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Optimal human ovarian follicle isolation: A review focused on enzymatic digestion

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Summary. The damage or depletion of ovarian reserves due to aging or cancer treatment can increase the need for fertility preservation techniques. One of the most common ways of supporting fertility in prepubertal girls and women who require immediate cancer treatment is through ovarian tissue cryopreservation and re-transplantation following cancer treatment. However, a more appropriate method should be employed in diseases such as leukemia, where malignant cells may be present in cryopreserved tissue, instead of ovarian tissue transplantation. Human ovarian follicle isolation for *in vitro* culture or the use of artificial ovaries for their growth can decrease the risk of reintroducing cancer cells into these individuals. Here we review the methods for the isolation of human ovarian follicles.

Key words: Human ovarian tissue, Follicle isolation, Enzymatic isolation, Artificial ovary, Live/dead

Introduction

Over the last few decades, there has been an increase in cancer incidence among women, with around 20 percent of women of childbearing age being affected (Maltaris et al., 2006; Akahori et al., 2019). Although anticancer treatments, including chemotherapy, radiation therapy, and other methods, have increased survival rates, the potential complications of these treatments were not fully understood until around 20 years ago (Meirow, 2000; Donnez et al., 2006; Resetkova et al., 2013; Smith et al., 2014). The ovaries are sensitive organs, and cytotoxic drugs such as alkylating agents and radiotherapy can damage a patient's reproductive ability and increase the risk of premature ovarian insufficiency (POI) (Demeestere et al., 2009; Peddie et al., 2012; Wenners et al., 2017; Buckenmeyer et al., 2023).

Fertility preservation is recommended when there is a high risk of POI in cancer patients.Among the different strategies available, ovarian tissue cryopreservation is the only one indicated for prepubertal girls or women who need to start cancer treatment as soon as possible and may not have sufficient time to undergo embryo or oocyte cryopreservation protocols (Wang et al., 2016; Donnez and Dolmans, 2017; Andersen et al., 2019; Silvestris et al., 2020).

Primordial and primary follicles found in the cortex area are preserved during ovarian tissue cryopreservation. Primordial follicles make up the majority of follicles in the ovarian cortex and are referred to as the ovarian reserve (Wallace and Kelsey, 2010). Furthermore, the small size, low metabolic rate, and absence of zona pellucida and cortical granules make primordial follicles more resistant to cryopreservation injuries (Gerritse et al., 2008). These small follicles are surrounded by collagen IV, laminin, and fibronectin (Heeren et al., 2015; Mouloungui et al., 2018).

In recent years, more than 200 live births have been achieved after the transplantation of cryopreserved ovarian tissue (Dolmans et al., 2020). However, the most critical concern about ovarian tissue transplantation is the possibility of cancer cells in the tissue and the recurrence of cancer. This issue is particularly relevant for cancers such as leukemia, Burkitt lymphoma, and neuroblastoma, which pose a higher risk (Dolmans, 2021). Therefore, researchers have been developing effective methods to isolate viable follicles from cancer

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Abbreviations. POI, Premature Ovarian Insufficiency; BM, Basement Membrane; GMP, Good Manufacturing Practices; DH, Dispase High; TM, Thermolysin Medium; TDE, Tumor Dissociation Enzyme

cells, either to grow them *in vitro* or to transplant them into an artificial ovary, to minimize the risk of cancer recurrence.

The purpose of this study was to review and compare the human ovarian follicle isolation methods.

Isolation of human preantral follicles

Why should we isolate ovarian follicles?

Acute lymphoblastic and myeloid leukemias are the most commonly occurring cancers in prepubertal girls (Ward et al., 2014). For these patients, ovarian tissue transplantation can increase the risk of cancer recurrence (Rosendahl et al., 2010). However, there are techniques available to identify cancer cells in the ovarian tissue, including real-time quantitative polymerase chain reaction, multicolor flow cytometry, and ovarian tissue xenografts (Mouloungui et al., 2018). Moreover, the basement membrane around the follicles serves as a physical barrier that protects them against malignant cells (Irving-Rodgers and Rodgers, 2006). Therefore, follicle isolation using approaches such as artificial ovaries (Soares et al., 2017; Chiti et al., 2018; Chen et al., 2021) or *in vitro* culture (Dolmans et al., 2006; McLaughlin et al., 2018; Wiweko et al., 2019) can be helpful for this group of patients.

How should we isolate ovarian follicles?

Numerous studies have investigated different techniques for isolating preantral follicles in humans, which are generally classified as either mechanical or enzymatic methods. In mechanical processes, ovarian fragments are cut using a tissue chopper or a sharp tool (e.g., scissors or scalpel) followed by dissection with needles. Although mechanical isolation is a safe method, human ovaries have a dense and fibrous structure, and using mechanical methods alone can be a hard and time-consuming approach. In one study, Langbeen et al. applied an Ultra Turrax T18 basic homogenizer instead of a tissue chopper in three ruminant species (bovine, caprine, and ovine) to decrease the length of the isolation procedure (Langbeen et al., 2015). In another study, some mechanical isolation methods including micro-dissection, tissue chopping followed by filtration, mincing, trypsinization and sonication were combined (Sharma et al., 2009). However, this technique was not used for human follicle isolation. Therefore, it seems that a combination of mechanical and enzymatic methods is typically employed to successfully isolate a substantial number of follicles. To the best of our knowledge, the only study comparing mechanical isolation followed by enzymatic digestion with that of mechanical isolation alone was performed by Dong et al. (Dong et al., 2014). The authors reported that the mechanical/enzymatic isolation method yielded a higher survival rate for primordial follicles after ten days of *in vitro* culture, while the survival rate of secondary follicles was higher with mechanical isolation alone. Interestingly, they found that culturing ovarian tissue for six days before follicle isolation led to larger follicles and higher levels of estradiol than with mechanical isolation alone. They explained that this was due to the ovarian tissue becoming looser after the six-day culture, allowing for easier follicle isolation with the mechanical method (Dong et al., 2014).

Considering the composition of preantral follicles, the basement membrane (BM) can be important in choosing the best enzyme. The follicular BM is made of laminin, collagenase IV, and fibronectin, and it maintains the structure of the developing follicle, protects the cellular structure, and regulates follicle growth and development by also regulating hormones and growth factors. These BM can be remodeled during follicle development (Heeren et al., 2015). On the other hand, BM can support capillaries, white blood cells, and nerve processes from the follicle compartment (Donnez et al., 2006). Therefore, it is essential to use an appropriate enzyme to isolate more viable follicles with a healthy BM. Enzymes, including various types of collagenases and liberases, and more recently, tumor dissociation enzyme (TDE), have been utilized over the years in numerous studies aimed at isolating human preantral follicles. During enzymatic digestion, sticky DNA ends released from damaged cells act as glue, causing isolated follicles to attach to other cells or remaining fragments of ovarian tissue, which can prevent the harvesting of fully isolated follicles (Wang et al., 2007). To circumvent this problem, DNase I is added to the enzymatic solution. All enzymes are produced under good manufacturing practices (GMP) for clinical practice.

Which materials could be useful to isolate human ovarian follicles?

Collagenase

Various types of collagenase (Ia, II, IX, XI) derived from Clostridium histolyticum can be used for isolating follicles, with or without DNase I (Lierman et al., 2015).

In 1993, Roy and Treacy (1993) reported the first successful isolation of primordial follicles from human ovaries. They dissected the ovarian tissue with a razor blade and then digested the fragments using a combination of collagenase type I and DNase. The authors reported that this protocol yielded a large number of isolated follicles, which were able to grow *in vitro*, as evidenced by their increased size and synthesis of estradiol, progesterone, and androstenedione after five days of culture (Roy and Treacy, 1993). It should be mentioned that shorter exposure to the enzyme, with lower or higher amounts of enzyme was not effective. Oktay et al. (1997) achieved high efficiency and viability rates by using collagenase type IA in combination with the mechanical method for partial disaggregation of ovarian tissue before or after

cryopreservation. However, electron microscopy revealed some damage following isolation and cryopreservation (Oktay et al., 1997). Several studies have utilized various types of collagenases for partial or complete isolation of human preantral follicles (Figueiredo et al., 1993; Hovatta, et al., 1999; Martinez-Madrid et al., 2004; Xu et al., 2009; Chen et al., 2022). For example, collagenase NB6, which is a combination of collagenase I and II frequently employed for isolating human umbilical cord cells, was used by Mouloungui et al. to isolate human ovarian follicles (Mouloungui et al., 2018). They isolated a high number of viable primordial follicles with an intact BM. Belen Martinez-Madrid et al. also applied collagenase IA for primordial follicle

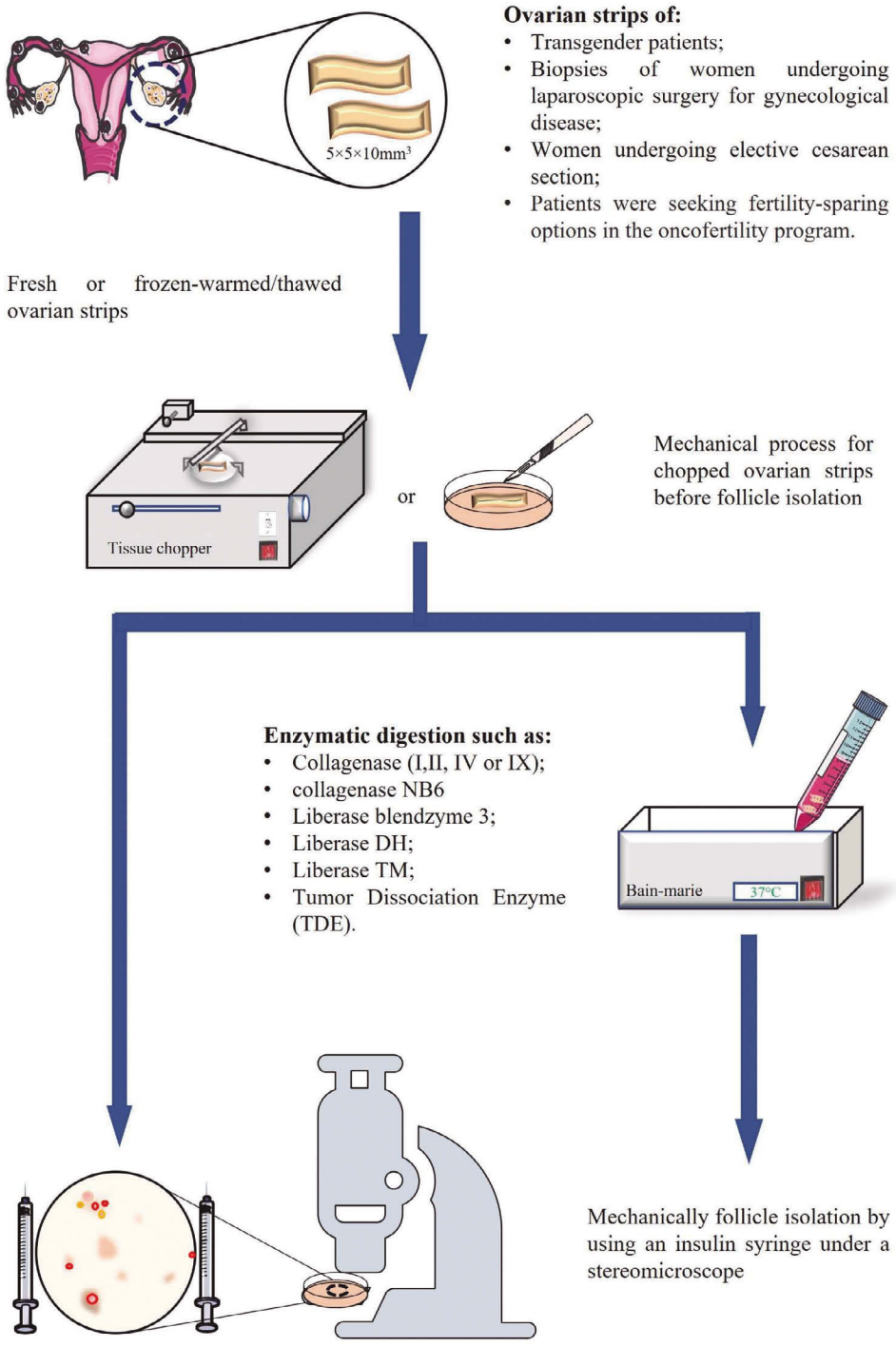

Fig. 1. Isolation of human ovarian follicules for research approaches.

Table 1. Summary of studies on the isolation of human preantral follicles using enzymatic digestion.

Sample (age)	Mechanical method	Enzymatic procedure	Diameter of the isolated follicles (*μ*m)	Aim	Main outcomes	Reference
Premenopausal women with regular menstrual cycles who were undergoing hysterectomy-oophorectomy (range 16 to 32 years of age)	Razor blade before and agitation during and at 4°C in a refrigerator for 36 hours after the enzymatic process for slow digestion	The mixture of collagenase and deoxyribonuclease (DNase) for 1 hour at 37°C and for 36 hours at 4°C	<90 µm	Long-term culture of isolated follicles	Successfully follicles isolation and long culture	Roy and Treacy, 1993
Biopsies of women undergoing laparoscopic (rage 25 to 32 years of age)	The 27-gauge insulin needles after enzymatic digestion	DNase and Collagenase IA	30-50 µm	Follicle isolation before and after cryopreservation	Success preantral isolation from fresh and frozen ovarian tissue	Oktay et al., 1997
Women undergoing elective cesarean section (range 5 to 33 years of age)	Tissue was chopped	Collagenase type IA and DNase I	-	Ontogeny FSHr expression in isolated follicles	FSHr expression in the early stages of follicle development	Oktay et al., 1997
Biopsies of women undergoing laparoscopic (rage 25-43 years of age)	Sliced in 0.3-1 mm before and using a 27-gauge needle after enzymatic digestion or just partially isolation for mechanical isolation group	Collagenase II with 1 or 0.5 mg/ml concentration for enzymatic group	20-80 µm	Culture of the ovarian follicle by two enzymatic and mechanical methods	Culturing the small tissue slices is better than partial isolation	Hovatta et al., 1999
Biopsies of women undergoing laparoscopic (range 26 and 31 years of age)	Using the tissue sectioner before, gentle agitation to mechanically disrupt digested tissue	Collagenase type IA	30-120 µm	Isolation of preantral follicles by ficoll density gradient method	Ficoll density gradient can reduce the manipulating time and increase the recovery of isolated follicles	Martinez-Madrid et al., 2004
Biopsies of women who underwent laparoscopic surgery for gynecological disease (range 20 to 32 years of age)	Using the tissue sectioner, and using a 130-*μ*m micropipette to transfer isolated follicles	Collagenase type IA *versus* Liberase blendzyme 3	40-80 *μ*m	Comparison between liberase blend enzyme and collagenase IA	Liberase shows high viability in isolated follicles and is a good alternative for collagenase IA	Dolmans et al., 2006
Biopsies of women undergoing laparoscopic surgery (range 23 to 32 years of age)	Using the tissue sectioner before, aspirated up and down during enzymatic digestion and aspirated up and down with a 10 ml pipette for pick up follicles	Liberase blendzyme 3	-	Short-term xenografting human isolated follicles	Isolated follicles can survive after 7 days of xenotransplantation by plasma clot	Dolmans et al., 2007
Biopsies of women undergoing laparoscopic (range 26 to 29 years of age)	Using the tissue sectioner before and using a 130 µm micropipette after enzymatic digestion	Collagenase IA *versus* liberase blendzyme 3 (PCB)	30-100 µm	Long-term xenografting human isolated follicles	Isolated follicles can survive after long-term xenotransplantation by plasma clot	Dolmans et al., 2008
Patients were seeking fertility-sparing options in the oncofertility program (range 16 to 39 years of age)	Ovarian cortical strips were cut into 1 mm^3 pieces before and 25-gauge needles in dissection media after enzymatic digestion	Collagenase and DNase	175±10 µm	*In Vitro* culture of secondary follicles	Successful culture of the secondary follicle in a bio-engineered system for 30 days	Xu et al., 2009
Biopsies of women undergoing laparoscopic (range 21 to 35 years of age)	Using the tissue sectioner, and using a 130-mm micropipette for the transfer of isolated follicles	Collagenase IA *versus* liberase DH	30-80 µm	Comparison between collagenase IA and liberase DH	The high number of follicles were isolated by liberase DH. Also, they can maintain their viability structure after *in vitro* culture	Vanacker et al., 2011
Biopsies of girls/women undergoing cryopreservation of the ovarian cortex (range 3 to 35 years of age)	Using the tissue sectioner before, and using a thin Pasteur pipette after enzymatic digestion	The mixture of Collagenase IV, Liberase TM, and DNase I	40-160 µm	Follicle isolation from the ovarian medulla	The medulla is full of preantral follicles, especially in young girls that can used for investigation and clinical approaches	Kristensen et al., 2011
Fresh and frozen-thawed ovarian tissue (range not mentioned)	1 ml syringe under an inverted microscope	Collagenase type I	The mean of preantral follicles isolated by collagenase IA combined with mechanical digestion after 6 days was 84.9±4.3 µm, mechanical alone was 84.9±4.3 µm	Comparison of isolation method on frozen-thawed ovarian follicles	There is no difference in the stage of isolated follicles before and after vitrification and the survival rate of secondary follicles is higher with mechanical isolation after 6 days of culture	Dong et al., 2014

isolation (Martinez-Madrid et al., 2004). They used a Ficoll density gradient to recover isolated follicles and discovered that this gradient can minimize the manipulation time and maintain high follicular viability.

While collagenase is a commonly used enzyme for ovarian follicle isolation, it contains a large amount of

Table 1. (Continued).

Frozen ovarian tissue of the transsexual person	Not mentioned	Liberase DH and Liberase TM alone or combined with collagenase IV *versus* collagenase IV alone	>60 μm (primordial follicles) to >200 μm (secondary follicles)	Comparison of five group enzymes for follicle isolation	The maturation status of follicles has a key role in response to enzymatic isolation	Lierman et al., 2015
Biopsies of women undergoing laparoscopic (range 22 and 36 years of age)	Using the tissue chopper, manually pipetting and transferring the follicles to fresh droplets using a 130-μm micropipette after enzymatic digestion	Liberase DH	-	Safe follicle isolation	Washing can eliminate malignant cells	Soares et al., 2015
Biopsies of women who underwent laparoscopic surgery for gynecological disease (mean 31.8± 8.36 years of age)	Using the tissue sectioner before and 29-gauge after enzymatic digestion	Liberase DH and DNase I	Mean 77.7±2.7 μm for fresh tissue, 77.2±3.2 μm, and 78.3±2.4 μm, from slow-freezed tissue and vitrified tissue, respectively	Comparison of slow freezing, vitrification, and fresh tissue after single follicle *in vitro* culture	There is no difference between slow freezing and vitrification	Wang et al., 2016
Tiny amounts of cortical tissues of cancer patients for cryopreservation (range 9 to 37 years of age)	Using the tissue chopper before and aspirating up and down the suspension to release the follicles into the media. Isolated follicles with red coloration were removed and washed twice in 378C DPBS supplemented with 10% FBS after enzymatic digestion	The mixture of Liberase TM, collagenase IV, and DNase I	≤60 μm to 120-250 μm	Culture of medulla isolated follicle in 3-D system	Secondary isolated follicles had better growth in comparison to smaller follicles after 7 days	Yin et al., 2016
Freeze-thawed biopsies of women undergoing laparoscopic (range 21 to 37 years of age)	Using the tissue sectioner before, and using a 130- µm micropipette after enzymatic digestion	Liberase DH	Between 38.8±6.3 µm to 48.8±2.5 µm	Survival and growth of xenotransplanted follicles by fibrin	It seems that fibrin can be used as a human artificial ovary	Paulini et al., 2016
Using vitrified ovarian strips (range not mentioned)	Using the tissue chopper before, filtration every 30 min for complete follicle isolation during, and 1 hour using a 130-μm micropipette after enzymatic digestion	Liberase DH and DNase I	30 to 129 µm	Design a modified protocol for follicle isolation	Increase the number and quality of isolated follicles	Chiti et al., 2017
Biopsies of women undergoing laparoscopic (range 25 to 37 years of age)	Manually pipetting and transferring the follicles using a 130-μm micropipette after enzymatic digestion	Collagenase IA, a GMP grade purified collagenase NB6 or Liberase DH	Mean for collagenase NB6 was 31.66±6.79 μm, for collagenase IA was 36.77±7.69 μm and for liberase DH was 36.77±7.69 μm	Follicle isolation without leukemic cells presenting	Follicles isolation was made by good manufacturing practices for cell therapy	Mouloungui et al., 2018
Fresh and frozen ovarian cortex fragments (range 29 to 39 years of age)	Mechanically cut	Tumor Dissociation Enzyme *versus* Liberase TM	-	Comparison of tumor dissociation enzyme with liberase TM	Isolated follicles with tumor dissociation enzyme had no necrosis and apoptosis	Schmidt et al., 2018a,b
Donated ovarian cortex from patients with various malignant diseases (range 15 to 37 years of age)	Using the tissue chopper	Mixture of Liberase TM and Collagenase IV	54.5-162.2 µm	Human ovary reconstruction	Xenotransplantation of decellularized ovarian cortical tissue can support the growth of human follicles	Pors et al., 2019
Biopsies of woman undergoing laparoscopic surgery (range 18 to 35 years of age)	Mechanically cut into ~0.5×1 to 1×1 mm^3 pieces by quick simultaneous movements of two scalpels no. 22, and using 125 µm V-denuded capillaries after enzymatic digestion	Tumor Dissociation Enzyme -cocktail *versus* Liberase DH/ /DNase	The mean for the tumor Dissociation Enzyme group was 40.5±4.1 µm, and Liberase DH group was 42±3.5 µm	Optimizing follicle isolation protocol	Tumor Dissociation Enzyme can used instead of liberase DH	Chen et al., 2022

endotoxin that can negatively impact follicle quality, in addition to the batch-to-batch variation that can affect reproducibility (Lierman et al., 2015). Furthermore, it has been reported that collagenase can cause damage to the follicle's basal lamina (Hovatta et al., 1999; Dong et al., 2014). To address these concerns, a purified collagenase blend or Liberase were used as alternatives for follicle isolation. Dolmans et al. compared Liberase Blendzyme 3 and collagenase type IA and found that the use of the former could be a suitable alternative to the latter (Dolmans et al., 2006, 2007, 2008). Due to using Liberase Blendzyme 3, not only was the follicular ultrastructure preserved but more than 86% of isolated follicles were viable, with sufficient capacity to grow *in vitro* or *in vivo* (Dolmans et al., 2006, 2008).

Liberase DH

Liberase DH (Dispase High) is a type of enzyme blend that contains collagenase I, collagenase II, a high concentration of Dispase, and a non-clostridial neutral protease. Studies comparing Liberase and collagenase for isolating preantral follicles have shown that Liberase DH produces better results in terms of the number and quality of preantral follicles, survival rates, *in vitro* culture outcomes, and structure of follicles (Vanacker et al., 2011). Mouloungui et al. (2018) conducted a comparison of collagenases NB6 and IA with Liberase DH for follicle isolation. However, their results did not show any significant difference in the number and survival rate of the isolated follicles using the three enzymes (Mouloungui et al., 2018).

Wang et al. (2016) used a combination of Liberase DH and DNase I to achieve the highest quality isolation and *in vitro* culture of follicles. Chiti et al. (2017) also used Liberase DH and DNase I in their study to enhance follicle isolation. Interestingly, they adapted the duration of the incubation in the enzymatic solution and reported that interrupting the enzymatic digestion every 30 min to retrieve the isolated follicles resulted in a greater number and better quality of isolated follicles compared with incubating the tissue in the enzymatic solution for 75 min. Moreover, this modification allows for the length of the isolation procedure to be adjusted based on the fibrous nature of the ovarian tissue (Chiti et al., 2017).

Liberase TM

Liberase TM (Thermolysin Medium) is a type of Liberase that contains collagenase I, collagenase II, and a medium concentration of Thermolysin (Schmidt et al., 2018b). Two studies by Kristensen et al. (2011) and Yin et al. (2016) used Liberase TM to isolate human preantral follicles from the ovarian medulla. While both groups used a mixture of 0.04 mg/ml Liberase TM and 0.2 mg/ml collagenase IV in warm McCoy's medium to isolate the follicles, Yin et al. added a 0.2 mg/ml DNase I to the solution. A comparison study by Lierman et al. shows that there was no significant difference between the number and type of follicles isolated with the low concentration of Liberase DH and Liberase TM combined with collagenase IV if they ignored the maturation status of the isolated follicles. However, Liberase DH and Liberase TM with collagenase IV work better than collagenase IV alone, while using collagenase IV is sufficient to isolate high-quality secondary follicles (Lierman et al., 2015).

Tumor Dissociation Enzyme (TDE)

Studies aimed at improving the isolation of human preantral follicle isolation have continued, with researchers such as Schmidt et al. (Schmidt et al., 2018a) and Chen et al. (2022) using a commercial enzyme cocktail called TDE to digest ovarian tissue and comparing it to Liberase TM and Liberase DH combined with DNase I, respectively. TDE is a highly purified enzyme that contains collagenase, protease, Dispase, and DNase, which aid the isolation of many follicles and prevent the digested tissue suspensions from becoming sticky (Schmidt et al., 2018a; Chen et al., 2022). According to these studies, TDE is an excellent alternative to different Liberases and is effective in reducing oxidative stress while maintaining follicle morphology. Additionally, the survival rate of follicles isolated with TDE can be higher than with Liberase and it decreases the risk of cancer cell reseeding (Schmidt et al., 2018a,b; Chen et al., 2022). Furthermore, the apoptosis rate does not increase with TDE (Schmidt et al., 2018a,b).

Discussion

Based on various studies, using a mechanical method to isolate secondary follicles in a two-step culture is sufficient, however, in the case of isolated follicle culture or transplantation, it is better to obtain both enzymatic and mechanical procedures (McLaughlin et al., 2018; Schmidt et al., 2018a,b).

Promising results have been achieved in the continued study of various enzymes for the isolation of human ovarian follicles. Among the first enzymes used for this purpose were those of the collagenase family (Figueiredo et al., 1993; Oktay et al., 1997; Martinez-Madrid et al., 2004). In addition, the Liberase family and TDE (Schmidt et al., 2018a,b) were employed for follicle isolation. Although, maintaining follicular structure is a crucial subject, obtaining healthy follicles with minimum damage to granulosa cells (GCs) and oocytes is very important (Dolmans et al., 2006). Therefore, choosing the best enzyme is essential. For example, the BM contains collagen IV (Heeren et al., 2015), and the enzyme collagenase IV can harm this barrier and reduce follicle quality (Kristensen et al., 2011). On the other hand, primordial follicles have a tighter contact with surrounding tissues but this connection will become looser over follicle development (Hovatta et al., 1999). It seems that complete separation

of primordial follicles during an isolation procedure may need a stronger enzyme, like collagenase IA; however, to recover the isolated follicles and select the viable ones, a Ficoll density gradient can be helpful (Kristensen et al., 2011). Most human studies used the Liberase family for preantral follicle isolation (Dolmans et al., 2008; Paulini et al., 2016).

In all studies, whether follicle isolation was for *in vitro* culture or for transplantation, assessing the viability of isolated follicles is crucial. To check this issue, Neutral Red is the best option to help isolate healthy viable follicles faster (Nemes et al., 1979). Nevertheless, using calcein AM and ethidium homodimer I can help classify isolated follicles based on their cell and oocyte damage by exhibiting a green and red fluorescent color (Paulini et al., 2016). Viability assessment of human follicles isolated by the Liberase enzyme blend (Dolmans et al., 2006) or Liberase DH shows these enzymes did not impair the isolated follicles (Paulini et al., 2016). Our study (unpublished data) also confirmed the viability of isolating human follicles by collagenase IA. Although, collagenase I is a common enzyme for follicle isolation, its effect has not been thoroughly investigated (Gosden, 1990; Telfer et al., 1990). It seems that partial follicle isolation by collagenase can lead to premature oocyte extrusion (Hovatta et al., 1999). Comparison between Liberases and collagenase confirms that although the number of fully isolated follicles is lower than with collagenase, it can protect the ultrastructure and morphology of the follicles. Because collagenase is a stronger enzyme and TEM analysis shows that it can harm follicle structures such as the follicular wall (Dolmans et al., 2006). Even so, there are different comparisons between various Liberase enzymes and it seems that Liberase TM is a better option for follicle isolation (Buckenmeyer et al., 2023). Also, TDE can be used instead of Liberase TM and Liberase DH; apoptosis and necrosis were not observed after follicle isolation (Schmidt et al., 2018a,b; Chen et al., 2022). However, there is speculation that the isolation process, regardless of the enzyme type, can impact cell-cell interactions within the follicle (Smith et al., 2014). Furthermore, comparison of some enzymatic protocols for follicular isolation shows that the maturation status of follicles plays a key role in response to enzymatic isolation (Lierman et al., 2015). As a result, there is a lack of comprehensive studies regarding the effects of common enzymes on isolated follicle structure and their consequences on further follicular *in vitro* culture or transplantation.

Conclusion

To conclude, in dense ovaries, such as human, Liberase TM is the best option for human ovarian follicular isolation. The Liberase family can maintain the follicular ultrastructure and reduce damage to GCs. On the other hand, TDE can be an alternative enzyme to decrease the apoptosis and necrosis of human ovarian follicles in enzymatic digestion.

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