

REVIEW

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Ovarian tissue freezing and activation after thawing: an update



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Abstract

Background: With the growth of women's age, ovarian failure can be caused by various factors. For the women who need chemotherapy because of cancer factors, the preservation of fertility is more urgent. The treatment of cancer is also a process in which all tissues and organs of the body are severely damaged, especially in the reproductive system.

Main body: As a new fertility preservation technology, autologous ovarian tissue cryopreservation and transplantation is developing rapidly and showing great potentiality in preserving ovarian endocrine function of young cervical cancer patients. Vitrification and slow freezing are two common techniques applied for ovarian tissue cryopreservation. Thus, cryopreserved/thawed ovarian tissue and transplantation act as an important method to preserve ovarian function during radiotherapy and chemotherapy, and ovarian cryopreservation by vitrification is a very effective and extensively used method to cryopreserve ovaries. The morphology of oocytes and granulosa cells and the structure of organelles were observed under the microscope of histology; the hormone content in the stratified culture medium of granulosa cells with the diameter of follicle was used to evaluate the development potential of ovarian tissue, and finally the ovarian tissue stimulation was determined by the technique of ovarian tissue transplantation.

Conclusions: Although there are some limitations, the team members still carry out this review to provide some references and suggestions for clinical decision-making and further clinical research.

Keywords: Ovarian tissue cryopreservation, PI3K activator, In vitro activation

Background

In recent years, with the improvement of people's living standards, the change of dietary structure, the increase of work and living pressure, and the acceleration of the pace of life of modern people, the incidence of this tumor disease has increased, and its occurrence has also become more and more young [1]. The treatment of tumor diseases by radiotherapy and chemotherapy will be accompanied by premature ovarian failure, menstrual disorders, high gonadotropin, and low estrogen levels, which may lead to a series of reproductive disorders for women. In order to preserve the fertility of cancer women and avoid the loss of gonadal function caused by

radiotherapy and chemotherapy in the process of cancer treatment, the technology of fertility preservation has brought hope for cancer women. For young women who wish to preserve their fertility prior to cancer treatment, several methods can be offered, including embryo, oocyte, or ovarian tissue freezing. Ovarian tissue cryopreservation technology can save many eggs at the same time, and it can be transplanted back to the female body timely through ovarian transplantation. For patients with premature ovarian failure, although there are still many dormant follicles in the ovary, these dormant follicles cannot grow spontaneously until the pregnancy ends. In view of this situation, in vitro activation (IVA) technology can artificially activate the remaining dormant follicles so that patients with premature ovarian failure can obtain their own eggs and eventually pregnancy [2].

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Main text

Cryopreservation of ovarian tissue

Application of ovarian tissue freezing technology

Considered to be of the most important reproductive organs of women, the ovary has two main functions: producing germ cells and secreting endocrine hormones. Its normal function directly affects women's fertility and happiness index. At present, the increasing incidence of tumor diseases has posed a severe challenge to the function of ovary. In recent years, ovarian tissue cryopreservation has rapidly developed as a successful method for preserving the fertility of girls and young women with cancer or benign conditions requiring gonadotoxic therapy and is now becoming widely recognized as an effective alternative to oocyte and embryo freezing when not feasible.

Laboratory research on ovarian cryopreservation and transplantation began in the 1950s leading to clinical studies in the 2000s [3]. According to the latest research data, about 1.3% women of childbearing age in China have ovarian tumor symptoms to varying degrees, and the number of infertile women caused by ovarian tumor is up to about 190,000 every year [4]. The ovary contains a large number of immature oocytes in its cortex, which is small in size, simple in structure, slow in metabolism, low in sensitivity to temperature, and not easy to be damaged by freezing. Therefore, the ovarian tissue freezing can avoid the loss of reproductive capacity caused by ovarian damage in the process of disease treatment [5].

Freezing and resuscitation of ovarian tissue

The technique of ovarian tissue cryopreservation is to keep the tissues and organs in low temperature and keep their activity. There are three methods for the cryopreservation of ovarian tissue, namely slow freezing, rapid freezing, and vitrification. Ovarian tissue should be treated before freezing. The ovarian tissue was transferred to fresh equilibrated Leibovitz's L-15 Medium, and after the medullary parts were removed by a surgical blade, the cortical tissue was cut into small fragments (approximately $2 \times 2 \times 1$ mm) under a sterile condition.

Slow freezing Cryopreservation of the primordial follicles in cortical tissue using slow programmed freezing has better results, with up to 65% of survival of follicles, thus is the current widely used protocol for this method. In the process of dehydration, cryoprotectant enters into the cells to dilute the concentrated electrolyte in the cells, so that the ice crystal damage and solute damage in the cells are minimized. Slow freezing scheme was first proposed by Oktay et al. [6]. In 1998, it was also the earliest basis of current slow freezing technology. Raffel, N., et al [7]. used the slow freezing method to freeze the ovarian tissue of sheep, and got successfully pregnant

after auto transplantation, which provided a good model for human ovarian tissue freezing and transplantation. Oktay et al. [6] used slow freezing method to freeze human ovarian tissue, through autologous transplantation to abdominal subcutaneous, and then used the oocyte produced by IVF technology to successfully obtain the embryo. These studies show that the slow freezing method can make ovarian tissue get better and more stable cryopreservation, and can obtain ovarian tissue with reproductive potential. However, this method is difficult to accept in general laboratory because of its complex operation, time-consuming process, and high cost of program cryostat.

Rapid freezing The rapid freezing method, also known as the ultra-fast freezing method, uses low or medium concentration of cryoprotectant and ultra-fast cooling rate to make the tissue or cell solution form a "glassy state". Usually, the tissue is placed in the liquid nitrogen steam for more than 12 h, and then put into the liquid nitrogen for preservation. Nikiforov, D. et al. [8] have used the method of ultra-freezing to preserve the ovarian tissue of adults, resulting in the destruction of the integrity of follicles in the tissue. At present, there are few reports about the rapid freezing of human ovarian tissue, and few cases of successful pregnancy.

Vitrification Vitrification is a method to minimize cell damage due to ice crystal formation by extremely rapid cooling using a high concentration of cryoprotectant. After rapid cooling, the high concentration of intracellular liquid changes from liquid to transparent and stable noncrystalline solidified state (i.e. glassy state, which can maintain the normal distribution of molecules and ions in the liquid). Vitrification, as a cryoprotective method for freezing cells or tissues at very high cooling rates, can avoid the formation of intracellular ice crystals that are potentially lethal during freezing and thawing, and maximally reduce the damage to cells. Lotz, L., et al. [9] reported the successful experiment of cryopreservation of mouse embryos by vitrification. Since then, vitrification has been widely used. Lantsberg, D [10]. used vitrified cryopreservation method to freeze human oocytes. After thawing, the woman was successfully pregnant through IVF and gave birth to a healthy female baby. With the continuous development and progress of vitrification technology, more and more researchers apply this technology to the study of ovarian tissue cryopreservation [11]. Isachenko, V. et al. [12] used nitrifications to freeze human ovarian tissue. After resuscitation, 86% of oocytes showed normal morphology. At the same time, the tissue mass was cultured in vitro to confirm that the cryo resuscitated tissue has endocrine function. At present, because the vitrification technology is convenient,

and the operation is relatively simple, it has been widely used in the laboratory.

Mechanism of ovarian tissue activation after thawing

The activation of ovarian tissue after resuscitation is the premise to ensure the normal function of ovarian tissue in patients with ovarian tissue cryopreservation due to poor ovarian function or ovarian dysfunction. At present, kit gametes, vascular endothelial growth factor, leukemia inhibitory factor, and other internal factors have been found to play an important role in the activation of dormant primordial follicles [13]. The phosphatase and tension homolog of chromosome 10 (PTEN) /phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) is a major signaling pathway governing primordial follicle recruitment and growth. In adult mouse oocytes, the deletion of phosphatase and tension homologous deletion gene (PTEN) on 10# chromosome can activate AKT phosphorylation, leading to the activation of primordial follicles [14].

In addition, inhibition of Hippo signaling pathway can activate follicular development. The Hippo signaling pathway is an evolutionarily conserved system that determines organ size by regulating apoptosis and cell proliferation [15]. YAP and TAZ are 2 key downstream effectors of the Hippo signaling pathway. In terms of function, YAP1 and TAZ are mainly transcription co activators and multifunctional intracellular connexins, which participate in intracellular signal transduction and transcriptional co-activation of their downstream target factors. Furthermore, we demonstrated that fragmentation of ovaries induced a transient increase in the polymerization of G-actin to F-actin, decrease in phospho-YAP levels, and increase in nuclear localization of YAP, resulting in the upregulation of downstream CCN growth factors and baculoviral inhibitors of apoptosis repeat containing (BIRC) apoptosis inhibitors. Fragmentation of ovarian cortex into small cubes changed cytoskeletal actin dynamics and induced disruption of the Hippo signaling pathway, leading to the production of CCN growth factors and anti-apoptotic BIRC [16]. Because there is a tumor suppressor pathway in the human body that can regulate the size of organs, the imbalance of this pathway will lead to the growth of tissues and organs. In the mammalian ovary, we demonstrated the expression of Hippo signaling pathway genes and showed that ovarian fragmentation increased actin polymerization, leading to YAP nuclear translocation and increased expression of cysteine-rich 61, connective tissue growth factor, and nephroblastoma overexpressed growth factors and baculoviral inhibitors of apoptosis repeat containing apoptosis inhibitors, followed by enhanced follicle growth [17].

Application of IVA in ovarian follicles

In clinic, the success rate of hormone therapy for ovarian dysfunction is not high. Furthermore, previous

studies have shown that the short-term in vitro activation of dormant ovarian follicles via the stimulation of the PTEN/PI3K/AKT/FOXO3 signaling pathway with a PTEN inhibitor and a PI3K activator enhances primordial follicle activation, with improved oocyte maturation, suggesting that modulating the PI3K/AKT signaling pathway in patients with diminished ovarian reserves may have clinical utility. Because treatment with PI3K activators stimulates dormant primordial follicles, whereas ovarian fragmentation stimulates secondary follicle growth, ovarian fragmentation and PI3K activator treatment were combined to activate residual follicles in ovaries of patients. The abnormal or dysfunctional bilateral ovarian tissues were taken out by laparoscopic surgery, and then cut into small pieces, which can be frozen by vitrification technology [18], thawed during use and cultured in vitro for 2 days in a vessel containing PI3K activator, and then transplanted under the abdominal cavity microscope to the serosa of fallopian tube. Among the 3 cases whose final pregnancy outcome has been reported after activation of ovarian tissue by the above methods, 1 case was aborted, and 2 cases were born healthy with IVA.

Monitoring of ovarian tissue activation after thawing

Form detection

After ovarian tissue resuscitation, the morphology of follicles and granulosa cells were observed and counted by histological microscope. The commonly used method is hematoxylin and eosin staining. This method is easy to operate, and the changes of follicle morphology can be observed intuitively. Yding Andersen, C. et al.'s [19] research shows that the changes of mitochondria and endoplasmic reticulum in the ultrastructure of primordial follicles can be observed objectively through the perspective microscope. Vanni, V. S. et al. [20], using immunofluorescence microscopy to determine the survival of cells through color change, so as to determine the survival rate of cells in ovarian tissue.

Detection of endocrine level

By detecting the secretion of hormones in the culture medium before and after freezing and thawing of ovarian tissue, we can judge whether the ovarian tissue returns to normal function, such as the secretion of E2, P, T, and other hormone indexes. In normal condition, E2 increases with the increase of secretion level over time, reaches a high peak on the eighth day, and begins to decline on the tenth day [21].

Immunohistochemical analysis

Immunohistochemistry is mainly used to detect the expression of proliferating cells, antigens, and apoptosis after resuscitation, while the proliferation of granulosa cells is generally considered as a marker of the activation

of primordial follicles [22]. Other studies have shown that adding different concentrations of angiotensin to the culture medium before and after freezing and thawing of ovarian tissue can inhibit the occurrence of apoptosis and promote the early recovery of blood supply of ovarian tissue. Follicles are the functional units of the ovary and consist of an oocyte and its supporting cells, such as granulosa cells, theca cells, and stromal cells. ICAM-1 expression on the surface of frozen/thawed VEC was detected at 4, 12, and 24 h after freezing/thawing with the immunohistochemical method [23].

Problems in the process of cryopreservation and activation of ovarian tissue

It has been nearly 20 years since the technology of ovarian tissue freezing was developed in foreign countries. Compared with China, the services of ovarian tissue cryopreservation and other related aspects need to be improved. Ovarian tissue cryopreservation can have several benefits including development of a large number of follicles in ovaries, no need for ovarian stimulation, no delay in the process of cancer treatment as well as no need for having a partner or gamete donation during cryopreservation; accordingly, investigators have used ovarian tissue cryopreservation for fertility preservation during recent years.

Apart from it still being an experimental approach to fertility preservation with no strict and clear recommendations for its use, it should also be noted that there is a great deal of follicular loss in the freeze/thaw process of ovarian tissues, especially in primary follicles. First of all, for patients undergoing ovarian tissue cryosurgery, it is the first problem to design the best operation plan according to their age, disease type, and operation time. In the process of cryosurgery, it may cause certain damage to ovarian tissue, such as vascular damage of ovarian tissue, and death of primordial follicle; so the selection of the best cryosurgery procedure is also the key point of clinical research work: how to obtain more follicle survival rate, transplantation success rate, activation of ovarian tissue, and stimulation of ovarian function after ovarian resuscitation will be verified by a large number of clinical practice after ovarian tissue resuscitation.

Conclusion

The *in vitro* activation of ovarian tissue is a new exploration in the treatment of ovarian dysfunction, which can increase the number of mature oocyte in patients with infertility. However, the success rate is not high at present, and further research is needed. A large number of experimental studies have shown that the ovarian tissue frozen by slow freezing method has a shorter function maintenance time, a lower follicle maturation rate, and a serious loss of follicles after transplantation for the

selection of ovarian tissue freezing scheme. Compared with fresh ovarian tissue, vitrified ovarian tissue has a better effect on ovarian function and follicular development, which will become the first choice for ovarian tissue freezing, providing a certain basis for the research of reproductive medicine [24–26].

Abbreviations

IVA: *In vitro* activation; PI3K: Phosphatidylinositol 3 kinase

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Author's contributions

The single author for this submission did all the conceptualizing, data collecting, and writing, among others. The author(s) read and approved the final manuscript.

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Competing interests

The authors declare that there are no conflicts of interest.

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