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Treatment with trypLE before freezing improves thawing integrity and functionality of sheep ovarian tissue

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Abstract

Objective To study innovative approaches to ovarian tissue cryopreservation, a critical issue for fertility preservation in pediatric cancer patients. Despite historical attempts, recent advances in cancer treatment have underscored the urgent need for more effective and reliable ovarian tissue cryopreservation methods. Our research aims to evaluate if decreasing the rigidity of stroma before cryopreservation by investigating pre-treatments with enzymes can enhance the quality of ovarian tissue post-thawing.

Design Our research evaluated the use of five commonly used enzymes to disaggregate tissue (trypLE, collagenase, dispase, accutase and hyaluronidase) before freezing ovarian tissue to decrease rigidity and facilitate cryopreservation. Sheep ovaries, with high similarity to human ovaries, were used as an animal model. Tissue structure, cell proliferation, apoptosis and viability were assessed before and after thawing.

Results Our findings showed that enzymatic treatment with trypLE before freezing offered immediate benefits post-thawing with the highest viability values and percentage of intact follicles. A decrease in viability was observed after thawing and culturing the samples. The pretreatment with accutase damaged the tissue severely with also the lowest viability values. Ki67-positive follicles and stromal cells were observed in fresh samples, but only trypLE and hyaluronidase maintained Ki67-positive antral follicles after 2 days culture. Besides, only trypLE maintained all follicles negative to caspase-3 after thawing, and 7 days after culture primordial follicles were apoptotic in all treatments apart from trypLE.

Conclusion our findings suggest that trypLE pretreatment could provide a beneficial approach for maintaining the functions and viability of cryopreserved ovaries after thawing. Further research is needed to fully understand their impact and optimize cryopreservation protocols in this important clinical context.

Keywords Cryopreservation, Ovarian tissue, Follicles, Fertility, Enzymes, Prepuberal cancer

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Introduction

Fertility preservation in girls with pediatric cancer is a crucial and challenging issue in modern medicine [1]. While fertility can be preserved by freezing oocytes or embryos in post-pubertal woman, these modalities are not an option for younger patients as they do not respond to hyperstimulation ovarian treatment due to lack of maturation of the hypothalamic-pituitary axis [2]. The primary fertility preservation option that exists for pre-pubertal girls or oncologic patients undergoing immediate gonadotoxic cancer treatment is ovarian tissue cryopreservation (OTC) and its subsequent transplantation (OTT) [3]. Currently, it is estimated that more than 10,000 girls and woman worldwide have undergone OTC [4] and more than 200 children have been conceived from OTT, with an estimated birth rate of around 47% [5]. There is loss of approximately 50% of follicles because of damage during preservation, ischemia and oxidative stress damage [6, 7].

It has been demonstrated that rigidity of stroma impacts the ability of follicles to accommodate and survive the current cryopreservation process [8]. Limited information about rigidity modulation and ovarian tissue preservation is available. Only one study conducted on mouse ovaries using collagenase as a facilitating enzyme has demonstrated that pretreatment of ovaries with collagenase before vitrification maintained ovarian reserve [8]. The underlying basis for the improved preservation is related to accommodating high osmotic solutions. Authors observed that brief treatment with collagenase before exposure to the hyperosmotic solution maintained the cadherin adhesions between the oocyte and granulosa cell layers. Consequently, secondary follicles collected from frozen-thawed ovaries that were pretreated with collagenase showed normal follicular development. Increasing cryoprotectant penetrations can play an important role in the preservation of cells within the tissue as it can decrease ice formation during cryopreservation of the tissues as they can depress the equilibrium freezing point and encourage supercooling; they have a relatively high glass transition temperature; and they can permeate cell membranes so that intracellular freezing can be achieved [9].

Ethical barriers and the limited availability of human ovarian tissue preclude the widespread use of human ovaries for research. Consequently, a robust animal model comparable with humans is needed. The sheep ovary is an adequate candidate for research due to the similar ovarian architecture, limited number of developed follicles, single ovulation with primordial follicles distributed superficially in the cortex and a comparable collagen-dense cortical layer [10–13].

In this study, we aimed to improve the ovarian tissue cryopreservation protocol by treating tissue briefly with 5 different enzymes (TrypLE, collagenase, dispase, accutase, hyaluronidase) before exposure to freezing media to determine the treatment that maintained the functions and viability of ovarian tissue after thawing.

Material and methods

Experimental design

A strategy for optimizing the cryopreservation of ovarian tissue by decreasing the rigidity of the tissue by treatment before freezing with 5 different enzymes (trypLE, collagenase, dispase, accutase, hyaluronidase) was studied, comparing with an untreated control. Sheep ovaries show similarities to human ovaries in terms of fibrous cortical tissue and were used as an animal model in this project [14]. Ovaries from adult sheep, 3–4 years old (approximately 40 ovaries), with small or no dominant follicles, were obtained from the abattoir and transported in PBS (Phosphate Buffer Solution) with antibiotics. Upon arrival at the laboratory, the medullary layer from the ovaries was removed to isolate the cortical area, i.e. the layer containing follicles. Samples of approximately 1 mm thick were cut into quadrilaterals of 0.5 cm × 0.5 cm. This study was approved by the Ethics Committee of Animal Experimentation from the University of Zaragoza, Zaragoza, Spain with the code of PI06/22NE.

Enzymatic pretreatment of samples

The effect of 5 enzymatic pretreatments was analyzed, including a control without enzymes. Four replicates were carried out per treatment and assessment time. The samples were evaluated before freezing, at 0 h of thawing and after 2 and 7 days of culture. The enzymatic treatments used were: 1) TrypLE (1X; 9 ml PBS and 1 ml of the commercial solution of the *TrypLE*® enzymatic cocktail at 10X concentration); 2) Collagenase Type I (0.83 mg/ml); 3) Dispase (2.5 mg/ml); 4) Accutase (*Acumax*®); 5) Hyaluronidase (25 mg/ml). Samples of 1 cm × 1 cm × 1 mm dimensions were subjected to the different enzymatic treatments for 10 min at 37 °C and then immediately washed three times with abundant PBS supplemented with 20% of FCS for enzyme inactivation before initiating the freezing process. Untreated sample with enzymes before freezing were considered as the control.

Freezing/thawing

All samples were frozen following the modified slow freezing protocol proposed by Herraiz et al. [15]. Briefly, 3 solutions were used: Solution 1: DMEM (*Dulbecco's Modified Eagle Medium*) + 5% FBS (fetal bovine serum); Solution 2: Solution 1 + 10% DMSO (dimethyl sulfoxide);

and Solution 3: Solution 1 + 12% DMSO. The samples for each treatment were deposited in tubes with 8 ml of Solution 1. Then, 8 ml of Solution 2 (final concentration of DMSO 5%) were added and incubated for 20 min. After incubation, 8 ml of liquid was removed, adding 8 ml of Solution 3 (final concentration of DMSO 8.5%) and incubating for 15 min. Then samples were transferred to the cryotube containing the final freezing solution (DMSO 8.5%) using forceps. This process was carried out at room temperature, next transferring the tubes to a cell freezer, where the temperature was decreased at a 1 °C/minute rate until reaching −80 °C (approximately 24 h). A cell freezer was used in this study as it is a cost-effective alternative to a programable freezer as demonstrated in a previous study [16]. Finally, the samples were stored in a liquid nitrogen tank at a temperature of −196 °C. For thawing, cryovials were immersed in a water bath at 42 °C and agitated until were completely thawed. Subsequently, three washes with PBS were performed to remove the cryoprotectants.

Assessment and culturing

The samples were evaluated before freezing, at 0 h post-thaw, and after 2 and 7 days of culture. DMEM medium supplemented with L-glutamine (1%) and fetal bovine serum (20%) was used as culture medium. Samples were covered with 2 ml of culture medium and placed in an incubator at 37 °C and 5% CO2 to meet the specified observation times. The next parameters were assessed: viability, tissue integrity and cell proliferation and apoptosis.

Viability

To assess tissue viability, calcein-AM (ThermoFisher, C3100MP) to stain alive cells and ethidium homodimer-1 (EthD-1) (ThermoFisher, L3224B) for dead cells were used [17]. A live/dead solution was prepared following the manufacturer's instructions, obtaining a solution with a final concentration of 2 µM calcein AM and 4 µM EthD-1 in PBS. The samples were incubated with the live/dead solution for 30 min at 37ºC and washed three times with PBS before clearing. Samples were cleared to eliminate the excess of pigments and improve visibility of tissue viability, following the protocol proposed by Lempereur et al. [18]. Briefly, 400 µl of 50% clearing solution (clearing solution stock MD+: PBS, 1:1) were added to the samples, incubating for 2 h. Next the samples were incubated for 45 min in 100% clearing solution. The clearing solution MD + contains a combination of 45% sucrose (w/v), 20% nicotinamide (w/v), 20% triethanolamine (w/v) and 0.1% TritonX-100 (v/v). Then the samples were visualized under a confocal microscope (*Nikon TI-E-c1 Confocal modular*), at 590/50 nm wavelength for calcein (green, labeling live cells) and 650LP nm for propidium iodide (red, labeling dead cells), with a fixed gain of 95 and 97 for red respectively. The samples were observed in frames of z-stack adjusted from the bottom to the top of the tissue slices. Images were exported from the EZ-C1 software into ImageJ/Fiji software and files were transformed from ids format to TIFF for viability assessment. In the figures shown in this manuscript, maximum orthogonal projections, no image manipulation was performed prior to export into ImageJ/Fiji software to generate the figures and quantifying green and red fluorescence intensity in arbitrary units (AU). More than 400 points per sample were analyze for green and red AU. These data were used to calculate the tissue viability percentage (green AU/Sum and red AU; %).

Morphological analysis

In order to evaluate morphological analyses of ovarian tissue, tissue samples were fixed in 4% PFA for 24 h at 4 °C and then processed in a rapid tissue processor (X-PRESS X50 processor, Sakura, Japan) until paraffin embedded. Once paraffin blocks were made, 2,5 µm sections were cut with a rotation microtome and paraffin sections were taken on glass slides. Slides were air dried at 37ºC overnight, and then, they were deparaffinized and rehydrated.

For Hematoxylin–eosin staining, tissue sections were immersied in Carazzi's Hematoxylin solution (Panreac; ref. 255,298) and eosin yellowish hydroalcoholic solution (Panreac; ref. 251,301). Hematoxylin and eosin-stained preparations were evaluated under a light field optical microscope for morphological study of ovarian follicles and stroma. The number of follicles were counted. Follicles classified as morphologically normal had an intact basement membrane, no pyknotic nuclei, a spherical oocyte, and no vacuoles present. Follicles were classified as abnormal when any of the previous criteria were no met. Intact stroma was considered when the connective and interstitial tissue did not exhibit white spaces, maintained good cellularization, and did not show focal areas of necrosis or apoptosis [19]. At least 40 follicles were counted per treatment.

Immunohistochemistry

Caspase-3 (AF835, R&D Systems) was used for apoptosis detection and Ki67 (IR626, Dako) was used for proliferation detection. For Inmunohistochemistry staining, antigen retrieval was performed by means of the PT Link (Dako). After retrieval, automated immunostaining was performed with a previously optimized protocol. For this process endogenous peroxidase was first blocked. Primary antibody was then used (Ki67 RTU; capasa-3 at 1/125 dilution) and after this, a visualization system for

signal amplification and 3,3'-Diaminobenzidine (DAB), were used for signal development after precipitation at primary antibody binding sites. Follicles at all stages were evaluated and categorized as apoptotic when the oocyte and/or > 50% of granulose were caspase positive. Follicles were classified into 2 categories: resting, when all granulosa cells were negative for Ki67 or proliferating, with at least 1 granulose cell was positive for Ki67 [20, 21]. At least 15 follicles were counted per treatment.

Statistical analysis of viability

Viability, follicular damage and immunohistochemistry data were analyzed using SPSS (*Statistical Package for the Social Sciences*) version 19.0 (SPSS, Chicago, IL). Normality of distribution was assessed using the Kolmogorov–Smirnov test, revealing a non-normal distribution in the data. Significant differences were considered at $p < 0.05$. Differences between treatments were compared using the Kruskal–Wallis test, followed by post-hoc analysis.

Results

Viability

Ovarian tissue clearing permits better visualization and analysis of tissue viability. In samples before freezing (fresh), the highest viability percentages were observed with dispase treatment, while the lowest were observed with accutase. Immediately after thawing, the highest viability was observed in samples treated with trypLE, while accutase showed the lowest viability values. When culturing tissue samples after thawing 7 days, the tripLE treatment continued to exhibit the highest viability values. In general, viability improvement compared to the control before freezing was observed in treatments with trypLE, collagenase, and dispase. However, immediately after thawing, which is typically when transplantation occurs, only trypLE treatment showed better viability results than control. After 2 days of culture, control exhibited the worst viability results, and after 7 days of culture, only tripLE treatment showed improvements in terms of viability compared to the control. Viability images used for the AU analysis with FIJI for all treatment at 0 h and 7 days after thawing are shown in Fig. 1. It is worth noting that in all treatments, a decrease in viability was observed after thawing and culturing of the samples (Table 1). Regardless of the treatment, viability decline could be observed by comparing the total viability values over time of assessment (Fig. 2).

Table 1 Tissue viability values (%) before freezing and at thawing (0 h. 2 days. and 7 days of culture). Values expressed as mean ± standard error. Different letters within the same column indicate significant differences between treatments ($p < 0.05$)

	Fresh	0 h	2d	7d
TrypLE	76.89 ± 0.21^{a}	77.85 ± 0.9^{a}	67.24 ± 0.08^{a}	67.26 ± 0.42^{a}
Collagenase	81.76 ± 0.5^{b}	66.71 ± 0.25^{b}	66.76 ± 0.2^{b}	53.32 ± 0.23^{c}
Dispase	87.58 ± 0.05^{c}	65.27 ± 0.2^{c}	62.04 ± 0.3^{c}	40.57 ± 0.26^{d}
Accutase	71.08 ± 0.44^{d}	47.79 ± 0.54^{d}	56.03 ± 0.35^{d}	45.06 ± 0.16^{d}
Hyaluronidase	71.49 ± 0.2^{d}	70.25 ± 0.3^{e}	61.81 ± 0.46^{b}	59.69 ± 0.21^{b}
Control	74.01 ± 034^{f}	70.32 ± 0.33^{e}	61.52 ± 0.22^{b}	60.06 ± 0.25^{b}
P-value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

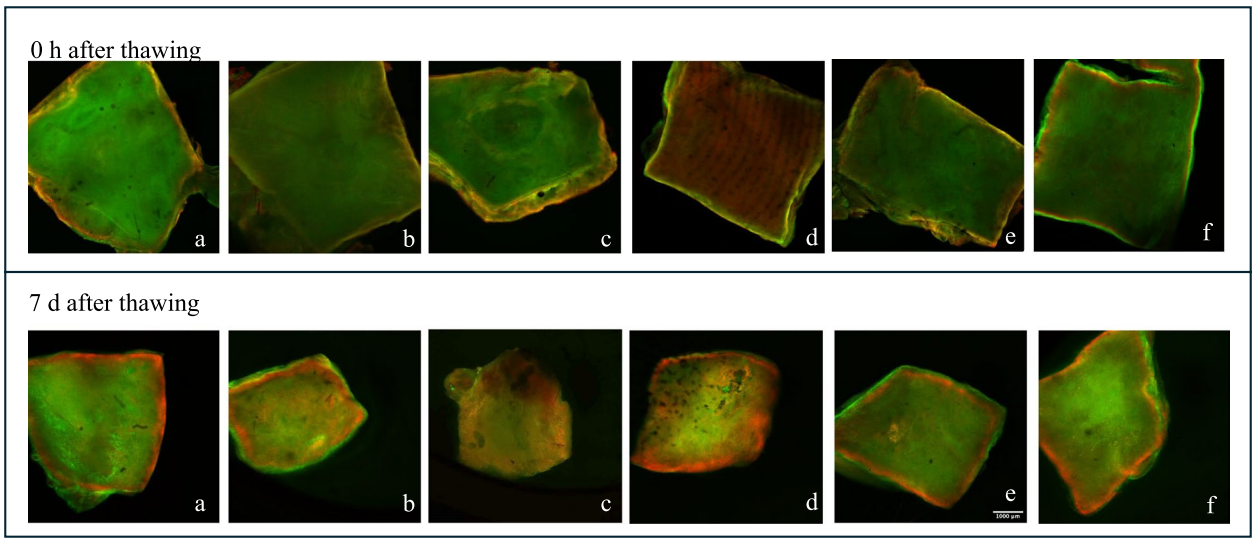

Fig. 1 Viability preparations in of ovarian tissue (0.5 cm × 0.5 cm × 1 mm squares of tissue) in TIFF format for FIJI analysis at 0 h after thawing and 7 d after thawing. **a** TrypLE. **b** Collagenase. **c** Dispase. **d** Accutase. **e** Hyaluronidase. **f** Control

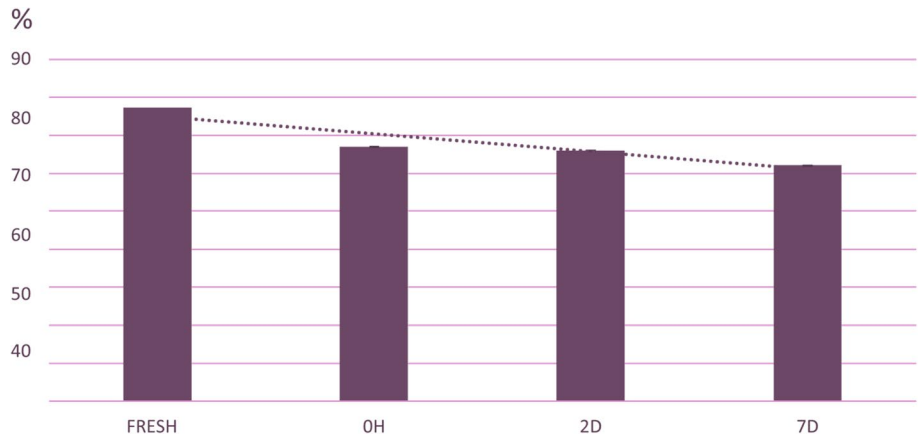

Fig. 2 Percentage of tissue viability measured as a percentage of the total treatments over time (Mean ± SEM)

Follicular and stromal integrity

In samples before freezing, most of the follicles were found intact (219 total follicles with only 1 damaged with dispase treatment and 1 in control; *P*-value = 0.93) and the stroma was intact.

The data of follicular damage is observed in Fig. 3. Significant differences were observed at 0 h after thawing for follicular integrity (*P*-value = 0.03). Follicular and stromal damage was observed after thawing, with separation of the granulosa area from the oocyte, as well as loss of shape of the basal plasma and nuclear membranes in the follicles, with appearance of decellularization in the stromal part (Fig. 4). TrypLE and hyaluronidase treatments maintained the integrity of the tissue better at 0 h of damage. The accutase treatment presented the worst results with multiple foci of pyknosis and necrosis coinciding with the lowest viabilities measured at 0 h. Around 50% of the follicles showed disintegration of the granulosa areas, vacuoles, and loss of shape in the treatments with accutase. Treatment with collagenase, and dispase produced stromal damage mainly at the edges of the tissue with more intact stroma in the central part. The control presented similar results of follicular alteration than collagenase and dispase with and some foci of decellularization and necrosis at the stromal level.

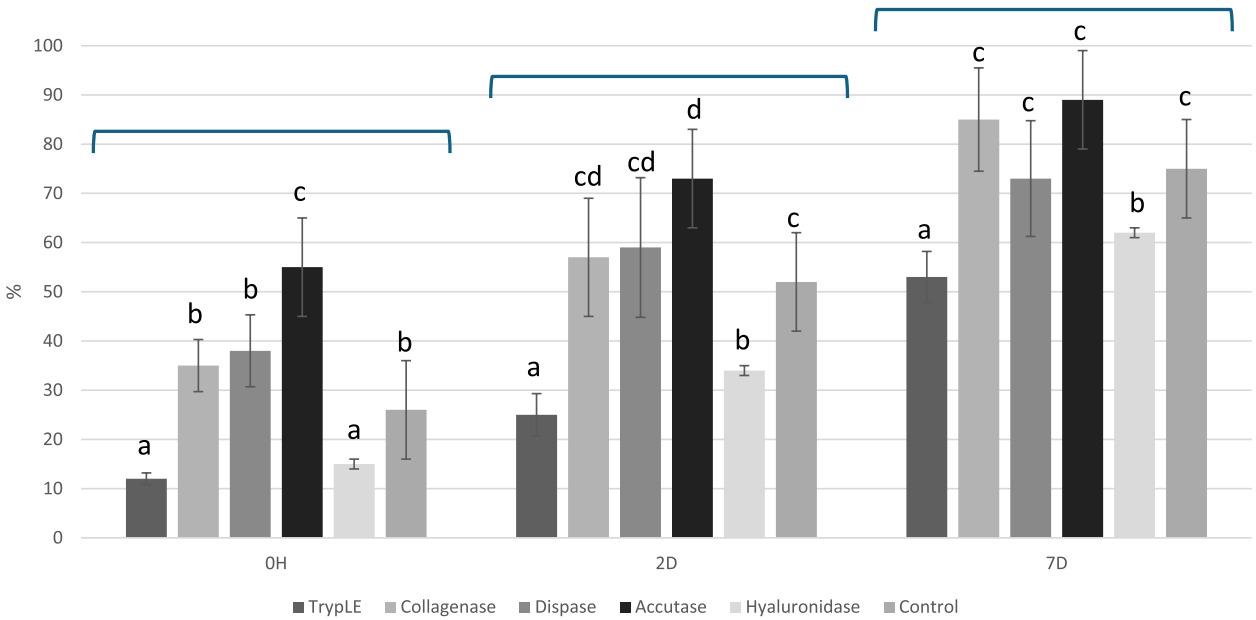

Fig. 3 Percentage of follicular damage at 0 h, 2 d and 7 d post-thawing in TrypLE, collagenase, dispase, accutase, hyaluronidase and control. (Mean ± SEM). Different letters indicate significant differences among treatments

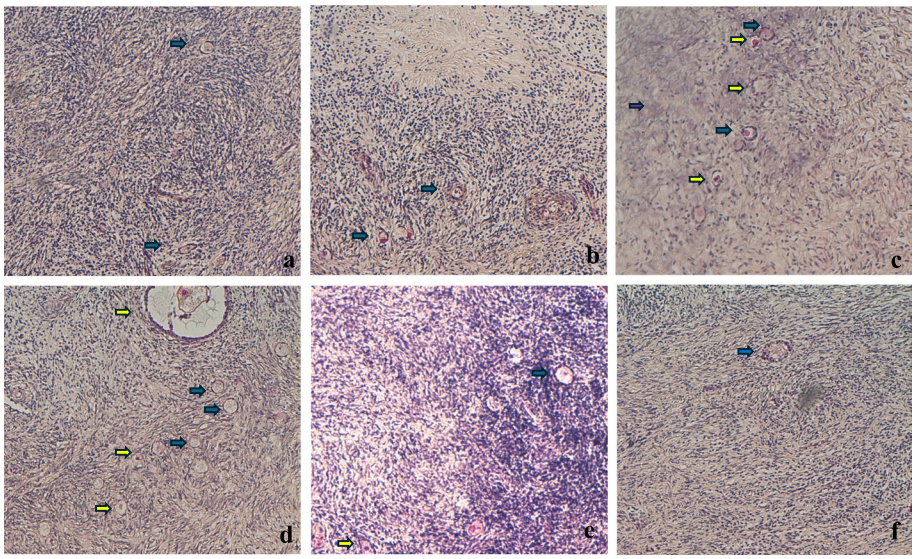

Fig. 4 H&E preparations of ovarian tissue at 0 h post-thawing. **a** TrypLE. **b** Collagenase. **c** Dispase. **d** Accutase. **e** Hyaluronidase. **f** Control. Blue arrows indicate healthy follicles. Yellow arrows indicate damaged follicles. Red arrows indicate areas of necrosis or pyknosis. Purple arrows indicate areas of decellularization

At 2 days of culture post-thawing, more damage in follicles and stroma was observed in all treatments, with more foci of necrosis and decellularization (Fig. 5). Significant differences were observed at 2 d after thawing for follicular integrity (P-value = 0.01). TrypLE treatment again maintained the tissue integrity better with few foci of necrosis and around 20% of the follicles altered. The hyaluronidase treatment also maintained stromal integrity in the inner part of the tissue, showing more decellularization at the edges; and similar percentages of follicular viability at the follicular level. In collagenase treatment and in control, around 50% of the follicles were damaged with more cellular disorganization at the stromal level. The dispase treatment showed significant tissue disorganization at the edges with some foci of necrosis, maintaining better integrity in the central area and with follicles positioned in the peripheral area altered. The accutase treatment exhibited the greatest damage at the

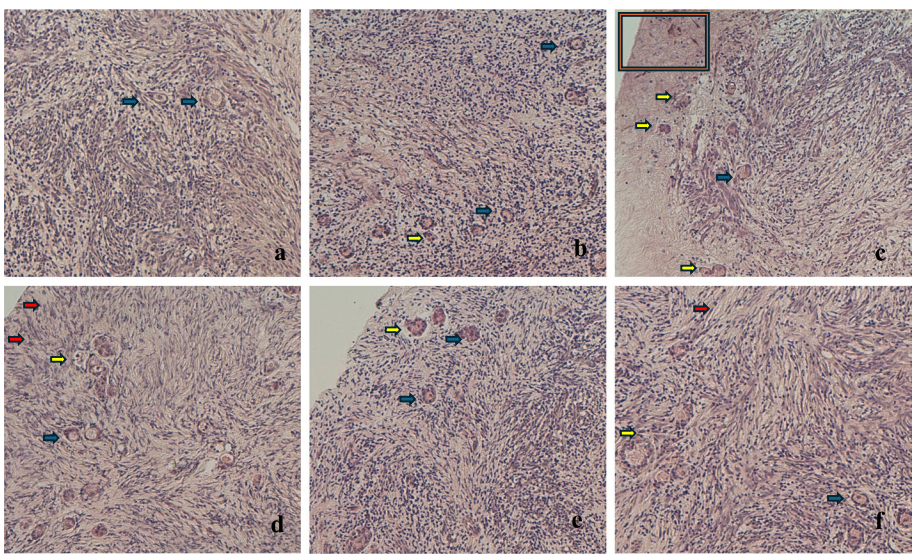

Fig. 5 H&E preparations of ovarian tissue at 2 days post-thawing. **a** TrypLE. **b** Collagenase. **c** Dispase. **d** Accutase. **e** Hyaluronidase. **f** Control. Blue arrows indicate healthy follicles. Yellow arrows indicate damaged follicles. Red arrows indicate areas of necrosis or pyknosis. Orange rectangles indicate damaged edges

follicular level (around 70%) with alterations mainly in the granulosa area of the follicles, multiple foci of pyknosis and higher decellularization.

At 7 days of culture post-thawing, an increase in foci of pyknosis and necrosis, with more decellularization and damage at the follicular level were observed in all treatments (Fig. 6). Significant differences were observed at 7 d after thawing for follicular integrity (*P*-value = 0.03). The trypLE treatment was the treatment that better maintained tissue integrity (follicular damage around 50%), while in the hyaluronidase treatment the edges of the tissue and the follicles were more damaged. In the dispase treatment and in the control, follicular damage increased to more than 70% with severe stromal alterations, primarily at the edges of the tissue in the case of dispase. The most damage results at 7 days were again found in the accutase and collagenase treatments, where most of the tissue was necrotic and the follicles were altered (around 80%).

Immunohistochemistry

Results for Ki67 and Caspase are shown in Fig. 7. Primordial and primary follicles were negative to Ki67 in all treatments and times. Only antral follicles were counted for Ki67. Antral follicles in fresh samples were mostly Ki67 positive in all treatments (104 follicles positive to Ki67; 3 follicles negative to Ki67) with no differences among treatments (*P*-value = 0.89). After thawing, antral follicles positive to Ki67 were observed in all treatments except in accutase (*P*-value = 0.20). Only trypLE and hyaluronidase maintained positive antral follicles to Ki67 after 2 days culture (*P*-value = 0.34). However, no positive Ki67 follicles were observed after thawing and 7 days culture in any treatment (103 follicles negative to Ki67).

In terms of apoptosis, caspase-3 was assessed in all follicles. Positive antral follicles were observed in fresh samples in all treatments (*P*-value = 0.67). At 0 h and 2 d after thawing tripLE showed the best results (*P*-value = 0.22; *P*-value = 0.16). After 7 days culture, all antral follicles were positive to caspase-3, and only trypLE maintained some primordial follicles negative to caspase-3 (*P*-value = 0.44). Images of Ki67 and caspase are shown in Fig. 8.

Discussion

Although the first experiments in ovarian tissue freezing date back to the eighteenth century, it is only recently that this technique has received more attention. Protocols from other types of tissue have been adapted and optimized through the study of various factors considered important to maintain tissue viability and functionality during freezing, such as CPAs and freezing rates. However, the low quality of follicles after grafting remains a problem [6, 7]. Conventional freezing negatively affects cell viability due to the formation of intracellular ice crystals [12]. Increasing cryoprotectant penetrations could decrease ice formation during cryopreservation of the tissues. Rigidity of the stroma impacts the ability of follicles to accommodate and survive the current cryopreservation processes and to recover once the tissues are transplanted in culture and in vivo. Measures to retain stromal structure facilitate resumption of follicular development,

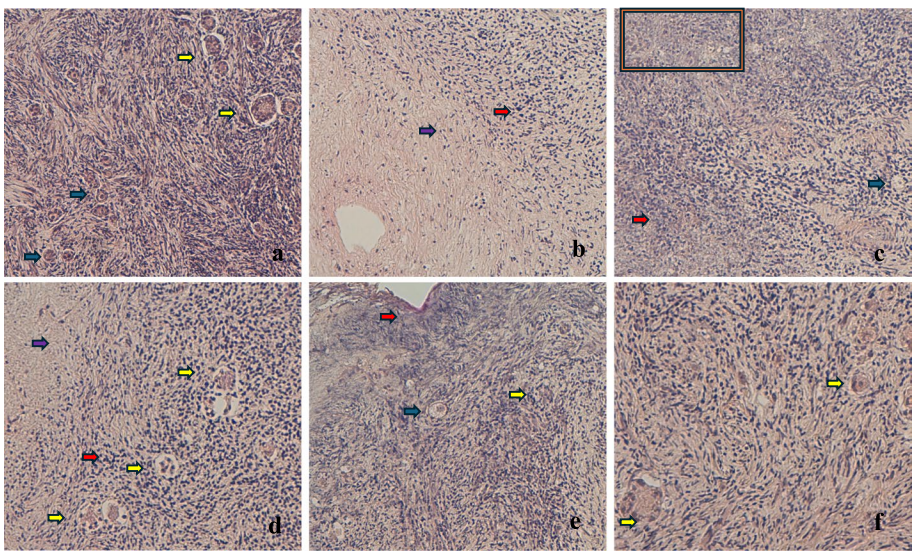

Fig. 6 H&E preparations of ovarian tissue at 7 days post-thawing. **a** TrypLE. **b** Collagenase. **c** Dispase. **d** Accutase. **e** Hyaluronidase. **f** Control. Blue arrows indicate healthy follicles. Yellow arrows indicate damaged follicles. Red arrows indicate areas of necrosis or pyknosis. Purple arrows indicate areas of decellularization. Orange rectangles indicate damaged edges

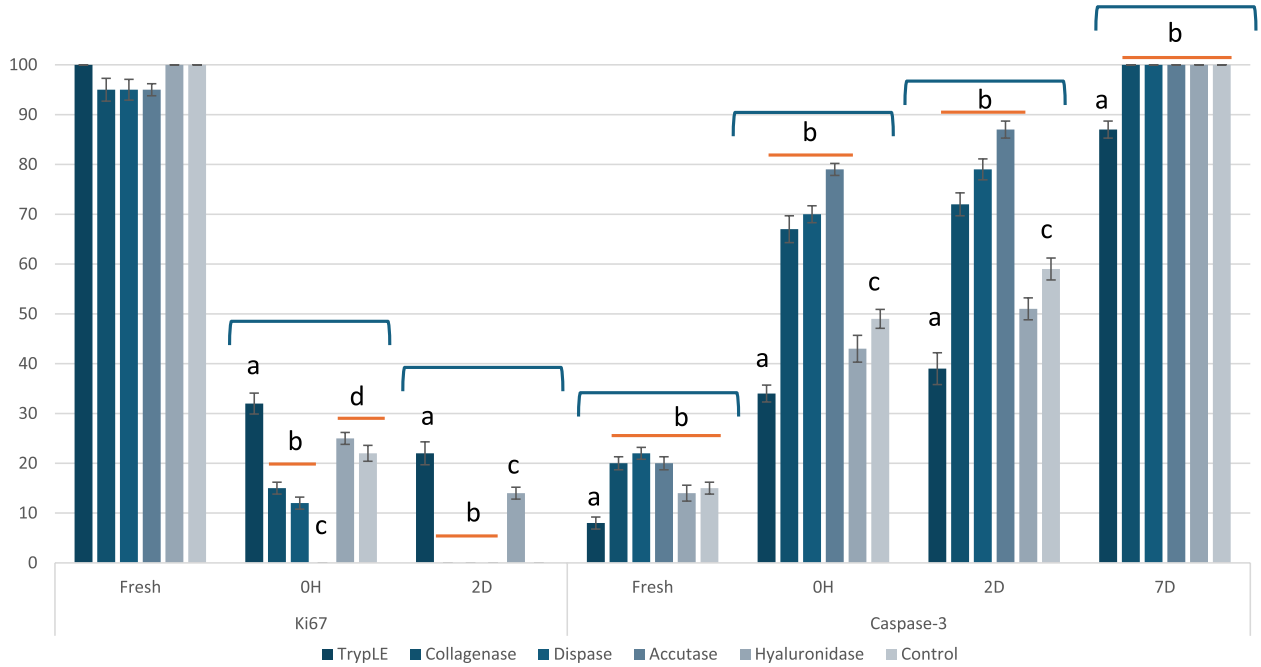

Fig. 7 Percentage of positive Ki67 at fresh and 0 h and 2 d after thawing in TrypLE, collagenase, dispase, accutase, hyaluronidase and control.. Percentage of positive Caspase-3 at fresh and 0 h, 2 d and 7 days after thawing in TrypLE, collagenase, dispase, accutase, hyaluronidase and control. (Mean ± SEM). Different letters indicate significant differences among treatments

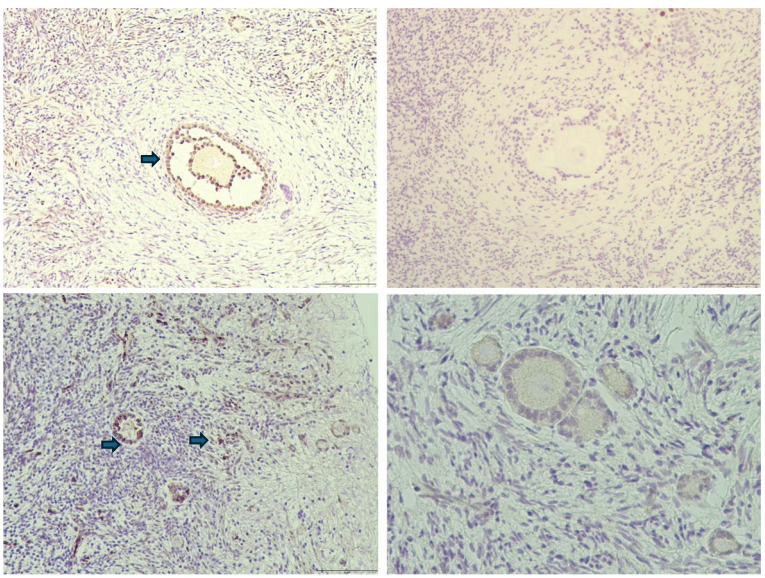

Fig. 8 Immunohistochemistry. **a** Follicle Ki67 positive in fresh tissue after dispase treatment. Blue arrows indicate cells in proliferation. **b** Follicle Ki67 negative in 0 h after thawing in accutase. **c** Follicle caspase-3 positive in 2 days culture after thawing in dispase treatment. Blue arrows indicate apoptotic cells. **d** Follicle caspase-3 negative in fresh sample after trypLE treatment

thus restoring fertility. This is the first study assessing the use of different enzymes to decrease rigidity in ovarian tissue before cryopreservation. Our findings showed that enzymatic treatment with trypLE before freezing could benefit post-thawing with the highest viability values and percentage of intact follicles maintaining Ki67-positive antral follicles and negative to caspase-3 compared to other treatments and control.

The ovarian ECM, predominantly made of collagen, provides scaffolding for stromal cells and follicles, impacts cell behavior via physical adhesion and mechanical tension, and sequesters important paracrine factors involved in cell-to-cell communication [22, 23]. Regarding pretreatment with enzymes, only one study describes the use of collagenase to partially disaggregate mouse ovarian tissue before freezing [8]. This study showed that during the vitrification process, there is a separation between the oocyte and the granulosa cells due to a loss of adhesion molecules without altering the extracellular matrix. However, with collagenase pre-treatment, the ratio between oocyte diameter and follicle diameter was maintained, preserving the adhesions. This would also translate into lower follicle atresia and therefore greater ovarian reserve. This study emphasizes the need to adapt this collagenase pre-treatment to different species. In this study, very low doses of collagenase (1–100 µg/ml) were used compared to our study (0.83 mg/ml), but it must be considered that mouse and human ovaries have many differences in terms of stromal characteristics and extracellular matrix, as well as in thicknesses. Our study showed little tissue disaggregation without affecting follicle quality after treatment at our selected collagenase concentration. Our hypothesis is that the enzymatic treatment causes partial disaggregation within the tissue allowing a better penetration of the cryoprotectants. The results obtained in both studies are difficult to compare since different cryopreservation techniques were performed, slow freezing in our study versus vitrification in the murine ovary study, with different assessment techniques and different animal species. Although the use of collagenase did not improve the viability of stromal and follicular morphology compared to the control, unlike the murine study [8], treating the tissue with trypLE resulted in improvements in tissue viability immediately after thawing. It is worth mentioning that viability measurements were taken from central sections of the tissue piece, avoiding the edges, to ensure similar sections. Severely damaged edges were observed after dispase and collagenase treatment.. A possible explanation is that paraffin sectioning were not completely flat, more bulging or protruding in the inner part, so the edges are more external than the central part and that is why only the edges appear damaged. Consistently these damages in the edges appear more in dispase treatments. Based on these sections, an improvement in stromal and follicular integrity was observed in treatments with trypLE and hyaluronidase.

This is the first study to compare the effect of different enzymes for ovarian tissue freezing. These enzymes were selected because they are commonly used enzymes for tissue treatment for cell isolation. Hyaluronidase enzyme is used in the denudation of oocytes (removing them from the cumulus oophorus) in assisted reproduction laboratories to facilitate sperm penetration in the in vitro fertilization process. However, it is used in much lower concentrations than those used in this study as direct contact with the oocyte is very damaging while higher concentrations are required to penetrate tissue in this study (0.08 mg/ml for oocytes vs 2.5 mg/ml for tissue) [24]. The trypLE enzyme complex is used to isolate cells such as mesenchymal stem cells for the analysis of their surface antigens and has not been used in ovarian tissue previously. The dispase enzyme has been used similarly to isolate different types of cells, such as lymphocytes labeled with certain antibodies or tumor cells. In ovarian tissue, it was used for follicular isolation for designing artificial ovaries [25]. As for the accutase enzyme, although it has been proven to be an excellent cell isolator in other types of tissues (fibroblasts, keratinocytes, vascular endothelial cells), this is the first study where it has been used in ovarian tissue and the results are not as promising, showing the lowest viability values and alterations in follicular and stromal morphology. Only one paper has documented the use of low doses of collagenase treatment to improve fertility outcomes in mice by reducing the rigidity while tissue is subjected to hyperosmotic solutions during cryopreservation [8]. The study presented herein documents the effect of pre-treatments with enzymes usually used to disaggregate tissues, to see if tissue quality was improved after freezing with this modulation of rigidity.

Cryopreservation procedure damages ovarian stromal and granulosa cells, leading to significant fibrotic areas after transplantation, asynchrony between granulosa cells and oocyte development, and thinner theca layers [26, 27]. In our results we have found a general decrease in viability in agreement with other authors [28, 29]. We would like to emphasize that this is the first study where the clearing technique has been used to assess the viability of ovarian tissue with satisfactory results. Some articles have been published where ovarian tissue viability was measured before and after freezing but without prior clearing [30, 31]. In the optimization of the ovarian tissue viability assessment protocol, we attempted to measure viability following standard protocols that do not include clearing, finding a lack of homogeneous distribution of fluorochromes and with many sections remaining unstained, complicating result assessment. The goal of clearing is to remove tissue pigments for better visibility under microscopy. In our case, it allowed us to assess viability satisfactorily by achieving a homogeneous distribution of calcein and propidium iodide fluorochromes, staining the entire sample. This clearing protocol has been used in other tissues [32, 33]. Similar clearing

protocols have been used to assess follicles in ovarian in mouse model but not in either sheep or human models [34, 35].

Furthermore, this is the first study where viability has been subjectively quantified in a piece of tissue without discriminating structures and after subjecting the tissue to clarification. This can be an interesting alternative to the typical follicle counting, which is often tedious due to the thickness, allowing us to have a joint viability value of stroma and follicles, both important for the functionality of the tissue. Other studies have assessed viability as mentioned earlier but not quantitatively [28, 30], limiting result interpretation. Zver et al. [36] described a method to quantify ovarian tissue before autograft by isolation of viable cells from human ovarian cortex to obtain an ovarian cell suspension analyzable by multicolor flow cytometry for viability and ovarian residual disease detection. However, our aim was not to obtain a cell suspension as we wanted to maintain the integrity of the tissue.

Immunohistochemistry data suggested a decrease in proliferation and increase in apoptosis after thawing. Few studies have used Ki67 to assess ovarian tissue after thawing. In a similar study with sheep ovarian tissue, a decrease in Ki67 expression was also observed after thawing [37]. Choi reported that ovarian tissue cryopreservation suppresses granulosa cell proliferation, and that this impairment is recovered within 48 h after culture [38]. Ayuandari et al. [20] observed an increase in Ki65 after 12 weeks of grafting in comparison to pregraft controls. Henry et al. [39] observed that granulosa cells associated with healthy follicles were able to proliferate as soon as 2 days of culture; thereafter, proliferation seemed to decrease, as observed after 6 days. In a recent study by Hossay et al. [40] proportions of Ki67 positive cells were greater on day 6 compared to just after thawing. In contrast, in our study, we could not find positive Ki67 cells after thawing either 2 or 7 days after thawing and culture, confirming that these cells are not in a growing phase. In the same study [40], caspase-3 was also used to assess apoptosis. Similarly, authors found a considerable increase in caspase-positive cells after day 6 of culture compared to just after thawing. It was demonstrated that cryopreservation-induced apoptosis in granulosa cells is mediated by activation of caspase-8, −9, and −3 dependent apoptotic pathways [41]. Other authors have found an increase in the expression of caspase-3 in granulose cells and stroma after thawing [42–44]. These results are in agreement with our study where we observed an increase in caspase-3 positive cells after thawing and culture at day 2 and 6.

Altogether, our results show that viability decreases over time after thawing as well as stromal and follicular integrity. For clinical applications, the tissue fragments must survive for months or years after transplantation by recruiting new blood vessels to the tissue fragments. This process is performed by growth factors present in the extracellular matrix, produced by ECM cells. In future studies we should study the effect of the enzymes in neo-angiogenesis. Further studies focused on other culture media and supplements should be studied in future research. as a recent study conducted on human tissue showed better primordial follicle reserve in the tissue cultured with the Chorioallantoic Membrane [40].

The next steps of this research line will focus on assessing the effect of these enzymes in human adult tissue. Results will be compared and their effect on human pre-pubertal tissues will be evaluated, to determine conclusively whether these enzymatic pretreatments should be included in the prepubertal ovarian tissue freezing protocol to develop a more effective fertility preservation method for girls with cancer.

Conclusions

Treatment with trypLE before freezing has beneficial effects after thawing ovarian tissue samples. Accutase treatment is not advisable before ovarian tissue cryopreservation. Further experimental studies are required to optimize the treatment with tripLE (concentration and time of incubation) before ovarian tissue cryopreservation to maintain ovarian tissue integrity and functionality as well as a study to transplant the pre-treated tissue, for evaluating probable enzymes side effects.

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Disclosure Statement

The authors have no conflict of interest to declare.

Attestation Statement

Data regarding any of the subjects in the study have not been previously published unless specified. Data will be made available to the editors of the journal for review or query upon request.

Capsule

Rigidity of stroma impacts on the ability of follicles to accommodate and survive the current cryopreservation process. Enzymatic treatment with trypLE before freezing maintained the highest viability and proliferation values, with the lowest apoptosis.

Authors' contributions

AM: investigation, methodology and writing original draft; MG: data analysis and writing original draft; JC: conceptualization, formal analysis and review and editing; MRC and AID: methodology, resources and visualization; ARR: investigation, conceptualization and editing; MFC: methodology; CM: conceptualization, formal analysis, investigation, methodology, project administration, supervision review and editing.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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