Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions

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BACKGROUND: Cryopreservation of follicles in ovarian cortical tissue has been suggested as a method for preserving fertility for young women who need to undergo cytotoxic therapy. Varying compositions of cryoprotectant solutions have been used to prevent tissue damage during cryopreservation and thawing. We compared human serum [20% (v/v)] and human serum albumin (HSA) (25 mg/ml) in cryoprotectant solutions containing propanediol and sucrose to evaluate whether serum-free medium could be used for this purpose.

METHODS: Biopsies of ovarian cortical tissue were obtained from 23 subjects after informed consent. Fourteen underwent Caesarean section and nine underwent sterilization by laparoscopy. The cortical tissue was cut into pieces of 1–1.5 mm³ and cryopreserved in cryoprotectant solutions containing serum or HSA. After thawing, a total of 1318 follicles were analysed using light microscopy, transmission electron microscopy (TEM) or live/dead assay.

RESULTS: Viability of the follicles was 99.3% in freshly dissected tissue. After thawing, 65% of the follicles and 75% of the oocytes were viable with serum, and 69 and 74%, respectively, with HSA. No significant differences were observed between results in solutions containing serum versus HSA. TEM showed similar results; however, poor survival of stromal tissue was evident in this analysis. The live/dead assay showed 82% viability after thawing for both groups. No benefits were seen from post-thawing culture for 4 h before histological preparation.

CONCLUSIONS: A cryoprotectant solution containing HSA was equally effective as one containing serum. Good viability of follicles was confirmed when using propanediol and sucrose as cryoprotectants, with a large number of follicles.

Key words: cryopreservation/follicles/human/oocytes/ovary

Introduction

Cryopreservation of follicles in ovarian cortical tissue has attracted considerable attention in recent years. The possibility of preserving the fertility of young women undergoing treatment against malignant disease by using this method may be an option for patients facing the necessity of a potentially sterilizing treatment (Hovatta et al., 1996; Newton et al., 1996; Abir et al., 2001; Gook et al., 2001; Oktay, 2001; Van den Broecke et al., 2001; Gosden et al., 2002).

Live births were first obtained in mice after autografting of frozen–thawed ovarian cortical tissue >40 years ago (Parrott, 1960). Restoration of fertility has also been achieved after these procedures in other species such as sheep (Gosden et al., 1994). Human ovarian function after cryopreservation, thawing and autotransplantation of ovarian tissue has been observed recently. Follicular development induced by FSH stimulation, estradiol production and ovulation has been confirmed (Oktay and Karlukaya, 2000). A temporary decrease in FSH levels and estradiol production (Callejo et al., 2001) in addition to follicular and endometrial development has also been observed (Radford et al., 2001). No pregnancies have as yet been reported in humans using this procedure.

Survival of human ovarian follicles after cryopreservation has been shown (Hovatta et al., 1996), using propanediol–sucrose (PrOH-S) and dimethylsulfoxide (DMSO) as cryoprotectants, and also by using ethylene glycol (Newton et al., 1996). The viability of the tissue has been shown in organ culture (Hovatta et al., 1997), after follicle isolation from thawed tissue (Oktay et al., 1997) and after follicle isolation with subsequent culture (Abir et al., 2001). Viability of thawed tissue has also been shown after transplantation to immunodeficient mice (Newton et al., 1996; Van den Broecke et al., 2001) where follicle and oocyte growth has been observed.

The cryopreservation protocols used for human ovarian tissue usually call for either DMSO at 1.5 mol/l as a penetrating
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Comparison of serum and HSA in cryopreservation of human ovarian cortical tissue
cryoprotectant (Hovatta et al., 1996; Newton et al., 1996; Oktay et al., 2000), sucrose at 0.1 mol/l as a non-penetrating cryoprotectant or PrOH-S at the same concentrations (Hovatta et al., 1996; Gook et al., 1999, 2001; Abir et al., 2001). Ethylene glycol at 1.5 mol/l has also been used successfully (Oktay et al., 1997). Table I gives further details.

Varying concentrations of serum and serum substitutes have been used for cryopreservation of ovarian tissue. To achieve live births in sheep, supplementation with 10% bovine calf serum was used (Gosden et al., 1994), and previously we have reported the use of 20% human serum for cryopreservation with PrOH-S or DMSO (Hovatta et al. 1996). Other authors report the use of human serum albumin (HSA) at a concentration of 10 mg/ml (Gook et al., 1999) and synthetic serum (Raz et al. 2002). No comparative studies regarding serum supplementation in cryopreservation of ovarian tissue have been carried out.

The necessity of evaluation and development of cryopreservation methods is apparent. Solutions prepared with serum are not subject to quality control in the same way as commercially available cryoprotectant solutions which are readily available at most fertility clinics. Bovine serum bears the risk of infection and is therefore not suitable for cryopreservation of human ovarian tissue. We have shown previously that HSA is more suitable than serum for the culture of human ovarian follicles in vitro (Hreinsson et al., 2002). At the same time, the necessity for using serum instead of commercially available HSA has not been clearly established in the cryopreservation of human ovarian cortical tissue. We decided to investigate this question systematically using the established PrOH-S protocol.

Post-thawing culture has been shown to improve viability of cryopreserved tissue after thawing (Paynter et al., 1999). Others have not used this method to evaluate the viability of

Figure 1. Flow chart of tissue handling in the study.
The mean age of the women was 34.3 years, range 28–39. Nineteen underwent Caesarean section and nine underwent sterilization by laparoscopy. Consent for participation in the study was obtained as donations from 23 women who had given informed consent for participation in the study. Fourteen of them underwent follicle aspiration after obtaining written consent. Small biopsies of ovarian cortical tissue, up to 5 mm in diameter, were obtained from 23 women 24 to 40 years of age, who had given written informed consent to participate in the study. The tissue was collected into warm, pre-equilibrated HEPES-buffered medium containing HSA (Game®; Vitrolife, Gothenburg, Sweden) and transported to the laboratory. The transport time was 5 min or less and the tissue was processed immediately. After removing most of the medullar tissue, the cortical tissue was cut into pieces of ~1–1.5 mm³ with a scalpel, keeping the tissue immersed in collection medium while working under a stereomicroscope.

Tissue pieces were divided between different types of analysis as shown in Figure 1.

**Cryopreservation**

The cryopreservation solution with HSA as obtained commercially (Freeze-Kit 1®; Vitrolife) consisted of a phosphate-buffered salt solution (PBS) supplemented with HSA at 25 mg/ml. In addition to this basic solution, the first cryo-solution contained 1.5 mol/l PrOH and the second cryo-solution contained 1.5 mol/l PrOH with 0.1 mol/l sucrose. Cryopreservation solutions with serum consisted of PBS (Dulbecco’s PBS, Invitrogen Inc., UK) supplemented [20% (v/v)] with pooled heat-inactivated serum obtained from women undergoing pituitary de-sensitization preceding fertility treatment at the Fertility Unit. The serum was stored at -20°C until the solutions were made up. This basic solution was supplemented with 1.5 mol/l PrOH for the first cryo-solution and 1.5 mol/l PrOH with 0.1 mol/l sucrose for the second cryo-solution (PrOH and sucrose were supplied by Sigma-Aldrich Corp., St Louis, MO). These solutions were sterile-filtered through a 0.2 μm filter (Gelman, Pall Life Sciences, Ann Arbor, MI) before use.

The tissue pieces were immersed in the basic solution for 5 min, and transferred to the first cryo-solution for 10 min and to the second cryo-solution for 5 min. All steps were carried out at room temperature.

The tissue pieces in 1 ml of the second cryo-solution were transferred to 1.8 ml Nunc cryovials (Nunclon®, Roskilde, Denmark) and placed in a programmable freezer (CryoLogic, Australia), cooled from room temperature to -6.5°C at a rate of 2.0°C/min, seeded with liquid nitrogen-cooled forceps during a 10 min holding period, cooled to ~-35°C at a rate of 0.3°C/min and plunged into liquid nitrogen for storage.

The tissue was stored in liquid nitrogen for 5–7 months before it was thawed for the second part of the study.

**Thawing**

The tissue was thawed using thawing solutions (Cryo-PBS) containing HSA (Thaw Kit 1®; Vitrolife) or serum, corresponding to the cryopreservation procedure, i.e. tissue cryopreserved using Freeze Kit 1® was thawed using Thaw Kit 1®, and tissue cryopreserved using serum for freezing solutions was thawed using the same source of serum for the thawing solutions.

Thawing solutions (TS) were as follows: TS 1 contained 1.0 mol/l PrOH and 0.2 mol/l sucrose, TS 2 contained 0.5 mol/l PrOH and 0.2 mol/l sucrose, TS 3 contained 0.2 mol/l sucrose and TS 4 only cryo-PBS.

All thawing steps were performed at room temperature. The cryovials were removed from the liquid nitrogen, air-thawed for 30 s and thawed in a water bath until the ice had melted. The tissue was transferred in the cryoprotectant to an empty organ culture dish (Falcon 3037, Becton-Dickinson, Franklin Lakes, NJ). The tissue pieces were immediately transferred to TS 1 and incubated for 5 min, and transferred to TS 2 and again incubated for 5 min. They were incubated in TS 3 for 10 min and finally in TS 4 for 10 min.

Two to three tissue pieces from six subjects were fixed directly after thawing for light microscopy analysis. All other tissue pieces were cultured in pre-equilibrated α-MEM (Invitrogen Inc.) supplemented with HSA (10%; Pharmacia, Stockholm, Sweden) and ascorbic acid (50 μg/ml; Sigma-Aldrich, Steinheim, Germany) (Thomas et al., 2001) for 4 h in a Nunc 24-well plate (Nunclon, Roskilde, Denmark). Culture was performed at 37°C and with 5% CO₂ in a humidified incubator to see if the normality of the tissue was restored.

**Light microscopy**

The tissue pieces were fixed in Bouin’s solution. Following paraffin embedding (Paraplast, Sherwood Medical, St Louis, MO), they were cut into 4 μm sections. To avoid double counting of follicles, at least 10 sections were discarded before the next one was mounted onto the slide. Staining was performed with haematoxylin and eosin for histological examination.

The developmental stages of the follicles were evaluated as defined previously (Gougeon, 1996). We defined primordial follicles as those containing a single layer of flat follicular cells around the oocyte, primary follicles as those with one or more cuboidal follicular cells, and secondary follicles as those where at least part of the oocyte was surrounded by two or more layers of cuboidal granulosa cells.

Follicles were considered atretic whenever any granulosa cell or oocyte had pyknotic nuclei and/or eosinophilic cytoplasm or clumping of the chromatin material (Gougeon 1986).

**Transmission electron microscopy (TEM)**

The tissue samples taken for TEM were first incubated for 2 h at room temperature in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1 mol/l sodium cacodylate buffer and 0.1 mol/l sucrose pH 7.4. The tissue was then stored in the fixative at 4°C for 3–4 weeks before further processing. The tissue blocks were embedded in LX-112 (Ladd Research Industries Inc., Burlington, VT), cut into 0.5 μm sections and stained with toluidine blue. Light microscopy was used to select sections with follicles for further examination. Thin sections, ~50 nm, were cut and stained with 2% uranyl acetate, followed by lead citrate and examined in a transmission electron microscope (Tecnai, Fei, Eindhoven, The Netherlands) at 80 kV. Micrographs were taken at low magnification (1400–2000×), and printed copies at a final magnification of 3200–4500× were used for evaluation.

Oocytes, granulosa cells and stromal cells were scored separately. An independent observer mixed all the micrographs and they were then evaluated in a blind fashion by two experienced electron microscopists. The ultrastructure of the different cell types was compared as described above.

**Comparison of serum and HSA in cryopreservation of human ovarian cortical tissue**

The tissue was cryopreserved using serum (Cryo-PBS) or HSA (Thaw Kit 1®; Vitrolife) directly in the thawing solutions. The development of human follicles was scored in a blind fashion by two experienced electron microscopists.

**Subjects and methods**

Small biopsies of ovarian cortical tissue, up to 5 mm in diameter, were obtained as donations from 23 women who had given informed consent for participation in the study. The tissue was collected into warm, pre-equilibrated HEPES-buffered medium containing HSA (Game®; Vitrolife, Gothenburg, Sweden) and transported to the laboratory. The transport time was 5 min or less and the tissue was processed immediately. After removing most of the medullar tissue, the cortical tissue was cut into pieces of ~1–1.5 mm³ with a scalpel, keeping the tissue immersed in collection medium while working under a stereomicroscope.

Tissue pieces were divided between different types of analysis as shown in Figure 1.

The tissue was stored in liquid nitrogen for 5–7 months before it was thawed for the second part of the study.
evaluated by using a scoring system. The nuclear content and membrane integrity, cristae of the mitochondria and their density, the density of the cytoplasm, the content of membrane vesicles and attachment to the granulosa cells were the criteria used for evaluation of the oocytes. In granulosa cells, the same parameters were judged, except for attachment. The stromal cells were evaluated only by their nuclear content and the integrity of the extracellular matrix.

Normal structures were scored as 2, small changes as 1 and severe damage as 0. The maximum additive score for the oocytes was 8.6 for the follicular cells and 4 for the stroma. The final total score of the individual cell types was divided by the maximum possible score of all evaluated cells to give a ratio reflecting the preservation of the cells. A perfectly preserved cell group would thus obtain a ratio of 1.0 (100%). A morphometric analysis was performed to evaluate the area and extent of vacuolization in the oocytes, and the number of pyknotic granulosa cells was counted.

**Live/dead assay**

Tissue pieces were digested with 1.5 mg/ml collagenase type II (Invitrogen, NY) in pre-equilibrated α-MEM (Invitrogen Inc.) with 10% human serum (obtained in the same way as described in the previous section) at 37°C and 5% CO₂ in a humidiﬁed incubator. After 1 h, the presence of isolated follicles was checked. If there were follicles floating in the medium, all the medium and tissue were filtered through a 100 µm filter (Gelman, Pall Life Sciences). Tissue pieces on the filter were collected for further digestion, and the flow through medium was centrifuged at 100 g for 5 min. Most of the supernatant was removed and the pellet with a small amount of medium was checked under an inverted microscope for follicle identification. Follicles were stored in culture medium in the incubator for later analysis. This procedure was repeated every 30 min as long as follicles were found in the medium. Usually after 2–3 h digestion, a clear view of the follicles in the stroma was possible and they could be stained along with fully isolated follicles. At this point, the medium containing the tissue was centrifuged at 100 g for 5 min and the pellet together with the individual follicles was resuspended in live/dead working solution [Live/Dead Viability/Cytotoxicity Kit® (L-3224), Molecular Probes, Eugene, OR]. The working solution was PBS containing 2 µmol/l calcine AM which is converted to calcine by intracellular esterases and produces an intense green fluorescence in live cells, and 4 µmol/l ethidium homodimer-1 (EthD-1), which enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, producing bright red fluorescence in dead cells. EthD-1 is excluded by the intact plasma membrane of live cells.

After 20–30 min incubation at room temperature, the cell suspension was briefly centrifuged again (100 g for 1 min) and most of the supernatant was removed. Partly and fully isolated follicles were mounted on glass slides. After the cover slides were mounted, the viability of the follicles was assessed under a fluorescence microscope (Olympus, Tokyo, Japan).

Only follicles showing exclusive esterase activity (green colour) were counted as viable in the assay, and follicles showing red colour in either the oocyte or surrounding granulosa cells were counted as dead.

**Statistical analysis**

The χ² test was used for statistical analysis of differences in the proportions of viable follicles between groups and the ratio (percentage) of pyknotic granulosa cells. With Statistica 6.0 (Stat Soft Inc., Tulsa, USA) we used the Mann–Whitney U-test to analyse the differences in scoring between groups in the TEM analysis. \( P < 0.05 \) was considered statistically significant.

**Results**

**Light microscopy**

A total of 693 follicles were analysed by light microscopy to assess the developmental stage and viability before and after cryopreservation and thawing. Table II gives further details on the number of follicles analysed and the proportion of atretic follicles.

In freshly dissected tissue, 99.3% of the follicles were judged to be viable. After cryopreservation and thawing, a significant reduction in viability was seen \( (P < 0.01 \text{ for all}) \) where 65.1% of the follicles and 75.2% of the oocytes were judged to be viable when cryopreserved in a solution containing serum, and 69.9% of the follicles and 74.4% of the oocytes were viable when cryopreserved in a solution containing HSA. No significant differences were observed between viability in the serum versus HSA groups. However, tissue cryopreserved with serum showed a significantly higher proportion of viable follicles before post-thawing culture compared with that after 4 h culture (80.2% versus 65.1%, \( P < 0.05 \)). A graphic presentation of the results is shown in Figure 2. Figure 4a and b shows follicles cryopreserved with serum and HSA after thawing.

**Transmission electron microscopy**

Utrastructural evaluation was performed on a total of 66 follicles. Figure 3 shows the relative score of oocytes, granulosa cells and stroma. Reduced viability after cryopreservation compared with controls was observed; oocytes, granulosa cells and stromal cells from the freshly prepared control specimens had significantly higher scores than the cryopreserved/thawed tissue \( (P < 0.05) \). The most noticeable

| Table II. Numbers and developmental stages of follicles analysed in the study |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Freshly          | Serum            | HSA             | Serum            | HSA             |
| Light microscopy                | dispersed tissue | 186              | 46              | 18              | 70              | 80              |
|                                 | Serum            | 82              | 18              | 7               | 14              | 22              |
| Secondary                       | 21              | 1               | 3               | 0               | 7               |
| Atretic                         | 2, 0.7%         | 16, 19.8%       | 8, 22.2%        | 45, 34.9%       | 47, 30.1%       |
| Live/dead assay                 | Viable          | 122             | 163             | 35, 17.7%       | 40, 17.4%       |
|                                | Atretic          | 9, 6.9%         | 35              | 35              | 35              |
| TEM                             | 25              | 26              | 15              |

A total of 1318 follicles were analysed.
A reduction in score compared with the control tissue was observed in the stromal tissue. The oocytes, follicular cells and stromal tissue showed no significant differences in score after cryopreservation with medium containing serum or HSA.

A morphometric analysis of the extent of vacuoles in the TEM images showed a vacuolization of 13% of all oocytes in fresh control tissue. After cryopreservation and thawing in solutions with serum, the vacuolization of the oocytes was 18%; with HSA, the vacuolization was also 18%. The number of pyknotic cell nuclei in granulosa cells in control tissue was 1/161 (<1%). With serum, this ratio was 16/91 (17.6%) and with HSA 13/50 (26.0%, NS).

A variety of cryoprotectants and serum supplements have been used for storage of ovarian tissue at liquid nitrogen temperatures. Table I gives details from a few selected references on the subject. It clearly demonstrates the need to standardize the methodology used in this field to obtain comparable results without confounding factors and to provide a stable platform on which progress may be based. Our efforts

The viability of the follicles was 93% in the control tissue, and this was reduced to 82% in both groups after thawing and culture (4 h), the difference being statistically significant (both P < 0.01). Figure 4f and g shows images of viable follicles cryopreserved and thawed using serum and HSA.

Live oocytes were seen in some follicles with dead granulosa cells. Completely dead follicles (both oocyte and all granulosa cells) were not found.

Discussion

We showed in this study that serum does not convey protective effects over HSA in cryopreservation of follicles in human ovarian tissue using the PrOH-S method.

The use of serum in cryoprotectant solutions has been recommended previously (George et al., 1992) for freezing of mouse oocytes, mainly to protect against zona hardening. A protective effect of certain macromolecules in serum has also been suggested (Carroll et al., 1993). This does not seem to be the case for oocytes in early follicles since the zona pellucida has not yet been formed.

The oocyte in the primordial follicle has several characteristics that should make it more likely to survive cryopreservation at this early stage than later in development. The most important of these are the small size of the oocyte and its support cells, which facilitates equilibration with cryoprotectants, low metabolic rate, absence of a zona pellucida and cell cycle stage (arrested in prophase of the first meiotic division) (Shaw et al. 2000). The inherent quiescence of primordial follicles may also make them more likely to survive the initial ischaemia following grafting before the tissue has been revascularized.

A variety of cryoprotectants and serum supplements have been used for storage of ovarian tissue at liquid nitrogen temperatures. Table I gives details from a few selected references on the subject. It clearly demonstrates the need to standardize the methodology used in this field to obtain comparable results without confounding factors and to provide a stable platform on which progress may be based. Our efforts
in this study were aimed at clarifying the question regarding serum supplementation. The live/dead assay used in our study is similar to that used by Oktay et al. (1997) and our results were comparable with theirs. Isolated follicles from thawed tissue have also been studied by Abir et al. (2001). It is possible that some follicles, especially completely degenerate ones, were lost during the digestion procedure. This may explain why we saw a higher proportion of viable follicles by the live/dead assay than by light microscopy. However, we estimate that the number of lost follicles was limited since most follicles were partly isolated. A few secondary follicles were seen; however, they all degenerated. We do not know if this was due to the cryopreservation or the isolation process. The proportion of live follicles was similar after cryopreservation using serum or HSA. Staining in tissue slides as described by Cortvrind and Smitz (2001) could eliminate this potential cell loss.

We observed some cytoplasmic vacuolization in the oocytes similar to that described by Gook et al. (1999) in cryopreserved/thawed tissue, but not as extensive. A shorter incubation time in the cryoprotectant solutions might be one explanation, since long incubation times may result in apoptosis in the tissue. The poor survival of the stromal cells in our system is similar to that described by Gook and co-workers, and the overall survival of the follicles can be considered comparable.

Conventional embedding in TEM involves many steps, including fixation, dehydration and embedding. One disadvantage with this technique is the poor preservation of lipid drops. Usually, lipids will be extracted during dehydration and it is difficult to predict if a vacuole in the cell cytoplasm is an extracted lipid or a vacuole. It is possible to preserve lipids for TEM by use of imidazole-buffered osmium tetroxide. However, in this investigation, such a method was not used. Therefore, we measured all vacuoles as vacuoles, regardless of origin.

We have attempted to quantify the results obtained from the TEM analysis in the same way as the results from the standard histological assessment by light microscopy and live/dead assay. Hence we applied a scoring system to achieve a ratio of preservation for the oocytes, granulosa cells and stroma in the cryopreserved and thawed tissue. We consider the relatively low scoring of the fresh control samples to show the strict scoring applied; the results after cryopreservation and thawing should be considered from this perspective.

The importance of culture after thawing has been investigated by Paynter et al. (1999), who showed that the morphological parameters of bovine follicles were improved after 4 h of culture, suggesting a certain degree of recovery of damage sustained by the follicles during the cryopreservation process. We did not observe this effect in our system; in fact we saw a higher proportion of viable follicles before the 4 h culture period when cryopreserving with serum.

Cryopreservation of follicles in ovarian cortical tissue is a promising method for preservation of fertility among young women undergoing cytotoxic therapy against cancer and for women at risk of premature ovarian failure (Oktay, 2001). These results so far have inspired cautious optimism regarding the possibility of offering this method for preservation of fertility for women at risk of premature ovarian failure. At our unit, patients are carefully informed about the experimental nature of the procedure before attempting cryopreservation of ovarian tissue for fertility preservation.

As other authors have observed previously (Gook et al., 1999; Siebzehnrubl et al., 2000), the immature oocytes alone do not present the most challenging problem as regards cryopreservation of ovarian cortical tissue; rather it is the simultaneous preservation of the oocytes, granulosa cells and the stroma surrounding the follicles. The current technique for cryopreservation of human ovarian cortical tissue has been influenced by the techniques used for cryopreservation of oocytes and pre-implantation embryos. Improvement regarding the preservation of stromal tissue surrounding the follicles remains a challenge.

In conclusion, we showed that the use of HSA in cryoprotectant solution instead of serum results in a good rate of follicle survival after cryopreservation and thawing and should be used primarily in the cryopreservation of human ovarian tissue. Optimizing the methods for concomitant survival of the surrounding stromal tissue along with the follicle remains a subject for further studies.

Figure 4. (a) A primordial follicle cryopreserved with serum, analysed by light microscopy after thawing. The oocyte has tiny vacuoles and the granulosa cells have lost contact in one part of the follicle, which also is slightly deformed. The diameter of the follicle is 40 μm. Toluidine blue was used for staining. (b) A primordial follicle cryopreserved with HSA, analysed by light microscopy after thawing. The oocyte is well preserved, as are the granulosa cells. The diameter of the follicle is 45 μm. Toluidine blue was used for staining. (c) A primordial follicle cryopreserved with serum, after thawing, analysed by TEM. Vacuolization is apparent in the oocyte, and two of the granulosa cells are pyknotic. The nuclear membrane and basal membrane are intact, but the cell membrane of the oocyte has lost attachment from the granulosa cells in large areas. In our scoring system, the oocyte scored 75%, granulosa cells scored 67% and stroma scored 25%. Original magnification 1900×, scale bar = 5 μm. (d) A primordial follicle cryopreserved with HSA, after thawing, analysed by TEM. A low degree of vacuolization is seen in the oocyte, and the nuclear membrane seems intact. Some of the granulosa cells are pyknotic. The oocyte scored 75%, granulosa cells scored 50% and stroma scored 0. Original magnification 2000×, scale bar = 5 μm. (e) A follicle analysed by TEM. No cryopreservation (control). Tiny vacuoles are seen in the oocyte, otherwise the follicle morphology is normal. The oocyte scored 88%, granulosa cells scored 100% and stroma scored 100%. Original magnification 1500×, scale bar = 5 μm. (f) A follicle cryopreserved with serum, analysed by the live/dead assay after thawing. The granulosa cells can be seen surrounding the oocyte, the outline of which is not very clear but can be seen between 10 and 2 o’clock. The follicle and oocyte are clearly viable as shown by the green fluorescence. Original magnification 400×. (g) A follicle cryopreserved with HSA, analysed by the live/dead assay after thawing. Granulosa cells are seen surrounding the oocyte. Original magnification 400×.
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References


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