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Female fertility preservation strategies: cryopreservation and ovarian tissue *in vitro* culture, current state of the art and future perspectives

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Summary

In the present review, the main strategies of female fertility preservation are covered. Procedures of fertility preservation are necessary for women who suffer from diseases whose treatment requires the use of aggressive therapies, such as chemotherapy and radiotherapy. These kinds of therapy negatively influence the health of gametes and their progenitors. The most commonly used method of female fertility preservation is ovarian tissue cryopreservation, followed by the retransplantation of thawed tissue. Another approach to female fertility preservation that has been actively developed lately is the ovarian tissue *in vitro* culture. The principal methods, advantages and drawbacks of these two strategies are discussed in this article.

Keywords: Cryopreservation, *In vitro*, Oncology, Ovary, Tissue.

Introduction

Female fertility preservation is one of the most common problems addressed by assisted reproductive technologies. Many women suffer from diseases like hormone-dependent malignancies (e.g. breast cancer) or autoimmune diseases (e.g. vasculitis), the treatment for which involves aggressive methods of therapy such as radiotherapy and chemotherapy. As these methods negatively influence the health of gametes and their progenitors, fertility preservation is required in these cases. Moreover, developing new techniques allowing us to preserve fertility may be necessary for women who want to postpone their childbearing.

Although oocyte cryopreservation is commonly used for fertility preservation, this method involves hormonal ovarian stimulation, which cannot be used in some groups of patients, e.g. prepubertal girls and patients who suffer from hormone-dependent types of cancer. This paper presents a review of the current literature and a discussion of the data regarding methods of fertility preservation that are applicable for patients who cannot undergo hormonal stimulation. These techniques refer to two main strategies of female fertility preservation that are being actively developed: ovarian tissue cryopreservation followed by retransplantation, and ovarian tissue *in vitro* culture.

Main strategies of female fertility preservation

Currently, patients who suffer from different kinds of hormone-dependent malignancies, such as breast cancer or autoimmune diseases like vasculitis, are offered an aggressive form of therapy, which negatively influences the health of both themselves and the gametes. For example, the treatment of breast cancer, which is a hormone-dependent kind of cancer, may involve a drastic method like oophorectomy. This

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procedure leads to a decrease in the production of sex hormones (Di Leva Gianpiero *et al.*, 2013). Moreover, treatment of other cancer types includes radio- and chemotherapy, which negatively affects the ovarian status. It is well known that most of the alkylating chemotherapeutic agents are gonadotoxic (Bokemeyer *et al.*, 1994; Thomson *et al.*, 2002). Use of these agents in a therapy protocol leads to the degradation of follicles and oocytes (Familiari *et al.*, 1993; Oktem & Oktay, 2007a). Moreover, such treatment may result in the formation of ovarian tissue fibrosis (Meirow, 1999; Behringer *et al.*, 2005). Chemotherapeutic agents induce apoptosis in ovarian stroma and in the follicular system (Verga Falzacappa *et al.*, 2012), which leads to a reduction in follicle numbers or, in other words, results in an increase in the risk of infertility (Oktem & Oktay, 2007b).

The use of radiation therapy is dangerous for women over the age of 30, because the effective sterilizing dose of radiotherapy decreases with age (Wallace *et al.*, 2005). At the same time, the risk of malignancy formation increases with age (Xu *et al.*, 2013). Given these facts, the use of aggressive antitumour therapy for women over 30 must be supplemented with procedures of fertility preservation.

In contemporary society, many women prefer to delay childbearing, but the number of follicles decrease and reproductive health becomes impaired with age, reducing the probability of normal fertilization and pregnancy (Wallace & Kelsey, 2010). Therefore, methods that allow us to preserve fertility could also help these women to bear healthy children.

In some cases, fertility preservation is assisted with a classical cycle of ovulation induction (Cardozo *et al.*, 2015; Sigismondi *et al.*, 2015). Obtained oocytes can be cryopreserved, or fertilized and the developed embryos can subsequently be cryopreserved. Use of such a protocol is possible only in cases in which the woman is absolutely healthy or when her disease was diagnosed as being at an early stage of development and does not depend on gonadotropic and steroid sex hormones. Otherwise, the use of this protocol may lead to growth of the tumour. Moreover, such a strategy of therapy is unsuitable for prepubertal patients (Mahajan, 2015).

At the present time, a patient requiring fertility preservation is offered cryopreservation of ovarian tissue, followed by retransplantation of thawed fragments into the patient's body (Meirow *et al.*, 2007). However, technologies of ovarian tissue *in vitro* culture are being actively developed (Roy & Treacy, 1993; Abir *et al.*, 1997, 1999, 2001; Nayudu *et al.*, 2003; Xu *et al.*, 2011b). The main aim of these technologies is to produce mature oocytes capable of fertilization from pieces of *in vitro* cultured ovarian tissue (Filatov *et al.*, 2015). Embryos obtained after the fertilization of oocytes may be cryopreserved and transferred into the patient's uterus after full recovery. Moreover, obtained embryos may be used in surrogacy programmes if the patient would like to have a child before the disease is cured.

Diseases whose treatment requires fertility preservation

Many diseases whose treatment requires aggressive methods of therapy and procedures of fertility preservation are known. These diseases can be divided into two groups: oncological and non-oncological diseases. Non-oncological diseases include autoimmune and haematological disorders that, like oncological ones, are treated with chemotherapy and radiotherapy (Donnez *et al.*, 2006a,b, 2010a,b). The most common diseases the treatment of which requires procedures of fertility preservation are represented in Table 1.

In many cases, diagnosis of the disease occurs at a late stage, when treatment must begin immediately. In these situations, the usage of standard protocols of assisted reproductive technologies, followed by the cryopreservation of embryos obtained after *in vitro* fertilization of oocytes received via ovulation stimulation, is not possible (Sigismondi *et al.*, 2015, Cardozo *et al.*, 2015). Moreover, the use of classical programmes of assisted reproductive technologies for fertility preservation is not possible for prepubertal girls (Mahajan, 2015). In these cases, the application of ovarian tissue cryopreservation technologies or the ovarian tissue *in vitro* culture technique seems to be the best strategy for female fertility preservation.

Methods of ovarian tissue isolation

Isolation of ovarian tissue for further cryopreservation or *in vitro* culture may be performed in two ways: surgical removal of the whole ovary or biopsy of ovarian tissue pieces. The most commonly used method for obtaining ovarian tissue is laparoscopy (Meirow *et al.*, 1999), but minilaparotomy also may be used for this purpose (Bolla *et al.*, 2012). There is no consensus on the amount of ovarian tissue that must be left in the patient's body after the procedure of ovarian tissue isolation. If treatment requires usage of an aggressive therapy that can negatively influence reproductive function, it is better to isolate larger parts of ovarian tissue (Rosendahl *et al.*, 2008; Harel *et al.*, 2011). Conversely, parts of ovarian tissue in the body must be preserved in order to provide the better survival of retransplanted fragments.

If the proposed fertility preservation strategy is the cryopreservation of ovarian tissue followed by retransplantation of isolated fragments, in most cases,

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Table 1 Diseases that require fertility preservation (according to Wallace *et al.*, 2005; Donnez & Dolmans, 2013)

Non-oncological	Oncological
Autoimmune:	Osteosarcoma
Systemic lupus erythematosus	Ewing sarcoma
Rheumatoid arthritis	Breast cancer
Behçet's disease	Cervical carcinoma
Wegener's granulomatosis	Vaginal carcinoma
	Vulvar carcinoma
Haematological:	Ovarian tumours
Sickle-cell anaemia	Hepatoblastoma
Thalassaemia major	Neuroblastoma
Aplastic anaemia	Medulloblastoma
	Melanoma
Other:	Bowel malignancy (cancer)
Inflammatory process in ovaries and fallopian tubes	Pelvic sarcoma
Amenorrhoea	Rhabdomyosarcoma
Multiple cysts' formation in ovaries	Sacral tumour
	Rectosigmoid tumour
	Wilms' tumour (nephroblastoma)
	Non-Hodgkin's lymphoma
	Leukaemia
	Thyroid cancer

the cortical part of ovarian tissue is extracted. Cortical ovarian tissue contains the bulk of the follicles, particularly the primordial and primary ones. Furthermore, it is supposed that after the procedure of ovarian tissue cryopreservation and thawing, small follicles survive better than do larger ones (Hovatta *et al.*, 1996, 1997; Newton *et al.*, 1996; Oktay *et al.*, 1997).

The ovarian tissue biopsy procedure allows the obtaining of fragments about 1 cm × 4–5 mm × 1–1.5 mm (Donnez *et al.*, 2006a, 2010a, 2013). Before cryopreservation, isolated fragments must be separated into small pieces about 0.3–2 mm wide (Hovatta *et al.*, 1996; Silber *et al.*, 2005; Silber, 2011). There are two main reasons to cut obtained ovarian tissue fragments into thin slices. First, after retransplantation, thin tissue slices stimulate better neovascularization than thicker ones do. This situation allows a decrease in ischaemia stress in retransplanted tissue (Silber, 2011; Andersen *et al.*, 2012; Silber & Barbey, 2012). Second, the thinness of fragments allows cryoprotectants to penetrate better into the tissue, resulting in less injury to the tissue during the procedures of freezing and thawing. It has been demonstrated that 2 mm thick slices thaw better than 4 mm thick slices (Ferreira *et al.*, 2010).

In some cases, a whole ovary instead of ovarian tissue fragments can be used for retransplantation (Leporrier *et al.*, 1987; Wang *et al.*, 2002; Hilders *et al.*, 2004; Martinez-Madrid *et al.*, 2004; Mhatre *et al.*, 2005; Imhof *et al.*, 2006; Silber *et al.*, 2008a,b). Whole ovary retransplantation decreases the risk of ischaemia in ovarian tissue because the immediate revascularization of the transplant is achieved via the vascular pedicle (Leporrier *et al.*, 1987; Wang *et al.*, 2002; Hilders *et al.*, 2004; Martinez-Madrid *et al.*, 2004; Mhatre *et al.*, 2005; Imhof *et al.*, 2006; Silber *et al.*, 2008a,b). There is a clinical case of a live birth after the transplantation of a non-cryopreserved whole ovary into the donor's body (Silber *et al.*, 2008b). However, cryopreservation of the whole ovary seems to be impossible because, in the event of microvascular anastomosis failure, all ovarian tissue may be lost with no chance of future fertility restoration (Courbiere *et al.*, 2009).

Ovarian tissue cryopreservation techniques

The two most commonly used methods of ovarian tissue cryopreservation are vitrification and slow freezing. The slow freezing technique was developed earlier than vitrification. The slow freezing protocol involves ovarian tissue impregnation with the cryoprotective agent, followed by a step-by-step temperature lowering. Usually, the slow freezing procedure involves the application of programmed freezers, which are special devices that allow the gradual lowering of temperature according to the program's settings. In most cases, tissue is cooled to −140°C and then placed into liquid nitrogen for

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storage (Newton *et al.*, 1996, 1998; Fuller & Paynter, 2004; Hovatta, 2005).

Both slow freezing and vitrification require the exposure of ovarian tissue to a cryoprotective solution. The principal difference between vitrification and slow freezing is the very rapid cooling of the sample in the former. Rapid cooling allows the avoidance of ice crystal formation in the tissue. The appearance of ice crystals inside cells during cryopreservation has a detrimental effect on their viability: ice has a lower density than water, meaning that frozen water takes up more volume than the liquid form. Ice crystals can impair a cell's membrane and, after thawing, a cell with a destroyed membrane may die.

The vitrification technique has demonstrated good results in the cryopreservation of oocytes and early embryos (Kuwayama *et al.*, 2005; Cobo *et al.*, 2009; Abedellhafez *et al.*, 2010; Cobo *et al.*, 2010; Edgar & Gook, 2012), but this method has grave disadvantages. Cryopreservation with a vitrification protocol requires the application of cryoprotective agents in high concentrations, which may negatively affect cryopreserved cells (Fahy *et al.*, 1984; Isachenko *et al.*, 2003, 2007; Gandolfi *et al.*, 2006; Keros *et al.*, 2009; Kagawa *et al.*, 2009; Amorim *et al.*, 2011;). Moreover, the high concentration of cryoprotective agents can cause osmotic shock in cells (Fahy *et al.*, 1990). Only two clinical cases of a successful pregnancy followed by a live birth after the vitrification and retransplantation of ovarian tissue are known (Kawamura *et al.*, 2013; Suzuki *et al.*, 2015). Brief descriptions of cryopreservation protocols whose usage led to pregnancies and live births are listed in Table 2. Comparison of the effectiveness of vitrification vs. slow freezing requires further studies, which are to be carried out in the near future.

Methods of ovarian tissue transplantation

There are three principally different methods of ovarian tissue transplantation: orthotopic retransplantation, heterotopic retransplantation and xenotransplantation. During orthotopic retransplantation, ovarian tissue is placed into the remaining ovary or (if that is not possible) into the pelvic cavity. In other words, ovarian tissue is transplanted into its native surroundings. In the case of heterotopic retransplantation, ovarian tissue is transplanted to a different site, e.g., under the skin of the forearm or under the abdominal wall (Oktay & Karlikaya, 2000; Callejo *et al.*, 2001; Oktay *et al.*, 2001; Radford *et al.*, 2001; Donnez *et al.*, 2004, 2005; Meirow *et al.*, 2005; Schmidt *et al.*, 2005; Demeestere *et al.*, 2006; Kim *et al.*, 2009; Grynberg *et al.*, 2012).

At present, two main orthotopic retransplantation techniques of ovarian tissue exist. In a case where some parts of the ovary remain in the patient's body, thawed pieces of cortical ovarian tissue can be attached to the remaining medulla. Where the medulla has not been saved, ovarian tissue pieces can be placed into the peritoneal window, in the region of small retroperitoneal vessels (Donnez *et al.*, 2012, 2013). The main advantage of orthotopic retransplantation as compared with the heterotopic method is that natural conception may occur when ovarian tissue is transplanted orthotopically. Moreover, in the case of the orthotopic technique, fragments are placed into an environment that is native for ovarian tissue. It is likely that tissue retransplantation into its original physiological surroundings may promote the development of transplanted tissue more effectively than retransplantation into a heterotopic site. The main disadvantage of orthotopic retransplantation is that it is often impossible to determine whether ovulation has occurred in the transplanted tissue or in the remaining ovary fragment, especially in the event of a natural conception.

Heterotopic retransplantation of ovarian tissue has other advantages over the orthotopic method. Firstly, it is easy to observe the actual condition of transplanted tissue. Secondly, heterotopic retransplantation of ovarian tissue does not require the usage of general anaesthesia. Thirdly, it is easier (when required) to remove the transplanted fragment than it is in the case of orthotopic retransplantation.

However, heterotopic retransplantation also has some principal drawbacks. First, conception may occur only with assisted reproduction technologies; moreover, the non-native surroundings of transplanted tissue probably reduce the ability to induce neovascularization in the ovarian fragment than in the case of orthotopic retransplantation. Taken together, these two factors reduce the probability of pregnancy (Oktay *et al.*, 2001, 2004; Partridge *et al.*, 2004; Sonmezer & Oktay, 2004; Kim *et al.*, 2009; Jemal *et al.*, 2010; Donnez *et al.*, 2013).

The method of Rosendahl *et al.*, (2006) shows that ovarian tissue xenotransplantation can also be applied. In this case, ovarian tissue is transplanted under the skin or under the renal capsule of immunodeficient mice (Sonmezer & Oktay, 2004; Chao *et al.*, 2008; Lan *et al.*, 2010). Immunodeficient mice do not develop an immune response to tissues transplanted from other species (Bosma *et al.*, 1983).

The main advantage of xenotransplantation is that the transplanted tissue is located outside the patient's body. In the case in which malignant cells have remained in the transplanted tissue, the tumour will grow in the mouse rather than in the patient. For this reason, this technique seems to be very

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Table 2 Protocols of cryopreservation procedures that led to live births after the retransplantation of thawed ovarian tissue

No.	Vitrification/Slow freezing	Cryoprotectors	Thawing	Reference
1	Slow freezing: cooling 2°C/min to −9°C, 5 min of soaking in cryoprotective solution, then manual seeding for ice crystal nucleation induction, 0.3°C/min to −40°C, 10°C/min to −140°C. Then samples were plunged into liquid nitrogen at −196°C. A programmable Planner freezer (Planner K10, Planner Ltd, UK) was used	Before the slow freezing procedure, pieces of tissue were transferred to 30 ml of 0.1 mol/l sucrose and 1.5 mol/l ethylene glycol in a PBS solution, and equilibrated for 30 min at 1°C on a tilting table	Samples were thawed rapidly in a 37°C water bath	Schmidt *et al.*, 2003; Andersen *et al.*, 2008
2	Slow freezing: 2°C/min to −8°C; at this temperature, ice crystal nucleation was induced manually, followed by 0.3°C/min to −30°C, 50°C/min to −150°C, before being stored in liquid nitrogen. A programmable Planner freezer (Planner K10, Planner Ltd, UK) was used	Tissue slices were rinsed briefly in a PBS solution containing 10 mg HSA per ml (base medium) followed by dehydration in 1.5 mol/l propanediol with 0.1 mol/l sucrose in a base medium at room temperature for 90 min	Samples were transferred to a water bath (+37°C) and thawed for 2–3 min	Gook *et al.*, 1999, 2005; Stern *et al.*, 2013
3	Slow freezing: cooling 2°C/min from 0°C to −8°C; ice crystal nucleation was induced manually by using forceps pre-cooled in liquid nitrogen; cooling 0.3°C/min to −40°C; cooling 30°C/min to −150°C. Then samples were stored in liquid nitrogen. A programmable Planner freezer (Planner K10, Planner Ltd, UK) was used	Fragments of tissue were placed into a Leibovitz medium containing 4 mg/mL human serum albumin and 1.5 mmol/L DMSO	Sample thawing was performed in the following way: at room temperature for 2 min, followed by ~2 min in a water bath (+37°C). Ovarian tissue pieces were rinsed in a Leibovitz medium three times	Donnez *et al.*, 2004
4	Slow freezing: the temperature was lowered from 4°C to −9°C at the rate of 2°C/min. After a manual seeding by touching the CryoTubes with liquid nitrogen pre-chilled forceps, samples were cooled to −40°C at the rate of 0.3°C/min, then to −140°C at 10°C/min. After stabilization of the temperature for 10 min, the samples were transferred to liquid nitrogen and stored until thawing. A programmable freezer (Planer Kryo 360/3.3) was used	Ovarian tissue pieces were cryopreserved in a solution of Leibovitz L-15 medium containing 1.5 mol/L dimethyl sulphoxide (DMSO), 0.1 mol/L sucrose and 10% decomplemented patient serum	After rapid thawing, strips were washed in decreased solutions of DMSO 1.5M (5 min), 1M (5 min), 0.5M (10 min) and in a solution of 0.05 mol/L sucrose in a Leibovitz L-15 medium supplemented with 10% decomplemented patient serum. The strips were then rinsed and transferred to the operating theatre for the graft in medium containing only 20% serum	Fauque *et al.*, 2007; Roux *et al.*, 2010

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Table 2 Continued

No.	Vitrification/Slow freezing	Cryoprotectors	Thawing	Reference
5	Slow freezing: −2°C/min to 1°C, −0.5°C/min to −5°C, −0.3°C/min to −9.3°C, 10 min of soaking in a cryoprotective solution. Then −0.3°C/min to −40°C and −10°C/min to −140°C, at which temperature the samples were plunged into liquid nitrogen at −196°C. The open freezing system CTE-920 was used	Pieces of ovarian tissue were equilibrated for 30 min in 1.5 mol/L ethylene glycol (or DMSO, or propanediol) and 0.1 mol/L sucrose in PBS solution on a tilting table on ice	Thawing occurred rapidly in a warm water bath (+37°C). The tissue fragments were released from the protective cryopreservation medium in reverse order, with the addition of 0.25 mol/L sucrose	Dittrich *et al.*, 2015
6	Slow freezing: after 5 min for equilibration at +4°C, cooling was performed at 0.5°C/min to −7°C, at which temperature seeding was automatically induced. Ovarian tissue was further cooled at a rate of 0.5°C/min to −50°C and subsequently at 5°C/min to −80°C, and finally at 8°C/min to −120°C. The tissue was then transferred to liquid nitrogen (−196°C) and stored until autografting. The programmable freezer CM25 was used	The tissue was equilibrated at 4°C for 10 min in a 0.7M dimethyl sulphoxide solution containing 20% human serum in an RPMI 1640 medium. Samples were then transferred to the freezing solution with a similar composition, but one containing 1.5M dimethyl sulphoxide, and were left to equilibrate for 10 min at +4°C	Thawing was performed in a water bath at +37°C until the ice was melted. The freezing medium was stepwise diluted over 7 min at +4°C to a final concentration of 0.14 M dimethyl sulphoxide, by adding a double volume of RPMI 1640 medium supplemented with 4% of HSA each minute. Ovarian tissue was then equilibrated over 3 min at +4°C, before replacing the diluted medium with the Flushing Medium. Thereafter, the ovarian tissue and Flushing Medium were equilibrated for 25 min at +37°C in a 5% solution of CO_2 to allow a more complete cell rehydration before grafting	Callejo *et al.*, 2001, 2013
7	Slow freezing: samples were cooled from +1°C at a rate of −2°C/min to −9°C. After manual seeding, cooling at a rate of −0.3°C/min was performed down to −40°C, followed by rapid cooling at a rate of −10°C/min down to −140°C. Samples were then transferred to liquid nitrogen. The programmable freezer Kryo10 was used	Ovarian tissue pieces were placed into the pre-cooled (+4°C) cryoprotectant solution: Leibovitz L-15 medium containing 1.5 M dimethyl sulphoxide (DMSO), 0.1 M sucrose and 10% (vol/vol) HSA	The vials with samples were thawed individually by agitating them in water at room temperature (~100°C/min). The cryoprotectant was quickly removed from the tissue by repeated washing in saline and a fresh quantity of the Leibovitz medium	Newton *et al.*, 1996; Revel *et al.*, 2009, 2011

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Table 2 Continued

No.	Vitrification/Slow freezing	Cryoprotectors	Thawing	Reference
8	Vitrification: the straw with sample was immersed vertically into liquid nitrogen (−196°C) for cooling	The vitrification solution (VS) contained H199 supplemented with 20% SSS, 5.64 M ethylene glycol, 5% (w/v) polyvinylpyrrolidone and 0.5 M sucrose. Three steps for the equilibration of ovarian tissue were used. Samples were placed in H199 supplemented with 20% SSS and 1.61 M ethylene glycol for 10 min, transferred to H199 supplemented with 20% SSS and 3.22 M ethylene glycol for 10 min and equilibrated in VS for 5 min. All procedures were performed at room temperature (+24–26°C). After equilibration in 1 ml VS for 5 min at room temperature, 0.2 ml of ovarian tissue cubes and vitrification solution were packed into 0.5 ml straws. Both ends of the straw were sealed	For warming, straws were warmed in water at +35°C for 10 s and the tissue cubes were expelled into 1 ml of H199 containing 20% SSS and 0.8 M sucrose (pre-warmed at +37°C). The tissue cubes were transferred to H199 containing 20% SSS and 0.4 M sucrose, and then to H199 containing 20% SSS for twice 5 min each at room temperature. After removal of the cryoprotectant, the ovarian sections were kept in H199 containing 20% SSS until transplantation	Suzuki *et al.*, 2012; Kawamura *et al.*, 2013

DMSO, dimethyl sulphoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; HSA, human serum albumin; PBS, phosphate-buffered saline; SSS, serum substitute supplement.

promising for patients who suffer from hormone-dependent types of cancer. Furthermore, it is very simple to realize monitoring of transplanted tissue and to obtain mature oocytes. However, the application of xenotransplantation in routine clinical practice is not possible, due to the insufficiency of data about disease transmission from animals to humans via ovarian tissue (Seli & Tangir, 2005). Thus, the ovarian tissue xenotransplantation technique may be applicable only in research studies until the question of infection transmission is resolved.

Orthotopic, heterotopic and xenotransplantation methods have several advantages and drawbacks, as discussed above. In summary, orthotopic ovarian tissue retransplantation currently seems to be more optimal than the other techniques. In the case of orthotopic retransplantation, a tissue fragment is placed in a native environment with adequate temperature, blood pressure, mechanical tension and normal hormone and paracrine regulation. Furthermore, in contrast with heterotopic retransplantation and xenotransplantation, natural conception may occur in the case of orthotopic retransplantation. At the present time, xenotransplantation cannot be used in routine clinical practice, and the effectiveness of heterotopic retransplantation still remains unclear: there is only one case of a live birth after IVF procedures with an oocyte obtained from heterotransplanted ovarian tissue (Stern *et al.*, 2013). In all other known cases of live births after ovarian tissue retransplantation, the orthotopic technique was used.

Effectiveness of ovarian tissue cryopreservation

The effectiveness of cryopreservation and thawing procedures can be estimated using different methods. Evaluation of a tissue's condition after cryopreservation and thawing can be performed via histological analysis. However, the only reliable way to prove that

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a cryopreservation and thawing technique is effective is to obtain mature oocytes capable of fertilization and further development, followed by the live birth of healthy baby.

At present, more than 25 cases of successful ovarian tissue cryopreservation that led to childbirth are known (Donnez *et al.*, 2004, 2013; Meirow *et al.*, 2005; Chung *et al.*, 2013). Most of these cases are listed in Table 3. However, although the data are very promising, it is not known exactly whether the oocytes ovulated from the retransplanted ovarian tissue or the remaining fragment of the ovary. In most cases, pieces of ovary remain in the patient's body after an ovarian tissue biopsy, which is required for better survival rates of retransplanted tissue. It is only in isolated instances that women need a total bilateral oophorectomy, so only in these cases is it possible to declare confidently that an embryo has developed successfully from an oocyte obtained from the thawed ovarian tissue.

Cryopreservation of ovarian tissue followed by retransplantation is not applicable in several cases. Many scientific groups (Meirow *et al.*, 2005; Silber *et al.*, 2005; Donnez *et al.*, 2006a, 2010a, 2013; Andersen *et al.*, 2008; Sanchez *et al.*, 2008; Schmidt *et al.*, 2011; Silber, 2011) agreed that, as a rule, 35 is the upper age limit for the cryopreservation of ovarian tissue. This situation is due to the fact that, after the procedure of cryopreservation of ovarian tissue, follicles are preserved mainly in the early stages of folliculogenesis (primary and primordial follicles), the number of which reduces significantly with age.

In the case of prepubertal girls, obtaining ovarian tissue, followed by its cryopreservation and retransplantation, will negatively influence the patient's health. Such procedures will provoke harmful hormonal stress. In these cases, it seems better to obtain mature oocytes from ovarian tissue cultured *in vitro* and use them in surrogate programmes.

In some instances, ovarian tissue cryopreservation cannot be used (for example, when the patient is a prepubertal girl) or is not very effective (when the patient is older than 35). In these cases, the ovarian tissue *in vitro* culture technique can be applied for female fertility preservation.

Ovarian tissue *in vitro* culture

There are two methods for an ovarian tissue *in vitro* culture technique: (i) two-dimensional (2D); and (ii) three-dimensional (3D) systems (Hirao, 2012). The principal drawback of 2D systems is the low correspondence between the microenvironment of the cultured fragment and the *in vivo* conditions. In the case of the 2D method, the ovarian tissue culture's stroma and follicular cells migrate along the plane, follicles lose their normal shape and oocytes lose their normal cellular environment. Regulation of the 2D system's conditions may be performed only by adding various chemical agents (growth factors, hormones, etc.) to the culture medium. Three-dimensional systems allow us to regulate the condition of cultivated ovarian tissue, not only by adding different chemical agents but also by variation of microenvironmental physical properties.

For *in vitro* culture, the whole ovary (Jin *et al.*, 2010), ovarian fragments or single follicles can be used (Xu *et al.*, 2006b). *In vitro* culture of a whole ovary or ovarian fragments has a significant disadvantage. This is due to the difficulties of nutrient delivery and oxygenation in the deep layers of cultivated tissue. Therefore, studies dedicated to isolated ovarian follicles in an *in vitro* culture seem to be the most effective. In the case of isolated ovarian follicles *in vitro* culture, all cultivated follicles receive required nutrients that penetrate into all of the follicular compartments via diffusion. Moreover, when single ovarian follicles are cultivated, it is possible to trace the development of each follicle, which is not possible in the cases of a whole ovary or ovarian tissue fragments *in vitro* culture.

Mammalian ovarian follicles *in vitro* culture can be performed using various substrates, e.g. agarose, collagen, Matrigel or alginates and their derivatives. The main parameters of different substrates used for ovarian follicles *in vitro* culture are listed in Table 4. Described substrates belong to the group of hydrogels, which are polymeric compounds that are powdery when dried. If water or another solvent is added to the dry hydrogel, its granules swell, resulting in the formation of a gel-like substance. This gel can be used further for the *in vitro* culture of various cell types, particularly for the culture of ovarian follicles.

The described substrates have various limitations in their applications. For instance, agarose gelation requires a high temperature but heating is very harmful to ovarian follicles. The most commonly methods used for hydrogel gelation (exposure to UV radiation, high temperatures and aggressive chemical treatment) are dangerous for the living objects.

Alginate hydrogel gelation does not require toxic agents or application of non-physiological conditions (high temperature, UV radiation, etc.). Only the supplementation of a solution containing cross-linking agents (Ca^{2+} or Mg^{2+} ions) is needed for alginate polymerization. The effect of the conditions of hydrogel preparation on living cells is less negative. This is because alginate hydrogel preparation does not require temperatures higher than 37°C (physiological temperature) and polymerization agents (divalent cations, added to most culture mediums) have no

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Table 3 Cases of successful ovarian tissue cryopreservation that led to live birth

No. of patient	Age at cryopreservation/age at ovarian tissue transplantation	Disease	Chemotherapy before ovarian tissue cryopreservation	Transplantation type	Conception type	Note	Reference
1	25/32	Granulosa cell tumour	No	Heterotopic	IVF	Bilateral oophorectomy. Fertilization by ICSI. Dichorionic diamniotic twins	Stern *et al.*, 2013
2	25/29	Hodgkin's lymphoma	No	Orthotopic	Spontaneous	Singleton	Donnez *et al.*, 2004
3	28/30	Non-Hodgkin's lymphoma	Yes	Orthotopic	IVF	Singleton	Meirow *et al.*, 2005
4	24/28 and 30	Hodgkin's lymphoma	Yes	Orthotopic and heterotopic	Spontaneous	Unilateral oophorectomy was performed. Two singletons childbirths have been observed after orthotopic ovarian tissue transplantation	Demeestere *et al.*, 2007, 2010
5	26/28	Hodgkin's lymphoma	Yes	Orthotopic	IVF	Unilateral oophorectomy. Singleton	Andersen *et al.*, 2008
6	27/30	Ewing sarcoma	No	Orthotopic	IVF and spontaneous	Whole left ovary and two-thirds of the right ovary were removed. Two singletons	Andersen *et al.*, 2008; Ernst *et al.*, 2010
7	20/22	Sickle-cell anaemia	No	Orthotopic	Spontaneous	Singleton	Roux *et al.*, 2010
8	36/38	Breast cancer	No	Orthotopic	IVF	Fertilization by ICSI. Twins	Sánchez-Serrano *et al.*, 2010
9	17/25	Metastatic neuroectodermic tumour	No	Orthotopic	Spontaneous	Singleton	Donnez *et al.*, 2011
10	20/23	Hodgkin's lymphoma	No	Orthotopic	Spontaneous	Singleton	Donnez *et al.*, 2011
11	27/31	Microscopic polyangiitis	Yes	Orthotopic	IVF	Singleton	Donnez *et al.*, 2011
12	27/32	Hodgkin's lymphoma		Orthotopic	Spontaneous	Singleton	Dittrich *et al.*, 2015
13	28/31	Breast cancer		Orthotopic	Spontaneous	Singleton	Dittrich *et al.*, 2015
14	35/37	Hodgkin's lymphoma		Orthotopic	Spontaneous	Singleton	Dittrich *et al.*, 2015
15	30/35	Ovarian cancer		Orthotopic	Spontaneous	Singleton	Dittrich *et al.*, 2015

Table 3 Continued

No. of patient	Age at cryopreservation/age at ovarian tissue transplantation	Disease	Chemotherapy before ovarian tissue cryopreservation	Transplantation type	Conception type	Note	Reference
16	21/29	β-Thalassaemia	Yes	Orthotopic	Spontaneous	Singleton	Revelli *et al.*, 2013
17	20/30	Dermoid cysts	No	Orthotopic	IVF	Bilateral oophorectomy. Fertilization by ICSI. Singleton	Callejo *et al.*, 2013
18	19/23	Transfusion-dependent but well chelated thalassaemia	No	Orthotopic	IVF	Singleton	Revel *et al.*, 2011
19	23/31, 32, 36	Ewing sarcoma	No	Orthotopic	IVF	Fertilization by ICSI. Singleton	Rodriguez-Wallberg *et al.*, 2015
20	18/26	Tubo-ovarian abscesses	No	Orthotopic	IVF	Both ovaries and tubes were removed. Fertilization by ICSI Singleton	Donnez *et al.*, 2012
21	29/29	Amenorrhoea	No	Orthotopic	IVF	Both ovaries were removed. Fertilization by ICSI. Singleton	Kawamura *et al.*, 2013
22	24/26	Thyroid cancer	No	Orthotopic	IVF	Singleton	Kiseleva *et al.*, 2014

ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

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Table 4 Substrates used for *in vitro* ovarian follicles' culture

Material	Origin	Method of gelation	Method of digestion	Interactions with cells	Organism	References
Collagen	Animal	Heating	Collagenase	Binds to integrins	Mouse	Itami *et al.*, 2011; Mochida *et al.*, 2013;
					Cow	Sharma *et al.*, 2009.
Matrigel	Animal	Heating	Collagenase	Binds to integrins	Mouse	Oktem & Oktay, 2007c; Oktem *et al.*, 2011; Guzel *et al.*, 2014
Agarose	Plant	Heating	Agarase	None	Dog/Cat	Fujihara *et al.*, 2013.
Alginate	Plant	Ionic cross-linking in the presence of divalent cations (i.e. Ca^{2+})	Alginate lyase or EGTA	None	Mouse	Xu *et al.*, 2006a; Choi *et al.*, 2014; Skory *et al.*, 2015
					Human	Amorim *et al.*, 2009; Xu *et al.*, 2009; Skory *et al.*, 2015
					Rhesus monkey	Hornick *et al.*, 2012
					Baboon	Xu *et al.*, 2011b

EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetra-acetic acid.

toxic effects on cells. Moreover, cultivated cells do not form contacts with an alginate hydrogel. The alginate hydrogel destruction procedure does not impair cultivated follicles, because substances that digest the alginate hydrogel are unable to destroy cell contacts.

One of the principal advantages of alginate substrates is their vegetative origins. Therefore, alginate hydrogels can be used in 'animal-free' projects. In some studies, e.g. human ovarian follicle culture, the application of substances of animal origin is forbidden because it may lead to the contamination of the culture medium by viruses, proteins and other molecules that are able to interact with the cultivated tissue. There is no consensus on horizontal gene transfer when tissues or substances from different species are cocultured.

Effectiveness of ovarian tissue *in vitro* culture

To date, the ovarian tissue *in vitro* culture technique is not a widely used technique in clinical practice because the developing methods are far from perfect. Furthermore, there are no standardized technique protocols for effectively obtaining oocytes capable of *in vitro* fertilization. There are two principally distinct strategies of ovarian tissue *in vitro* culture: *in vitro* culture of ovarian cortical tissue fragments (strips) and *in vitro* culture of isolated ovarian follicles.

The cortical compartment of an ovary contains a large number of primordial follicles. Most commonly, ovarian tissue fragments are obtained by dissection of the cortical tissue into strips, with their subsequent culturing *in vitro.* This approach allows to support the growth of primordial follicles (Telfer *et al.*, 2008).

Another approach to culture of ovarian tissue *in vitro* is based on the culture of isolated ovarian follicles. Commonly, primary and secondary follicles are used for this technique. For the ovarian tissue *in vitro* culture technique, researchers use various substrates that allow the stimulation of follicle growth and oocyte maturation, e.g., alginate (Xu *et al.*, 2006a, 2009, 2011b; Amorim *et al.*, 2009; Hornick *et al.*, 2012; Choi *et al.*, 2014; Skory *et al.*, 2015), collagen (Sharma *et al.*, 2009; Itami *et al.*, 2011; Mochida *et al.*, 2013), Matrigel (Oktem & Oktay, 2007c; Oktem *et al.*, 2011; Guzel *et al.*, 2014) and agarose (Fujihara *et al.*, 2012). There are promising results showing that the ovarian tissue *in vitro* culture technique allows not only to stimulate follicular growth but to obtain mature oocytes that are competent for development after fertilization (Xu *et al.*, 2006b, 2011a, 2013; Shikanov *et al.*, 2009, 2011; Jin *et al.*, 2010; Choi *et al.*, 2014).

In comparison with the method of ovarian tissue cryopreservation followed by retransplantation, the method of *in vitro* culturing of ovarian tissue allows to obtain mature oocytes not only from primary and primordial follicles that can be cryopreserved but also from follicles at advanced stages of development, e.g., from the secondary follicles (Mochida *et al.*, 2013).

It has been shown that the *in vitro* culturing of murine ovarian follicles can lead to the formation of

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mature oocytes that are able to undergo successful fertilization and further development. Furthermore, after the transfer of these embryos to a surrogate mother, the live birth of healthy pups has been obtained (Xu *et al.*, 2006b). However, live births of healthy pups after *in vitro* culturing of ovarian tissue have been shown in only a few studies (Yamamoto *et al.*, 1999; Hasegawa *et al.*, 2006; Xu *et al.*, 2006b; Mochida *et al.*, 2013; Higuchi *et al.*, 2015). Such significant results motivate researchers to develop more advanced systems that will allow us to obtain mature oocytes more effectively.

The latest studies of the ovarian tissue *in vitro* culture technique are dedicated to the reconstruction of the ovarian corticomedullary structure. During folliculogenesis, *in vivo* growing follicles migrate from the more rigid ovarian cortex to the soft ovarian medulla (Xu *et al.*, 2013). Therefore, in a natural environment, mechanical stress reduction occurs in follicular cells. Reconstruction of the corticomedullary ovarian structure *in vitro* can be performed using combined hydrogels; for example, a fibrin-alginate hydrogel can be used (Shikanov *et al.*, 2009, 2011; Jin *et al.*, 2010). A growing follicle secretes proteases that destroy fibrin, resulting in mechanical stress decreasing around the follicle and thus promoting its development (Shikanov *et al.*, 2011).

Another approach to reconstruct the ovarian corticomedullary structure *in vitro* is using an alginate-collagen system. The drop in which a single follicle is cultivated contains two compartments. The centre of the drop consists of collagen, and its periphery is formed with alginate. This technique allows to create a system with heterogenic mechanical tensions. Collagen is softer than alginate, so collagen imitates the medulla and alginate imitates the cortex (Choi *et al.*, 2014). It has been shown that follicular development up to the late antral stage occurs in 28% of cases when an alginate–collagen system is used (Choi *et al.*, 2014). Preparation of such a complex culture system can be performed using microfluidic technologies (Streets & Huang, 2014). These technologies allow to create drops consisting of various substances in the required proportions by using directed microflows of liquids. The type of microflow is determined by the microfluidic chip configuration.

Technologies of 3D ovary reconstruction *in vitro* are being actively developed (Vanacker *et al.*, 2012; Luyckx *et al.*, 2013). The main conception of this technology is the encapsulation of ovarian follicles with other ovarian cells into the matrix. At the present time, this technique is at the first stage of its development but it seems to be very promising in the future. These systems will allow better reconstitution of *in vivo* conditions, resulting in the better stimulation of follicles' growth and maturation.

Rodent ovarian follicles are most commonly used to develop ovarian tissue *in vitro* culture techniques. However, it is impossible to create an effective technology for medical practice on the basis of rodent ovarian tissue. Firstly, rodents have shorter ovarian cycles than the human female's one. Moreover, the ovarian tissue of primates has a higher density than the rodent one. Therefore, for the development of medical technology, studies on primate ovarian tissue *in vitro* culture are needed.

There is little scientific research related to primate ovarian follicles *in vitro* culture. However, the obtained results seem to be very promising. It has been shown that baboon follicles can produce mature oocytes that are capable of fertilization and can develop until the morula stage after fertilization via the intracytoplasmic sperm injection (ICSI) procedure (Xu *et al.*, 2011b).

Other research that was performed on rhesus monkeys has demonstrated that more rigid alginate hydrogels allow for better development of follicles than softer ones do (Hornick *et al.*, 2012). Moreover, mature oocytes capable of fertilization and further development up to the morula stage after fertilization by using the ICSI procedure have been obtained during a study on rhesus monkey ovarian follicles, using a fibrin-alginate hydrogel culture system (Xu *et al.*, 2013).

Research studies dedicated to human ovarian follicles *in vitro* culture are few in number. However, there are very encouraging results among that research. In the early works on human ovarian follicles *in vitro* culture, follicles were encapsulated into the agar (Roy and Treacy, 1993) or collagen gel (Abir *et al.* 1997, 1999, 2001), and they maintained their morphology. Moreover, some of the follicles developed until the early antral stages. A recent study has shown that alginate hydrogel permits the growth of encapsulated human ovarian follicles, corresponding to the normal folliculogenesis *in vivo.* Furthermore, an increase of oestradiol, progesterone and inhibin A during the period of cultivation has been observed (Xu *et al.*, 2009). These hormones are required for normal follicular development.

A scientific group from Belgium has shown that human ovarian follicles can grow in alginate hydrogel. Moreover, in 90% of cases, follicular cells and oocytes retained their viability (Amorim *et al.*, 2009).

In more recent work, researchers from the USA developed an alginate culture system that imitates follicles' microenvironment *in vivo.* Moreover, this scientific group (Skory *et al.*, 2015) has developed alginate hydrogel ovarian follicles *in vitro* culture system that reconstitutes natural conditions. Hormone levels (17β-oestradiol, progesterone, inhibin A, inhibin B, activin A and anti-Müllerian hormone) of cultured follicles were observed by an enzyme-linked immunosorbent

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assay. When the level of 17β-oestradiol increased and stabilized on the highest value, ovulation was induced by the addition of human chorionic gonadotropin (hCG) and epidermal growth factor (EGF) into the culture medium. Due to the monitoring of follicular hormone secretion, researchers imitated menstrual cycle conditions *in vitro* by adding hormones and growth factors to the culture medium.

The human ovary contains a large portion of connective tissue, the amount of which varies greatly from patient to patient. Therefore, it appears impossible to develop a standard protocol of a primordial follicle's isolation from human ovarian tissue. This is due to the fact that the period of enzymatic treatment of human ovarian tissue for the obtaining of primordial follicles varies noticeably in different patients. Moreover, the loss of the integrity of primordial follicles with surrounding somatic cells, which may occur during this procedure, negatively influences their condition (Laronda *et al.*, 2014). Therefore, for better female fertility preservation, both techniques can be used: ovarian tissue cryopreservation in the case of primordial follicles, and ovarian follicle *in vitro* culture for follicles of advanced stages.

A two-step culture system is another approach that can also be performed for primordial follicle preservation. In these systems, small fragments of ovarian cortical tissue that contain primordial follicles are first cultured in order to stimulate follicular development. After the cultivation, follicles of advanced stages of folliculogenesis (primary, secondary, etc.) are isolated from the ovarian tissue. Subsequent cultivation is carried out with the obtained follicles (Telfer *et al.*, 2008; McLaughlin *et al.*, 2014).

Akt-signalling pathway inactivation allows to stimulate growth of primordial follicles *in vitro.* The Akt-pathway suppressor is PTEN (phosphatase and tensin homologue). It has been shown that PTEN inactivation results in an increase in the number of growing ovarian follicles in the cases of humans (McLaughlin *et al.*, 2014) and mice (Jagarlamudi *et al.*, 2009).

Nowadays, mature oocytes that are capable of fertilization can be obtained not only from rodents but also from primates. Furthermore, these oocytes can develop after fertilization. Designing human ovarian tissue *in vitro* culture systems is a very complicated problem. Firstly, it is more difficult to obtain human ovarian tissue for the experiments than in the case of animal models. Secondly, human follicular development *in vivo* is longer than that of rodents, for example. Due to these facts, human ovarian tissue *in vitro* culture systems are at the very earliest stages of their development. However, the growth of human ovarian follicles *in vitro* has still been observed. It is likely that findings that will allow the obtaining of mature human oocytes from ovarian tissue using *in vitro* culture may appear in the near future.

Conclusion

The first papers dedicated to the cryopreservation of mammalian ovarian tissue were published in 1994 (Aubard *et al.*, 1994; Harp *et al.*, 1994), more than 20 years ago. However, to date, we know of only about a few dozen live births after ovarian tissue orthotopic retransplantation and one live birth after heterotopic retransplantation. In many cases, after ovarian tissue retransplantation, it is impossible to determine whether ovulation occurred in the transplanted tissue or in a remaining fragment of the ovary, especially in the event of natural conception. Therefore, the efficacy of ovarian tissue cryopreservation followed by retransplantation is low. Moreover, the ovarian tissue retransplantation technique cannot be applied in many cases. Retransplantation of ovarian tissue is an additional surgical procedure that can be dangerous for patients who have undergone chemo- or radiotherapy. Furthermore, it is necessary to detect the absence of malignant cells in transplanted tissue. For this reason, several pieces of tissue should be used for analysis that cannot then be transplanted, which reduces the probability of pregnancy. For a time after retransplantation, ovarian tissue is subjected to weak nutrition until angiogenesis is complete. A deficit of nutrients and oxygen leads to apoptosis and atresia in transplanted tissue, which also reduces the probability of pregnancy.

In comparison with the cryopreservation of ovarian tissue followed by retransplantation of thawed fragment ovarian tissue, the *in vitro* culture technique has a few principal advantages. Firstly, the retransplantation of ovarian tissue is not needed. Secondly, mature oocytes can be obtained and fertilized *in vitro,* and then embryos can be cryopreserved or transferred into the patient's uterus or into the womb of a surrogate mother. In the case where ovarian tissue cryopreservation is applied for fertility preservation, surrogate mother programmes require additional procedures such as hormonal stimulation and follicular punctures. These procedures can have a negative effect on patients' health. An ovarian tissue *in vitro* culture programme of fertility preservation allows to avoid the risky procedures mentioned above. Moreover, this method permits use of all obtained tissue for the culture. Analysis for the presence of malignant cells in the culture can be performed after tissue culturing. Therefore, the ovarian tissue *in vitro* culture technique increases the probability of pregnancy when compared with ovarian tissue cryopreservation. The ovarian

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Table 5 Female fertility preservation strategies' comparison: ovarian tissue *in vitro* culture vs. cryopreservation

Ovarian tissue cryopreservation followed by tissue retransplantation	Ovarian tissue *in vitro* culture
A second surgical operation is required for the transplantation of thawed tissue	Surgical intervention is needed only for tissue isolation
Part of the tissue should be analysed for malignant cells' absence	All obtained tissue can be used for the culture; analysis for the presence of malignant cells can be performed after the tissue culture
It is difficult to observe each individual follicle	It is easy to observe the growth and development of each individual follicle
It is difficult to provide good nutrition for the ovary during angiogenesis interruption	Follicles can be cultured individually, facilitating the supply of follicles with nutrients
Technologies for ovarian tissue cryopreservation have low efficacies	Ovarian tissue *in vitro* culture allows to obtain mature oocytes capable of fertilization. Oocyte and embryo cryopreservation technologies are more effective in comparison with the cryopreservation of ovarian tissue
Age limitations – less than 35 years old	No age limitations

tissue *in vitro* culture method allows observation of each follicle individually, and the effective provision of them with necessary nutrients.

Although the ovarian tissue *in vitro* culture is not currently a well developed technique that is applied widely in clinical practice, there are many results showing the efficacy of this method (Xu *et al.*, 2011a; Jin *et al.*, 2010; Xu *et al.*, 2006b; Choi *et al.*, 2014; Xu *et al.*, 2013; Shikanov *et al.*, 2011; Shikanov *et al.*, 2009). The ovarian tissue *in vitro* culture technique allows to obtain mature oocytes capable of fertilization and further development, not only in the case of rodents (Xu *et al.*, 2006b), but also for primates (Xu *et al.*, 2011b; Xu *et al.*, 2013). Moreover, the growth of human follicles was observed when using this method (Xu *et al.*, 2009).

Therefore, the ovarian tissue *in vitro* culture technique seems more promising than ovarian tissue cryopreservation followed by thawed tissue retransplantation. The principal advantages and drawbacks of the methods mentioned above are listed in Table 5. In contrast with cryopreservation, the ovarian tissue *in vitro* culture technique allows the avoidance of secondary surgical intervention and patient hormonal stimulation. Moreover, in the case of the ovarian tissue *in vitro* culture technique, it is easier to provide better nutrition for follicles and to observe their growth and development. All of these advantages help practitioners to yield better results.

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None.

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