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Follicles of various maturation stages react differently to enzymatic isolation: a comparison of different isolation protocols

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Abstract. Isolation of human follicles is based on digestion of the tissue by combinations of enzymes. Follicle vitality and morphology are often based on the analysis of pooled

follicles of different maturation stages. Information is therefore lacking on the effect of the isolation protocol to individual follicles of different maturation stages. A study was conducted using five protocols combining different enzymes and varying concentrations. Isolated follicles were classified according to their maturation stages, counted and characterized for vitality, morphology, early apoptosis and organization of transzonal projections. No statistical differences were found between the protocols when outcome parameters were analysed on a pool of follicles regardless of their maturation status. Differences were observed in quality when the follicles were analysed separately according to their maturation status. Combining morphologic characteristics and vitality, both Liberase DH and Liberase TM combined with collagenase IV were better at isolating high-quality primordial follicles, compared with collagenase IV. No statistical difference between the isolation protocols was found for primary follicles. If only high-quality isolated secondary follicles are needed, collagenase IV is found to be most advantageous. Follicles of different maturation stages react differently when enzymatic isolation protocols are compared.

Introduction

Ovarian tissue cryopreservation is a standard technique to preserve fertility in cancer patients. Transplantation of cryopreserved tissue, which is the only option today to restore both endocrine function and fertility, has led to over 24 live births (Donnez et al., 2013). Although promising and effective, there is a risk of reintroducing malignant cells upon transplantation of tissue originating from patients with certain types of cancer (blood cancer), Ewing sarcoma (Abir et al., 2010) or those suffering from metastatic cancers (Dolmans et al., 2010; Rosendahl et al., 2010). Isolating follicles from tissue, resulting in a suspension that is free from malignant somatic cells, can be an alternative for grafting. Vanacker et al. (2012) have shown that alginate hydrogel beads are a promising tool to embed grafted follicles. Isolating human follicles is particularly challenging because of the fibrous and dense nature of the human ovarian stroma. Therefore, a mechanical isolation of the tissue using needles or an isolation protocol using enzymes is needed to isolate the follicles effectively from the surrounding tissue. Different types of collagenase (Ia, II, IX, XI) have been used for this purpose, either alone or in combination with DNase, which avoids sticky DNA ends, making the digested tissue difficult to work with (Abir et al., 1999, 2001, 2008; Huntriss et al., 2002; Osborn et al., 1997; Roy and Treacy, 1993; Schröder et al., 2004). Collagenase, which is a crude preparation derived from *Clostridium histolyticum*, contains high endotoxin levels that could severely impair culture and grafting outcomes (Berney et al., 2001). Additionally, the enzyme shows batch-to-batch variation in effectiveness (McShane et al., 1989). Hovatta et al. (1999) reported higher amounts of premature oocyte extrusions from follicles that had been partially isolated using collagenase (type II). Although

enzymatic disruption using collagenase is an effective method, it could potentially impair follicle quality. Liberase, a purified mammalian tissue-free enzyme, is a blend of highly purified enzymes that has been recognized as a powerful tool to improve the quality of pancreatic islet isolation (Nikolic et al., 2010). Anatomical integrity of the islets was better preserved and increased viability was shown when tissue preparations were treated with Liberase compared with the collagenase preparation. Moreover, the possibility of using a purified endotoxin free enzyme is desired when used in a good manufacturing practice (GMP) setting required for autologous transplantation. Dolmans et al. (2006) described a first attempt to analyse different isolation protocols with Liberase DH compared with collagenase IA. Liberase DH showed isolated vital follicles with good morphology compared with collagenase IA. Later, it was shown that preantral follicles isolated with Liberase DH showed developmental competence after in-vitro culture (Vanacker et al., 2011). Kristensen et al. (2011) observed that a mixture of Liberase TM and collagenase IV revealed intact vital follicles derived from the medulla, and that this method was far less aggressive than enzymatic digestion with collagenase IV alone. These previously published studies using different enzymatic protocols describe the health of the follicles after isolation. The collected follicles are pooled and randomly selected for vitality analysis. As follicles of different maturation stages could react differently to the stringent conditions of an enzymatic isolation procedure, information could be lost when pooling follicles of different stages for different outcome parameters. The aim of the study was to compare different protocols for enzymatic follicle isolation in varying compositions and concentrations, and their effect on the quality of follicles of different maturation stages separately. Isolated follicles were counted and classified

according to their maturation status, and follicles of each maturation stage were analysed separately for follicle health, morphology integrity, absence of early apoptosis and intact oocyte–granulosa cellular interaction network.

Material and methods

Experimental design

Thirty ovarian tissue strips were evenly distributed over five different isolation protocols, for which the follicles were counted, classified and analysed for vitality and morphology. Immunostaining for apoptosis and confocal microscopy analysis of oocyte-granulosa cell connection was carried out for each isolation protocol.

Source of human ovarian tissue

Cryopreserved human ovarian tissue was obtained from three consenting female-to-male transsexual persons (mean age: 28.0 ± 2.0 years), and was kept frozen in liquid nitrogen. All consenting individuals were taking testosterone treatment before tissue preservation (Patient 1: oral testosterone undecanoate for 9 months; Patient 2: intramuscular testosterone enanthate for 4 years; patient 3: intramuscular blend of four esterized testosterone compounds for 7 years). Our centre has experience in working with ovarian tissue obtained from female-to-male transsexual persons. The follicles in these tissues have the potential to mature and grow as shown in previous studies from our group (Soleimani et al., 2010; Van den Broecke et al., 2002). For cryopreservation, the ovaries were bisected and the medulla removed. Residual medulla was scraped from the cortex until a minimum 1-mm thickness remained. The tissue was cut into fragments of about 5

× 5 mm under sterile conditions. The tissue pieces were transferred to freezing medium containing Leibovitz L-15 medium supplemented with 10% human serum albumin (HSA) (Rode Kruis, Belgium) and 1.5 M dimethylsulphoxide (DMSO). The tissue was transferred to cryogenic vials containing 800 □l of freezing medium and incubated for 20 min on ice water while gently shaking. Cryopreservation took place through slow controlled freezing (Planer Biomed, Sunbury, Middlesex, UK) by lowering the temperature at 2°C/minute from 4°C to −9°C; manual seeding at −9°C; cooling at –0.3°C/minute to – 40°C; and a final step to –140°C at a rate of 10°C/min. After programmed slow freezing, the vials were transferred to liquid nitrogen and kept in these conditions for long-term storage. The thawing protocol consisted of swirling the vials in a warm water bath (37°C) for 1 min. The cryoprotective agent was then removed by rinsing in Leibovitz L-15 medium three times. For this study, frozen ovarian tissue of transsexual persons who consented to scientific research on cryopreserved tissue were used. Studies have shown no difference in viability and developmental competence between fresh or frozen-thawed tissue (Abir et al., 1999; Hovatta et al., 1997; Oktay et al., 1997). For these reasons, we felt confident in using the frozen tissue. This study was approved by the Ghent University Hospital Ethical Committee on 13 November 2012 (UZ Ghent reference: 2012/780 Belgian registration number: B6702 01215468).

Histology and follicle counting

Histological analysis was carried out on a frozen–thawed tissue fragment of each consenting patient. The tissue was fixed in 4% buffered formalin and embedded in paraffin, serially sectioned at 5 □m and stained with haematoxylin and eosin. The sections were examined for the presence of follicles and apoptosis before follicle

isolation.

Isolation of follicles

From each patient ovarian tissue fragments 5 × 5 mm were distributed over five different isolation protocols. This experiment was carried out in triplicate. Each piece of ovarian tissue was cut into small pieces before it was placed in one of the five enzyme solutions. The five protocols are shown in Table 1. Liberase TM (Medium Thermolysin concentration) contains neutral protease Thermolysin, which is a thermo stable neutral protease that disrupt the extracellular matrix of the tissue. Liberase DH (High Dispase concentration) contains neutral protease dispase, which cleaves fibronectin in the tissue. Collagenase IV is a crude preparation derived from *Clostridium histolyticum*, that breaks the peptide bond in collagen. All enzyme solutions were dissolved in phosphate buffered saline (PBS) (Sigma, Belgium), DNase was not added to any of the solutions. Incubation was carried out in a water bath at 37°C with gentle agitation until the tissue was completely digested, assessed by visualization of a homogeneous suspension. During that time, the ovarian digest was mechanically disrupted by pipetting up and down every 15 min. Digestion was terminated by the addition of an equal volume of PBS supplemented with 10% HSA (Rode Kruis, Belgium). The suspensions were easy to handle and no stickiness was observed during the recovery of the isolated follicles. For the Liberase treatment, Liberase TM and Liberase DH (Thermolysin Medium and Dispase High; Roche Diagnostics GmbH, Mannheim, Germany) concentrations were calculated as described in http://www.roche-applied science.com/, in order to obtain a digestive power equivalent to 1 mg/ml (0.15 Wünsch Units) collagenase type IA (Life technologies, Belgium). After enzymatic digestion, the resulting suspension was transferred to a petri

dish for follicle identification using a stereomicroscope. The follicles were picked up using a polycarbonate micropipette (MXL-200, Origio, Belgium) and placed in PBS supplemented with 10% HSA at room temperature, until the process of follicular retrieval was completed.

Classification and morphology of isolated follicles

The diameter of the isolated follicles was measured using an inverted microscope with a calibrated eyepiece graticule and classified according to diameter: <60 µm (primordial), >60 µm to ≤75 µm (primary), >75 µm to >200 µm (secondary). The isolated follicles were classified according Gougeon's classification (Gougeon, 1996); (i) primordial follicle, oocyte surrounded by a single layer of flattened pre-granulosa cells; (ii) primary follicle, oocyte showing a single layer of cuboidal granulosa cells; (iii) secondary follicle, with at least two complete layers of granulosa cells; and (iv) antral follicle, with development of an antral cavity. Additionally, isolated follicles were classified into four categories depending on their morphology (M) and their granulosa cell layer integrity (Dolmans et al., 2006): M1, spherical shape with complete granulosa cell layer; M2, irregular shape with complete granulosa cell layer; M3, irregular shape with less than 10% granulosa cell loss; and M4, totally atypical shape with 10–50% granulosa cell loss, or an extruded oocyte (Figure 1).

Viability assessment with fluorescence dye

Follicle viability was assessed by double fluorescent labelling with calcein AM and ethidiumhomodimer stains (Live/ Dead Viability Assay kit, L-3224, Invitrogen, Belgium) as described by Dolmans et al. (2006). Briefly, isolated follicles were incubated in 1 ml

PBS containing 1% HSA, 1 µM calcein AM and 2 µmM ethidiumhomodimer for 45 min at 37°C in the dark. Non-fluorescent cell-permeant calcein AM enters the cell and is cleaved by esterase in living cells giving an intense uniform green fluorescence. Ethidiumhomodimer-I enters cells with damaged membranes binding to DNA with high affinity, resulting in a bright red fluorescence. After exposure to fluorescent dyes, the isolated follicles were washed in PBS containing 1% HSA and observed under an inverted fluorescence microscope (Axioplan II; Zeiss, Zaventem, Belgium). Green fluorescence was visualized in vital cells (ex/em ~495/ ~ 515 nm) and red fluorescence in dead cells (ex/em ~495/ ~ 635 nm). The isolated follicles were classified into four categories depending on the percentage of dead granulosa cells (Dolmans et al., 2006): V1, live follicles: follicles with the oocyte and GCs viable; V2, minimally damaged follicles: follicles with <10% of dead GCs; V3, moderately damaged follicles: follicles with 10–50% of dead GCs; and V4, dead follicles: follicles with either the oocyte or greater than 50% GCs dead (Figure 2).

Early apoptosis detection by TUNEL

In-situ TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling analysis was carried out according to the manufacturers' instructions (In Situ Cell Death Detection Kit – Fluorescein; Boehringer Mannheim, Germany). Briefly, isolated follicles were fixed in 3.7% paraformaldehyde and washed in PBS. For the positive control, isolated follicles were treated with 3000 units/ml DNase I recombinantin50mMTris-HCl (pH7.4−8.0) for 30 min at 37°C to induce non-specific breaks in DNA and washed twice in PBS. All isolated follicles were incubated for 1 h at 37°C with 50 µl TUNEL reaction mixture in the dark. Follicles were incubated with 50 µl reaction buffer without TdT as a

negative control. The reaction was stopped by washing the sections in PBS three times. Vectashield mounting medium was used to preserve fluorescence and prevent rapid photo-bleaching. All follicles were examined using a fluorescence microscope (Axioplan II; Zeiss, Zaventem, Belgium) equipped with an epi-illumination single band emitter filter cassette for the illumination of green (FITC). Fluorescein-12-dUTP, once conjugated to the 3′-OH ends of fragmented DNA, stains the nuclei of apoptotic cells green (Figure 3). For the tissue section, the TUNEL procedure was performed according to Liu et al. (2002). Follicles were classified as TUNEL-positive when the oocyte, the granulosa cells, or both, show a positive staining.

Actin-F immunofluorescence staining

Isolated follicles were fixed in a modified buffer originally described by Mattson and Albertini (1990). Briefly, follicles were fixed and extracted in a microtubule-stabilizing buffer (0.1 M PIPES, pH 6.9, 5 mM $MgCl_2O.6H_2O$, 2.5 mM EGTA, 0.01% [v/v] aprotinin, 1 mM dithiothreitol, 0.1% [v/v] Triton X-100, 1 μM taxol, 50% [v/v] deuterium oxide [D_2O], and 2.0% [v/v] formaldehyde) for 45 min at 37°C. Fixed follicles were washed three times (15 min each) at 37°C in a blocking solution consisting of PBS with 0.2% sodium azide, 2% normal goat serum, 1% bovine serum albumin, 0.2% powdered milk, 0.1 M glycine, and 0.01% Triton X-100. Follicles were stored in the same blocking solution at 4°C for up to 4 weeks until immunocytochemical staining. To visualize filamentous actin (F-actin), follicles were incubated with Alexa-Fluor 568-phalloidin (dilution 1:40 in blocking solution; Molecular Probes, Belgium) at room temperature for 90 min. Labelled follicles were washed and mounted in Moviol as an anti-fading reagent. Slides were stored in the dark at 4°C until analysis. Images of

labelled follicles were observed using a Nikon A1r confocal microscope mounted onto an inverted Ti Eclipse epifluorescence body (Nikon Instruments, Paris, France) with a 60x oil Plan Apo objective (NA = 1.4). Z-stacks were acquired with a pinhole setting of 1 Airy unit at 0.5–0.75 µm steps. Image annotation was performed with Image J freeware (rsbweb.nih.gov/ij/). The organization of the transzonal projections (TZP) was classified as complete (no contact lost between oocyte and granulosa cells [i.e. a dense F-actin ring]); partial loss (gaps between the oocyte and the granulosa cell); or total loss (no physical connections between oocyte and granulosa cells).

Statistical analysis

GraphPad Instat version 3.10 was used for statistical analysis. Comparisons between percentage of viable follicles, morphology and DNA fragmentation in all groups were analysed by chi-square test. $P < 0.05$ were considered to be statistically significant.

Results

Histology and follicle counting

Before starting the isolation of follicles, the presence of follicles in the tissue was examined through histological evaluation by counting 10 sections per tissue fragment. A patient was included in the study for isolation of follicles if follicles were present in these sections. A total of 30 ovarian tissue pieces were used in this study. The mean weight of each tissue fragment was 0.13 g ± 0.09 g (0.02 g to 0.399 g). These fragments contained a total of 922 isolated human follicles, which were analysed. The mean number of follicles was 30.73 ± 19.62 (4–77) per tissue fragment. Most of the follicles were observed at the primordial stage (*n* = 416; 45.1%), primary (*n* = 284; 30.8%) and secondary stage (*n* = 222; 24.1%) (Table 2). In all isolation protocols, follicles were classified according to their size and were further assessed for morphology and viability. No statistical differences were found between the isolation protocols concerning number of different types of follicles.

Classification and viability assessment of isolated follicles

All follicles were examined and classified for viability using the fluorescence probes calcein-AM and ethidium homodimer-1 (Table 3). The V1 represented undamaged follicles, V2 the minimally damaged follicles, V3 the moderately damaged follicles and V4 the severely damaged follicles (Figure 2). The percentages of viable follicles (V1 + V2 with 0 to less than 10% dead granulosa cells) were not statistically different between the different enzymes isolation protocols when isolated follicles were analysed as a pool, regardless of their maturation status: protocol 1: 100% (149/149); protocol 2: 98.2%

(216/ 220); protocol 3: 98.2% (221/225); protocol 4: 96.0% (169/ 176) and protocol 5: 98.5% (135/137), respectively. Additionally, the viability was assessed in relation to the follicular stage. It was shown that the enzyme protocol based on Liberase DH with the concentration of 0.01 mg/ml and 0.05 mg/ml had a higher percentage of undamaged primordial follicles (56.3% [80/142] and 55.1% [49/89]) compared with the enzyme protocol with collagenase IV (31.8% [47/148]; $P < 0.0001$; $P = 0.0004$) (Table 4).

Morphology

During the analysis of the vitality staining, follicles were simultaneously examined for morphology. Follicles showing a morphology of M1 or at least M2 were considered as very well preserved morphologically with granulosa cell integrity. Follicles showing morphology M3 were having a poor morphologic shape and M4 were follicles showing an extruded oocyte after isolation. In general, the morphology was found to be well preserved in all isolation protocols as shown in Table 5; however, some differences were observed between the protocols analysed. Isolation protocol 2 and protocol 3 showed a significantly lower percentage of M1 and M2 morphology ($P = 0.0002$) compared with the other isolation protocols when morphology was based on pooled follicles: protocol 1: 100% (149/149); protocol 2: 92.7% (204/220); protocol 3: 94.2% (212/225); protocol 4: 99.4% (175/176) and protocol 5: 97.8% (134/137), respectively.

The remaining follicles were classified as poor morphology (M3 and M4). The proportions of follicles with a totally atypical shape or an extruded oocyte (M4) were high in the isolation protocol 3 (collagenase IV [1 mg/ml]) and protocol 2 (Liberase TM [0.05 mg/ml] plus collagenase IV [0.2 mg/ml]). Additionally, the morphology was

assessed in relation to the follicular stage. It was shown that the enzyme protocol based on Liberase DH with the concentration of 0.01 mg/ml and 0.05 mg/ml had a higher percentage of normal shaped primordial follicles (51.0% [75/147] and 52.4% [55/105]) compared with the enzyme protocol with collagenase IV (33.7% [56/159]; $P = 0.0053$; $P = 0.0057$) (Table 6). Unfortunately, histological sections of isolated follicles of each follicular stage were not produced to study the morphology in more detail. When combining the results of the morphology with the vitality scoring, it was clear that depending on the follicular stage, primordial follicles with a morphology of at least M2-score and a vitality of at least V2-score, showed in the enzyme protocol based on Liberase DH with the concentration of 0.01 mg/ml and 0.05 mg/ml a higher percentage of normal shaped primordial follicles (54.4% [92/169] and 51.9% [69/133], respectively) compared with the enzyme protocol with collagenase IV (32.4% [68/210]; $P = 0.0002$; $P = 0.0010$) (Table 7). No statistical difference was observed in high-quality primordial follicles isolated with the Liberase DH or Liberase TM combination protocols, and all protocols were able to isolate high-quality primary follicles. For isolation of secondary follicles, surprisingly, collagenase (32.9% [69/210]) seemed the method of choice, as it yielded the highest proportion of vital secondary follicles in comparison with Liberase TM (0.01 mg/ml) combined with collagenase IV (0.2 mg/ml) (8.7% [13/149]) or Liberase DH (0.01 mg/ml) (14.8% [25/169]; $P < 0.0001$). No statistical difference were found for high-quality secondary follicles between collagenase IV (32.9% [69/212]) and Liberase DH (0.05 mg/ml) (23.3% [31/133]).

Early apoptosis detection

In the five isolation protocols, about 15 follicles were analysed at random for early

apoptotic cells regardless of their follicular stage, although most of the follicles analysed for apoptosis were at primordial and primary stages. If positive, DNA fragmentation detection was found. It was only seen in a small number of granulosa cells (1–5 cells/follicle) but not in the oocyte (Table 8). The isolation protocol 3, the collagenase IV enzyme solution, gave a higher DNA fragmentation rate compared with the protocol 2, and protocol 5 (Liberase DH), which did not show any DNA fragmentation, although the difference was not statistically significant. Generally, the Liberase DH derived isolation enzyme protocols tended towards lower DNA fragmentation in the granulosa cells and the oocyte, than the collagenase IV and the combined enzyme solutions.

Actin-F immunofluorescence staining

Initial loss of developing follicles may be related to oocyte death and also to the loss of contact between cellular compartments. Because follicle growth and survival is dependent on communication between the granulosa cells and the oocytevia TZP, we determined if TZP in different isolation enzyme protocols were preserved. A selection of isolated follicles from each isolation protocol was fixed and stained to examine the organization of TZPs, partial or total loss, by confocal microscopy. In the five isolation protocols, about 15 follicles were examined randomly, regardless of their follicular stages. A good connection could be determined for all isolation protocols and a good connection between oocyte and surrounding granulosa cells. No extracellular spaces or partial loss between the oocyte and the granulosa cells of TZPs were detected. All isolated follicles contained a round oocyte with a typical, dense ring of actin-rich TZP surrounding the growing oocyte (Figure 4).

Discussion

For cancer patients who cannot benefit from transplantation of cryopreserved ovarian tissue because of risk of transmission of malignant cells, the only option is the use of isolated follicles for transplantation or possibly in the future in-vitro maturation. As follicles of different maturation stages comprise of different cell layers, it is plausible that different enzymatic isolation protocols influence the health of follicles at different maturation stages in different ways. In the present study, different enzymatic protocols to isolate human ovarian follicles from cryopreserved ovarian tissue were compared. Vital follicles were successfully isolated from all digestion protocols, and our results indicate that follicle morphology and the granulosa cell layer are well preserved in all types of follicles. No statistical difference was observed in total number of isolated follicles between the protocols. Isolation with collagenase IV (1 mg/ml), however, showed follicles with a suboptimal morphology and damaged shape, where in some cases, an extruded oocyte. This was in accordance with previous reports (Dolmans et al., 2006; Kristensen et al., 2011; Vanacker et al., 2011). In our study, we could additionally show that different enzyme treatments had different effects on the primordial follicles. Fewer primordial follicles had a spherical shape and a complete granulosa cell layer in the collagenase IV enzyme protocol compared with the Liberase DH protocol. Oocyte and follicle growth are dependent on these structures for survival; however, being composed of F-actin and microtubules (Carabatsos et al., 1998), they are sensitive to changes in temperature and sheer stress, and thus vulnerable during cryopreservation (Vanhoutte et al., 2004). The TZP network is a good biomarker of follicle health, because of its necessity for bidirectional communication between somatic cells and the oocyte. In the

present study, no abnormalities were observed in the TZP network, indicating that these important structures were well preserved during the isolation protocol. Early apoptosis in the follicles plays a role in developmental competence, both *in vivo* and *in vitro*. Apoptosis staining by TUNEL of the isolated follicles showed low numbers of apoptotic cells in some of our isolation protocols. The Liberase DH enzyme isolation protocols showed a lower DNA fragmentation than the collagenase IV enzyme solution and the combined (Liberase TM plus collagenase IV) enzyme solution protocol. It has been shown that some apoptosis was present in frozen–thawed ovarian tissue (Martinez-Madrid et al., 2007); however, apoptotic cells and follicles might be lost in the enzymatic isolation procedure and were perhaps therefore not observed in our study. The histological sections of frozen– thawed ovarian tissue fragments confirmed the TUNEL staining results of the isolated follicles, showing no apoptosis in the tissue after freezing and thawing. A recent study of Vanacker et al. (2013) showed that follicles isolated from frozen-thawed ovarian tissue appear to grow faster than cryopreserved isolated follicles. Keeping the follicles in their cortex during freezing would, therefore, possibly be a good clinical approach. When combining morphologic characteristics and vitality, our data showed that both Liberase DH and Liberase TM combined with collagenase IV were capable of isolating high-quality primordial follicles, compared with collagenase IV. No statistical difference was found between the isolation protocols when primary follicles were analysed. For isolation of high-quality secondary follicles, several protocols can be used, such as collagenase IV, liberase TM (0.05 mg/ml) combined with collagenase IV or Liberase DH (0.05 mg/ml). Finally, our study showed that different enzyme solutions react differently to follicles depending on their follicular maturation stage. Although

experimental studies are needed to assess the functionality of isolated follicles from different isolation protocols after xenotransplantation, in-vitro culture, or both, our observations could be of importance when using isolated follicles for grafting or in-vitro maturation studies. Our data show no statistical difference between isolation protocols when outcome parameters were analysed on a pool of follicles regardless of their maturation status. Differences were observed, however, in quality when the follicles were analysed separately according to their maturation status. Taking our observations into account, we indicate that Liberase DH (0.05 mg/ml) allows isolation of a high number of vital follicles of primordial, primary and secondary status, where other enzyme solutions showed a negative effect on vitality of primordial or secondary follicles. Additionally, all follicles, regardless of their maturation stage, maintain their morphological integrity, there are no signs of apoptosis and good granulosa-cell connection with the oocyte is shown. In conclusion, our data show that follicles of different maturation stages react differently to enzymatic isolation protocols.

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Figures

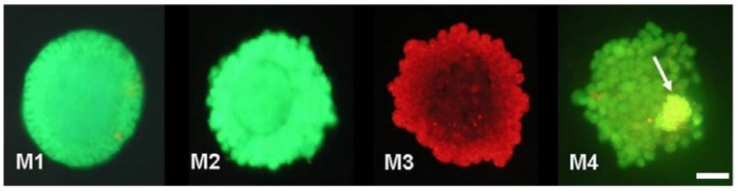

Figure 1 Morphology classification of isolated follicles was carried out during the viability staining. The red and green staining has no meaning for interpretation of the follicle morphology. The isolated follicles were classified into four categories depending on their form and granulosa cells. M1, spherical form with complete granulosa cell layer; M2, irregular form with complete granulosa cell layer; M3, irregular form with less than 10% loss; M4, totally atypical form with 10–50% granulosa cell loss or an extruded oocyte (see arrow); bar = 28 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

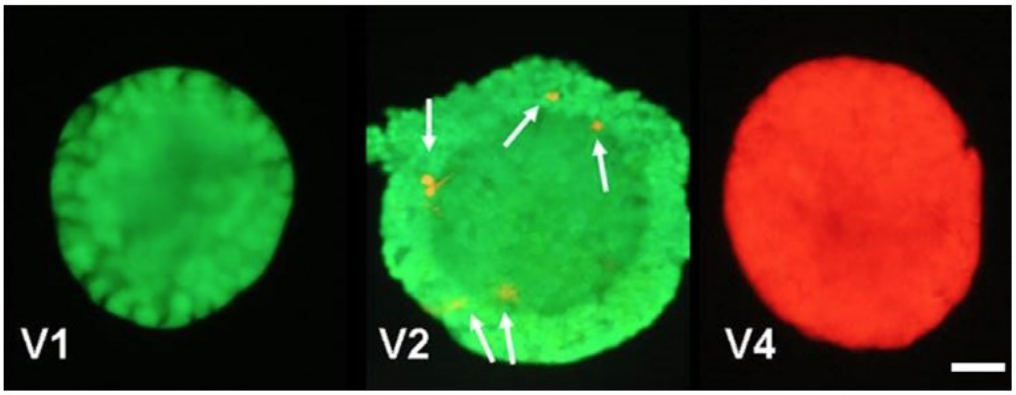

Figure 2 Viability classification of isolated follicles into four categories depending on the percentage of dead granulosa cells and oocyte viability: V1, live follicles (follicles with the oocyte and all the granulosa cells viable [stain green]); V2, minimally damaged follicles (follicles with less than 10% of dead granulosa cells [stain red]); V3, moderately damaged follicles (follicles with 10–50% of dead granulosa cell [not shown]); V4, dead follicles (follicles with both the oocyte or over 50% of granulosa cells dead); bar = 27 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

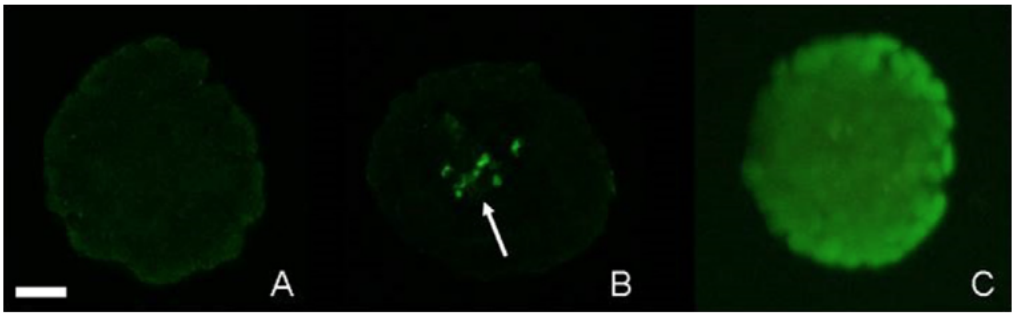

Figure 3 Apoptotic cells were detected in the isolated follicles Tdt (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL). TUNEL-positive cells are stained green. (A) Negative control, no TUNEL labelling; (B) some TUNEL positive cells are detected in the granulosa cells (see arrow); (C) isolated follicle treated with DNase to serve as a positive control for TUNEL labelling; bar = 23 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

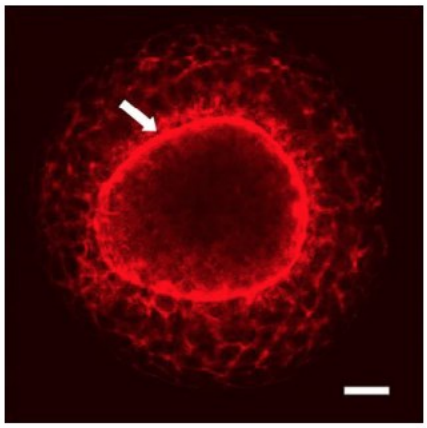

Figure 4 Confocal laser scanning analysis of actin-cytoskeleton stained by phalloidin in an isolated follicle. Single section of a secondary follicle, the contact between oocyte and cumulus granulosa cells (see arrow) is well preserved (dense actin ring); bar = 18 μm.

Tables

Table 1 Isolation protocols.

Protocol 1	Liberase TM 0.01 mg/ml plus collagenase IV 0.2 mg/ml; to obtain a more mild isolation, a lower concentration of the Liberase TM was chosen.
Protocol 2	Liberase TM 0.05 mg/ml plus collagenase IV 0.2 mg/ml; (Kristensen et al., 2011).
Protocol 3	Collagenase IV 1 mg/ml; (Maltaris et al., 2006 and Vanacker et al., 2011).
Protocol 4	Liberase DH (0.01 mg/ml); to obtain a more mild isolation, we chose to lower the concentration of the Liberase DH.
Protocol 5	Liberase DH (0.05 mg/ml) (Vanacker et al., 2011).

Table 2 Classification of isolated follicles per isolation protocol.

Enzyme protocol	*Follicles* (n)	*Primordial (%)*	*Primary (%)*	*Secondary (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	149	83 (55.7)	53 (35.6)	13 (8.7)
Protocol 2 Liberase TM (0.05 mg/ml) + collagenase IV (0.2 mg/ml)	224	97 (43.3)	60 (26.8)	67 (29.9)
Protocol 3 collagenase IV (1 mg/ml)	236	73 (30.9)	79 (33.5)	84 (35.6)
Protocol 4 Liberase DH (0.01 mg/ml)	176	93 (52.8)	58 (33.0)	25 (14.2)
Protocol 5 Liberase DH (0.05 mg/ml)	137	70 (51.1)	34 (24.8)	33 (24.1)

% = percentage on the total number of follicles per protocol group.

Table 3 Viability assessment of isolated follicles per isolation protocol.

Enzyme protocol	*Follicles (n)*	*V1 (%)*	*V2 (%)*	*V3 (%)*	*V4 (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	149	117 (78.5)[a,b]	32 (21.5)[d,e]	0 (0.0)	0 (0.0)[i]
Protocol 2 Liberase TM (0.05 mg/ml) + collagenase IV (0.2 mg/ml)	220	160 (72.7)	56 (25.5)[f]	2 (0.9)	2 (0.9)[j]
Protocol 3 Collagenase IV (1 mg/ml)	225	148 (65.8)[a,c]	73 (32.4)[d,g]	4 (1.8)	0 (0.0)[k]
Protocol 4 Liberase DH (0.01 mg/ml)	176	142 (80.7)[c]	27 (15.3)[f,g,h]	0 (0.0)	7 (4.0)[i,j,k]
Protocol 5 Liberase DH (0.05 mg/ml)	137	89 (65.0)[b]	46 (33.6)[e,h]	0 (0.0)	2 (1.5)

[a–k]Values with the same superscripts are significantly different between the isolation protocols ($P < 0.05$).
% = Percentage of the total number of follicles per protocol group. V1 = number of follicles with no dead granulosa cells; V2 = follicles with a high viability but with less than 10% dead granulosa cells; V3 = the moderate damaged follicles with 10–50% dead granulosa; V4 = follicles that are completely dead (oocyte and granulosa cells).

Table 4 Viability assessment (V1) per isolation protocol according to follicular stages.

Enzyme protocol	*V1 (n)*	*Primordial (%)*	*Primary (%)*	*Secondary (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	117	68 (58.1)[c]	41 (35.0)	8 (6.8)[e,f,g,h]
Protocol 2 Liberase TM (0.05 mg/ml) + Collagenase IV (0.2 mg/ml)	160	79 (49.4)[a]	41 (25.6)	40 (25.0)[e,i]
Protocol 3 Collagenase IV (1 mg/ml)	148	47 (31.8)[a,b,c,d]	52 (35.1)	49 (33.1)[f,j,k]
Protocol 4 Liberase DH (0.01 mg/ml)	142	80 (56.3)[d]	41 (28.9)	21 (14.8)[h,i,k]
Protocol 5 Liberase DH (0.05 mg/ml)	89	49 (55.1)[b]	23 (25.8)	17 (19.1)[g,j]

[a–k]Values with the same superscripts are significantly different between the isolation protocols ($P < 0.05$).
% = Percentage of the total number of V1 follicles per protocol group; V1 = number of follicles with no dead granulosa cells.

Table 5 Morphology assessment of isolated follicles per isolation protocol.

Enzyme protocol	*Follicles (n)*	*M1 (%)*	*M2 (%)*	*M3 (%)*	*M4 (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	149	126 (84.6)[a,b]	23 (15.4)[f]	0 (0.0)[j,k]	0 (0.0)
Protocol 2 Liberase TM (0.05 mg/ml) + collagenase IV (0.2 mg/ml)	220	132 (60.0)[a,c,d,e]	72 (32.7)[f,g,h,i]	14 (6.4)[j,l,m,n]	2 (0.9)
Protocol 3 Collagenase IV (1 mg/ml)	225	159 (70.7)[b,c]	53 (23.6)[g]	8 (3.6)[k,l,o]	5 (2.2)
Protocol 4 Liberase DH (0.01 mg/ml)	176	147 (83.5)[d]	28 (15.9)[h]	1 (0.6)[m,o]	0 (0.0)
Protocol 5 Liberase DH (0.05 mg/ml)	137	105 (76.6)[e]	29 (21.2)[i]	2 (1.5)[n]	1 (0.7)

[a–o]Values of the same superscripts are significantly different between the isolation protocols ($P < 0.05$).
% = Percentage of total number of follicles per protocol group. M1 = the number of follicles with a spherical shape with complete granulosa cell layer; M2 = follicles with irregular shape with complete granulosa cell layer; M3 = irregular shape with less than 10% granulosa cell loss; M4 = the follicles that showed a totally atypical shape with 10–50% granulosa cell loss, or an extruded oocyte.

Table 6 Morphology assessment M1 per isolation protocol according to the follicular stages.

Enzyme protocol	*M1 (n)*	*Primordial (%)*	*Primary (%)*	*Secondary (%)*
Protocol 1 Liberase TM (0.01 mg/ml)	126	74 (58.7)[b,d]	40 (31.7)	12 (9.5)[f,g,h,i]
Protocol 2 Liberase TM (0.05 mg/ml)	132	60 (45.5)[a,b]	40 (30.3)	32 (24.2)[f]
Protocol 3 collagenase IV (1 mg/ml)	159	56 (33.7)[a,c,d,e]	59 (35.5)	44 (27.7)[g]
Protocol 4 Liberase DH (0.01 mg/ml)	147	75 (51.0)[e]	52 (35.4)	20 (13.6)[i]
Protocol 5 Liberase DH (0.05 mg/ml)	105	55 (52.4)[c]	29 (27.6)	21 (20.0)[h]

[a–i]Values with same superscripts are significantly different between the isolation protocols ($P < 0.05$).
M1 = the number of follicles with no dead granulosa cells; % = percentage of the total number of M1 follicles per protocol group.

Table 7 Follicles of high quality with morphology of at least M2 and vitality V2 per isolation protocol according to the follicular stages.

Enzyme protocol	*Follicles* (n)	*Primordial (%)*	*Primary (%)*	*Secondary (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	149	83 (55.7)[a]	53 (35.6)	13 (8.7)[e,f,g]
Protocol 2 Liberase TM (0.05 mg/ml) + collagenase IV (0.2 mg/ml)	201	92 (45.8)[b]	57 (28.4)	52 (25.9)[e,h]
Protocol 3 Collagenase IV (1 mg/ml)	210	68 (32.4)[a,b,c,d]	73 (34.8)	69 (32.9)[f,i]
Protocol 4 Liberase DH (0.01 mg/ml)	169	92 (54.4)[c]	52 (30.8)	25 (14.8)[h,i]
Protocol 5 Liberase DH (0.05 mg/ml)	133	69 (51.9)[d]	33 (24.8)	31 (23.3)[g]

[a–i]Values with the same superscripts are significantly different between the isolation protocols ($P < 0.05$).
% = Percentage of the total number of follicles with a morphology of at least M2 and a vitality of at least of V2 per protocol group.

Table 8 Early apoptosis detection in isolated follicles.

Enzyme protocol	*Follicles* (n)	*DNA fragmentation negative (%)*	*DNA fragmentation positive (%)*
Protocol 1 Liberase TM (0.01 mg/ml) + collagenase IV (0.2 mg/ml)	14	13 (92.9)	1 (7.1)
Protocol 2 Liberase TM (0.05 mg/ml) + collagenase IV (0.2 mg/ml)	15	15 (100)	0 (0.0)
Protocol 3 Collagenase IV (1 mg/ml)	15	11 (73.3)	4 (26.7)
Protocol 4 Liberase DH (0.01 mg/ml)	16	15 (93.8)	1 (6.3)
Protocol 5 Liberase DH (0.05 mg/ml)	14	14 (100)	0 (0.0)

% = Percentage on the total number follicles per protocol group.