only from secondary follicular stages onwards [39], study of its production in culture allows for the evaluation of growth to secondary stages [19, 35, 37]. Since our histological studies identified very few secondary follicles, the E_2 production might have been due to secondary follicles in tissue levels that were not examined histologically, or to follicles between primary and secondary stages or to E_2 secretion from primary follicles, although to the best of our knowledge this has not been reported previously. Moreover, by contrast to the histological findings suggesting a benefit for alginate scaffold on Matrigel, the lack of significant difference in E_2 production in the spent media samples between the two groups might have been due to the sharp variability in E_2 secretion levels, as represented by the high SD values.

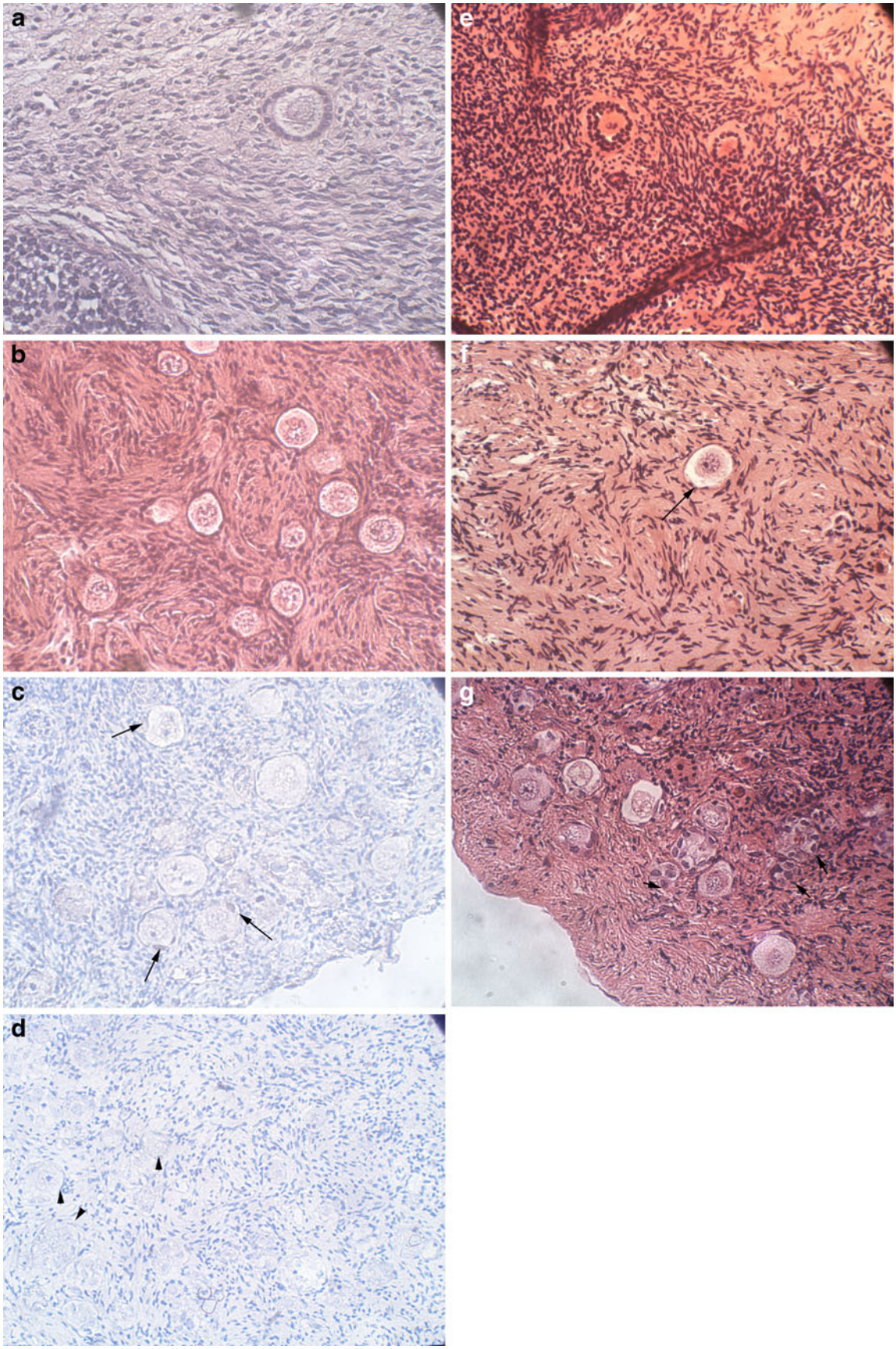
The significant decrease in E_2 after 2 weeks in both groups may be attributable to either a deterioration in follicular quality during culture or by intersample variation [5, 19, 35, 40]. Although we evaluated different ovarian slices from individual patients at various time points (prior to culture, week 1 of culture and week 2 of culture), it is possible that the initial follicular density differed among the individual samples. Moreover, in an earlier study, Telfer *et al.* [20] reported a decline in E_2 secretion after 4 days of culture. These findings suggest that while surviving follicles seem to continue growth in culture their hormonal function *in vitro* needs to be further optimized.

In previous studies, isolated ovarian follicles were encapsulated in alginate hydrogel beads and cultured [26–31, 43–45]. Culturing of isolated follicles in this manner removes them from their natural environment, thereby limiting the interactions between neighboring follicles and most importantly limiting the diffusion rate of large proteins in the hydrogels [46] and restricting follicular growth [47]. Moreover, culturing follicles in alginate hydrogel beads might potentially restrict their ability to grow to large antral sizes. Therefore, we suggest that culturing isolated follicles in alginate macroporous scaffolds similar to those used in the present study would provide a new alternative. This scaffold has a high porosity and large pore size providing a suitable culture environment for numerous isolated follicles, while imitating and maintaining follicle-follicle communication. In addition, both the porous structure and the pore size of the alginate scaffold can be easily manipulated by judiciously selecting various parameters during fabrication [36, 48], so it can be adjusted to follicular size.

The development of a successful culture system for primordial follicles poses a technological and scientific challenge, and studies are only at the initial stages. It is possible that a two-step culturing system is the optimal strategy for human follicle culture [20]. This approach has been utilized for murine [43, 49], bovine [21] and human [20] primordial follicles. The primordial follicles were initially cultured in organ culture without a supporting matrix [20, 21]; and thereafter secondary follicles were isolated and further cultured. So far the *in vitro* follicular growth from primordial stages to the development of functioning oocytes has only been achieved in mice [43, 49] with production of live young [49].

Telfer *et al.* [20] showed that human primordial follicles within cortical pieces could undergo activation to secondary stages within 6 days, as the physical setting of the follicles within the cortical tissue seems to influence the growth capacity [21]. Taking the present promising results into account, we propose that the activation of primordial follicles might be enhanced in the first culture step (organ culture) if the tissue is cultured on alginate scaffolds combined with improvements in their culture medium [10, 19, 35]. The macroporous alginate scaffolds would maximize the tissue's surface area for gas exchange and the supply of nutrients, while providing a support system that more closely resembles the ovary, such that the intact follicle is maintained and its contact with the surrounding stroma cells is preserved. Further follicular growth might then be achieved by isolating the secondary follicles followed by their culture in alginate hydrogel beads [26–31], collagen gels [22, 23], or other systems [20, 38]. Moreover, the alginate scaffold culturing system might be improved in future studies by use of fresh rather than cryopreserved-

Fig. 2 Histological and PCNA immunostained sections of the uncultured and cultured ovarian samples. **a** Uncultured freshly dissected ovarian section from a 19-year-old woman. Note the primary follicle. Hematoxylin and eosin (H & E) staining, original magnification X400. **b** Uncultured thawed ovarian section from a 13-year-old girl. Note the primordial follicles. H & E staining, original magnification X400. **c** Section of ovarian sample with PCNA immunohistochemical staining from a 17-year-old girl cultured on an alginate scaffold for 1 week. Note the primordial and primary follicles, with the *light red-brown* staining indicating PCNA expression in a portion of the oocytes and the GCs (*arrows*). Original magnification X400. **d** Negative control for PCNA from the same ovarian sample as in Panel C. Note the primordial and primary follicles (*arrow heads*) with overall blue staining and lack of red-brown PCNA staining. Original magnification X400. **e** Section of ovarian sample from the same woman as in panel A cultured on alginate scaffold for 2 weeks. Note the two primary-secondary follicles. H & E staining, original magnification X400. **f** Section of ovarian sample from ovarian sample from the same patient as in panels C and D cultured on Matrigel for 1 week. Note the primordial follicle (*arrow*). H & E staining, original magnification X400. **g** Section of ovarian sample from a 20-year-old woman cultured on Matrigel for 2 weeks. Note the primordial, primary and atretic follicles (*small arrows*). H & E staining, original magnification X400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thawed tissue for culture and by incorporating growth factors in its backbone or in special controlled release microspheres [24, 50, 51].

Acknowledgment The authors are greatly indebted to Ms. Gloria Ganzach from the Editorial Board of Rabin Medical Center, Beilinson Hospital for the English editing.

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