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Comparison of survival and apoptosis parameters of ovarian tissue follicles in two vitrification methods of needle-immersion and cryo-support in cancer patients

Kanser hastalarında needle-immersion ve cryo-support olmak üzere iki vitrifikasyon yönteminde over dokusu foliküllerinin sağkalım ve apoptoz parametrelerinin karşılaştırılması

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Abstract

Objective: Ovarian tissue (OT) cryopreservation is a useful technique for preserving fertility potential in women with cancer. Vitrification is a relatively new method. Several devices were discussed. Among these methods, cryo-support and needle-immersion vitrification (NIV) stand out as particularly popular. The aim of this study is the comparison of these devices in terms of follicle quality and DNA status.

Materials and Methods: The OT from 20 cancer patients was transferred with Dulbecco's Phosphate-Buffered Saline supplemented with 5% serum and maintained at 4 °C for 1 hour. After preparation of OT vitrified by two freezing solutions with different concentrations of cryoprotectant, small fragments of OT (~5×1×1 mm) were attached to an insulin needle, and in another group, the tissue fragments (~5×5×1 mm) were loaded onto the Ova Cryo Device Type M, also called cryo-support, and placed in liquid nitrogen. After warming, small tissue pieces were prepared to assess the quality of primordial, primary, and secondary follicles using eosin-hematoxylin staining. An immune-histochemical investigation, including anti-p53 and anti-Caspase 3, was conducted to examine the apoptosis pathway.

Results: The data showed a larger number of high-quality follicles in the cryo-support group ($p<0.05$). Also, the level of apoptosis (p53) molecules is higher in the NIV method ($p<0.05$). However, the levels of caspase 3 were not significantly different between the NIV approach and the cryo-support vitrification group.

Conclusion: A recent study revealed that the cryo-support device is more effective in increasing high-quality follicles and reducing p53, which is associated with early stages of apoptosis. This device may improve clinical outcomes and may be recommended for cryopreservation programs.

Keywords: Ovarian follicle, fertility preservation, vitrification, histological techniques, apoptosis

PRECIS: The study shows cryo-support vitrification better preserves high-quality ovarian follicles and reduces early apoptosis (p53 expression) compared to conventional method, needle-immersion, suggesting improved outcomes for fertility preservation in cancer patients.

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Öz

Amaç: Over dokusu (OD) kriyoprezervasyonu, kanserli kadınlarda doğurganlık potansiyelini korumak için yararlı bir tekniktir. Vitrifikasyon, son zamanlarda kullanıma sunulan nispeten yeni bir yöntemdir. Cryo-support ve needle-immersion vitrifikasyon (NIV) yöntemlerinin özellikle popüler olduğu çeşitli cihazlar tartışılmıştır. Bu çalışmanın amacı, bu cihazların folikül kalitesi ve DNA durumu açısından karşılaştırılmasıdır.

Gereç ve Yöntemler: Yirmi kanser hastasından alınan OD, %5 serum eklenmiş Dulbecco Fosfat Tamponlu Salin ile transfer edildi ve 1 saat boyunca 4 °C'de tutuldu. Farklı kriyoprotektan konsantrasyonları içeren iki dondurma solüsyonuyla vitrifiye edilen OD'nin hazırlanmasının ardından, küçük OD parçaları (~5×1×1 mm) bir insülin iğnesine tutturuldu ve diğer bir grupta, doku parçaları (~5×5×1 mm), cryo-support olarak da adlandırılan Ova Cryo Device Type M'ye yüklenerek sıvı nitrojene yerleştirildi. Isıtma işleminden sonra, eozin-hematoksilen boyama kullanılarak primordial, primer ve sekonder foliküllerin kalitesini değerlendirmek için küçük doku parçaları hazırlandı. Apoptoz yolunu incelemek için anti-P53 ve anti-Kaspaz 3'ü içeren bir immünohistokimyasal inceleme gerçekleştirildi.

Bulgular: Veriler, cryo-support grubunda daha fazla sayıda yüksek kaliteli folikül olduğunu gösterdi (p<0,05). Ayrıca, apoptoz (p53) moleküllerinin sayısı NIV yönteminde daha yüksekti (p<0,05). Ancak, kaspaz 3, NIV yaklaşımı ile cryo-support vitrifikasyon grubu arasında anlamlı bir fark göstermedi.

Sonuç: Yakın zamanda yapılan bir çalışma, cryo-support cihazının yüksek kaliteli follikülleri artırmada ve apoptozun erken evreleriyle ilişkili olan p53'ü azaltmada daha etkili olduğunu ortaya koymuştur. Bu cihazın kullanımı klinik sonuçları iyileştirebilir ve kriyoprezervasyon programları için önerilebilir.

Anahtar Kelimeler: Yumurtalık folikülü, doğurganlığın korunması, vitrifikasyon, histolojik teknikler, apoptoz

Introduction

Ovarian tissue cryopreservation (OTC) is a highly effective and promising approach for preserving a considerable number of primordial and primary follicles when the risk of premature ovarian failure is high (>30-50%)[1]. While treatments like chemotherapy, radiation, and bone marrow transplants lead to full recovery for 90% of children with cancer, there's a risk of ovarian failure due to these treatments[2]. In adolescent cancer cases, it is feasible to remove and freeze the ovaries before the depletion of ovarian follicles occurs[3,4]. Immature oocytes possess unique traits that make them ideal for freezing, including their small size (<20 µm), minimal granulosa cells, and inactive metabolism[5,6].

A meta-analysis conducted by Pacheco and Oktay[7] found that using cryopreserved ovarian tissues (OT) for autologous transplants, resulted in a live birth rate of 37.7%, demonstrating a satisfactory level of success in clinical settings. Given the positive outcomes from OTC and ovarian tissue transplant (OTT), the American Society for Reproductive Medicine Committee advocates for this approach as standard practice for young cancer patients aiming to preserve their fertility[8]. Despite these successes, there's a need to enhance follicle viability post-cryopreservation, increase the live birth rate, and extend the lifespan of ovarian auto-grafts, which currently last an average of 27 months per transplant[9].

Several studies have compared vitrification with slow freezing[10,11]. Abir et al.[12], 2017 examined the combination of slow freezing and needle-immersion in terms of proliferation, apoptosis, and follicles in OT, and deemed the slow method a superior technique. In addition, Sugishita et al.[13] investigated cryopreservation by slow freezing. Their results showed that both methods were remarkably similar. According to a recent meta-analysis, vitrification may be more successful than slow freezing in terms of primordial follicular DNA strand breakage and stromal cell preservation[14]. Vitrification is a rapid freezing technique that involves subjecting samples to high concentrations of cryoprotectant solutions and freezing at extremely low temperatures (-130 °C). This method prevents ice crystal formation, which could damage cells during freezing. Several vitrification techniques are available, such as the cryo-support technique, identified by cryo-support (Kitazato Ova Cryo Device Type M, Japan) and the needle-immersion vitrification (NIV) method[15]. The cryo-support device consists of four fine stainless needles and is designed for the cryopreservation of OT. A recent report found no differences in intact primordial follicle survival, DNA damage, and apoptosis rates between cryopreservation support and slow-freezing methods[13].

The purpose of this study was to examine two NIV cryo-support devices in terms of the number of follicles, survival, apoptosis, and DNA breakage. To the best of our knowledge, the above devices have not been examined in detail yet.

Materials and Methods

Samples

A total of 20 cancer patients under the age of 38 years were referred to the Department of Gynecology, Shahid Sadoughi Hospital, Yazd, Iran, and enrolled in this study. The female patients were diagnosed with moderate forms of cervical and uterine malignancies, and were seeking fertility preservation. An ovarian biopsy or OT resection was performed during therapeutic surgery by laparotomy or laparoscopy. These patients underwent OT removal, and the fine ovarian morphology showed no signs of metastasis. Following the completion of the surgical procedure, a portion of the OT was collected and transported to the fertility preservation laboratory with Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 5% human serum albumin maintained at 4 °C for 1 hour. The OT underwent three washes in the DPBS medium to ensure the absence of blood. Subsequently, the medulla tissue was excised, and the ovarian cortex was cut into small pieces for freezing. OT were randomly placed in one of the cryo-support or NIV groups (n=10 for each group). This research protocol was reviewed and approved by the Ethics Committee of the Yazd Reproductive Sciences Institute (approval number: IR.SSU.RSI.REC.1401.008 date: 04.09.2022).

Vitrification Procedure

The Kagawa et al.[16] method was used for vitrification/warming with some modification in tissue loading. Two freezing solutions were used in this method. First, the cortical pieces were submerged in a balancing medium containing Tissue Culture Medium 199 (TCM199) (Life Technologies, CA, USA) supplemented with 20% serum albumin [handling medium (HM)], 7.5% ethylene glycol (EG), and 7.5% dimethyl sulfide (DMSO) for 25 min. The second stage involved freezing the tissue in the vitrification solution (VS), which contains HM with 20% EG, 20% DMSO, and 0.5 M sucrose. Tissue pieces were immersed in VS medium for 15 min. Both stages were done on the shaker at 4 °C. The tissue pieces were separated into two groups.

Needle-immersion Vitrification Method

After freezing, small fragments of OT (~5×1×1 mm, in length, width, and thickness, respectively) were attached to an insulin needle and placed in liquid nitrogen (LN2) with a minimum freezing protection medium[9]. The frozen fragments were transferred into 2.0 mL cryogenic vials (BD Bioscience, San Jose, CA, USA) and stored in the nitrogen tank (Figure 1).

Cryo-support Device

The tissue fragments (~5×5×1 mm) were loaded onto the cryo-support device (Ova Cryo Device TypeM®; Kitazato BioPharma Co., Shizuoka, Japan). Then, they were placed into a cryogenic vial, inserted into the LN2, and subsequently stored in a nitrogen tank (Figure 2).

Thawing of Ovarian Tissue

First, the cryo-vials were removed from the LN2 and placed at room temperature for 1 min. The tissues were transferred into the first medium, containing 40 mL of HM supplemented with 1.0 mol/L sucrose, for 1 min. Then, they were placed in the second medium containing 15 mL HM with 0.5 M sucrose for 5 min and washed three times in the HM for 10 min. After thawing/warming, the ovarian cortical pieces were cut into 1×1 mm pieces for further evaluation to ensure the equal size of tissues in both groups. Small tissue pieces were placed in 100 µL of culture media containing TCM199 (Life Technologies, CA, USA) supplemented with 10% HAS, 100 IU penicillin/mL, and 100 µg streptomycin/mL (Life Technologies). Tissue pieces were incubated in a humidified atmosphere with 5% CO_2 at 37.0 °C for 4 hours. This period enabled the activation of pathways related to DNA damage and programmed cell death. After culture, the studied groups were kept in 10% formalin for 48 hours. These samples were used for hematoxylin-eosin (H&E) staining and immunohistochemistry.

Histological Studies

H&E staining was performed to assess the condition and density of primordial (oocytes surrounded by less differentiated squamous granulosa cells), primary (oocytes surrounded by a single layer of cuboidal granulosa cells), and secondary follicles, which consist of more granulosa cells and contain small accumulations of fluid in the intracellular spaces called follicular fluid. The OT was fixed in 10% buffered formalin (Sigma-Aldrich, Germany) for 2 days. The tissues were then subjected to tissue processing, which included dehydration in increasing concentrations of ethanol and paraffin (Sigma-Aldrich, Germany), followed by embedding. The ovarian cortex was processed by slicing it into 5 µm thick sections. Each slice was stained with H&E (Vetec, São Paulo, Brazil) for light microscopic examination (Olympus, Tokyo, Japan) at 400x magnification. The quality of the follicles was determined by their morphology, which included the nucleus, cytoplasm, and granulosa cells. The follicles were classified into two categories: good quality follicles, with an intact oocyte, well-organized granulosa cells, and no condensed nuclear chromatin; otherwise, they were considered bad quality morphology[17].

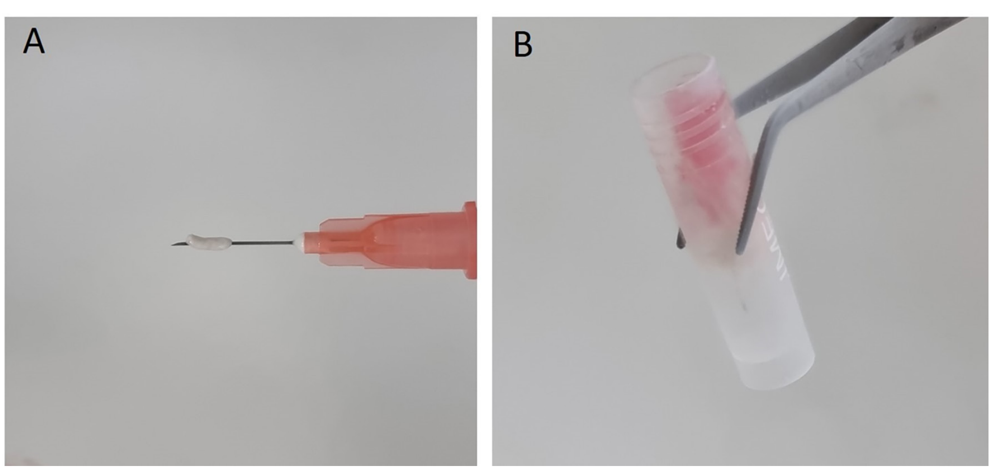

Figure 1. Needle-immersion vitrification method. A: A small fragment of OT (~5×1×1 mm) attached to an insulin needle, and B: It was transferred into 2.0 mL cryogenic vials and stored in the nitrogen tank

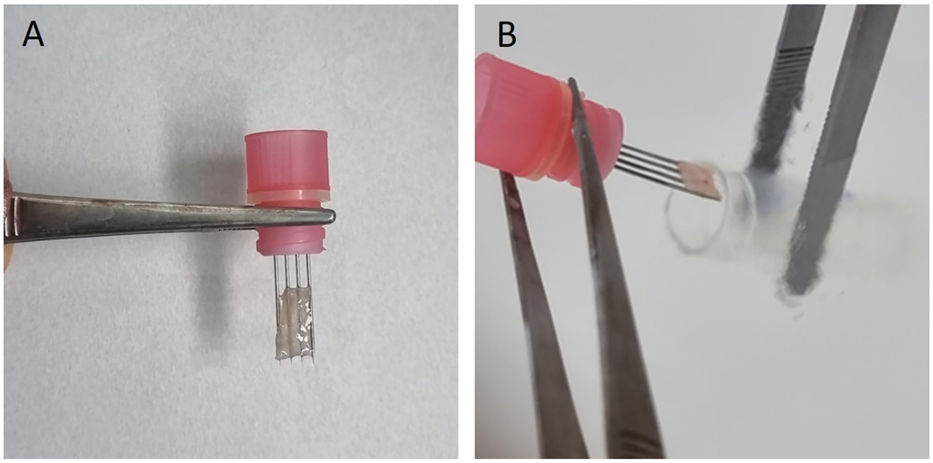

Figure 2. Cryo-support device. A: The tissue fragment (~5×5×1 mm) was loaded onto the cryo-support device, and B: It was inserted into the LN2, placed into a cryogenic vial, and subsequently stored in a nitrogen tank

Immuno-histochemical Dyeing

The initial process and end of apoptosis were investigated using an immunohistochemical approach. The p53 antibody was used to identify the early apoptotic pathway. Antibody Caspase 3 was used to identify the final pathway that leads to DNA damage. Two antibodies were utilized for immune-histochemical investigation, including anti-p53 (Santa Cruz Biotechnology, Sc-126) and anti-Caspase 3 (CPP324-1-18) (Santa Cruz Biotechnology, sc-56052). The slices were incubated for 30 min at 97 °C, then hydrated in a series of alcohol solutions, and finally, they were immersed in 1% hydrogen peroxide in methanol for an additional 30 min. After that, it was washed with Phosphate-Buffered Saline - Tween buffer (PBS-Tween 0.05%), and the antigens were restored with citrate buffer for 30 min at 97 °C. As all of these proteins are cytoplasmic, Triton X-100 was used to penetrate the cell membrane for 10 minutes. To prevent the unexpected response, a 10% goat serum solution was used for 30 min. The slices were then treated with primary antibodies overnight at 4 °C. The tissue slices were washed multiple times. The samples were then treated with secondary antibodies (Master Diagnosis, MAD-000237Q) for 2 h at 37 °C. After washing, DAB solution (3,3' diaminobenzidine) was added to the slices. Then they were stained with hematoxylin, dehydrated using an ascending series of alcohols, clarified in xylene, and mounted for examination at a magnification of 40×.

Statistical Analysis

Normality was analyzed using the Kolmogorov-Smirnov test and then assessed using the Independent samples t-test for parametric data and the Mann-Whitney U test non-parametric data. The data were analyzed using SPSS software version 23. The results were reported as mean ± standard deviation, maximum, and minimum, with a significance threshold of $p<0.05$. All charts were also drafted using the GraphPad Prism V8 application.

Results

There are no significant differences in female parameters in terms of age (p=0.31), anti-Müllerian hormone (p=0.80), follicle stimulating hormone (p=0.58), and obtained fragments of each ovary (p=0.72) between the two groups (Table 1). There was no significant difference in the number of primordial, primary, and secondary, follicle types between the NIV and ova-cryo devices (Table 2). The follicular granulosa cell arrangement and oocyte baseline integrity were better preserved in ova cryo-devices compared to NIV groups (Figure 3). However, compared to the group undergoing NIV, those who underwent cryo-support freezing showed a greater number of high-quality follicles (Table 3).

Our data showed that the quantity of positive apoptosis (p53) molecules was significantly higher in the NIV method than

Table 1. Comparative evaluation of laboratory characteristics of cancer female between NIV and cryo-support groups

Groups Female parameters	NIV group (n=10)	Cry-support group (n=10)	p-value
Age (year)	32.8±5.9	33.7±6.9	0.31*
AMH (ng/mL)	2.08±0.56	2.14±0.47	0.80#
FSH (IU/L)	6.7±0.81	6.5±0.5	0.58#
Mean number of fragments	5.5±2.4	5.1±2.4	0.72#

Data were presented as mean ± standard deviation. *: Statistical analysis was performed based on Mann-Whitney U test, #: Based on independent sample t-test, AMH: Anti-Müllerian hormone, FSH: Follicle stimulating hormone, NIV: Needle-immersion vitrification in the cryo-support freezing group in terms of primordial (p=0.03), primary (p=0.007), and secondary follicles (p=0.002) in Figure 4.

Immuno-histochemical results revealed that caspase 3 was not significantly different in the freezing groups using the NIV approach, compared to the cryo-support-freezing group in terms of primordial (p=0.13), primary (p=0.22), and secondary follicles (p=0.8) (Figure 5).

Discussion

This study aimed to investigate two methods of OTC: NIV and cryo-support, which are conventional procedures in the vitrification process. The results demonstrated that a cryo-support device could greatly enhance the quality and number of surviving follicles. In this procedure, the expression of the apoptosis-promoting molecule (p53) in follicles was significantly decreased compared to the immersion method with a needle, in cryo-support. This suggested a potential role of cryo-support in minimizing apoptosis during the cryopreservation process.

Currently, there are two common procedures for OT freezing: slow freezing and vitrification. Studies have shown over 150 recorded live births through OTC until 2021[18]. The five largest European centers reported a pregnancy rate of 29% and a live birth rate of 23%[7,19].

The vitrification method is a relatively new approach in OTC, and only a few live births have been documented so far.

Table 2. Comparison of the number of primordial, primary, and secondary follicles in two freezing groups using the NIV and cryo-support groups

Groups	Number of primordial means ± SD (min-max)	Number of primary means ± SD (min-max)	Number of secondary means ± SD (min-max)
Cryo-support	**27.30±6.63 (15-35)**	**19.50±5.93 (13-27)**	7.6±2.31 (6-11)
Needle-immersed	**27.20±5.84 (19-35)**	**19.90±4.22 (14-26)**	7.8±2.04 (5-11)
p-value	**0.97**	**0.86**	0.84

Data were presented as mean ± SD. Statistical analysis was performed based on an Independent sample t-test. P-value was considered <0.05, NIV: Needle-immersion vitrification, SD: Standard deviation, min: Minimum, max: Maximum

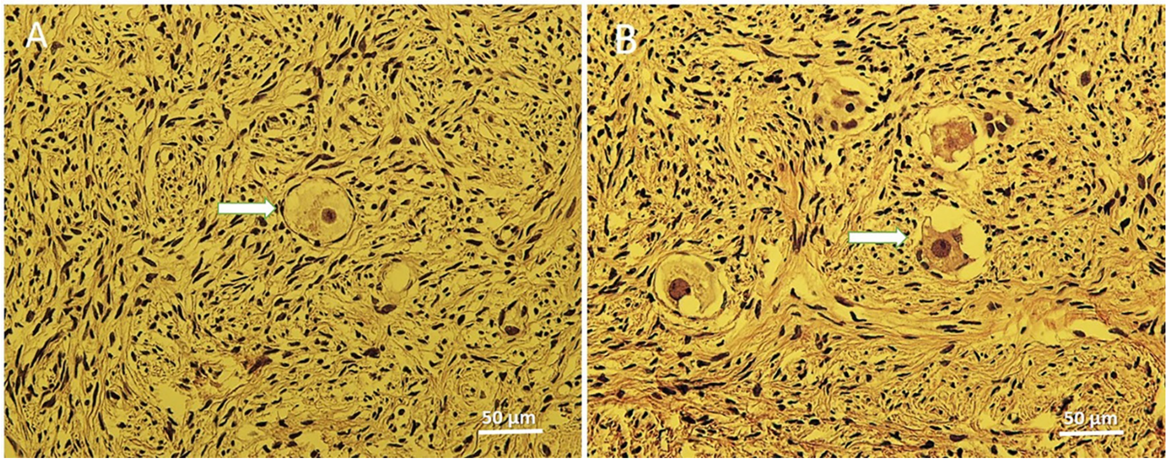

Figure 3. The quality of follicles after vitrification/warming in both groups. A: A primordial follicle with an intact oocyte and well-organized granulosa cells and no condensed nuclear chromatin were categorized as good quality morphology, and B: Primordial and Primary follicles were considered as bad quality morphology. The arrows show primordial follicles

Table 3. Comparison of the number of good quality follicles in two freezing groups NIV and cryo-support groups

Groups	Good quality primordial means ± SD (min-max)	Good quality primary means ± SD (min-max)	Good quality secondary means ± SD (min-max)
Cryo-support	16.60±4.40 (10-23)	6.70±2.31 (4-11)	4.5±1.26 (3-7)
Needle-immersed	12.80±2.82 (9-19)	4.30±1.70 (2-7)	3.3±0.94 (2-5)
p-value	**0.034#**	**0.017#**	0.028*

Data were presented as mean ± SD. *: Statistical analysis was performed based on Mann-Whitney U test, #: Based on Independent sample t-test. P-value considered <0.05, NIV: Needle-immersion vitrification, SD: Standard deviation, min: Minimum, max: Maximum

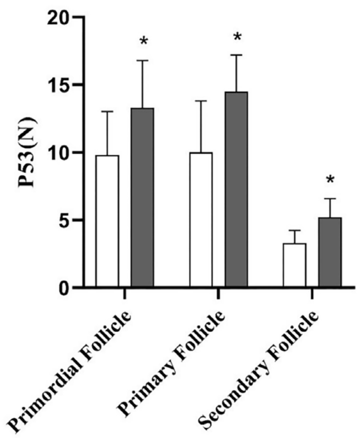

Figure 4. Comparison of the number of primordial, primary, and secondary follicles containing the apoptosis (p53) progressive molecule in two freezing groups, needle-immersion vitrification and cryo-support. All values are presented as the mean ± standard error of the mean. P-value is considered <0.05. Primordial (p=0.03), primary (p=0.007), and secondary follicles (p=0.002)

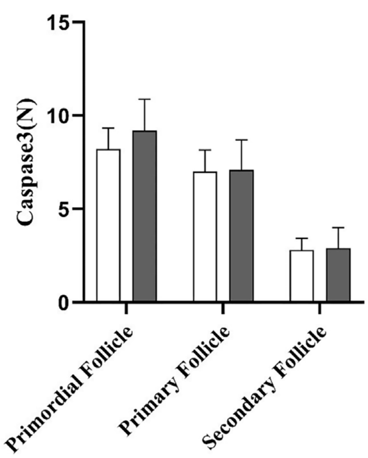

Figure 5. Comparison of the number of primordial, primary, and secondary follicles containing the leading molecule caspase 3 in two freezing groups, needle-immersion vitrification and cryo-support. All values are presented as the mean ± standard error of the mean. P-value is considered <0.05. There was no significant difference between the two groups

The benefits of vitrification include its brief duration during the freezing process and the absence of specific equipment requirements. New research comparing the two methods has shown that there is no substantial difference in clinical outcomes between vitrification and slow freezing methods(7,8).

In a meta-analysis study, Zhou et al.(20) evaluated the number of surviving primordial follicles after vitrification and slow freezing. They concluded that no significant differences were observed in the number of follicles in the freezing groups.

Comparisons of vitrification and slow freezing groups revealed a significant increase in apoptosis levels in both freezing groups, compared to the control. However, there were no significant differences between the freezing groups(21). In 2015, it was demonstrated that vitrification is a superior method for preserving follicle survival compared to slow freezing. The study found that follicle survival improved to 83.6% in the vitrification group compared to 80.7% in the slow freezing group. Furthermore, there was no significant difference in the amount of DNA breakage between the two groups(22).

In general, a vitrification technique can be classified into open and closed methods(8,23). An NIV method is a type of open method in OTC. In this method, the OT is placed on a special needle and then immersed in LN2. This method ensures the survival of both follicular and stromal cells(11,15).

This method is simple and requires only a small needle, such as an insulin needle. However, the small size of the OT may create challenges during transplantation. Moreover, a lack of tissue support during direct LN2 exposure can compromise tissue integrity. Cryo-support, another open vitrification method, addresses this issue by offering a stable platform and broader surface area for freezing. It results in better conditions for OTT and improved angiogenesis post-transplantation. By reducing ischemia-related follicle loss, cryo-support may enhance graft viability and functionality(24).

The primordial follicle count is a significant biomarker used to evaluate a woman's ovarian reserve or fertility potential. Since primordial follicles are more resistant to cold damage during the freezing and warming process, their morphology and integrity are often used to assess the quality of OT post-freezing(25). In our study, we assessed the post-freezing quality of OT and found that the cryo-support method better preserved primordial follicle structure and overall cortical tissue integrity compared to NIV. Additionally, a significant reduction in p53 expression further supported reduced apoptosis in the cryo-support group.

It's worth noting that various factors—including freezing method, cryopreservation media composition, cryoprotective agents, processing time, temperature, and freezing devices—can all affect the quality of OT follicles during freezing and thawing(26-28).

Reducing the thickness of the OT cortex minimizes ice crystal formation. Additionally, thinner fragments allow for deeper penetration of cryoprotectants, leading to proper hydration and cryoprotection(29). Since the tissue is positioned on stainless steel, cryo-support ensures uniform and rapid exposure to ultra-low temperatures when immersed in LN2, promoting optimal vitrification.

In 2021, Sugishita et al.(13) compared vitrification and slow freezing for preserving primordial and primary follicles. They reported significant follicle loss in freezing groups, though survival and DNA strand breakage rates were not significantly different between open and closed vitrification methods.

Also, we observed that the cryo-support group had a lower expression level of p53 compared to the NIV group. This process, which involves complete tissue trimming and mounting on cryo-support needles, facilitates even and controlled freezing in LN2. The effects of vitrification on the quantity of apoptosis in ovarian follicles are ambiguous. Although the vitrification method and kind of device are typical for freezing oocytes and embryos, their efficacy for freezing OT has been questioned, and the results are controversial[30].

Recently, Gupta et al.[31] assessed the influence of several vitrification techniques on apoptosis and growth-related gene expression. Their study showed that the percentage of viable pre-antral follicles obtained after vitrification was significantly lower than before freezing. In addition, the expression of the apoptotic gene BAX was recorded as higher in vitrification interventions with different protocols, and the expression of other apoptosis-related genes, such as BCL2 and Caspase 3, did not show any significant differences between the groups. Their results confirm that freezing induces cellular apoptosis. The presence of intracellular caspase is a clear marker of apoptosis, because caspases, particularly caspase 3, are exclusive proteases of apoptosis.

Kometas et al.[32] concluded that there was no significant difference in the quantity or form of OT follicles between vitrification and slow freezing. Vitrification protocols involve using high concentrations of permeable cryoprotectant like EG and DMSO as well as impenetrable cryoprotectant like sucrose, to facilitate cellular dehydration and prevent the formation of ice crystals inside and outside the cell.

A meta-analysis conducted by Shi reviewed 14 studies to evaluate the efficacy of tissue vitrification, despite several research findings showing a decrease in the percentage of apoptosis with vitrification. The study revealed that the effects of freezing were influenced by various parameters and component size, and that vitrification caused significantly less DNA follicular damage compared to slow freezing. Additionally, it was observed that vitrification had a positive impact on the ovary's preservation and the rate of cooling. It may be more successful than slow freezing in terms of preserving stromal cells and OT[14].

The Sugishita et al.'s[13] study utilized γH2AX to evaluate DNA damage and AC3 antibodies to analyze caspase 3 levels, comparing the levels of apoptosis in primordial and primary follicles among different groups, including the cryo-support group. The study concluded that there were no significant differences in the rates of DNA damage between the cryo-support and the other groups.

Study Limitations

One limitation of this study was the small sample size due to the challenge of obtaining healthy OT for comparison from cancer patients. Nonetheless, the consistent improvements in follicle quality observed with cryo-support emphasize its potential clinical value.

Conclusion

Currently, no studies directly compare NIV and cryo-support methods for vitrification. Our study fills this gap and highlights the benefits of cryo-support in preserving follicular integrity. A recent investigation revealed that the cryo-support device preserved higher-quality follicles and reduced p53 expression, indicating a decrease in apoptosis. These findings suggest that cryo-support may be a promising tool for optimizing OTC and improving clinical outcomes.

Ethics

Ethics Committee Approval: This research protocol was reviewed and approved by the Ethics Committee of the Yazd Reproductive Sciences Institute (approval number: IR.SSU.RSI.REC.1401.008 date: 04.09.2022).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: M.H.T., F.S.K.A., S.A.D.B., **Concept:** M.S., M.A.K., S.D., F.A., Design: M.S., M.A.K., S.D., F.A., Data Collection or Processing: M.S., M.H.T., F.S.K.A., S.A.D.B., F.A., Analysis or Interpretation: M.S., S.A.D.B., F.A., Literature Search: M.S., F.A., Writing: M.S., M.A.K., M.H.T., S.D., F.S.K.A., S.A.D.B., F.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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