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TECHNOLOGICAL INNOVATIONS

In vitro development of human primordial follicles to preantral stage after vitrification

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Abstract
Purpose The aim was to culture primordial follicles in vitro to reach preantral stage in vitrified human ovarian tissue.
Methods Ovarian tissue samples were obtained from six women. Tissue strips were vitrified by infiltration with a cryoprotectant followed by mounting on a stainless steel carrier. After culturing for 7 days the morphology and developmental stages of follicles enclosed in fresh and vitrified groups were analyzed.
Results High proportion of viable follicles in vitrified ovarian strips was obtained. After culturing for 7 days the percentage of secondary and preantral follicles increased significantly ($P<0.05$) whereas primordial and transitory follicles showed a significant decrease ($P<0.05$) compared to their respective counterparts at day 0 of culture.
Conclusions Vitrification of ovarian strips with an improved carrier device and culturing of follicles in ovarian strips after warming yielded developed follicles with high viability and morphological integrity that may be suitable for use in fertility preservation among cancer patients.

Keywords Carrier device · In vitro culture · Preantral follicles · Preservation of fertility · Primordial follicles

Capsule In vitro culture of vitrified ovarian strips yielded follicles with high morphological integrity and viability suitable for fertility preservation in cancer patients.

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Introduction

Cryopreservation of ovarian tissue before cancer therapy to provide opportunities for future fertility is one of the most challenging procedures in assisted reproductive techniques (ART) [1]. With the improved rate of cancer survivors in recent years, there is an increasing demand for cryopreservation of fertility [2]. The need for this approach is particularly eminent among young women without a male partner and in women who cannot postpone their cancer therapies or in prepubertal girls to whom embryo cryopreservation is not applicable. Presently, two techniques are commonly employed for cryopreservation: a conventional one which involves slow freezing and the other which involves vitrification. To date 24 live births have been reported after cryopreservation and transplantation of ovarian tissue strips with the slow freezing procedure [3]. In slow freezing lower concentrations of cryoprotectant are used. Slow freezing had been the preferred method for cryopreservation of human ovarian tissue until now. There are conflicting results on vitrification versus slow freezing in studies carried out by various groups. With the slow freezing procedure, some authors have noted the negative effects of ice crystal formation on certain ovarian components [4, 5]. On the other hand the vitrification procedure requires more efficient cooling rate and higher concentrations of cryoprotectant that may cause osmotic damage. During

vitrification fluid in the cell is converted to the solid state, avoiding cellular damage caused by the formation of intracellular ice crystals [6]. Vitrification also does not induce apoptosis in ovarian tissue after warming [7, 8]. Recent studies reported that the use of vitrification yielded higher oocyte survival rates compared to slow freezing [4, 9]. Vitrification is a relatively inexpensive procedure and does not require any special instrument [10]. Ovarian tissue vitrification has been performed in mice [11–13], domestic animals [14–17], non-human primates [18–20], and in humans [21–24]. To date, no pregnancy has been reported in humans after transplantation of vitrified tissue [3]. The carrier system is considered one of the most important factors that can influence the cooling rate and vitrification outcome [25]. Various carrier tools have been used for ovarian tissue vitrification such as Pasteur pipettes [18, 26], copper grids [27], cryovials [28], acupuncture needles [29], 4 fine stainless needle [17, 19] and the solid surface method [25].

The ovarian cortex contains a large number of follicles in various stages of development. Primordial follicles are the most populated ovarian follicles each of which contains an immature oocyte in meiotic arrest [30]. There is evidence that primordial follicles are more tolerant to cryopreservation owing to their small size, less differentiation, quiescence of their oocyte, and the lack of zona pellucida and cortical granules [31]. In vitro culture of cryopreserved primordial follicles embedded in ovarian strips to developed and growing follicles would allow the transfer of developed follicles for transplantation, eliminating the potential risk of reseeding cancer with ovarian tissue grafting [32].

There are different ways to evaluate the quality of the cryopreservation technique such as assessment of follicles immediately after thawing [33] and after post-thawing in vitro culture [34, 35], evaluation after post-thawing xenotransplantation [22, 36], and culture of follicles on embryonic chorio-allantoic membrane [37, 38]. Other investigators have studied the effect of human ovarian cryopreservation on viability and development of follicles and on xenotransplantation [39–42]. Different strategies have been used to culture immature follicles in vitro using fresh or cryopreserved cortical tissue in situ [43–46]. It has been shown in a recent study that, after 6 days in culture, the number of primordial follicles decreased and the number of preantral follicles increased in cortical strips [46].

Recently the neutral red (NR) dye has been employed as a tool to quantify viable follicles in situ without compromising viability and development of follicles before and after tissue cryopreservation [47, 48]. However for assessment of viability of stromal cells in ovarian strips fluorescent staining such as Calcein AM has been used [29]. In view of the information mentioned above, the aim of the present study was to assess the in vitro development of primordial follicles to preantral stage in ovarian fragments after vitrification using an improved carrier device made of stainless steel mesh and to compare the results with those obtained with the fresh (non-vitrified) group.

Materials and methods

Use of human ovarian tissue for this study was approved by the Human Ethics Board of Queen's University, Kingston, Ontario, Canada. Ovarian tissue samples were obtained with consent from six women aged 27–42 years old who were candidates for oophorectomy for benign gynecologic conditions. The reason of oophorectomy for the 27 years old woman was due to sex reassignment. All chemicals used in this study were purchased from Sigma-Aldrich unless stated otherwise. Freshly collected ovarian tissue was immediately placed at room temperature in a 50-ml sterile falcon tube containing 10 ml of Leibovitz medium (GIBCO) supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (3 mg/ml), penicillin G (75 mg/ml), streptomycin (50 mg/ml) and ascorbic acid (50 mg/ml) [46]. The tissue sample was then transferred within a few minutes to a Petri dish containing fresh Leibovitz medium with supplements as described above and the cortical ovarian tissue was trimmed with a scalpel into small pieces of about 0.5×2×2 mm in size under a dissecting microscope (Leica). Two ovarian strips prepared from each cortical tissue were fixed in Bouin's solution for histological evaluation. All the cortical tissues were divided randomly in two groups designated, respectively, as the vitrified group and the fresh or non-vitrified group which served as the control.

Vitrification

The vitrification procedure was performed according to the method of Kagawa et al. (17) with minor modifications. Ovarian strips were equilibrated for 15 min at room temperature in a mixture solution containing 7.5 % dimethyl sulphoxide (DMSO) and 7.5 % ethylene glycol (EG) in HTCM (TCM-199 with HEPES-buffer, GIBCO) supplemented with 10 % human serum albumin (HSA) (Life Global). This was followed by immersion in a vitrification solution containing 15 % DMSO, 15 % EG, 2.5 % polyvinylpyrrolidone (PVP) [22], 10 % HSA with 0.5 mol/L sucrose in HTCM for 7 min or until all strips descended to the bottom of the falcon tube.

Ovarian strips prepared from each ovary were placed on a stainless steel mesh (Fig. 1) which was vitrified by plunging into liquid nitrogen. Individual pieces of stainless steel mesh carrying the ovarian strips were transferred into a 1.8 ml-cryovial pre-filled with liquid nitrogen and were stored in a liquid nitrogen tank for at least 1 week before they were further processed.

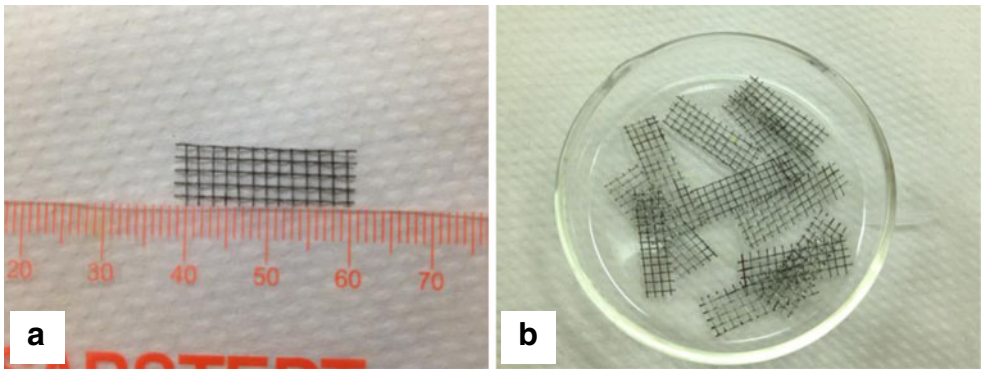

Fig. 1 Photographs showing the actual dimensions of a stainless steel mesh for carrying ovarian strips for vitrification (**a**) and how they appear in a petri-dish (**b**)

Warming

After opening the cryovial the stainless steel mesh was immediately transferred into 20 ml of pre-warmed (37 °C) HTCM solution supplemented with 10 % HSA and 1.0 mol/L sucrose for 3 min. Then the samples were transferred into 10 ml HTCM solution supplemented with 10 % HSA and 0.5, 0.25, 0.125 mol/L sucrose, respectively, for 5 min each at room temperature. Afterwards they were washed twice for 10 min with HTCM supplemented with 10 % HSA. Two ovarian strips were fixed in Bouin's solution and processed for histological evaluation. The warmed strips were incubated in fresh medium at 37 °C in 5 % CO_2.

In vitro culture (IVC)

Fresh and vitrified tissue samples were cultured for 7 days in 24-well culture plates containing 300 μl of McCoy's 5a culture medium supplemented with bicarbonate (GIBCO), HEPES (20 mM), HSA (0.1 %), glutamine (3 mM), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 mg/ml), selenium (5 ng/ml), insulin (10 ng/ml) and ascorbic acid (50 mg/ml). The plates were incubated in 5 % CO_2 at 37 °C. Half of the culture medium was replaced with fresh one every other day [46]. After 7 days two ovarian strips from each group (fresh and vitrified) were fixed in Bouin's solution and processed for histological evaluation.

Evaluation of follicles in situ with neutral red

Neutral Red (NR) is a weak cationic dye which penetrates the plasma membrane and accumulates in the lysosomes of viable cells [49]. It has been previously used to assess the viability of porcine oocytes, granulosa and theca cells [50, 51]. In the present study, the viability of follicular cells in tissue strips was assessed, respectively, at day 0 and at day 7 of in vitro culture in both fresh and vitrified-warmed groups. The strips were incubated for 5 h at 37 °C in 5 % CO_2 in 10 ml of pre-warmed culture medium supplemented with 50 μg/ml of neutral red solution [47, 48].

Isolation of follicles

Ovarian strips ($n=120$) of both fresh and vitrified groups after 7 days of IVC were transferred to 50-ml falcon tubes containing 10 ml of McCoy's 5a culture medium supplemented with 50 μg/ml of NR solution and incubated for 5 h. Thereafter, for enzymatic digestion of the strips, a mixture of Liberase TM (0.08 mg/ml) (Roche) and Collagenase IV (0.2 mg/ml) was added to the culture medium and the ovarian strips were incubated for 90 min at 37 °C in 5 % CO_2. The strips were agitated every 15 min by gentle pipetting. The enzymatic digestion was completed by the addition of 10 ml of cold (4 °C) PBS containing 10 % FBS (GIBCO) [48]. The enzyme-digested strips were mechanically disrupted with a thin pasture pipette to release follicles from the stroma into the medium. Under a stereomicroscope NR-positive follicles with red color and unstained follicles from each group were transferred with a thin pasture pipette into a culture dish containing fresh culture medium. The number of reddish-staining, viable follicles and their corresponding stages of follicular development were recorded using an inverted microscope (IX 70 Olympus).

Evaluation of viability of stromal cells and isolated follicles

Vitrified ovarian strips after IVC were evaluated for viability of stromal cells and follicles using fluorescent double staining with Calcein-AM and Ethidium homodimer-1(Invitrogen) [14, 52]. Briefly, ovarian strips and isolated follicles were incubated for 40 min in an incubator at 37 °C in 5 % CO_2 in 1 ml of McCoy's 5a medium containing 1 % HSA, 2 μM Calcein AM and 5 μM Ethidium homodimer-1. Non-fluorescent Calcein-AM enters follicles and is cleaved by esterase in live follicles to become fluorescent Calcein. Ethidium homodimer-1 enters follicles with disrupted membranes producing red fluorescence in dead follicles. Ovarian strips and isolated follicles were washed with McCoy's 5a

culture medium containing 1 % HSA. Images were taken on an inverted fluorescent microscope (IX 70 Olympus).

Histological procedure and evaluation

Ovarian strips after 24 h of fixation in Bouin's solution were dehydrated in ascending concentrations of ethanol (70 %–100 %) and then embedded in paraffin wax. Five micron-thick serial sections were cut. Every sixth section was stained with hematoxylin and eosin and then examined under a light microscope (Nikon). The follicles were classified into 6 categories according to their stage of development based on morphology of the granulosa cells surrounding the oocyte [30, 46, 53, 54].

1. Primordial follicles: a single layer of flattened granulosa cells
2. Transitory follicles: appearance of one or more cuboidal granulosa cells
3. Primary follicles: a complete layer of cuboidal granulosa cells
4. Secondary follicles: two complete layers of cuboidal granulosa cells
5. Preantral follicles: more than two layers of cuboidal granulosa cells
6. Atretic follicles: oocyte with a pyknotic nucleus and pyknotic granulosa cells.

To avoid double counting of the follicles, follicles were counted when focused on one plane and only follicles displaying an oocyte were counted. A follicle was considered normal when the follicle displayed well organized granulosa cells of spherical shape, an intact layer of theca interna, and a spherical oocyte with a normal nucleus. Atretic follicles were characterized by the presence of a pyknotic nucleus in the oocyte and an uneven distribution and disorganization of granulosa cells. The percentage of various stages of follicular development was determined for both fresh (non-vitrified) and vitrified ovarian strips.

Statistical analysis

Statistical analyses were carried out using the SPSS 16 program (SPSS 16, Chicago, IL). Number and percentage of follicles in different stages were expressed as mean ± SD. Mean values of measurements were compared using t-test. Values of $p<0.05$ were considered statistically significant.

Results

Histological analysis

Ovarian strips in fresh (non-vitrified) ($n=12$) and vitrified groups ($n=12$) after fixation were compared and the morphology and developmental stages of follicles in both groups were evaluated (Fig. 2). A total of fifty-two follicles in the non-vitrified strips and forty-three follicles in the vitrified strips were counted. Atretic follicles were found to be 9.61 % in the

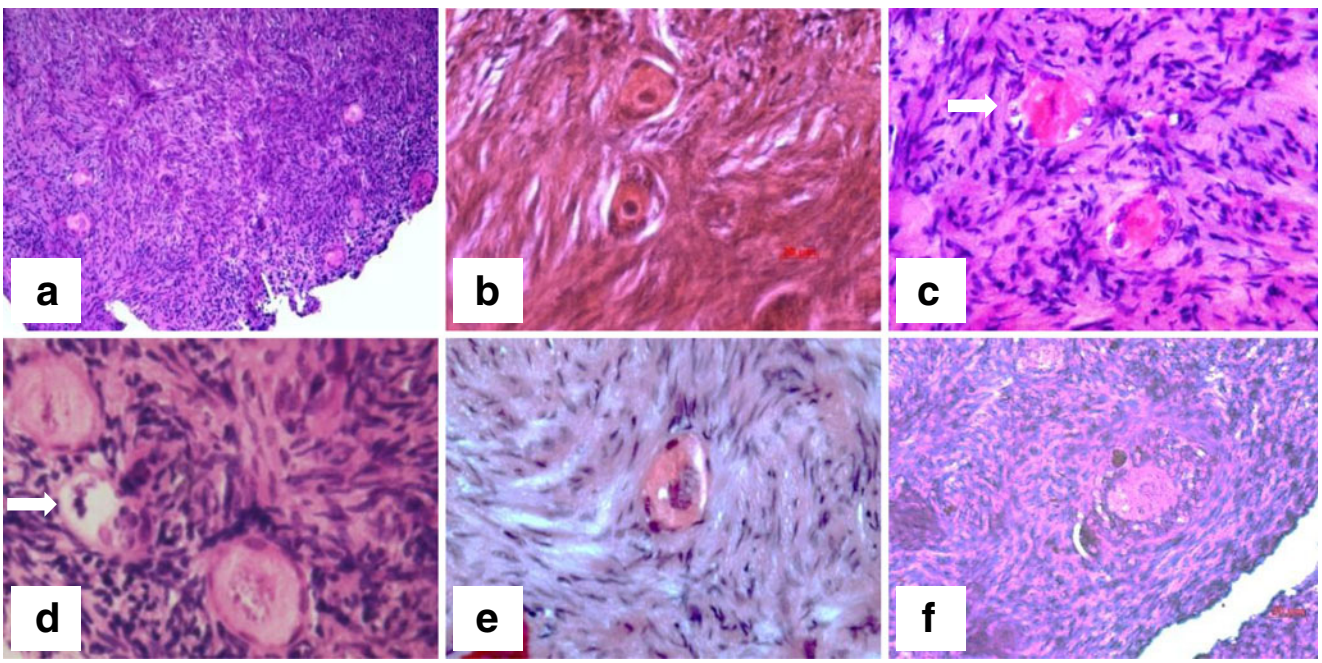

Fig. 2 Light photomicrographs showing hematoxylin and eosin stained human ovarian follicles in fresh ovarian strip and after vitrification/warming. **a** and **b**: fresh follicles; **c**: follicles after IVC; **d** and **e**: primordial follicle after vitrification; **f**: preantral follicle after vitrification and 7 days of IVC. Abnormal follicles are indicated by *arrows* in **c** and **d**. Bars: **a** = 100 μm, **b** and **f** = 20 μm, **c**-**e** = 50 μm

Table 1 Histological analysis showing the percentage of follicles in different stages of development in ovarian strips of the fresh and vitrified groups

	Primordial	Transitory	Primary	Secondary	Atretic
Non-vitrified (n=12 strips)	28.85	36.54	19.23	5.77	9.61
Vitrified (n=12 strips)	27.91	30.23	18.60	2.33	20.93

Table 2 Histological analysis showing the percentage of follicles in different stages of development in ovarian strips of the fresh and vitrified groups after in vitro culture for 7 days

	Primordial	Transitory	Primary	Secondary	Preantral
Non-vitrified (n=12 strips)	14	16	24	20	12
Vitrified (n=12 strips)	15.91	20.45	18.18	13.64	6.82

fresh strips and 20.93 % in the vitrified group. In the fresh strips 65.39 % of the normal follicles were primordial and transitory follicles while 25 % of the follicles were at primary and secondary stages. In the vitrified strips the percentage of primordial and transitory follicles was 58.14 % and the percentage of primary and secondary follicles was 20.93 % (Table 1 and Fig. 3).

Histological evaluation after IVC

After 7 days of in vitro culture (IVC) the morphology and developmental stages of follicles in fresh (n=12) and vitrified strips (n=12) were compared (Fig. 2). Fifty follicles in the fresh strips and 44 follicles in the vitrified fragments were counted. After 7 days of IVC the percentage of normal primordial and transitory follicles (undeveloped follicles) in fresh ovarian strips decreased to 30 % compared to 65.39 % before IVC and the percentage of developed follicles (primary, secondary and preantral) increased to 56 % compared to 25 % before IVC. In the vitrified group developed follicles increased to 38.64 % compared to 20.93 % before IVC and undeveloped follicles decreased to 36.36 % compared to 58.14 % before IVC. After 7 days of IVC the percentage of atretic follicles in the non-vitrified group was 14 % compared to 9.61 % at day 0. In the vitrified group the percentage of atretic follicles increased from 20.93 % at day 0 to 25 % at day 7 of IVC (Table 2 and Fig. 4).

Evaluation of isolated follicles with NR

Follicles from fresh (non-vitrified) (n=60) and vitrified (n=60) ovarian strips after in vitro culture were stained with NR and then isolated. Only well-organized, spherical follicles with an intact basement membrane were considered. A total of 120 follicles in non-vitrified and 100 follicles in vitrified strips were counted. 81.64 % of the isolated follicles in the fresh strips and 72 % in the vitrified strips after IVC were positively stained with Neutral Red. The developmental stages of live follicles in both fresh and vitrified strips were also evaluated. There were more primary, secondary and preantral follicles in fresh IVC strips than those found in the vitrified group after IVC. 41.65 % of the follicles in the fresh group were developed follicles as compared to 32 % in the vitrified group (Table 3 and Fig. 5).

Staining of viable follicles in situ with NR

Two ovarian strips each from the fresh group and the vitrified group and both after 7 days of culture were placed in NR dye for 5 h. The reddish-staining viable follicles at different stages of development were detected in both groups although the

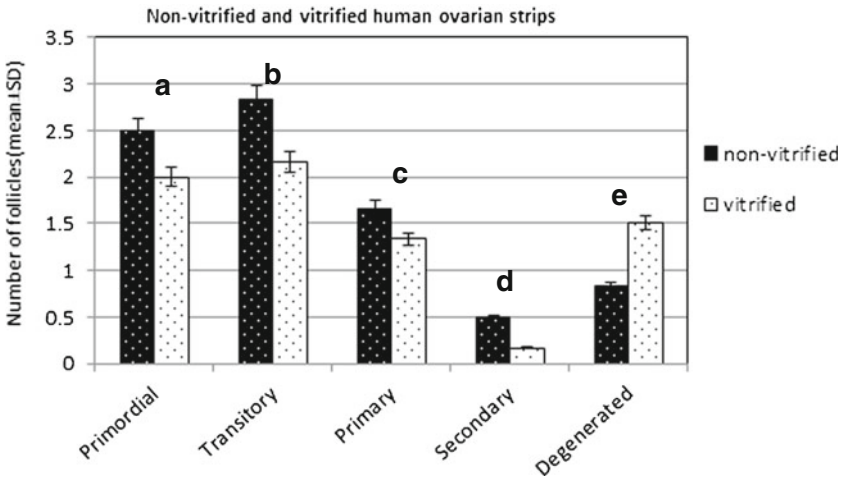

Fig. 3 Histogram showing the number of follicles (mean ± SD) in non-vitrified and vitrified ovarian strips. There is no significant difference between the vitrified and fresh (non-vitrified) groups. ^{a}p=0.503, ^{b}p=0.140, ^{c}p=0.496, ^{d}p=0.260 and ^{e}p=0.11

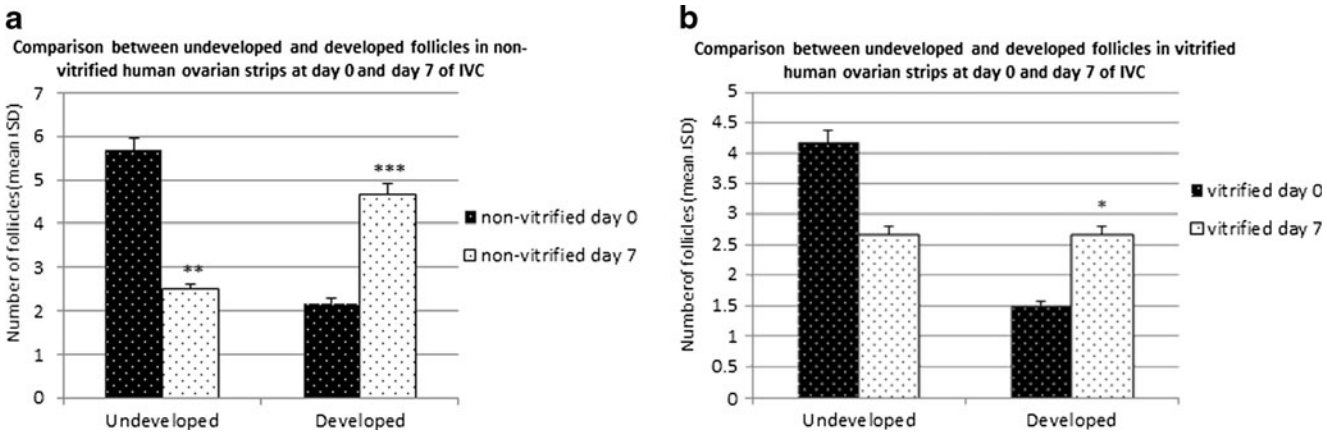

Fig. 4 Histogram showing the number of developed and undeveloped follicles (mean ± SD) at day 0 and day 7 after IVC. **a**: fresh (non- vitrified group); **b**: vitrified group. * indicates significant difference ($p<0.05$), **indicates significant difference ($p<0.01$) and ***indicates significant difference ($p<0.001$) between day 0 and day 7

numbers of viable follicles after vitrification decreased significantly. After vitrification more live primordial and transitory follicles were detected as compared to primary and secondary follicles. Dead follicles were unstained but they could be visualized under the inverted microscope in thinner regions of the ovarian strips. After 7 days of culture there was a decrease in the number of NR-positive primordial and transitory follicles in both fresh and vitrified groups while the proportion of NR-positive secondary follicles was found to be higher than that of the follicles in other stages of development (Fig. 6).

Assessment of viability of ovarian stromal cells and isolated follicles

Fluorescent staining was performed in vitrified ovarian strips after 7 days of IVC. Live follicles and stromal cells indicated by green fluorescence were observed in most of the ovarian strips. Statistical analysis was not performed in this particular experiment as the thickness of ovarian strips varied and the location of follicles was not in the same level among different ovarian strips making it difficult to carry out a meaningful statistical analysis. However, the number of viable isolated follicles was recorded after vitrification and IVC for a qualitative assessment. The follicles which displayed green fluorescence in their entirety were counted as viable and follicles with red fluorescence counted as dead ones. Based on this premise, 65 % of the isolated follicles following vitrification and IVC were found to be viable (Fig. 6). As shown in Fig. 6d, most of the follicles and the surrounding stroma did not show any damage but displayed green fluorescence in full.

Discussion

In recent years ovarian tissue cryopreservation especially with the slow freezing method and transplantation have become a promising approach with great potential to preserve fertility among cancer patients [1, 55]. In spite of this potential, transplantation of ovarian tissue suffers from the risk of reintroducing malignant cells of certain types of cancer [32]. In addition, a large number of grafted follicles could be lost due to ischemia that might occur in post-grafted fragments [56]. An alternative option is cryopreservation of ovarian tissue whereby immature follicles can be isolated from the thawed tissue and then allowed to grow in vitro to the desirable developmental stage for subsequent transplantation. As follicles in earlier stages of development do not contain any small blood vessels, capillaries or white blood cells, grafting would be much safer [57]. In vitro culture has been generally used to evaluate the viability of vitrified ovarian follicles [58].

In the present study we used a simple carrier device made of stainless steel mesh which is inexpensive and suitable for

Table 3 Percentage of isolated live follicles (neutral red stained)

IVC of human ovarian strips					
	Primordial	Transitory	Primary	Secondary	Preantral
Non-vitrified ($n=60$ strips)	16.66	23.33	16.66	16.66	8.33
Vitrified ($n=60$ strips)	17	23	17	9	6

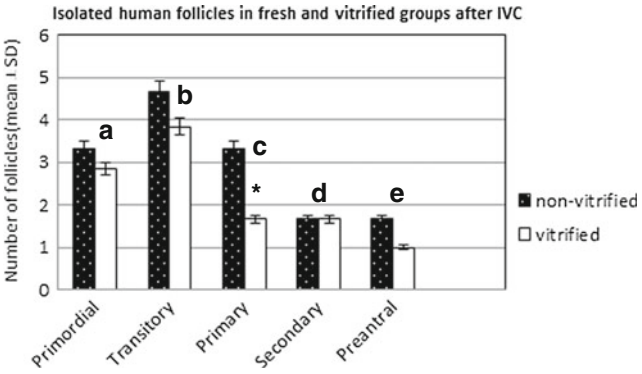

Fig. 5 Histogram showing the number of isolated follicles (mean ± SD) in non-vitrified and vitrified ovarian strips after 7 days of IVC. There is no difference between the isolated follicles in non-vitrified and vitrified groups except for primary follicles ($^{c}p=0.011$). $^{a}p=0.360$, $^{b}p=0.096$, $^{d}p=1.000$ and $^{e}p=0.206$

carrying relatively large number of ovary fragments. The stainless steel mesh consists of criss-crossed strands of metal wires with large pores allowing drainage of excess vitrification solution. It also provides a roughened surface for the support of ovarian tissue fragments, and increases the cooling rate. For vitrification we added PVP as an additional non-permeable cryoprotectant to the vitrification solution to increase glass formation [22] and viscosity [59]. Hashimoto et al.[20] reported that adding PVP to the vitrification solution increases the number of follicles with normal morphology in ovarian tissues obtained from monkeys. An optimal exposure of ovarian strips to the cryoprotectant solution is an important factor for successful vitrification [60]. The exposure time and the size of ovarian tissue strips are closely related to each other in the vitrification procedure.

Various sizes of human ovarian strips and different exposure times were employed in previous studies. Thus, it would be difficult to compare results of the present study with those of previous studies. For example Chang et al. (2011)[60] reported that optimal exposure time for vitrification of human ovarian fragments with 1 mm cube thickness is 10 min [60]. On the other hand Kagawa et al. (2009) [21] used 1×10×10 mm ovarian fragments with 15 min exposure time and reported this method as a successful procedure for human vitrification [21]. Our histological evaluation showed that the proportion of developed follicles (primary, secondary and preantral) increased and undeveloped follicles (primordial and transitory) decreased significantly in both fresh and vitrified groups albeit there were more growing follicles in the fresh group than the vitrified group. These results showed that after vitrification follicles have the potential to grow from undeveloped towards developmental stages. In contrast to our results Choi et al. (2007) [61] reported that in vitrified mouse ovarian samples after in vitro culture for 5 days the percentage of primordial follicles was significantly higher than that of developing follicles. They concluded that cryopreservation caused the death of ovarian cells through

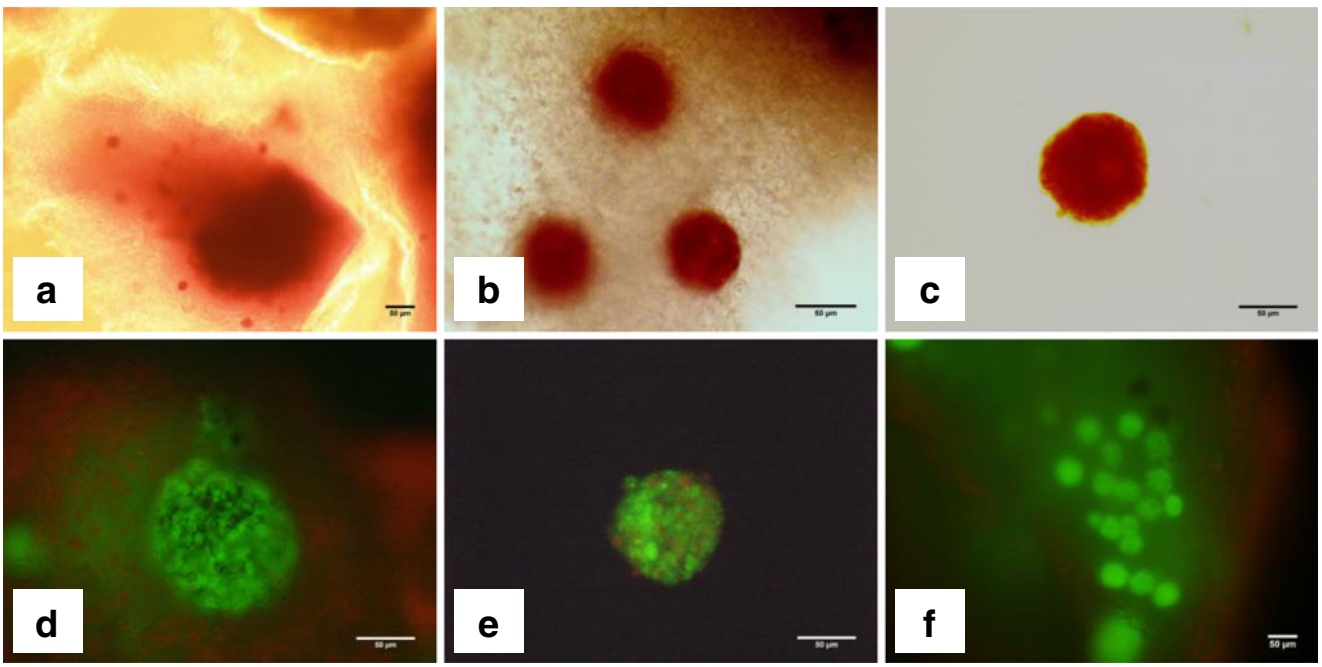

Fig. 6 Light photomicrographs showing ovarian strips (**a** and **b**) and isolated follicle (**c**) stained with Neutral Red after 7 days of IVC, and isolated follicles (**d** and **e**) as well as vitrified/warmed ovarian strips (**e**) stained with Calcein AM and Ethidium homodimer-1 after 7 days of IVC. **a**: fresh ovarian strips; **b**: vitrified/warmed ovarian strips; **c**: isolated follicle; **d**: viable isolated follicle (*green*) and dead stromal cells (*red*); **e**: an isolated follicle with several dead granulosa cells (*red*); **f**: ovarian strip showing numerous live stromal cells and follicles within the strip. Bars=50 μm

apoptosis and thus inhibiting further development of primordial follicles [61]. However a study by Mazoochi et al.(2008) [62] carried out in the mouse showed that there was no sign of apoptosis in vitrified and cultured follicles [62]. A study carried out with human ovarian tissue also reported that vitrification has no effect on apoptosis of follicles [63, 64]. In the present study, the number of viable isolated follicles obtained with fluorescent staining in the vitrified/IVC group appeared to be smaller than that obtained with NR staining in the same group (65 % versus 72 %). This discrepancy may be due to the criteria for evaluation of viable follicles between the two staining methods. In the fluorescent staining method, we considered follicles that displayed green fluorescence without any red coloration of granulosa cells as viable and those with any red coloration of granulosa cells as dead follicles. This evaluation method might have skewed the viable follicles to a smaller number as determined by the fluorescent staining method compared to NR staining. To the best of our knowledge only a few studies on cryopreservation of ovarian tissue have been carried out culturing tissue strips in vitro for more than 24 h after thawing to evaluate the survival and development of follicles [25, 44, 61, 65, 66]. Finally, results of the present study showed that stainless steel mesh can be used as a carrier device in the vitrification procedure. This procedure makes it possible to restore folliculogenesis in in vitro culture allowing immature follicles to further develop to preantral stage after 7 days in organ culture. This provides a new and potential avenue, in the future, for transplanting a suspension of developed follicles in patients who, otherwise, may be at risk of transmission of malignant cells after transplantation of ovarian fragments. Clearly, more investigations will be necessary to evaluate the feasibility of transplanting these follicles in patients who opt for the preservation of fertility. For example, it will be important to demonstrate whether the developed follicles obtained from vitrified human ovarian tissue strips can further grow and become mature eggs in an appropriate milieu such as the three-dimensional in vitro culture system. The latter is currently being investigated in our laboratory.

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