


Original Research Article

Towards the best enzymatic treatment prior to ovarian tissue cryopreservation. A statistical and model-based analysis

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ABSTRACT

The objective was to study an innovative approach based on a mathematical model to analyze the potential benefits of using enzymatic pre-treatment of ovarian tissue to increase its permeability. Our approach aims to elucidate whether enzymatic pre-treatment would enhance the penetration of cryoprotectants in the ovarian tissue, leading to better preservation and giving best results for obtaining primordial and primary follicles without damage. The effect of five enzymatic treatments (TrypLE, collagenase, dispase, accutase, hyaluronidase) over time on sheep ovarian tissue was evaluated, focusing on penetration depth and tissue damage. First results showed that up to 45 min of incubation, all enzymes penetrated similarly, but longer treatments revealed differences. Dispase showed the greatest penetration at 180 min but was highly aggressive, causing rapid tissue lysis. TrypLE and hyaluronidase were found to be the most effective in minimizing follicular damage while ensuring sufficient tissue penetration, particularly when applied for 10 min. TrypLE caused significant edema after 10 min, but this was reduced after 15 min. Hyaluronidase caused minimal edema and had excellent penetration, making it ideal for enhancing ovarian tissue permeability. The present study showed that pre-treatments with hyaluronidase and TrypLE for 10 min are potential candidates for enhancing ovarian tissue permeabilization, offering the best balance between penetration and minimal tissue damage. These findings could improve fertility preservation techniques and future cryopreservation strategies.

1. Introduction

Ovarian tissue cryopreservation (OCT) has gained attention in recent years as an effective method for fertility preservation in women [1]. Cryopreservation has been successfully applied to a range of tissues, but ovarian tissue faces several difficulties. It is an extremely complex tissue, with follicles in various stages of development, and these structures can be damaged during the process. Preservation of ovarian tissue is of great importance in cases where women have to undergo cancer therapy such as chemotherapy and radiotherapy. By cryopreserving ovarian tissue before treatment, it may be possible to transplant the tissue back after cancer treatment, thus restoring fertility [2].

The main techniques used for OCT are slow-freezing and vitrification. Unfortunately, current techniques face several limitations, such as ice crystal formation during freezing or thermal stress in the thawing

process, leading to cellular damage and viability decrease of the preserved tissue [3–5].

One of the possible alternatives to improve cryopreservation protocols is to ensure that cryoprotectants penetrate well into the tissue. For this purpose, one option would be to perform an enzymatic pre-treatment to reduce the rigidity of the stroma, enhancing the ability of the enzymes to penetrate the tissue. This approach has already been done in mouse ovarian tissue. Results showed that the addition of collagenase increased the number of intact follicles and the viability of the offspring when compared to the control without collagenase [6]. Previous innovative work in our group studied the effect of different enzymes prior to cryopreservation of ovarian tissue [7]. Five commonly used enzymes to disaggregate tissue before freezing ovarian tissue to decrease rigidity and facilitate cryopreservation, TrypLE, collagenase, dispase, accutase and hyaluronidase, were studied. Our findings showed

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that enzymatic treatment with TrypLE before freezing offered immediate benefits post-thawing, with the highest viability values and percentage of intact follicles. Although this type of pre-treatment could be of great help in preserving ovarian tissue, this option has not been explored in depth. One of the main reasons is that access to such samples can be difficult, especially if they are derived from human tissue. Fortunately, *in silico* models can now be developed and can be used to predict the enzymatic mechanisms that may help cryoprotectants to adequately penetrate the tissue, using data obtained from *in vitro* models.

Mathematical models allow the estimation of the enzyme penetration into the tissue *in silico*, avoiding ethical issues associated with human and animal testing. These models can rapidly generate results and predict optimal conditions for tissue permeabilization, which can then be tested and validated in the laboratory [8–10]. By considering the solution to the diffusion equation, we were able to discard diffusion as the main mechanism of enzyme penetration, unveiling other mechanisms related with tissue integrity. This could help to explore a wide range of conditions regarding enzyme concentration and time of incubation, significantly accelerating the development of more effective tissue permeability protocols.

The aim of the present work was to analyze the best optimal enzymatic pre-treatment in terms of enzyme concentration and incubation time to preserve intact follicles. The first step was to study the effect of TrypLE, hyaluronidase, dispase, collagenase and accutase in an ovarian tissue *in vitro* model. Sheep ovaries show similarities to human ovaries in terms of fibrous cortical tissue and were used as an animal model in this project [11]. Once we obtained the data regarding tissue penetration of the different enzymes over time, we explored whether an *in silico* model is able to explain the experimental results, and therefore could be valuable to predict which pre-treatment would be the option of choice to enhance tissue permeability without damage. In addition, the effect of enzymes, in terms of tissue penetration, percentage of damaged follicles and edema, were studied using statistical techniques.

2. Materials and methods

2.1. Sheep ovarian tissue samples

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, for sample preparation, medullary tissue was eliminated by scraping with a surgical scalpel so that the tissue would have a maximum thickness of 1 mm, isolating only the cortical zone. Samples were cut into quadrilaterals of 1 cm × 1 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.

2.2. Enzymatic treatment

The effect of five enzymatic treatments was analyzed, including a control without enzymes. The enzymatic treatments performed were as follows: TrypLE (1X; 9 mL PBS and 1 mL of the commercial solution of the TrypLE enzymatic cocktail at 10X concentration (A127177-01 Gibco), collagenase Type I (0.83 mg/mL, 17100017 Gibco), dispase (2.5 mg/mL, 17105041 Gibco); accutase (Acumax, 00-4666-56 Invitrogen™) and hyaluronidase (25 mg/mL, H3506 Merck). DMEM F12 medium (L0091, Biowest) supplemented with L-glutamine 1 % (25030081 Gibco) and fetal bovine serum 20 % (10270106, Gibco) was used as basal culture medium. Samples of 1 cm × 1 cm × 1 mm dimensions were subjected to the different enzymatic treatments in time intervals of 0, 5, 10, 15, 30, 45, 60, 120 and 180 min at 37 °C. The final volume of enzymatic solution used was 2 mL. Four replicates were prepared per treatment. Then samples were washed three times with DMEM supplemented with 20 % FCS for enzyme inactivation. Untreated

samples were considered as the control. An average number of follicles were studied for each treatment: 49.52 ± 5.32 primordial follicles, 25.97 ± 3.18 primary follicles, 6.87 ± 2.11 secondary follicles and 1.77 ± 0.32 antral follicles. Morphological assessment was carried out in each treatment (enzyme/time) [7].

2.3. Morphological assessment of ovarian tissue

Morphological analyses of ovarian tissue were evaluated by haematoxylin and eosin histological staining. Four replicates per treatment were prepared. Briefly, ovarian tissue samples were fixed in 4 % PFA for 24 h at 4 °C, after which they were processed in cassettes (215-01-10LM, Sandon Diagnostics Limited) using a standardized protocol for 16 h (automated tissue processor; Magnus, Milestone). The blocks were made with the block-making unit (Leica 14039387257) after which they were solidified on a cold plate. The paraffin-embedded samples were cut into 3 µm thick sections using a microtome (Microm GmbH, Type HM325). They were placed in a tempered bath from where they were collected with slides (D100004, Deltalab). Six sections per replicate were cut and placed in the slides. The slides were placed on vertical racks and left to dry overnight at 37 °C. A deparaffinization and dehydration process was carried out as follows: 2 h in formalin, 25 min in ethanol 70°, 1 h in ethanol 100°, 1 h and 15 min in ethanol 100°, 1 h and 30 min in ethanol 100°. Samples were then placed for 2 h in xylene and 55 min, 50 min in isopropanol and 5 h in liquid paraffin. For haematoxylin and eosin staining, a standard protocol was used for ovarian tissue slices as follows: the nuclei of the cells in tissue sections were stained by immersing in haematoxylin (8240907024282001, VENTANAa) for 7 min. Sections were rinsed in tap water for 5 min. Cell cytoplasm was stained by immersing in eosin solution ((240)06544304001, VENTANA) for 2 min and dipped in water for 1 min. After eosin staining, sections were dehydrated by immersion in ascending alcohol solutions (70 %, 96 % and absolute alcohol 100 %) for 15 s each. Finally, all sections were cleaned in xylene for 15 s and mounted with a cover slipping reagent (Leica CV Mount) using glass coverslips. The staining process was made in a stainer workstation (VENTANA HE 600, Roche). Haematoxylin and eosin-stained preparations were evaluated under a light field optical microscope for morphological study of ovarian follicles and stroma. Micrographs for dispase, TrypLE and hyaluronidase pre-treatment are provided in [Supplementary 1, 2 and 3](#), respectively. Different parameters (penetration (µm), lysis (µm), edema (µm) and % of damaged primordial and primary follicles) were analyzed to compare each enzymatic treatment in the ovarian tissue. In the cortex area, penetration was assessed with the appearance of loose tissue, lysis with the appearance of damaged tissue and edema when fluids accumulate in the tissue. The number of follicles were counted. Intact follicles were considered to be follicles maintaining the integrity of membranes and zones (theca zone, basal lamina, granulosa zone, zona pellucida, plasma membrane, nuclear membrane). 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.

2.4. Mathematical model

To evaluate if enzyme penetration follows a diffusion process, as reported in other studies on enzyme penetration [12], we used as a standard reference the solution field for a diffusion process in a half-plane, $x \geq 0$, with a fixed concentration at the boundary [13]. The concentration of the enzyme at point x and time t is:

$$C(x, t) = C_0 \operatorname{erfc} \left(\frac{x}{2\sqrt{Dt}} \right), \quad (1)$$

where C_0 is the enzyme concentration at the boundary $x = 0$, D is the diffusion coefficient and erfc is the complementary error function

$$\operatorname{erfc}(u) = \frac{2}{\sqrt{\pi}} \int_x^{+\infty} e^{-u^2} du, \quad (2)$$

By fixing a detection threshold C^* , the penetration at time t , $x^*(t)$, can be computed as,

$$x^* = 2K\sqrt{Dt}, \quad (3)$$

where $K = \operatorname{erfc}^{-1}\left(\frac{C^*}{C_0}\right)$ is a constant for each enzyme (provided that the boundary condition is the same for each experiment). Therefore

$$X^* = (x^*)^2 = \kappa t, \quad (4)$$

with $\kappa = 4K^2D$ a fitting parameter. For each enzyme, denoting by x_i^* the mean value of the penetration at time t_i (so that $X_i^* = (x_i^*)^2$) and by σ_i its standard deviation we obtain the value of κ by solving the Weighted Linear Least-Squares (WLLS) minimization problem with loss function

$$L(\kappa) = \sum_{i=1}^N \left(\frac{X_i^* - \kappa t_i}{\sigma_i} \right)^2, \quad (5)$$

with N the number of data points. The diffusion model is rejected if $L \gg N - 1$, being $N - 1$ the number of degrees of freedom or similarly if $R^2 \ll 1$.

2.5. Statistical analysis

For estimating the maximum penetration time without damage, for each enzyme and each measurement time we compared the proportion of damaged primordial follicle between the control and the enzyme treatment using a unilateral t -student parametric test. The first time for which the null hypothesis $\mu_{treated} = \mu_{control}$ was rejected in favor of $\mu_{treated} > \mu_{control}$, with a significance level of 5% was considered to be the maximal penetration time without damage. A similar approach was used for the maximum penetration time without lysis.

To assess whether the treatment induced significant damage in the tissue, we evaluate the proportion of damaged primordial and primary follicles. A student t -test was used to compare for each enzyme and its associated maximum penetration time (that is, for each pair enzyme – associated maximum penetration) the proportion of damaged primordial and primary follicle between the treatment and the control data,

using a unilateral hypothesis test.

The different treatments were compared by evaluating the discrepancy between the different relevant variables related to the tissue integrity, as they are the proportion of damaged primordial, primary and secondary follicles, as well as the extent of the edema and the enzyme penetration in the tissue. Statistical significance was evaluated with a bilateral t -test.

3. Results

3.1. Analysis of enzymatic penetration in vitro as a function of time

Samples of ovarian tissue were treated with TrypLE, hyaluronidase, collagenase and dispase and the enzyme penetration was analyzed from 0 min to 180 min at 37 °C. Results showed that all enzymes went through the ovarian tissue in an equivalent manner when samples were treated for 45 min (Fig. 1). The diffusion model was rejected for all the treatments, showing that other transport mechanisms drive the enzyme penetration, related with the tissue integrity. In other words, a diffusion model was not able to capture the penetration for any enzymes individually. This is a pertinent question for future work, but with the current data and the large variability between samples, a fit with more parameters would surely result in overfitting.

When samples were treated from 60 to 180 min, different effects were found. Dispase penetration in the ovarian tissue was gradually increased, reaching $1490 \pm 40 \mu\text{m}$ depth in the tissue at 100 min and its effect was maximum at 180 min when penetration was $1720 \pm 60 \mu\text{m}$ depth. The opposite effect in penetration was found when the tissue was treated with TrypLE, since its effect started to be lower when incubation times with this enzyme went from 60 to 120 min. This loss in enzymatic progression through the tissue was restored at 180 min of enzymatic treatment. Hyaluronidase, collagenase and accutase enzymatic penetration followed a similar trend once they reached the maximum enzymatic penetration, meaning that longer periods of enzymatic treatment did not change the penetration into the tissue (Supplementary 4).

3.2. Maximum enzymatic penetration points without follicle damage

After analyzing the time required for each enzyme to penetrate the ovarian tissue in the in vitro model, the resulting tissue damage was assessed for each treatment. Results showed that the best time for avoiding primordial follicle damage during the enzymatic treatment

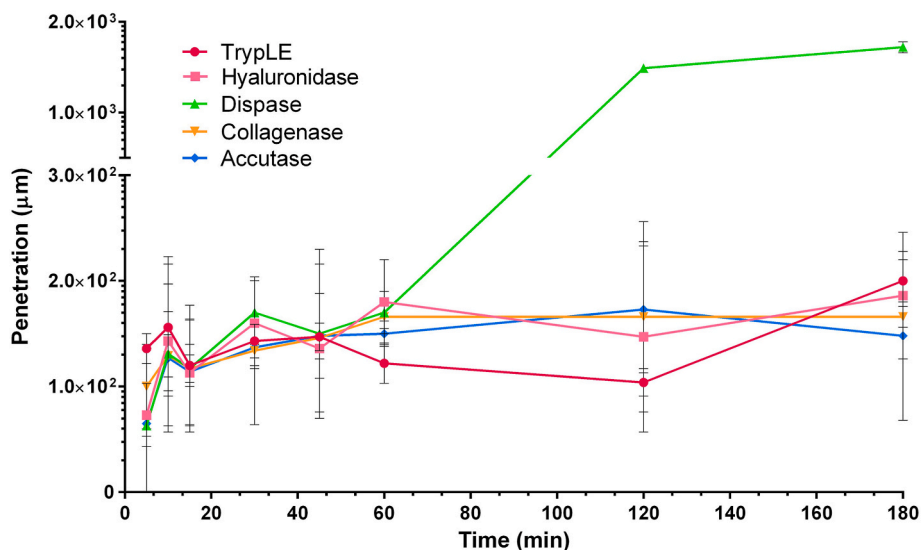


Fig. 1. Penetration of the different enzymes at different times. TrypLE, hyaluronidase, collagenase and dispase were used for ovarian tissue treatment from 0 to 180 min. Results showed that all enzymes penetrated in the tissue in a similar manner up to 45 min. Mean \pm SE.

with TrypLE, hyaluronidase, and accutase was 10 min, in contrast to 15 min for TrypLE and 30 min with collagenase (Table 1). It is also relevant to note that time without lysis of the tissue was calculated, providing information about the aggressiveness of the enzymatic treatment. Dispase was the most aggressive treatment since signs of lysis were estimated to appear just 5 min after exposure to the enzyme. Collagenase produced lysis after 30 min, which corresponds to the time without damage in primordial follicles. Accutase, TrypLE and hyaluronidase produced the lysis of the tissue when longer periods of incubation with the enzymes was performed, from 120 to 180 min. It is important to point that each enzyme was used at a concentration for tissue digestion or cell culture disaggregation.

In connection with the best time for each enzyme for avoiding damage in primordial and primary follicle, the percentage of damaged primordial and primary follicles for each treatment was analyzed (Fig. 2). Results showed that percentage of damaged primordial follicles was $26.79 \pm 15.53\%$, $36.67 \pm 21.34\%$ and $26.59 \pm 11.55\%$ when tissue was treated with accutase, dispase and collagenase, respectively, but no significant differences were found (Fig. 2a). Similar results were obtained for damaged primary follicles where the percentage of damage with accutase, dispase and collagenase was $25.00 \pm 14.43\%$, $24.48 \pm 15.03\%$ and $27.2 \pm 17.18\%$, respectively (Fig. 2b). Although hyaluronidase and TrypLE showed a decrease in primordial and primary follicle damage, only the treatment with hyaluronidase for 10 min significantly reduced the percentage of damaged primordial ($10.71 \pm 6.84\%$) and primary follicles ($8.57 \pm 5.08\%$) compared to the control without enzymatic treatment.

TrypLE and hyaluronidase were the enzymes that best maintained the stroma; severe damage was not observed during incubation. In TrypLE treatment, moderate damage was observed after 30 min, whereas in hyaluronidase, severe damage was not observed until 1 h of incubation. In collagenase treatments, moderate damage was observed after 30 min, and severe damage was observed after 45 min of incubation. In treatment with accutase and dispase, moderate damage was observed after 45 min and 1 h, and severe damage after 2 h of incubation.

3.3. Comparison between the three optimal enzymatic treatments

Finally, enzymatic treatment with TrypLE, hyaluronidase and accutase was compared in the ovarian tissue after 10 and 15 min of exposure to each enzyme, in terms of damage in primordial follicle, primary follicle, and stroma, as well as the penetration in the ovarian tissue (Fig. 3). These three enzymes were thoroughly evaluated since they gave the best results in terms of reducing tissue damage after treatment. Results showed that enzymatic treatment with accutase for 15 min caused the most significant damage in primordial ($49.65 \pm 5.08\%$) and primary ($45.07 \pm 6.24\%$) follicles when compared with the other treatments, especially with TrypLE. This enzyme caused the lowest proportion of damaged primordial and primary follicles, being $4.06 \pm 2.01\%$ after 10 min treatment and $11.11 \pm 6.42\%$ after 15 min for primordial follicles. The same effect in primary follicles was found, where the proportions of damage were 0.00% and $10.61 \pm 6.43\%$, respectively (Fig. 3a and b).

Table 1

Maximal time reported for each enzyme without significant damage in primordial follicle and without lysis. The best time for avoiding primordial follicle damage during the enzymatic treatment was 10 min (TrypLE, hyaluronidase and accutase), 15 min for TrypLE and 30 min for collagenase.

Enzyme	Time without damage in primordial follicle (min)	Time without lysis (min)
TRYPLE	15	180
HYALURONIDASE	10	180
DISPASE	10	5
COLLAGENASE	30	30
ACCUTASE	10	120

Apart from studying the effects of enzymatic treatment in primordial and primary follicles, two additional parameters were analyzed: edema and penetration in the ovarian tissue. TrypLE treatment for 10 min produced more edema when compared with other treatments, where the ovarian tissue presented an increase in volume of $62.50 \pm 18.67 \mu\text{m}$. Surprisingly, this effect was reduced when the treatment was applied for 15 min, when the edema resulted in a volume increase of $22.50 \pm 1.90 \mu\text{m}$. On the other hand, hyaluronidase treatment for 15 min produced less edema, being only $1 \pm 0.81 \mu\text{m}$ when compared with the other enzymes (Fig. 3c). Finally, penetration of all enzymes in the ovarian tissue was studied. TrypLE and hyaluronidase treatment for 10 min penetrated more when compared to the other enzymes, being 156.00 ± 5.78 and $142.75 \pm 8.46 \mu\text{m}$, respectively. After 15 min of treatment, there was no increase in tissue penetration for any of the enzymes compared with 10 min of treatment. (Fig. 3d).

Having analyzed all these results together, hyaluronidase or TrypLE treatment for 10 min would be two good potential candidates for improving ovarian tissue permeability, since they are the enzymes that produced the best results, not only in terms of primordial and primary damage but also in penetration. Regarding edema, TrypLE produced this artifact in the ovarian tissue when it was treated for 10 min. Hyaluronidase would allow the cryoprotectants to penetrate and thus preserve the tissue in the best possible condition.

4. Discussion

Cryopreservation of ovarian tissue has gained attention in recent years to preserve fertility in young women after chemotherapy treatment, among others [14]. Current methods such as vitrification and slow freezing allow the preservation of ovarian tissue in which the follicles are located [15]. They are the main structures to be properly preserved for subsequent transplantation. The present study was based on the need to improve these techniques so that cryoprotectants can optimally penetrate the tissue, by reducing the rigidity and potentially increasing successful fertility preservation by ovarian tissue transplantation, a technique that allows fertility recovery in patients [16–18]. Previous work showed that enzymatic pre-treatment could provide a better functionality and viability of cryopreserved ovarian tissue after thawing. Therefore, the effect of different enzymes commonly used and accessible in the laboratory was analyzed to study their effect on the tissue at different times of incubation and their possible use as a pre-treatment before cryopreservation. The results of this study reveal important differences in the ability of five commonly used enzymes for tissue digestion and cell disaggregation (TrypLE, hyaluronidase, accutase, dispase and collagenase) to penetrate and affect ovarian tissue over time [7].

All enzymes penetrated ovarian tissue similarly up to 45 min, but the behaviour diverged significantly at longer treatment times. Dispase showed a higher penetration at 100 min, reaching its maximum at 180 min (Fig. 1). However, this effect can be related to the rapid onset of tissue lysis after only 5 min of exposure (Table 1). In shorter times and up to 10 min, TrypLE was the one that reached the maximum penetration point and no lysis was found at that time.

It is particularly relevant that, in terms of preserving the integrity of primordial and primary follicles, hyaluronidase excelled with a significant reduction in damage when used for 10 min when compared to control (Fig. 2). The same effect was found with TrypLE used for 15 min but no significant differences were obtained when compared to control. These findings highlight the importance of time-controlled enzymatic treatment in minimizing tissue damage, a crucial consideration in preparing tissues for cryopreservation. The reduction of edema with hyaluronidase also supports its use, as less edema may contribute to better structural preservation of ovarian tissue (Fig. 3c). Hyaluronidase treatment for 10 min not only provides sufficient penetration to allow subsequent penetration of cryoprotectants but also minimizes damage to follicles and reduces the incidence of edema. This could have important clinical implications in improving cryopreservation techniques, thereby

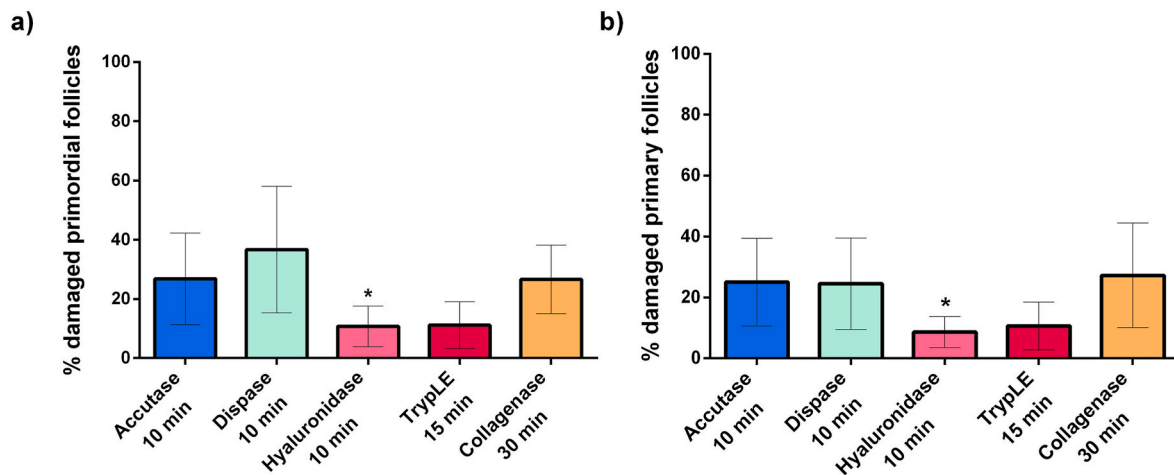


Fig. 2. (a) Proportion of damaged primordial follicle for each optimal treatment. (b) Proportion of damaged primary follicle for each optimal treatment. Hyaluronidase treatment for 10 min significantly reduced the percentage of damaged primordial and primary follicles compared to the control without enzymatic treatment. * means $p < 0.05$ when compared to control.

improving tissue survival rates and post-thawing reproductive function. On the other hand, TrypLE should also be considered as a good candidate for pre-treatment before cryopreservation. This mixture of enzymes was the one that had the best results in terms of percentage of damaged primordial and primary follicles and reached the maximum penetration point when the three best treatments were applied for 10 min (Fig. 3a, b, d). It is also important to point out that TrypLE produced edema when compared to the other treatments and this fact should be also considered since it could be a major disadvantage for cryopreservation. Previous experimental work performed in vitro showed that pre-treatment with TrypLE prior to cryopreservation enhances ovarian tissue viability after thawing, while accutase is not recommended. Further studies in vitro using ovarian tissue grafts are needed to optimize TrypLE incubation conditions to preserve not only ovarian tissue integrity but also functionality.

There are many studies where improvements in vitrification and slow-freeze methods have been made but only one on ovarian tissue in mice included an enzymatic pre-treatment [6]. This study showed that pre-treatment of the ovarian tissue with collagenase improved the fertility rate after transplantation. However, differences in the enzyme concentration used make it difficult to compare these results with our study. Moreover, our results indicate that collagenase would not be the best option for enzymatic pre-treatment when compared to other enzymes included in the study. Hyaluronidase is used before Intracytoplasmic Sperm Injection (ICSI) since it facilitates the denudation of the oocyte [19–21]. The concentration required for this process is much lower than the concentration needed for ovarian tissue digestion. For that reason, and as in the case of collagenase, it is difficult to compare the effect of this enzyme in previous studies in ICSI to our results. Liberase (Dispase High), was used for follicular isolation to design an artificial ovary [22], while TrypLE and accutase are commonly used enzymes for cellular disaggregation but none of them were used for enzymatic treatment of the ovarian tissue.

At this point it is clear that computational model-based analysis would be highly beneficial for predicting the effect of different enzymes on ovarian tissue, since there are very few, if any, studies that have explored the potential of using this type of enzymatic pre-treatment. Indeed, computational models are invaluable tools both for understanding and predicting the effect of the different enzymes in the tissue. On the one hand, computational modelling is key for elucidating the physical underlying mechanisms related to enzyme penetration and tissue integrity. For instance, in the present study, diffusion was rejected as the process driving enzyme penetration, unlike results reported for other CPAs [23]. This stimulates a search for other physio-chemical

mechanisms related to tissue integrity, and to decide whether a treatment is fit for purpose. On the other hand, reliable computational models may be used for predictions that could help in the design of new cryopreservation protocols. Actually, in spite of their use in the study of cryopreservation processes [24,25] or toxicity assessment [26], computational models have not yet been fully exploited in the field of fertility preservation, although there are some incipient studies [27]. In addition, new monitoring techniques offer the possibility of extracting richer data to obtain more robust and accurate models [27]. Interesting aspects such as the penetration depth, duration of the process, toxicity, or the efficiency of each treatment could be investigated without the need for the design of experiments for each one of them, thus saving time and resources. Finally, the combination of penetration models and data assimilation techniques could help in the design and optimization of CPA perfusion protocols, taking into account toxicity, vitrification efficiency, or the duration of treatment [26].

Despite the positive results, this study also has certain limitations that should be considered. For example, the study focused on specific times and enzymes, which leaves open the possibility that other times or enzyme concentrations could provide different results. Future research could explore the optimization of these variables, as well as evaluating the long-term effects of these treatments on post-cryopreservation ovarian tissue viability and functionality. It would also be interesting to perform comparative studies with other tissues and explore the application of these results in vivo models. However, these preliminary data open the door to the study of this type of enzymes for pre-treatment methods to be implanted prior to cryopreservation. The great advantage of combining the data with mathematical models is that it considerably reduces the economic cost, time and ethics required to carry out in vivo experiments.

5. Conclusions

The results show that hyaluronidase or TrypLE applied for 10 min are the best options for ovarian tissue preparation prior to cryopreservation due to their ability to effectively penetrate the tissue with minimal follicular damage and edema. These findings could significantly improve current practices in fertility preservation and have the potential to be applied in future cryopreservation strategies.

CRedit authorship contribution statement

Alodia Lacueva-Aparicio: Writing – original draft, Investigation, Conceptualization. **Jacobo Ayensa-Jiménez:** Software, Methodology,

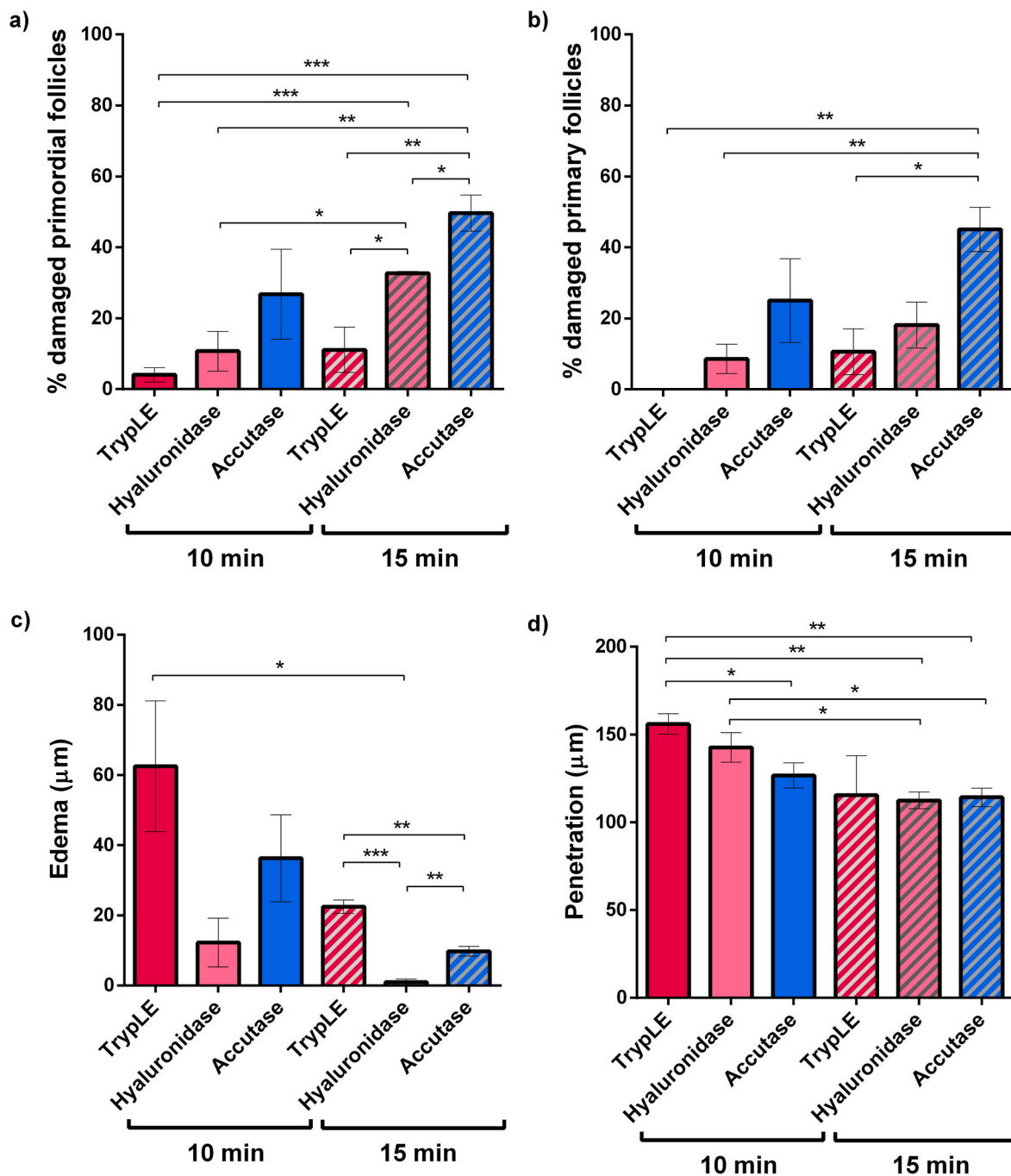


Fig. 3. Comparison of the tissue state at the 4 optimal treatments. (a) damaged primordial follicle; (b) damaged primary follicle, (c) stroma damage. (d) penetration in the ovarian tissue. TrypLE caused the lowest percentage of damaged primordial and primary follicles after 10 and 15 when compared to hyaluronidase and accutase. Edema generation was also analyzed, showing that TrypLE would cause this effect in the ovarian tissue after 10 min of treatment and reaching the maximum penetration possible in the tissue. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Formal analysis. **María Francisca Perulero:** Investigation. **Dácil Diaz:** Investigation. **Sigrid Zalaya:** Investigation. **Emilio Ignacio Abecia:** Software, Methodology, Formal analysis. **Clara Malo:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Conflict of interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2025.117521>.

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