

Evaluation of Liberase, a purified enzyme blend, for the isolation of human primordial and primary ovarian follicles

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BACKGROUND: The purpose of this study is to evaluate the effectiveness of a standardized mixture of purified enzymes (Liberase), for the isolation of human ovarian follicles. **METHODS:** This is an experimental prospective study. Ovarian biopsies were obtained from eight young women undergoing laparoscopy for benign gynaecological disease. Follicles were isolated by Liberase or collagenase enzymatic digestion. Follicle quality was assessed by evaluating their general morphology and viability after fluorescent staining, and the ultrastructure by electron microscopy. **RESULTS:** The number of fully isolated follicles recovered from the Liberase-treated group was lower than from the collagenase group (156 versus 263) despite equal-sized biopsies being taken. A high proportion of follicles (98.6%, 70/71) were viable after Liberase isolation and most follicles were of good morphology with a complete granulosa cell layer (70.4%, 31/44). Ultrastructural studies indicated that Liberase-isolated follicles showed signs of atresia only occasionally and that the oolemma–follicular cell interface was well preserved. **CONCLUSIONS:** Liberase treatment allows the isolation of highly viable follicles from human ovarian tissue, with an unaltered morphology and ultrastructure. This purified endotoxin-free enzyme preparation is a promising alternative to impure collagenase preparations for the reproducible isolation of intact primordial and primary follicles for culture and grafting purposes.

Key words: collagenase/fertility preservation/human follicles/isolation/Liberase

Introduction

Progress in the management of childhood malignancy has significantly improved the long-term survival rate of young cancer patients. Unfortunately, the ovaries are very sensitive to cytotoxic treatment, often resulting in the loss of both endocrine and reproductive functions. By retrieving and cryopreserving ovarian tissue prior to the initiation of cancer treatment, there is now the possible option of restoring fertility by implanting the frozen–thawed ovarian tissue once the patient has recovered (Donnez and Bassil, 1998; Schmidt *et al.*, 2003a; Donnez *et al.*, 2004). Successful transplantation of frozen–thawed ovarian tissue cryopreserved before cancer treatment has been described in a patient with Hodgkin's lymphoma (Donnez *et al.*, 2004).

Although safe transplantation of ovarian tissue from lymphoma patients has been reported in severe combined immunodeficient (SCID) mice (Kim *et al.*, 2001), the possibility of reintroducing tumour cells into cancer patients by autografting of ovarian tissue cannot be excluded (Shaw *et al.*, 1996; Meirou, 1999). To avoid transferring malignant cells, ovarian tissue culture with *in vitro* follicle maturation could be performed.

Culturing isolated follicles from the primordial stage is a particularly attractive proposition since they represent >90% of

the total follicular reserve and show high cryotolerance (Smitz and Cortvrindt, 2002). However, isolated primordial follicles do not grow properly in culture (Hovatta *et al.*, 1999; Abir *et al.*, 2001) and further studies are clearly needed to identify factors sustaining follicular maturation and growth in humans (Smitz and Cortvrindt, 2002) and to assess the contribution of stromal cells to these processes. Encouraging results were achieved by Hovatta (2004) when human primordial follicles were grown in organ culture. But follicle isolation (Abir *et al.*, 1999, 2001), or partial follicle isolation (Hovatta *et al.*, 1999; Abir *et al.*, 2001), severely impairs follicular viability in culture. After isolation, primordial and primary follicles degenerate within the first 24 h of culture (Abir *et al.*, 2001) and only more advanced, multilaminar preantral follicular stages can survive in short-term culture, a few reaching the early antral stage (Roy and Treacy, 1993; Abir *et al.*, 1997).

Another approach could be to transplant a suspension of isolated follicles. As the follicular basal lamina encapsulating the membrana granulosa excludes capillaries, white blood cells and nerve processes from the granulosa compartment (Rodgers *et al.*, 2003), grafting fully isolated follicles could be considered safer. Moreover, this would allow the introduction of a high and

known number of follicles into the host, obtaining faster angiogenesis and minimizing ischaemic and reperfusion damage (Laschke *et al.*, 2002). Transplantation of frozen–thawed isolated primordial follicles has indeed been successfully achieved in mice (Carroll and Gosden, 1993), yielding normal offspring.

For human primordial follicles, however, mechanical isolation is not possible due to their size (30–40 μm) and their fibrous and dense ovarian stroma. Collagenase is currently used for the enzymatic isolation of ovarian follicles. Different types of collagenase (Ia, II, IX, XI) have been employed for this purpose, either alone (Hovatta *et al.*, 1999; Martinez-Madrid *et al.*, 2004) or in combination with Dnase or mechanical isolation (Roy and Treacy, 1993; Oktay *et al.*, 1997; Osborn *et al.*, 1997; Abir *et al.*, 1999, 2001; Huntriss *et al.*, 2002; Schröder *et al.*, 2004). However, the drawback of collagenase, which is a crude preparation derived from *Clostridium histolyticum*, is that it may contain high endotoxin levels that could severely impair culture and grafting outcomes, as shown for pancreatic islets (Berney *et al.*, 2001). Moreover, collagenase shows substantial variations in effectiveness between batches (McShane *et al.*, 1989), that may explain the discrepancies in results between different groups. Abir *et al.* (2001) found more lipid droplets in the granulosa cells of collagenase-isolated follicles than in intact cortical tissue, but Oktay *et al.* (1997) did not find any signs of ultrastructural damage after collagenase isolation. Other studies have also revealed alterations in the basal lamina of collagenase-isolated mouse follicles (Eppig, 1994), and Hovatta *et al.* (1999) reported more markedly premature oocyte extrusions from follicles that had been partially isolated using collagenase. This appears to indicate that some crude collagenase preparations may contain components interfering with follicle quality.

Liberase is a blend of highly purified enzymes that has been recognized, since 1997, as a powerful new tool to improve the quality of human pancreatic islet isolation (Linetsky *et al.*, 1997). The anatomical integrity of the islets is better preserved, and their viability superior, with Liberase (Linetsky *et al.*, 1997; Lakey *et al.*, 1998; Jahr *et al.*, 1999; Georges *et al.*, 2002), compared to collagenase preparations.

In order to enhance the chances of follicular survival and reproductive function restoration, enzymatic digestion procedures for human ovarian tissue need to be optimized and standardized. The poor results obtained with some collagenase batches thus prompted us to set up a new follicle isolation protocol using Liberase. The present study was designed to assess human follicle integrity and viability after Liberase enzymatic treatment.

Materials and methods

Collection and dissection of ovarian tissue

The use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. After obtaining written informed consent, ovarian biopsies were taken from eight women (between 20 and 32 years of age). The patients were all undergoing laparoscopic surgery for non-ovarian benign gynaecological disease, such as myomas, peritoneal endometriosis or tubal ligation.

Tissue was immediately transported from the operating room to the research laboratory in HEPES-buffered modified Eagle's medium

(HEPES–MEM) (Gibco, UK) on ice. The medullar part was removed from the biopsies using surgical scissors. The size of the biopsies varied between 5×5 and 10×8 mm. Each biopsy was cut into two equal parts: one for collagenase digestion and the other for Liberase digestion. The two groups were processed separately during all the manipulation procedures, by two operators with equal experience. The cortical portions were placed in a tissue sectioner (McIlwain Tissue Chopper, The Mickle Laboratory, Guildford, UK), adjusted to 0.5 mm. The cutting procedure was swift (<5 min), and uniform-size pieces of 0.5×0.5×1 mm were obtained.

Enzymatic digestion of ovarian tissue

The fragments were transferred to 50 ml conical flasks (Greiner Bi-one, Belgium) containing 10 ml of Dulbecco's phosphate-buffered saline (PBS) medium (Biochrom AG, Berlin, Germany) supplemented with 1 mg/ml collagenase type IA (Sigma, St Louis, MO, USA) for the collagenase group, and 0.04 mg/ml Liberase blendzyme 3 (Roche, Indianapolis, USA) for the Liberase group. Incubation was performed in a water bath at 37°C with gentle agitation for 60 min (collagenase group) or 75 min (Liberase group). The ovarian digest was shaken every 15 min with a pipette to mechanically disrupt digested tissue. Digestion was terminated by the addition of an equal volume of PBS medium at 4°C supplemented with 10% fetal bovine serum (FBS) (Sigma).

Preliminary studies were conducted to determine optimal concentrations and incubation times for both enzymes in order to obtain fully isolated follicles. For these preliminary studies, ovarian cortical biopsies were obtained from four women (aged 22–35 years) after informed consent. For the collagenase treatment, concentrations of 0.5, 1 and 1.5 mg/ml and incubation times of 30, 60 and 90 min were tested. A collagenase concentration of 1 mg/ml and incubation time of 60 min were subsequently selected. The lower concentration of 0.5 mg/ml and shorter incubation time of 30 min were not sufficient to isolate follicles, since many dense undigested fragments were obtained, while the 90 min incubation time and the 1.5 mg/ml collagenase concentration yielded many damaged follicles (with extruded oocytes). For the Liberase treatment, the blendzyme 3 Liberase product and 0.04 mg/ml starting concentration were selected. This starting concentration was calculated as described in http://www.roche-applied-science.com/prodinfo_fst.htm?collagenase/prod02.htm, in order to obtain a digestion power equivalent to 1 mg/ml (0.15 Wunsch Units) collagenase type IA. A higher Liberase concentration of 0.08 mg/ml was also tested but failed to yield more isolated follicles. The best results were obtained after 75 min of Liberase treatment, since 60 min of incubation was clearly not sufficient to isolate follicles and 120 min yielded a proportion of fully isolated follicles similar to that at 75 min.

Recovery of isolated follicles

After enzymatic digestion, the resulting suspension was centrifuged at 50 g for 10 min at 4°C. The supernatant was then discarded and the pellet was further processed as described below.

The pellets were transferred to Petri dishes and investigated for follicles under a stereomicroscope (Leica, Van Hopplynus Instruments, Brussels, Belgium). The follicles were picked up using a polycarbonate micropipette (Flexipet™ micro-manipulation pipettes 130 μm inner diameter; Cook Ob/Gyn, Spencer, Indiana, USA), taking care to avoid the stromal cells (Figure 1), and placed in PBS medium supplemented with 10% FBS at 4°C, where they were left until the process of follicular retrieval was completed for both groups.

Outcomes

Follicles recovered from each enzymatic treatment were randomly allocated to one of three groups in order to evaluate their morphology,

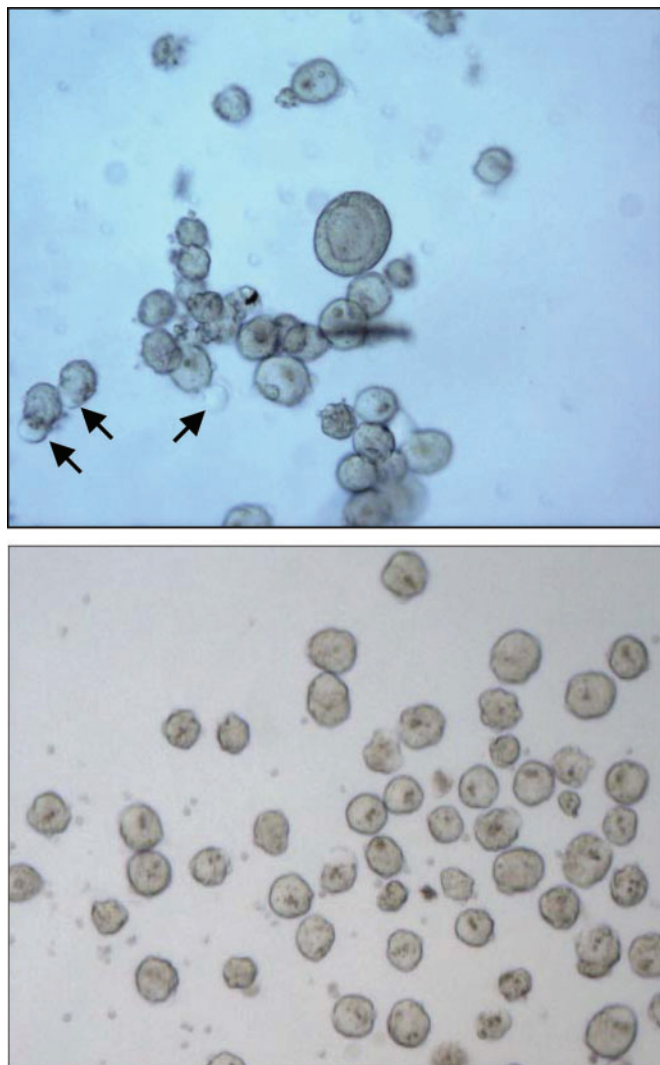


Figure 1. Enzymatically isolated human follicles. (a) Collagenase-isolated follicles, 30–100 μm in size, under the microscope. The arrows show three freshly extruded oocytes. (Original magnification $\times 200$.) (b) Follicles fully isolated by Liberase digestion (Original magnification $\times 200$.)

viability and ultrastructure. Results were evaluated blindly by two observers.

Morphology (M)

Using a polycarbonate micropipette, follicles were transferred to Eppendorf tubes containing 10% FBS (10 follicles per 200 μl medium). Entire follicles were then projected onto Superfrost Plus slides (Menzel-Glaser, Germany) by the cytospin technique (Thermo Electron Corporation, Pittsburgh, Pennsylvania, USA) (55 g, 5 min). The slides were subsequently dried for 2 h at room temperature, fixed in formalin for the next 2 h, and rinsed once in PBS before storage at -20°C . The fixed follicles were stained with a special formulation of mounting medium containing 4',6-diamidino-2-phenylindole (Vectashield[®] Mounting Medium with DAPI; Vector Laboratories, Burlingame, USA). DAPI binds to DNA and may also stain RNA, producing a blue fluorescence (excitation at 360 nm and emission at 460 nm). After 10 min incubation in the mounting medium in the dark, the follicles were observed under a fluorescence microscope (Leica). They were classified into four categories depending on their morphology and their granulosa

cell layer integrity: M1, spherical shape with complete granulosa cell layer; M2, irregular shape with complete granulosa cell layer; M3, irregular shape with $<10\%$ granulosa cell loss; M4, totally atypical shape with 10–50% granulosa cell loss, or an extruded oocyte (Figure 2).

Viability (V)

For follicular viability assessment, follicles were transferred using a polycarbonate micropipette to 100 μl of PBS containing 2 $\mu\text{mol/l}$ of calcein-AM and 5 $\mu\text{mol/l}$ of ethidium homodimer-I (Molecular Probes, Leyden, The Netherlands). Follicles were incubated with the fluorescent dyes for 45 min at 37°C in the dark (Cortvrindt and Smits, 2001). Non-fluorescent cell-permeant calcein-AM enters the cell and is cleaved by esterases in living cells, producing calcein which is well retained within live cells and gives an intense uniform green fluorescence. Ethidium homodimer-I enters cells with damaged membranes and then binds to DNA with high affinity, resulting in a 40-fold enhancement of fluorescence and producing a bright red fluorescence in dead cells.

After exposure to fluorescent dyes, the follicles were washed in PBS and observed under an inverted fluorescence microscope (Leica). Green fluorescence was visualized in live cells (ex/em 495/515 nm) and red fluorescence in dead cells (ex/em $\sim 495/\sim 635$ nm). The follicles were classified into four categories depending on the percentage of dead granulosa cells: V1, live follicles: follicles with the oocyte and all the granulosa cells (GC) viable; V2, minimally damaged follicles: follicles with $<10\%$ of dead GC; V3, moderately damaged follicles: follicles with 10–50% of dead GC; V4, dead follicles: follicles with both the oocyte or $>50\%$ GC dead (Martinez-Madrid *et al.*, 2004) (Figure 3).

Ultrastructure

The integrity of collagenase- and Liberase-isolated follicles was further investigated by light microscopy (LM) and transmission electron microscopy (TEM), by evaluating and comparing a series of structural and ultrastructural parameters. Because studies have shown alterations in the basal lamina of collagenase-isolated mouse follicles (Eppig, 1994), which could be deleterious for their further development, we placed particular emphasis on the assessment of basal lamina integrity of both collagenase- and Liberase-isolated follicles.

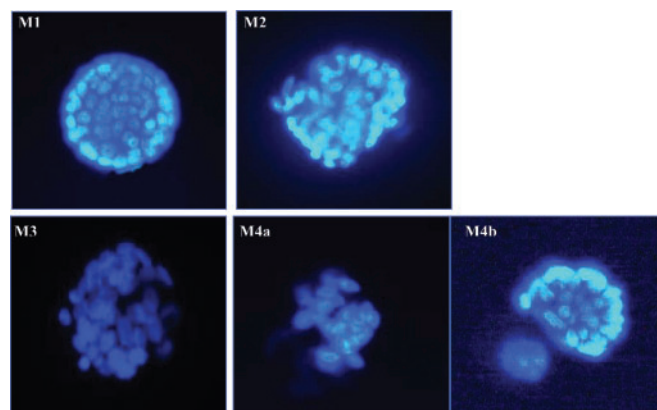


Figure 2. Morphology classification of isolated follicles into four categories depending on their form and number of granulosa cells: M1, spherical form with complete granulosa cell layer; M2, irregular form with complete granulosa cell layer; M3, irregular form with $<10\%$ granulosa cell loss; M4, totally atypical form with 10–50% granulosa cell loss (M4a), or an extruded oocyte (M4b).

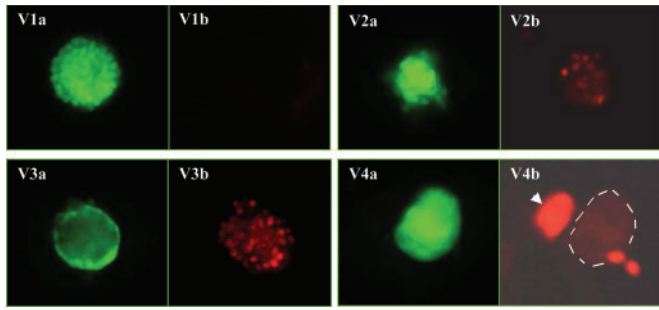


Figure 3. Viability classification of isolated follicles into four categories depending on the percentage of dead granulosa cells and oocyte viability: V1, live follicles: follicles with the oocyte and all the granulosa cells (GC) viable (**a** and **b** show the images obtained for the same follicle with two filters to visualize green fluorescence or red fluorescence for live or dead cells respectively); V2, minimally damaged follicles: follicles with <10% of dead GC; V3, moderately damaged follicles: follicles with 10–50% of dead GC; V4, dead follicles: follicles with both the oocyte and >50% GC dead. V4b shows, using the filter to visualize red fluorescence, an extruded dead oocyte (arrowhead) in a collagenase-isolated follicle with live granulosa cells, visible in V4a.

Enzymatically isolated follicles were fixed in 1.5% glutaraldehyde in PBS solution containing 5% FBS. After fixation for 2–5 days at 4°C, the samples were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar Scientific) in PBS, and rinsed again in PBS. Follicles were then embedded individually under a stereomicroscope in small blocks (width: 5 mm; height: 1 mm) of 1% agar (Nottola *et al.*, 1991), dehydrated through an ascending series of ethanol, immersed in propylene oxide (solvent substitution), embedded in Epon 812 and sectioned using a Reichert–Jung Ultracut E ultramicrotome. Semithin sections (1 µm thick) were stained with toluidine blue, examined by LM (Zeiss Axioskop, Germany) and photographed using a digital camera (Leica, DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids and contrasted with saturated uranyl acetate followed by lead citrate. They were examined and photographed using Zeiss EM109 and Zeiss EM 10 electron microscopes at 80 kV.

Statistical analysis

Analyses were carried out using the SPSS 11.5 program. A comparison was made between the collagenase and Liberase groups with regard to the percentages obtained for each morphology and viability category. Differences between the two groups were calculated by the Mantel–Haenszel χ^2 -test, after performing Breslow–Day's odds ratio equality test. $P < 0.05$ was considered statistically significant.

Results

A total of 419 isolated human follicles was analysed. The mean number of recovered fully isolated follicles per mm³ varied from 0.27 to 2.72.

In all, 263 follicles were recovered after collagenase treatment and 156 after Liberase treatment. In the collagenase group, 103 follicles were assessed for morphology, 92 for viability and 68 for ultrastructure evaluation. In the Liberase group, 44 follicles were assessed for morphology, 71 for viability and 41 for ultrastructure evaluation.

Morphology

The morphology was found to be well preserved in the Liberase-isolated follicles.

A total of 147 isolated follicles was examined for morphology after DAPI staining. As shown in Table I, after Liberase isolation, a high proportion (70.4%) of follicles showed a good morphology [M1 (22.7%) and M2 (47.7%)] and had a complete granulosa cell layer. The percentage of good morphology follicles was significantly lower (34%) after isolation with our current collagenase preparation [M1 (10.7%) and M2 (23.3%) respectively]. The remaining follicles were classified as poor morphology (M3 and M4). The proportion of follicles with a totally atypical shape or an extruded oocyte was found to be lower in the Liberase group (9.1%) than the collagenase group (38.8%) ($P < 0.003$). The rate of oocyte extrusion (M4b) among fully isolated follicles was found to be 12 and 3% for collagenase- and Liberase-isolated follicles respectively (Figure 1).

Viability

A total of 163 isolated human follicles was examined for viability using the fluorescent probes calcein-AM and ethidium homodimer-1 (Table II). V1 and V2 represented follicles with high viability (maximum 10% of dead granulosa cells) and V3 and V4 follicles with poor viability (10–100% of dead granulosa cells or a dead oocyte). The collagenase group showed

Table I. Morphological score of isolated follicles in the collagenase and the Liberase groups

Experiment no.	Collagenase ($n = 103$)				Liberase ($n = 44$)			
	M1	M2	M3	M4	M1	M2	M3	M4
1	0	0	1	11	0	3	3	1
2	0	3	0	1	0	1	0	0
3	1	2	3	4	0	2	2	0
4	2	7	4	2	0	6	1	1
5	2	4	3	5	0	0	0	2
6	2	3	3	1	0	0	0	0
7	2	3	10	15	9	9	3	0
8	2	2	4	1	1	0	0	0
Total no. of follicles	11	24	28	40	10	21	9	4
%	10.7 ^a	23.3 ^b	27.2	38.8 ^c	22.7 ^d	47.7 ^e	20.5	9.1 ^f

^{a,b}Significantly different from ^{d,c}($P < 0.001$) ^cSignificantly different from ^f($P < 0.003$).

Table II. Viability score of isolated follicles in the collagenase and the Liberase groups

Experiment no.	Collagenase ($n = 92$)				Liberase ($n = 71$)			
	V1	V2	V3	V4	V1	V2	V3	V4
1	7	1	2	0	4	1	0	0
2	0	1	2	1	0	1	0	0
3	0	3	0	4	7	0	0	0
4	6	17	4	0	19	2	0	0
5	5	10	2	0	0	0	0	0
6	1	2	0	1	0	0	0	0
7	3	10	8	1	30	5	1	0
8	0	2	0	0	1	0	0	0
Total no. of follicles	22	46	17	7	61	9	1	0
%	23.9 ^a	50 ^b	18.5	7.6	85.9 ^c	12.7 ^d	1.4	0

^{a,b}Significantly different from ^{c,d}($P < 0.001$).

significantly lower viability than the Liberase group, with 73.9% (23.9 + 50 V1 and V2 respectively) of high viability follicles, compared to 98.6% (85.9 + 12.7 V1 and V2 respectively) in the Liberase group ($P < 0.001$). The remaining poor viability follicles (V3 + V4) accounted for 26.1% in the collagenase group with 7.6% of dead follicles (V4), whereas in the Liberase group, only V3 follicles were found (1.4%) and no dead follicles (V4) were encountered ($P < 0.001$).

Ultrastructure

Light microscopy generally showed collagenase- and Liberase-isolated follicles to be uniformly rounded structures varying in diameter from 40 to 80 μm , surrounded by a single, uninterrupted layer of follicular cells. Irregularly shaped follicles with an incomplete follicular wall were also found in both groups.

Intact follicles were classified either as primordial or primary on the basis of their general features. Primordial follicles were composed of an oocyte surrounded by a layer of flattened follicular cells. The oocyte organelles were usually condensed into a crescent region of the cytoplasm immediately around the nucleus (corresponding to Balbiani's vitelline body). In primary follicles, the follicular cells were cuboid in shape and the oocyte organelles appeared to be scattered in the cytoplasm (Figure 4a, b). Areas of detachment between follicular cells and the oocyte were occasionally detected in collagenase-isolated follicles (Figure 4e).

By TEM, both collagenase- and Liberase-isolated follicles were seen to be surrounded by a discontinuous basal lamina (Figures 4c, d). Numerous connective tissue fibres were found on the outer aspect of the residual basal lamina of the Liberase-isolated follicles (Figure 4d).

Follicular cells had a voluminous nucleus surrounded by an indented nuclear membrane, containing a well-developed nucleolus and isolated peripheral patches of heterochromatin. Large confluent patches of heterochromatin were more frequently detected in the nuclei of follicular cells of the collagenase-isolated follicles (Figure 4c). Numerous elongated mitochondria, elements of endoplasmic reticulum and scattered lipid droplets of variable electron density were found in the follicular cell cytoplasm (Figures 4c, d).

Close interdigitations between follicular cell projections and oocyte microvilli were observed in all the follicles studied (Figures 4c–f). However, as also revealed by LM, focal discontinuities at the oolemma–follicular cell interface were frequently detected in follicles isolated with our collagenase preparation (Figure 4e). Oocytes of both primordial and primary follicles generally contained a large, irregularly rounded nucleus, in which chromatin aggregates were seen around the nucleoli. Irregularities in the nuclear profile were most evident in collagenase-isolated follicles compared to Liberase-isolated follicles (Figure 4c).

Among the oocyte organelles, numerous rounded mitochondria, membranes of endoplasmic reticulum, free ribosomes and, sometimes, multivesicular bodies were found in the cytoplasm of oocytes in both the collagenase- and Liberase-treated groups. Clusters of lipid droplets, small vacuoles and myelin-like structures were also encountered in the oocytes of collagenase-isolated follicles (Figure 4e).

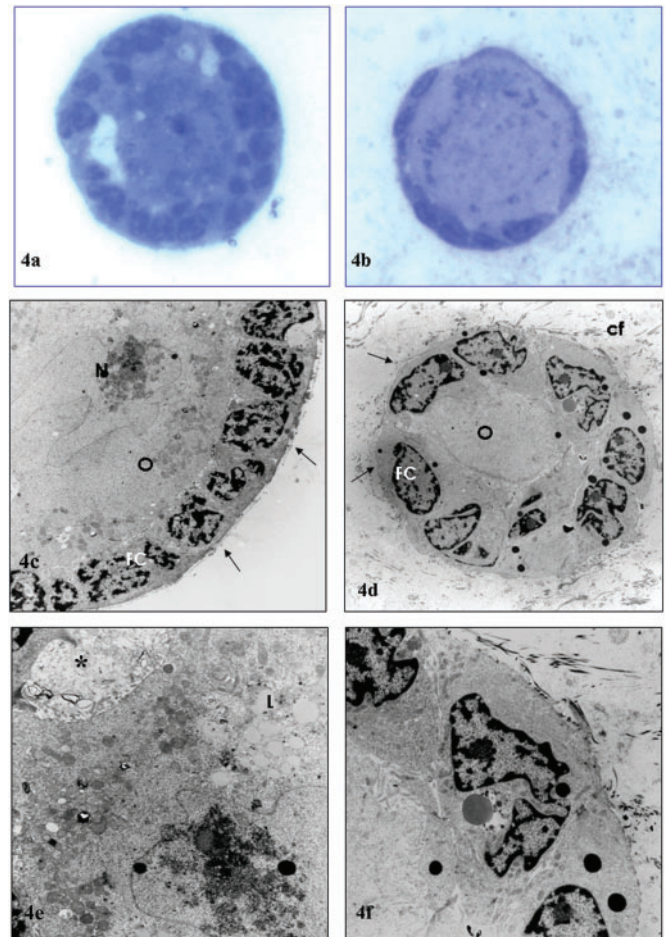


Figure 4. Transmission electron microscopy: (a) Collagenase-isolated primary follicle. A continuous layer of cuboid follicular cells surrounds the oocyte. LM: $\times 400$. (b) Liberase-isolated follicle, in transition between the primordial and primary stage. A continuous layer of flattened/irregular cuboid follicular cells surrounds the oocyte. Balbiani's vitelline body is recognizable in the ooplasm. LM: $\times 400$. (c, d) Collagenase- and Liberase-isolated primary follicles, seen in a peripheral section (c and d respectively). The oocyte (O) is surrounded by a single layer of cuboid follicular cells (FC), bordered by a discontinuous basal lamina (arrows). Follicular cells show indented nuclei containing large patches of heterochromatin, more evident in c. Close interdigitations are observed between follicular cell projections and oocyte microvilli. Note the presence of an irregularly shaped nucleus (N) in the oocyte shown in c. cf.: fibres of connective tissue (d). TEM: $\times 2500$. (e, f) Collagenase-isolated (e) and Liberase-isolated (f) primary follicles. An area of detachment between the follicular cells and oocyte (asterisk), as well as numerous lipid droplets (L) in the ooplasm, are illustrated in e. TEM: $\times 6300$.

Discussion

In the present study, a new enzymatic digestion protocol using Liberase, a purified endotoxin-free enzyme blend, was initiated for the disruption of ovarian tissue. Our results demonstrate that Liberase is able to dissociate the dense cortex of human ovaries, allowing isolation of follicles from the surrounding stromal cells. However, the number of fully isolated follicles recovered from the Liberase group was found to be lower than from the collagenase group ($n = 156$ versus $n = 263$), although the size of the biopsies in both groups was the same. Indeed, in

the Liberase suspension, we often observed clusters of partially isolated follicles still surrounded by stroma. This may explain the lower number of fully isolated follicles in this group and be due to the presence of fewer different enzymes in the Liberase preparation. Indeed, it is known that crude collagenase preparations contain a mixture of ~30 different enzymes, some of which are not present in the Liberase preparation, that may be more powerful for extracellular matrix disruption. This is borne out by the fact that higher Liberase concentrations and longer incubation times failed to increase the proportion of fully isolated follicles after digestion.

In this study, 419 fully isolated follicles were obtained from eight ovarian biopsies. This may be considered a low follicle yield, but can be explained by several factors. First of all, the size of the biopsies was small. Moreover, our follicular density results are in line with other studies in the literature. Lass *et al.* (1997), who studied follicular density in ovarian biopsies obtained from 60 women, found a range from 0 to 160 follicles/mm³, with a median of 8 follicles/mm³. Schmidt *et al.* (2003b) found a huge difference in follicular density, not only from patient to patient, but also between different fragments of the same ovary. Martinez-Madrid *et al.* (2004) (our previous study on follicle isolation) reported a mean number of recovered follicles per mm³ varying from 0.4 (the youngest patient in the study, 26 years old) to 21.3 (a 31 year old patient). This is comparable with our present results.

Quality assessment of the isolated follicles was based on the general follicle morphology, on follicle viability analysed by vital fluorescent dyes, and on ultrastructural follicle integrity investigated by TEM.

The results of the present study clearly indicate that follicle morphology and granulosa cell layer integrity are well preserved in Liberase-isolated follicles (70.4% of good morphology follicles).

The viability of follicles was found to be high after Liberase treatment (98.6%) whereas, after collagenase treatment, it appears to vary considerably from study to study. Indeed it ranges from 70.4% (present study) and 71.6% (Oktay *et al.*, 1997) to 93% (Hreinnsson *et al.*, 2003) and 95.8% (Martinez-Madrid *et al.*, 2004) (our previous studies). This may be explained by lot-to-lot variations, as demonstrated for pancreatic islet isolation (McShane *et al.*, 1984). However, an apparently viable oocyte does not necessarily reflect a healthy oocyte which is functional.

Ultrastructural investigation provides us with additional qualitative information on follicle morphology after enzymatic isolation using Liberase. When sections were observed by LM and TEM, both primordial and primary follicles were found. The majority showed a uniformly rounded profile and a continuous follicular cell layer in both Liberase- and collagenase-isolated groups. This is in accordance with the ultrastructural findings reported by Oktay *et al.* (1997) on follicles partially disaggregated with collagenase and then completely isolated by microdissection. Thus, a certain structural follicular integrity may be preserved in both experimental groups, despite the discontinuities observed in the basal lamina.

Scattered lipid droplets, characteristic of more advanced stages of follicular growth (Motta *et al.*, 2003), were also

observed by TEM in both categories of follicles (collagenase- and Liberase-treated groups). This suggests that the enzymatic treatments may induce slight morphofunctional changes in the follicular cell compartment. However, an increase in lipid droplets in follicular cells does not appear to affect follicular viability and growth, as shown by Abir *et al.* (2001).

In the present study, ultrastructural markers of atresia were occasionally found in Liberase-isolated follicles. Similar observations were made by Oktay *et al.* (1997) after collagenase isolation. However, in the present study, we found that collagenase-isolated follicles, despite an absence of obvious alterations in shape or extensive disruption to the follicular wall, frequently showed ultrastructural signs of early atresia in all follicle compartments. These included the presence of large patches of heterochromatin in the follicular cell nuclei, focal areas of detachment between follicular cell prolongations and oocyte microvilli (with a consequent structural/metabolic uncoupling inside the follicular unit), irregularities in the oocyte nuclear membrane, and the presence of lipid droplets, vacuoles and myelin-like structures in the ooplasm (Familiari *et al.*, 1993; de Bruin *et al.*, 2002; Motta *et al.*, 2003). This may indicate that, as observed in studies on pancreatic islet isolation, crude collagenase preparations may contain factors that alter cell viability by inducing apoptosis (Berney *et al.*, 2001).

Different forms of crude bacterial collagenase are derived from cell culture supernatants and typically contain a mixture of protease enzymes (exhibiting both collagenolytic and non-specific proteolytic activities) and non-protease components (e.g. fermentation by-products, media components, pigments, enzymes such as phospholipase, and endotoxins). Liberase blendzyme 3 contains only purified proteases and a neutral protease (thermolysin; US Pat. No. 5,830,741), and is endotoxin-free. Endotoxins may be one of the detrimental components of collagenase extract, inducing apoptosis in culture (Jahr *et al.*, 1999) and strongly impairing grafting outcomes after pancreatic islet transplantation (Berney *et al.*, 2001).

Isolation of primordial and primary follicles has several potential applications, including *in vitro* culture and grafting. However, ensuring good quality and consistent follicular preparations that are free of contaminants, such as endotoxin, is essential before attempting any such approaches in humans. Moreover, enzymatic follicle isolation procedures may also provide isolated follicles and stromal cells for molecular biology analyses.

Concerning *in vitro* approaches, preliminary studies indicate that primordial follicles obtained after isolation degenerate in culture (Hovatta *et al.*, 1999; Abir *et al.*, 2001), possibly reflecting their initial health status or a lack of factors essential to sustaining their survival and growth. Further studies are required to identify these factors (Smitz and Cortvrindt, 2002) and to determine whether culture of primordial follicles as isolated entities is a feasible option or whether contact with the stromal cell compartment is required.

Progress in human primordial follicle organ culture (Hovatta *et al.*, 2004) and the application of highly sensitive molecular biology approaches (Quennell *et al.*, 2004) should provide a better understanding of early human follicular development *in vitro*.

The grafting approach has, however, been successfully applied in mice (Carroll and Gosden, 1993) and preliminary studies

conducted in our laboratory indicate that human follicles obtained after Liberase digestion are viable and grow after transplantation into nude mice (data not shown). Grafting isolated follicles could well be an option for preserving fertility in patients where the risk of reintroducing the disease by grafting exists. Indeed, if follicles are fully isolated up to the follicular basal lamina, the capillaries are excluded and the suspension may be considered safe for transplantation. Large quantities of connective tissue fibres, but no cell contamination, were found in follicle preparations obtained after Liberase treatment, confirming that this isolation technique may be adequate for purging tissue of malignant cells before grafting cryopreserved ovarian follicles into patients affected by cancers. Another possibility is to purge the entire ovarian tissue suspension of cancer cells by selective immunolabelling, as recently described by Schröder *et al.* (2004).

The present Liberase protocol, besides producing fully isolated follicles of good morphology and viability, may also be considered as a new method of obtaining partially isolated follicles swiftly and easily. Indeed, follicles present in partially digested stroma can be clearly seen under the stereomicroscope due to the mucified aspect of the stroma after digestion. This is not the case for dense stromal clumps obtained after collagenase treatment. Grafting these partially isolated follicles could provide a solution to the revascularization problem encountered in fragment reimplantation (Baird *et al.*, 1999; Nisolle *et al.*, 2000) in patients suffering from non-haematological diseases.

In conclusion, we were able to obtain good quality isolated human follicles (good morphology and integrity and high viability) using Liberase. Obtaining good quality isolated follicles is an absolute prerequisite for the further successful processing of these follicles, either for culture or transplantation. The purified endotoxin-free enzyme preparation, Liberase, is a promising alternative to impure collagenase preparations for the reproducible isolation of intact primordial and primary follicles from human ovarian tissue.

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