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Alginate beads as a tool to handle, cryopreserve and culture isolated human primordial/primary follicles ☆

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ABSTRACT

Background: One major concern of grafting cryopreserved ovarian tissue to restore fertility in cancer patients is the possibility of reintroducing tumor cells. Cryopreservation of isolated primordial/primary follicles (PFs) may circumvent this problem. The aim of our work was to compare dimethyl sulfoxide (ME_2SO) and ethylene glycol (EG) as cryoprotectants (CPAs) for slow-freezing of isolated human PFs in alginate.

Methods: Ovarian biopsies from four women were processed for follicle isolation. PFs were embedded in alginate (5–15 per group). Follicles were frozen-thawed using 1.4 M ME_2SO or 1.5 M EG as CPAs. Fresh and cryopreserved isolated follicles were *in vitro* cultured (IVC) for 7 days. At different time periods (after isolation, cryopreservation and IVC), follicles were evaluated with live/dead assay (using fluorescent probes) and diameter measurement. Follicle viability was calculated according to the percentage of dead follicular cells and the presence of a live/dead oocyte.

Results: A total of 841 PFs were isolated, embedded in alginate and cryopreserved with ME_2SO ($n = 424$) or EG ($n = 259$), or used as controls ($n = 158$). After 7 days of IVC, a significant increase in follicle size was observed in the fresh and ME_2SO groups, but not in the EG group. The percentage of totally viable PFs was not significantly different before or after seven days of culture in fresh (100% and 82%) or ME_2SO (93.2% and 85.1%) tissue. The EG group showed significantly lower viability before (63.9%) and after IVC (66.2%) than the fresh and ME_2SO groups.

Conclusions: Our results show that 1.4 M ME_2SO yields better preservation of isolated PF viability after thawing and 7 days of IVC than 1.5 M EG. Alginate constitutes an easy, safe hydrogel matrix to handle and cryopreserve isolated human follicles using ME_2SO as a CPA.

Introduction

Cryopreservation of human primordial/primary follicles has several advantages over embryo and mature oocyte freezing for fertility preservation purposes. Primordial follicles represent the resting follicular reserve and are more cryoresistant, as they have a low metabolic rate, contain fewer organelles, are smaller and lack a *zona pellucida* and cortical granules [21].

Primordial/primary follicles may be cryopreserved enclosed in ovarian tissue or as isolated entities. In both cases, after cryopreservation, they may be *in vivo* or *in vitro* matured [17]. Of these two options, cryopreservation and autografting of ovarian cortical fragments is currently the only possible strategy to restore endocrine and reproductive functions in humans, yielding 21 live births to date ([18] for review). Despite these promising results, this technique has some limitations. One is the difficulty to quantitatively and qualitatively evaluate the follicle population present in ovarian fragments before cryopreservation [1], due to heterogeneous follicular distribution in human ovarian cortex [20,33].

Another serious limitation in some types of cancer, mainly hematologic, is the possibility of reintroducing malignant cells after transplantation of frozen-thawed tissue [15,30,35].

To avoid the risk of malignant cell transmission, individually isolated primordial follicles may be cryopreserved and matured either *in vitro* [26,36] or *in vivo* [14]. Indeed, follicles are unlikely to contain metastasis, as the follicular basal lamina encapsulating

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the *membrana granulosa* excludes capillaries, white blood cells and nerves from the granulosa layer [31].

Research into this field is very limited in humans, however, probably due to technical difficulties manipulating isolated follicles and lack of knowledge on *in vitro* procedures to grow individual follicles from the primordial or primary stage [4]. In humans, a study was previously conducted on isolated secondary follicle cryopreservation [7]. In this paper, the authors showed that isolated human secondary follicles are able to survive slow-freezing and can be *in vitro* cultured for 6 days. However, as primordial follicles represent more than 90% of the follicle population [22], procedures to cryopreserve and grow single-layered human primordial and primary follicles clearly need to be developed.

Investigations in different animal species have demonstrated the feasibility of isolated primordial or primary follicle cryopreservation [mice: 10, 11; cats: [24]; sheep: 2, 3; goats: 32; marsupials: [13]; rats: [40]].

We recently set up a protocol to cryopreserve human primordial/primary follicles embedded in a 3-dimensional (3D) alginate hydrogel system to facilitate their manipulation [39]. This protocol was compared with our current ovarian tissue slow-freezing procedure using 1.4 M ME_2SO, and yielded similar follicle survival after 7 days of IVC [39].

In the present study, two cell-permeable CPAs, ME_2SO (1.4 M) and EG (1.5 M), were compared to find the best means of cryopreserving isolated human primordial/primary follicles embedded in alginate. These two CPAs were chosen based on the successful results obtained after cryopreservation of human primordial/primary follicles enclosed in ovarian tissue [6,16].

Materials and methods

Collection and dissection of ovarian tissue

Use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. After obtaining written informed consent, ovarian biopsies were taken from four women (between 21 and 31 years of age).

The tissue was immediately transported from the operating room to the laboratory on ice. The medullar part was removed from the biopsies using surgical scissors. The size of the biopsies ranged from 117 mm^3 to 400 mm^3.

Experimental design of the study

Four series of experiments were carried out with ovarian biopsies from four patients. As detailed in Fig. 1, the tissue was processed for follicle isolation by Liberase DH digestion and alginate encapsulation. After alginate embedding, three beads were taken from each patient: one was processed for viability assays and diameter measurement (freshly isolated follicle group); the other two were cultured for 7 days and follicle viability and diameter were measured at the end of *in vitro* culture (IVC fresh follicle group).

The remaining beads were assigned to two groups for freezing: one using ME2SO as a CPA (ME2SO group) and the other using EG (EG group).

After thawing, the beads were *in vitro* cultured for seven days. Embedded follicles were tested for viability and follicle diameters were measured immediately after thawing (ME_2SO and EG frozen follicle groups) and at the end of the culture period (IVC ME_2SO and IVC EG frozen follicle groups).

Enzymatic tissue digestion and follicle recovery

Single-layered preantral follicles were isolated as described by Vanacker et al. [37]. Briefly, the ovarian cortex was cut into small pieces and placed in a tissue chopper (McIlwain Tissue Chopper, Mickle Laboratory, Guildford, UK) adjusted to 0.5 mm. The small pieces of ovarian cortex were then transferred to 50 ml conical tubes containing 10 ml of Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium (Lonza, BioWhittaker, Verviers, Belgium), supplemented with 0.28 Wunsch unit/ml Liberase DH (Roche Diagnostics, Vilvoorde, Belgium). Incubation was performed in a water bath at 37 °C with gentle agitation for 75 min. The suspension was shaken with a pipette every 15 min to mechanically disrupt the digested tissue. Digestion was completed by the addition of an equal volume of DPBS medium without calcium and magnesium (Lonza) at 4 °C, supplemented with 10% fetal bovine serum (FBS) (Gibco, Ghent, Belgium).

The resulting suspension was then centrifuged at 50*g* for 10 min at 4 °C. The supernatant was discarded and the pellets were transferred to plastic Petri dishes and examined for follicles under a stereomicroscope (Leica, Van Hopplynus Instruments, Brussels, Belgium). The follicles were picked up using a 135 µm-diameter stripper tip (Mid-Atlantic Diagnostics, Inc., Mount Laurel, NJ, USA) linked to a tubing set with a sterile filter (Swemed Lab, Billdal, Sweden). Follicle diameter was evaluated under an inverted microscope with a stage micrometer. All single-layered follicles with a diameter up to 50 µm were included in the study. The follicles were then washed three times at 4 °C in DPBS without calcium and magnesium, but supplemented with 10% FBS, in order to avoid introduction of stromal cells into the alginate matrix.

Isolated follicle embedding in calcium alginate

The protocol for embedding isolated follicles in an alginate matrix was previously described by Amorim et al. [4]. A 1% (w/v) solution of sodium alginate (alginic acid, Sigma, St Louis, MO, USA) was prepared and autoclaved. The isolated follicles were randomly transferred with the stripper tip to 20 µl droplets of alginate solution. To form the beads, the droplets were gently released into a plastic Petri dish containing a 0.1 M $CaCl_2$ solution (Sigma). Beads containing follicles (5–15 follicles/bead) were washed three times

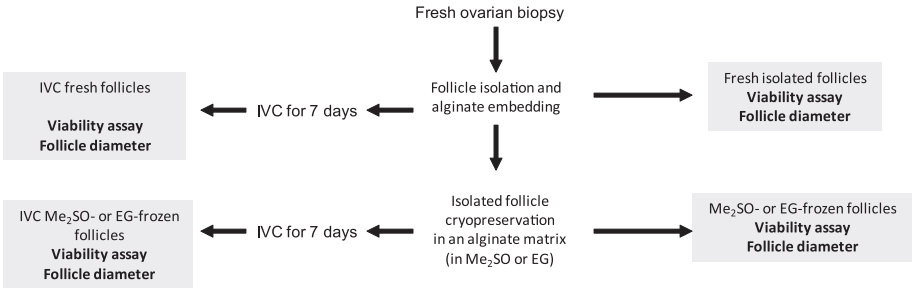

Fig. 1. Experimental design for the study of cryopreservation and IVC of isolated human primordial/primary follicles.