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Vitrification versus slow freezing for human ovarian tissue cryopreservation: a systematic review and meta-analysis

Qingquan Shi^{1,2}, Yidong Xie^{1,2}, Yan Wang^{1,2} & Shangwei Li^{1,2}

Vitrification is a well-accepted procedure for cryopreservation of gametes and embryos. Less is known, however, about its performance in preserving ovarian tissue, for which slow freezing is the current convention. Increasing interest is being focused on vitrification, but there are as yet no standard protocols for its use with ovarian tissue. In part, this is because of the variety of cell types and complex nature of ovarian tissue. We performed a meta-analysis of 14 studies that compared vitrification with slow freezing for cryopreservation of ovarian tissue. In the pooled analysis, there was no significant difference between the two methods in terms of the proportion of intact primordial follicles, but vitrification was associated with significantly less DNA damage. Secondary endpoints included the number of stromal cells, significantly higher with vitrification, and primordial follicle density, which did not differ between the two methods. The present meta-analysis suggests that vitrification may be more effective than slow freezing, with less primordial follicular DNA strand breaks and better preservation of stromal cells. These advantages should lead to improved ovarian function after transplantation.

Thanks to improvements in early cancer diagnosis and therapy, survival rates for women with cancer have increased by more than 1.6% during the past 5 years^{1,2}. However, gonadotoxic treatment can induce great damage to ovarian reserve, frequently leading to premature ovarian failure, with loss of both steroidogenic and gametogenic functions. Additionally, the increasing number of women postponing childbearing for social or financial reasons^{3,4} will likely increase demands for fertility preservation⁵.

There are several methods used to preserve female fertility, including ovarian tissue cryopreservation (OTC) and cryopreservation of embryos and oocytes. At present, cryopreservation of embryos and oocytes is an accepted, clinically established procedure, whereas OTC has not been endorsed by the American Society of Reproductive Medicine and is still considered experimental⁶. Initially, there were only a few case reports. Successful human ovarian transplantation was first reported by Silber *et al.* with cortical-tissue grafting in monozygotic twins who were discordant for premature ovarian failure⁷. Subsequently, Donnez *et al.*⁸ reported what is deemed to be the first human live birth from orthotopic transplantation of frozen human ovarian tissue in 2004, with another successful live birth achieved by Meirou in 2005.⁹ However, it appears now that there is a worldwide live birth rate of over 30 to 70%, with more than 70 babies¹⁰. In the opinion of many pioneers, there is now enough evidence to support OTC and to stop considering it an experimental or investigational approach¹¹.

There are two methods for OTC: slow freezing and vitrification. Slow freezing has been the conventional technique for years, despite reports of extensive loss of the follicular pool and excessive damage to stromal cells¹². To date, only two live birth has been reported after vitrification of ovarian tissue; all other live births have resulted from slow-frozen ovarian cortex^{13,14}. However, interest in vitrification is increasingly a focus of investigation^{13,15,16}. Vitrification seems to be an emerging alternative to slow freezing, as it has been successfully applied for preservation of human blastocysts and oocytes, and good results have been reported for ovarian tissue from rodents, domestic animals, and non-human primates^{15,17,18}. Vitrification prevents the formation of ice crystals, reducing the risk of mechanical injury to cells. Compared with slow freezing, vitrification also has the advantages of being

¹Department of Reproductive Medical Center, West China Second University Hospital, Sichuan University, Chengdu, 610000, China. ²Key Laboratory of Birth Defects and Related Disease of Women and Children, West China Second University Hospital, Sichuan University, Chengdu, 610000, China. Qingquan Shi and Yidong Xie contributed equally to this work. Correspondence and requests for materials should be addressed to S.L. (email: lswivf@163.com)

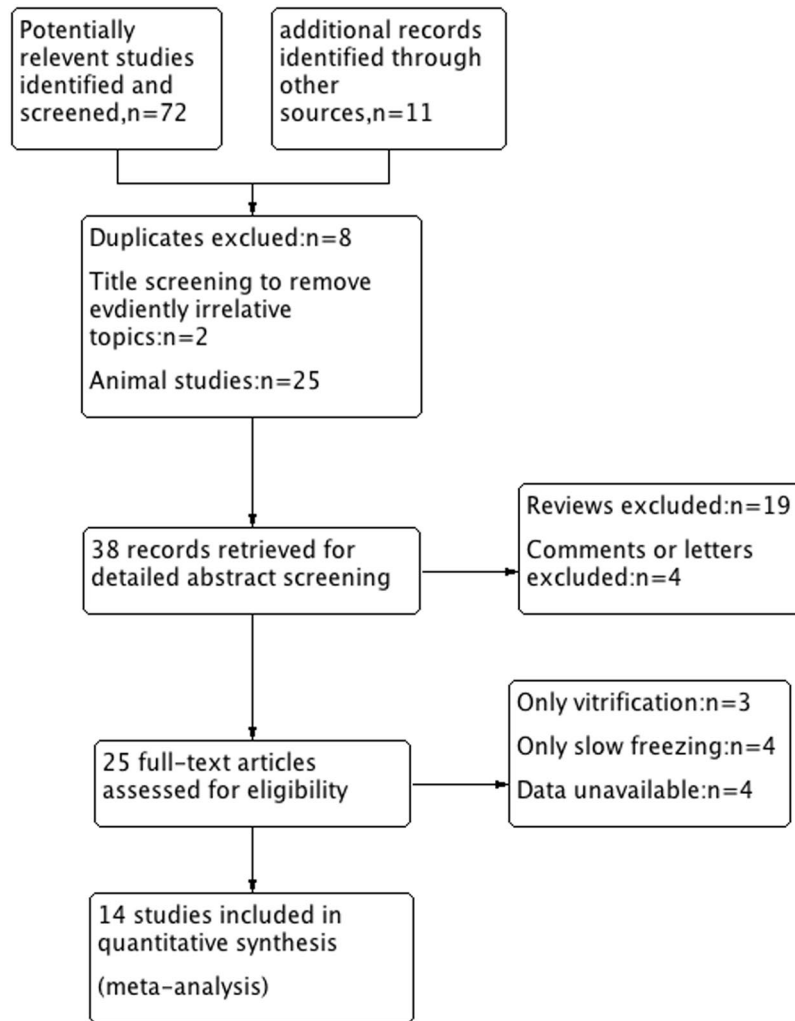


Figure 1. Flow diagram of the study selection process.

a time-saving, simple process, requiring no special or expensive equipment. It is also reported that vitrification preserves the morphologic integrity of stromal cells better than slow freezing^{19–22}. Compared with conventional slow-frozen tissues, Rahimi *et al.*²³ found a higher percentage of apoptotic cells in vitrified ovarian tissues. Also as the originators of ovarian tissue vitrification, Silber S. compared the viable oocytes in vitrified tissue and slow freezing tissue, suggesting that vitrification might provide better results after transplantation²⁴. However, Oktem *et al.* reported lower primordial follicle density and viability after vitrification²⁵. In contrast, other studies failed to show significant differences between the two methods in terms of the proportion of morphologically intact follicles and apoptotic cells^{26,27}. Because there is as yet no optimal protocol for vitrification, data on human ovarian tissue vitrification are still limited and conflicting. Thus, whether vitrification is superior to slow freezing for OTC remains under debate. The purpose of the present meta-analysis is to evaluate the efficacy of ovarian tissue vitrification and to seek to identify which is the better method for OTC.

Results

Study characteristics. The search identified 83 reports, of which 58 were excluded following removal of duplicates and review of titles and abstracts. A further 10 studies were excluded after reading the full text (Fig. 1). Data were extracted from the remaining 14 non-randomized comparative studies 19AQ1^{20,21,25–35} and analyzed. The number of study patients ranged from 3 to 26 with ages ranging from 14 to 43 years. Ovarian tissues were retrieved by laparoscopy or laparotomy performed for benign gynecologic conditions or at the time of cesarean section or cancer patients who have oophorectomy, ovarian cystectomy, and ovary transposition. The vitrification protocols vary from tissue carrier and cryoprotectant. The number of primordial follicles ranged from 176 to 1015, among which 134 to 611 were morphologically intact primordial. Additionally, total number of the evaluation of DNA fragmentation in primordial follicles ranged from 56 to 781, among which 22 to 245 were primordial follicle with DNA fragmentation. Overall, included studies that assessed the stromal cells ranged from 56 to 781, among which 22 to 245 were normal stromal cells. A summary of the main characteristics and outcomes of the identified studies is presented in Table 1.

| First author (publication year) | Patients | Age | Vitrification method | | | primordial follicle | | DNA fragmentation evaluation | | Evaluation of stromal cells | | Outcomes |
|---------------------------------|----------|------------------|---------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|-----------------|------------------------------|---------------------------------|-----------------------------|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| | | | ovarian tissue carrier | equilibration solution | vitrification solution | Total | Intact follicle | Total | follicle with DNA fragmentation | Total | normal stromal cells | |
| Fabrizi (2016) | 6 | Range:14–34 | open plastic home-made support | 2 M propylene glycol + 3 M EG + 0.2 M Sucrose for 30 mins | 3 M propylene glycol + 5 M EG + 0.5 M Sucrose + 15%HS 30 mins | 642 | 352 | NA | NA | NA | NA | estimate follicular density, Follicle intact morphology, intracellular ROS localization and levels states of ovarian follicles and stroma |
| Sanfulippo (2015) | 5 | Mean: 28 (1.1) | dropped directly into liquid nitrogen | step 1: 0.37 M PrOH + 0.37 M EG for 5 mins step 2: 0.75 M PrOH + 0.75 M EG for 5 mins | 1.5 M PrOH + 1.5 M EG + 0.5 M raffinose for 10 mins | 856 | 685 | 93 | 22 | NA | NA | estimate follicular density Follicle intact morphology DNA fragmentation in the follicles by TUNEL |
| Klocke (2015) | 23 | Mean: 29.9 (5.0) | acupuncture needle | 7.5% EG + 7.5%DMSO + 20%HSA for 15 mins | 15% EG + 15% DMSO + 0.5 M sucrose for 2 mins | NA | NA | NA | NA | NA | NA | the proportion of high-quality follicles Evaluation of the integrity of the follicles after tissue culture Estradiol production in the ovarian culture system |
| Herraiz (2014) | 8 | Mean: 27 | metallic grids | 7.5% EG + 7.5% DMSO for 25 mins | 20% EG + 20% DMSO + 0.5 M sucrose + 20%synthetic serum substitute for 15 mins | 1015 | 526 | 510 | 182 | NA | NA | follicular densities Morphometric Study Cell Proliferation and Vascularization DNA Strand Breaks by TUNEL fibrotic surface area |
| Xiao (2013) | 3 | Range:20–36 | acupuncture needle | 7.5% EG + 7.5% DMSO for 10 mins | 13.5% EG + 13.5% DMSO + 0.5 M sucrose for 2 mins | 271 | 180 | 294 | 115 | NA | NA | abnormal primordial follicles apoptotic primordial follicles |
| Amorim (2012) | 7 | Range: 30–41 | Protocol 1: Solid-surface protocol 2: open cryostraws | protocol 1: Step1, 5% DMSO + 5% EG 5 mins step2, 10% DMSO + 10% EG 5 mins protocol 2: Step1,2.5% DMSO + 6.5% EG 5 mins step2, 5% DMSO + 13% EG 5 mins | protocol 1: 20% DMSO and 20% ethylene glycol 10 mins protocol 2: 10% DMSO and 26% ethylene glycol 1 min | 287 | 172 | 56 | 14 | NA | NA | Morphologically normal follicles Follicular proliferation and apoptosis |
| Oktem (2011) | 15 | Range: 18–37 | cryovials | 15% propanediol + 15% EG + 0.2 M sucrose for 10 mins | NR | NA | NA | NA | NA | NA | NA | Follicle density AMH and estradiol levels in culture fluid |
| Chang (2011) | 11 | Mean: 31.9 | cryovial | Group1, 20% EG for 5 mins; group2, 20% EG for 10 min; group3: 20% EG for 20 mins | 40% EG, 18% Ficoll, and 0.3 M sucrose 5 mins | 268 | 161 | 270 | 127 | 600 | 319 | morphologically intact follicles Detection of apoptotic follicle by TUNEL |
| Xiao (2010) | 10 | Range: 21–36 | acupuncture needle | 7.5%EG + 7.5%DMSO for 10 mins | Group3: 2.69 M EG (15%) + 2.11 M DMSO (15%) + 0.5 M sucrose 2 mins Group3: 2.42 M EG (13.5%) + 1.90 M DMSO (13.5%) + 0.5 M sucrose 2 mins Group C: 2.15 M EG (12%) + 1.69 M DMSO (12%) + 0.5 M sucrose 2 mins | 760 | 611 | 781 | 245 | 800 | 376 | assess morphologic damage of the primordial follicles Oocytes, granulosa cells of primordial follicles, and stromal cells were analyzed using TEMTUNEL Assay for primordial follicle and stromal cells Detection of Apoptosis |
| Keros (2009) | 20 | Range: 20–41 | Hand-cut cryostraws | Group 1: Step 1: 0.35 M DMSO + 0.38 M PrOH + 0.38 M Eg for 5 mins step 2: 0.7 M DMSO + 0.75 M PrOH + 0.75 M EG for 5 mins Group 2: Step 1 0.35 M DMSO + 0.38 M PrOH + 0.38 M EG for 10 mins step 2: 0.7 M DMSO + 0.75 M PrOH + 0.75 M EG for 10 mins | Group 1: 1.4 M DMSO + 1.5 M PrOH + 1.5 M EG + polyvinylpyrrolidone for 5 mins Group 2: 1.4 M DMSO, 1.5 M PrOH, 1.5 M EG + polyvinyl-pyrrolidone for 10 mins | 279 | 144 | NA | NA | 26763 | 13513 | The number of follicles of different developmental stages was evaluated. intact the follicles proportion of intact stromal cells assessing the structures of oocytes, granulosa cells and the stroma |
| Wang (2008) | 5 | Range: 21–37 | acupuncture needle | 7.5% EG + 7.5% DMSO for 10 mins | 15% EG + 15% DMSO + 0.5 M sucrose 2 mins | 272 | 225 | NA | NA | 400 | 169 | follicle morphology; The oocytes, the granulosa cells of the primordial follicles and the stromal cells surrounding follicles were evaluated separately; Viability assay |
| | | | Pasteur pipette. | 10% EG + 10% DMSO for 5 mins | 20% EG + 20% DMSO 5 mins | | | | | | | |
| Huang (2008) | 26 | Mean: 29.9 (3.4) | Solid-surface vitrification | Step 1: 5% DMSO + 5% EG for 5 mins Step 2: 10% DMSO + 10% EG for 5 mins | Step 1: 15%(DMSO) + 15% (EG)10 mins Step 2: 20%(DMSO) + 20% (EG)10 mins | 623 | 528 | NA | NA | NA | NA | The percentage of morphologically normal primordial follicles; Assessment of apoptosis in primordial follicles and stromal cells; Production of hormones by warmed/thawed ovarian tissue |

Continued

| First author (publication year) | Patients | Age | Vitrification method | | | primordial follicle | | DNA fragmentation evaluation | | Evaluation of stromal cells | | Outcomes |
|---------------------------------|----------|--------------|---------------------------------------------|-------------------------------------|------------------------------------------------------------------|---------------------|-----------------|------------------------------|---------------------------------|-----------------------------|----------------------|---------------------------------------------------------------------------------------|
| | | | ovarian tissue carrier | equilibration solution | vitrification solution | Total | Intact follicle | Total | follicle with DNA fragmentation | Total | normal stromal cells | |
| Li (2007) | 15 | Mean: 33.1 | Minimum drop size directly plunged into LN2 | 2 M DMSO + 0.1 M sucrose for 5 mins | 2 M DMSO + 2 M (PROH) + 0.2 M sucrose 5 mins | 176 | 134 | NA | NA | NA | NA | Proportions of normal primordial follicle; concentration of estrogen and progesterone |
| Gandolfi (2006) | 3 | Range: 26–33 | Straws directly plunged into LN2 | 0.64 M EG + 20% FBS for 30 mins | 5.64 M EG + 5% polyvinylpyrrolidone + 0.4 M trehalose for 2 mins | 550 | 277 | NA | NA | NA | NA | Normality of follicular structures |

Table 1. Basic characteristics and outcomes of studies included. EG = ethylene glycol, DMSO = dimethyl sulphoxide, PrOH = 1,2-propanediol, NA = not applicable, NR = not reported, M = mol/L *Data were presented as range and/or mean (standard deviation).

Quality assessment. The average Newcastle–Ottawa Quality Assessment Scale score of included studies was 7.07 (range 6–8), which suggested that the majority of eligible studies were of relatively high quality (Table 2). All the articles had clear descriptions of the intervention and measured the outcomes with objective and/or subjective methods. Overall, the results indicated that the studies were of good quality.

Proportion of intact primordial follicles. Intact primordial follicles were measured in 12 studies, of which two^{28, 33} indicated that vitrification was associated with a significantly higher proportion of intact primordial follicles compared with slow freezing. The remaining 10 studies did not observe any differences in the number of intact primordial follicles between the two methods. There was significant evidence of heterogeneity across the 12 studies ($I^2 = 69\%$), therefore, a random effect model was used for the pooled estimates. The pooled OR showed no significant difference in the proportion of intact primordial follicles after slow freezing or vitrification (OR = 0.98; 95% CI, 0.74–1.28; $P = 0.86$; Fig. 2)

DNA fragmentation in primordial follicles. Six studies reported DNA strand breaks in primordial follicles. Two studies by Xiao^{20, 33} showed that vitrification was associated with significantly less DNA fragmentation in primordial follicles compared with slow freezing. However, the remaining four trials did not show any differences in terms of follicular DNA damage. Overall, the pooled analysis of these studies revealed significantly less DNA damage in primordial follicles with vitrification than with slow freezing (RR = 0.71; 95% CI, 0.62–0.80; $P < 0.00001$; Fig. 3).

Proportion of normal stromal cells. Six studies evaluated stromal cells, but adequate data were available in only 4^{19–21, 35}. When ovarian tissues were pretreated with equilibration solution for 10 minutes before vitrification, pooled analysis showed that vitrification protected more stromal cells than did slow freezing. However, if ovarian tissues were pretreated for 5 minutes, there was no significant difference in the proportion of normal stromal cells between the two methods. Overall, the pooled subgroup analysis of these studies revealed significantly more normal stromal cells after vitrification than after slow freezing (RR = 1.69; 95% CI, 1.47–1.94; $P < 0.00001$; Fig. 4).

Primordial follicle density. Only three studies reported data on primordial follicle density^{25, 29, 35}. Herraiz indicated the vitrification was associated with significantly greater primordial follicle density than slow freezing³². However, the other two studies did not find any difference^{25, 29}. The pooled analysis revealed no significant differences in primordial follicle density between vitrification and slow freezing (IV = 3.44; 95% CI, –5.09–11.98; $P = 0.43$; Fig. 5).

Discussion

Due its excellent outcomes, vitrification has replaced conventional slow freezing as the primary approach to cryopreservation of gametes and embryos^{17, 21, 36}. However, since the ovary is an organ composed of diverse cell types, OTC is more complicated. Additionally, the outcomes of cryopreservation depend on multiple factors including the cryoprotectants, the size of ovarian fragments, and the speed of cooling^{15, 37}. OTC requires balancing of effects on the oocytes, granular cells, and stroma. Whether vitrification is a better procedure for preserving ovarian fragments is still controversial, so vitrification and slow freezing have been used recently to investigate its effects on stromal cells, as well as the morphologic integrity of primordial follicles. Early studies indicated that the slow-frozen ovarian cortex was better preserved than vitrified ovarian tissue²⁸. However, vitrification has more recently been suggested to have positive effects on granulosa cells and ovarian stroma, providing equivalent or better results than slow cooling for preserving ovarian tissue^{19, 21, 27, 32, 33, 38}. The purpose of this meta-analysis of 14 studies was to evaluate the efficacy of ovarian tissue vitrification compared with slow freezing, including evaluation of stromal cells as a secondary outcome, and to identify which method is better for OTC. And we find vitrification may be more effective than slow freezing, with less primordial follicular DNA strand breaks and better preservation of stromal cells.

In the frozen ovarian cortex, the appearance and quality of follicles may in part predict the possibility of restoring reproductive function. Primordial follicles are the primary type in cryopreserved human ovarian fragments, accounting for more than 90% of all follicles. Thus, when it comes to assessing the efficacy of cryopreservation,

| | Fabbri (2016) | Sanfulippo (2015) | Klocke (2015) | Herraiz (2014) | Xiao (2013) | Amorim (2012) | Oktem (2011) | Chang (2011) | Xiao (2010) | Keros (2009) | Wang (2008) | Huang (2008) | Li (2007) | Gandolfi (2006) |
|-----------------------------------------------------------------------------------------|---------------|-------------------|---------------|----------------|-------------|---------------|--------------|--------------|-------------|--------------|-------------|--------------|-----------|-----------------|
| Were the exposed cohorts somewhat representative in the community? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Were the non exposed cohorts drawn from the same community as the exposed cohorts? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Did the ascertainment of exposure have a secure record? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Demonstration that outcome of interest was not present at start of study? | yes | yes | yes | yes | yes | yes | NA | yes | yes | yes | yes | yes | NA | yes |
| Was the intervention clearly described in the study? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Were additional interventions (co-interventions) clearly reported in the study? | yes | NA | no | no | yes | no | NA | yes | yes | yes | yes | yes | NA | yes |
| Were relevant outcomes appropriately measured with objective and/or subjective methods? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Was follow-up long enough for outcomes to occur? | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Was the loss to follow-up reported? | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| NOS score | 8 | 7 | 7 | 7 | 8 | 7 | 6 | 7 | 7 | 7 | 8 | 7 | 6 | 7 |

Table 2. The detail NOS score of studies included. NA = not applicable

a common endpoint is morphologic intactness of primordial follicles. Such investigations were performed by Gandolfi *et al.* on human, bovine, and porcine ovarian tissues. They concluded that conventional freezing results in much better preservation of all follicle types than does vitrification²⁸. Conversely, Xiao *et al.* reported that a novel vitrification technique was comparable to slow freezing with respect to preserving primordial follicles in human ovarian tissue. The percentage of morphologically abnormal primordial follicles was significantly lower with vitrification than with slow freezing³³. But Oktem *et al.* found that vitrified human ovaries have fewer primordial follicles and produce less anti-Müllerian hormone than slow-frozen ovaries²⁵. In this meta-analysis, 12 studies recorded data on intact primordial follicles, and the overall pooled analysis showed no difference between vitrification and slow freezing for this endpoint. Additionally, the primordial follicle density of ovarian tissue was evaluated in three studies, and no significant difference between the two methods was found.

Although follicles may be morphologically intact after cryopreservation, their ability to develop and produce an oocyte to be fertilized, with the ultimate achievement of pregnancy and live birth, may be affected by cryopreservation, regardless of the freezing method. Apoptosis has been suggested as a marker of the developmental capacity of primordial follicles after cryopreservation³⁹. Rimon *et al.* observed that apoptosis was already present in follicles that appeared normal when examined only morphologically³⁹. They concluded that apoptosis might be a more definitive indicator of the viability of follicles than the general morphologic appearance. In the investigation by Xiao *et al.*, the incidence of TUNEL-positive primordial follicles was lower after vitrification than after slow freezing³³. Overall, analysis of pooled data in the present review demonstrated that vitrification yielded a lower proportion of DNA fragmentation in primordial follicles.

Vitrification is an ultra-rapid cooling technique with no ice crystal formation. Conversely, the faster cooling rate means a higher concentration of cryoprotectant, which may be toxic to living cells. Thus, a variety of cryoprotectants have been used in combination to reduce the toxicity of individual agents while still achieving a highly viscous solution^{15, 19–27}. To date, however, there is still no optimal vitrification protocol. In the studies we reviewed, vitrification was routinely achieved by initial exposure of ovarian tissues to concentrations of permeating cryoprotectants (commonly combinations of ethylene glycol, Dimethyl Sulphoxide, propylene glycol and propyl alcohol) similar to those employed prior to conventional slow cooling. The duration of exposure to the high concentrations before loading onto a carrier differed among the studies.

Since the ovary is an organ composed of heterogeneous cellular components, diffusion rates of the cryoprotectants and the potential for ice crystal formation vary for each cell and tissue type⁴⁰. Thus, OTC involves a compromise between effects on the oocytes, follicular cells, and stroma, the latter comprising blood vessels, nerves,

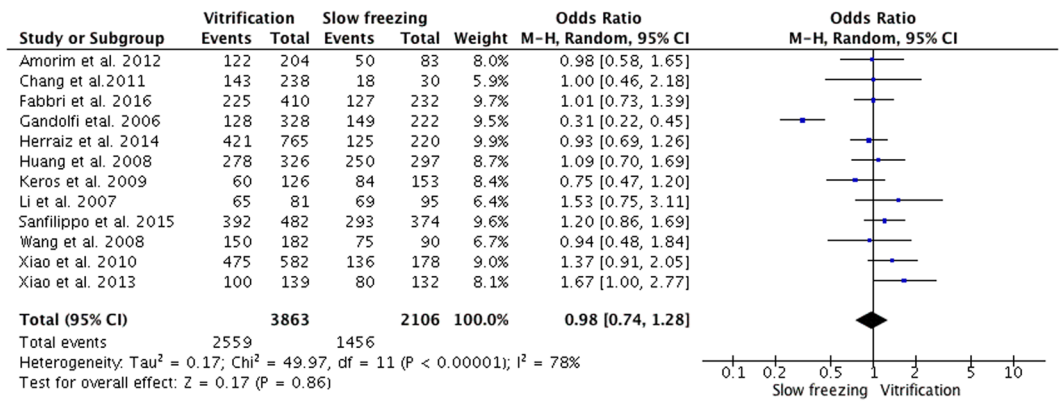


Figure 2. Random effect model of odds ratio with 95% CIs of the proportion of intact primordial follicles: slow freezing versus vitrification.

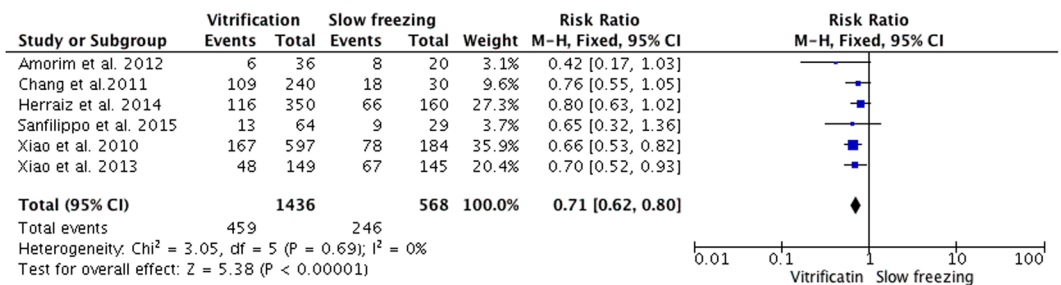


Figure 3. Fixed model of risk ratio with 95% CIs of the DNA fragmentation in primordial follicles: vitrification versus slow freezing.

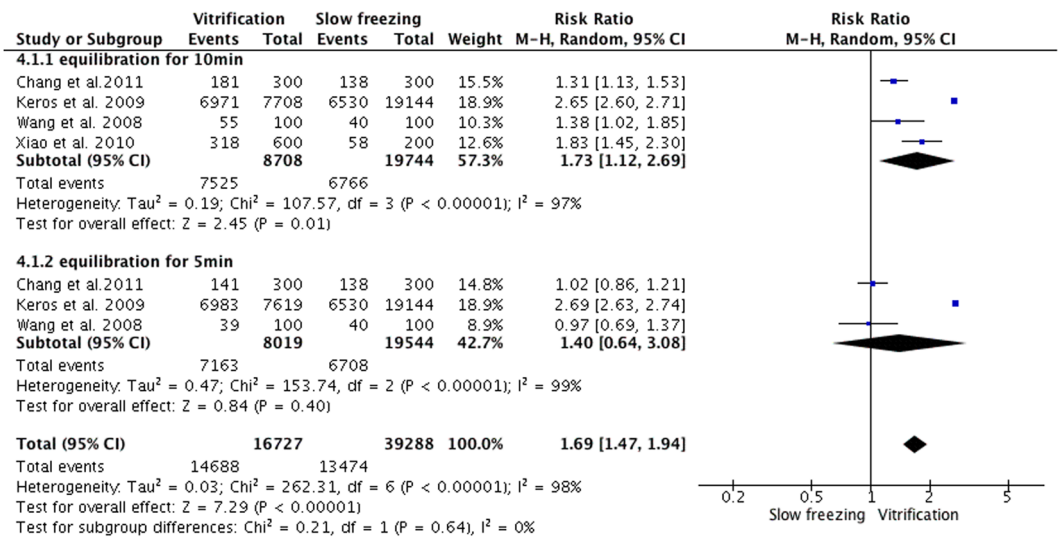


Figure 4. Random effect model of risk ratio with 95% CIs of the proportion of normal stromal cells: slow freezing versus vitrification.

and extracellular matrix. The stromal cells can transform into the theca interna and externa outside the follicular basal lamina, which is believed to positively affect granulosa cell proliferation and differentiation¹⁹. So, to evaluate the effect of cryopreservation, investigations of stromal cells are as essential as those of the follicles. However, we found only four studies that evaluated stromal cells. These explored the best concentrations of cryoprotectant and exposure times in the vitrification protocol. In those four studies, the protocol was a two-step process including equilibration and vitrification. We therefore performed a subgroup analysis of the effect on stromal cells

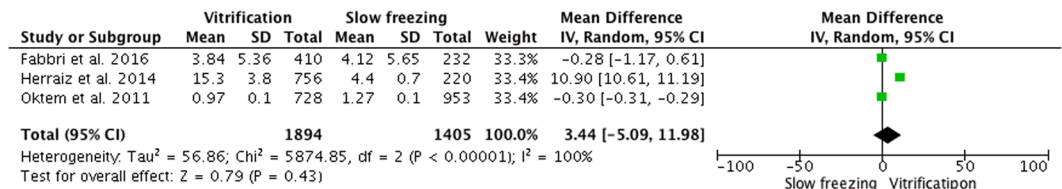


Figure 5. Random effect model of the mean difference with 95% CIs of the primordial follicle density: slow freezing versus vitrification.

according to equilibration time. Overall, the pooled subgroup analysis of these studies revealed that vitrification resulted in significantly more normal stromal cells than did slow freezing.

There are limitations of this analysis that need to be considered. The studies included varied terms of in the vitrification procedure and cryoprotectants used. We were unable to examine the effect of different vitrification protocols because of insufficient data. In addition, the outcome was only based on morphologic examination and cell vitality, including DNA strand breaks. We did not have data on the endocrine function of the ovarian fragments. Lastly, we assessed only the primordial follicles, not growing follicles. Since a larger number of primordial follicles does not necessarily mean better results in terms of pregnancy, the potential of these primordial follicles to develop into primary or secondary follicles, mature, and produce oocytes capable of fertilization should also be evaluated. In the future, a different approach to OTC might involve removal of small antral follicles, followed by *in vitro* maturation. Immature oocytes can also be collected from antral follicles in ovarian tissue at the time of cryopreservation, matured *in vitro*, and then cryopreserved to await fertilization⁴¹.

Conclusion

Based on the data available, the present meta-analysis suggests that vitrification may be more effective than slow freezing for OTC, resulting in with fewer primordial follicular DNA strand breaks and better preserved stromal cells, which should lead to improved tissue function after transplantation. However, the included studies varied in terms of the vitrification protocol used. These findings must now be validated in prospective randomized studies, with healthy live births as the primary endpoint.

Methods

Design and Search Strategy. This systematic review and meta-analysis was conducted in accordance with PRISMA guidelines⁴². A comprehensive and systematic literature search was performed independently by Q.S. and Y.X. Online electronic databases, including PubMed, EMBASE, and the Cochrane database, were searched for studies published from their inception until December 2016. To identify any potentially relevant studies, the following MeSH terms were used: (((ovarian cortex) OR ovarian tissue) OR ovarian strips) OR whole ovary) AND (((vitrification) OR ultra-rapid freezing) OR rapid cooling) OR rapid freezing) OR ultra-rapid cooling) AND (((slow cooling) OR slow freeze) OR conventional slow freezing) OR slow-frozen) OR slow cryopreservation)). The references of all identified studies were also searched. Two investigators evaluated all identified trials and separately assessed the methodologic quality of the studies. Any discrepancies were resolved by mutual discussion.

Inclusion and Exclusion Criteria. The following criteria were used for study selection: 1) studies were randomized controlled trials/cohort/observational studies; 2) comparison of human ovarian tissue which were preserved by vitrification and conventional slow freezing; 3) outcomes included at least one of the following endpoints: morphologically intact primordial follicles/DNA fragmentation in primordial follicles/stromal cell/primordial follicle density.

Exclusion criteria were as follows: 1) letters, comments, editorials, case reports, and reviews; 2) studies with no comparison of vitrification with slow freezing; 3) no quantitative primary or second outcome data reported; 4) animal studies.

Outcome Measures. The primary outcome measure was the proportion of morphologically intact tissue and DNA fragmentation in primordial follicles after cryopreservation. The secondary outcomes were the proportion of normal stromal cells and primordial follicle density (follicles/mm²).

Study Quality Assessment. For RCTs we will use the risk bias assessment According to the criteria of the Cochrane Handbook for Systematic Reviews of Interventions, for observational/cohort/case-control studies, the Newcastle–Ottawa Quality Assessment Scale, a rating tool that evaluates the quality of non-randomized studies from three perspectives (selection, comparability and outcome), was used to assess the validity⁴³. This rating system's score ranges from 0 to 9, and studies with a score of more than 7 were assumed to be of high quality.

Data Extraction and Statistical Analysis. Two reviewers extracted the relevant data independently, and these data were then cross-checked. The following information was extracted from each study: study design, year of publication, population characteristics, and relevant outcome data. Any disagreements that could not be reconciled by discussion were considered by a third person (SW.L.). This meta-analysis was performed in accordance with the recommendations of the Cochrane Collaboration⁴³. Statistical analyses were conducted using Review Manager 5.1.0. The Mantel-Haenszel χ^2 test and I^2 test were used to assess statistical heterogeneity. When the I^2

value was less than 50%, heterogeneity was considered to be acceptable⁴³. A fixed effect model was used for calculations in the absence of evidence of heterogeneity; otherwise, a random effect model was applied. Risk ratios (RR) were used to evaluate dichotomous variables, while mean differences were used to evaluate continuous variables; both were accompanied by 95% confidence intervals (CI). For these trials, a P value < 0.05 was considered to be statistically significant.

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Author Contributions

L.S. conceived of the study and the critical revision of the article. S.Q. and X.Y. searched and selected the studies, analyzed the data, prepared figures and drafted the article. W.Y. participated in the acquisition of data and statistical analysis. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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