Chapter 3

Gamete Preservation

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Introduction

With the increase in survivorship following cancer for women in their reproductive years, as well as an increase in survivorship with childhood cancers, there is a demand for perfecting current fertility preservation methods and generating new options for patients who are unable to pursue the conventional course of fertility treatments. Cryopreservation using a slow-cooling method for embryos is currently the standard-of-care for women wishing to preserve their fertility; other options, such as oocyte cryopreservation and embryo vitrification, have become increasingly accepted methods of fertility preservation.

As important as oocyte and embryo cryopreservation methods are for preserving fertility, some patients may be too sick or too young to undergo fertility treatments or have hormone-sensitive cancers that preclude standard approaches; therefore, other preservation options must be explored. Ovarian tissue cryopreservation followed by ovarian tissue transplant and follicle cryopreservation followed by in vitro follicle maturation are experimental techniques that have shown promising results. This chapter will discuss current methods of gamete preservation as well as new, experimental options to preserve immature gametes. An overview of ovarian biology, as well as the science of cryobiology, is discussed to help the reader better understand the circumstances under which different techniques may be used.

Organization of Gametes in the Human Ovary

The human ovary is divided into three main areas (Fig. 3.1): the hilum, the inner medulla, and the outer cortex. The hilum is the area attached to the mesovarium and it is the entry point for nerves and blood vessels serving the ovary. The medulla is the

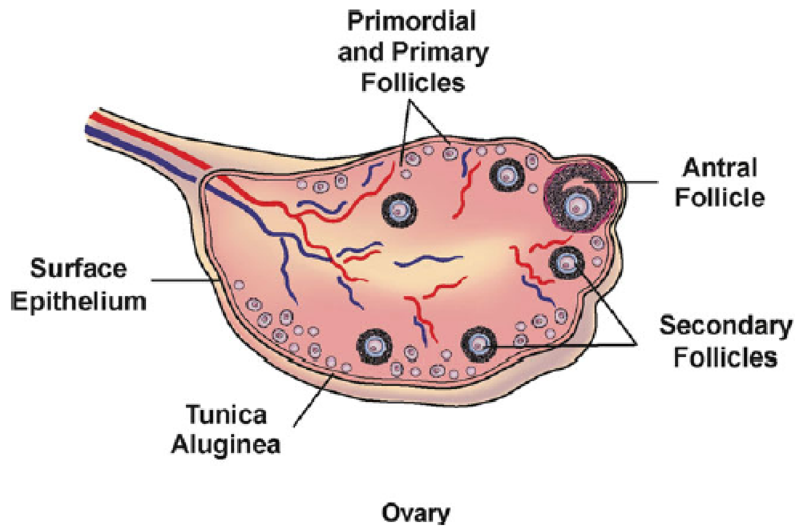

Fig. 3.1 A human ovary showing the blood supply feeding the arrangement of follicles within the ovarian cortex

central vascular region of the ovary that nourishes immature and growing follicles with necessary hormones and gas exchange. The ovarian cortex, which is approximately 2–3 mm thick and surrounds the central medulla, contains all immature and growing germ cells as well as supportive and endocrine hormone-producing cells. The dense outer layer of the cortex, just under the surface epithelium, is called the tunica albuginea and is filled with dense connective tissue that gives support to the primordial follicle population.

At the time of birth, the human ovary contains the total number of germ cells it will need and use through the reproductive years [1–4]. At this time, all germ cells exist as immature oocytes inside primordial follicles, the smallest functional units of the ovary. Each follicle is comprised of an oocyte surrounded by few interconnected squamous epithelia cells and a basal lamina. In a prepubescent ovary, primordial follicles are scattered throughout the cortex with the majority of the follicles residing near the outer edge of the ovarian cortex [5]. During childhood, low levels of gonadotropins stimulate some of the follicles to exit the arrested primordial state and begin the process of follicle growth; however, these follicles become atretic and do not produce mature oocytes [6, 7]. It is not until the onset of puberty that selected primordial follicles undergo follicle growth, or folliculogenesis, in a cyclic fashion.

In folliculogenesis, the growth of selected follicles proceeds from the primordial to primary follicle stage, during which the squamous epithelium becomes cuboidal in shape and proliferates to form one complete layer of follicle cells. At this time, the central oocyte also begins to grow and secrete proteins on its surface, generating a protective shell called the zona pellucida [8]. The follicle continues to grow with proliferation of follicle or granulosa cells to form multiple cell layers surrounding the oocyte, which in turn stimulate the growth of the oocyte by producing paracrine factors and forming physical cell–cell interactions. As the follicle increases in size, the antral cavity forms, separating the follicle cells into two distinct types of supportive cells, the mural and

cumulus granulosa. The mural granulosa remain toward the outer edge of the follicle and express an abundance of luteinizing hormone (LH) receptor, which is essential for ovulation in response to the LH surge. These cells, in conjunction with the theca cells that surround the follicle externally, are essential for aromatization (or conversion) of testosterone to estradiol [9]. The cumulus granulose cells stay connected to the oocyte and support and nourish the growing oocyte by transporting necessary molecules such as amino acids [10, 11], cAMP [12, 13], glucose [14], and pyruvate [15] through gap junctions. As follicles grow, the fluid filled antral cavity expands, pushing outward and reaching preovulatory sizes of 12–15 mm in diameter. These mature follicles contain oocytes that are capable of resuming meiosis and undergoing fertilization [4, 16]. For an excellent review on hormone regulation in follicular development, see Zeleznik et al. [17].

Cryopreservation Techniques

Cryopreservation holds tissues at temperatures between –140 and –200!C, at which no biological activity can occur, producing a state of "suspended animation" of tissues that can be maintained indefinitely [18]. It is the process of cooling and warming, not long-term cryo-storage, that harms cells or tissue [19]. Currently, there are two main approaches for cryopreservation: slow-cooling and vitrification. The success of either of these approaches depends upon the tissue, the cryoprotectant, and the freezing vessel used.

The most important hurdle in cryopreservation is avoiding the phase transition between water and ice inside the cell. Because water is everywhere in a cell and is important for the function of macromolecules and larger structures such as lipid membranes, the formation of large ice crystals upon cooling destroys cellular components and ruptures intact membranes [20]. Interestingly, pure water will supercool substantially and form ice only at approximately –39!C [18], which is much lower than the temperature at which ice is thermodynamically stable (<0!C). Impurities, such as dust, act as ice nucleators, initiating large crystal formation at temperatures well above –39!C [18]. However, allowing ice crystals to form at temperatures more than a few degrees below zero during cooling has been shown to damage embryos and oocytes [21]. To initiate crystal formation in a solution at higher temperatures, the solution must be seeded with ice either by the addition of an ice crystal or by touching it with something colder, such as a forceps dipped in liquid nitrogen, as is done in the laboratory. As ice crystals grow, the volume of the unfrozen solution decreases, thus increasing the concentration of solutes (e.g., salt), which not only helps to reduce ice formation inside the cell but also severely dehydrates cells and can cause cell damage and death [22–24].

Cryoprotectants are defined functionally as any compound that increases cell survivability when used in a cryopreservation method. There are many different types of cryoprotectants, including alcohols, sugars, oils, and starches, and each type acts through different mechanisms. However, a good cryoprotectant is one that can preserve cell structures and is not toxic. James Lovelock first described the mechanism of action of cryoprotectants in his experiments showing that erythrocytes (red blood cells) freeze at

lower temperatures when combined with glycerol. He found that the increase in salt concentration resulting from ice crystal formation causes hemolysis [25, 26]. His work highlights the delicate balance between the many factors that must be considered when developing a successful cryopreservation method.

Currently, there are three widely used permeating cryoprotectants in fertility preservation: dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PG). These cryoprotectants have similar properties: solubility in water at low temperatures, cell permeability, and relatively low toxicity. However, each of these cryoprotectants also has different degrees of membrane permeability, as has been shown with mammalian oocytes [27–29]. Mouse oocytes seem very hearty and are capable of being cryopreserved using several different cryoprotectants; however, evidence suggests that EG is less toxic to mouse oocytes that have resumed meiosis and reached metaphase-II (MII) [30–32]. In contrast, rhesus monkey oocytes are much more sensitive to cryoprotectants and have been shown to be less permeable to glycerol than to PG, EG, and DMSO, and at room temperature, the oocytes are more permeable to PG than to the other cryoprotectants [28, 33]. Interestingly, it was found that PG causes potentially lethal effects when used in human oocyte cryopreservation protocols designed for DMSO. By incubating oocytes in PG at a higher incubation temperature for a shorter time, it may be possible to prevent oocyte lysis [34].

Conventional cryopreservation is the process of slow-rate freezing in which a relatively low concentration of cryoprotectant is used (!1.5 (M)olar), showing little toxicity to cells or tissue [35]. As cryoprotectant is added to cells, it results in initial cellular dehydration followed by a return to isotonic volume with the permeation of cryoprotectant and water. Generally, cells are cooled slowly using a controlledrate freezing machine, which allows samples to be cooled at various rates; ovarian tissue is generally cryopreserved at 2"C/min prior to ice seeding and 0.3"C/min after crystallization to ensure the tissue is dehydrated before intracellular ice formation occurs. Optimal rates to minimize intracellular ice formation vary among cell and tissue types; for example, stem cells survive better at a freezing rate of 1"C/min and red blood cells at a rate of 1,000"C/min [35].

As mentioned above, extracellular ice nucleation is triggered manually and must be performed above the temperature of intracellular ice formation. The temperature at which optimal nucleation is performed is determined by the cryoprotectant used as well as the characteristics of the cells [36]. It has been shown that the optimal seeding temperature for human oocytes [36] is different from that for primate [37] and mouse oocytes [38, 39] and varies depending on the meiotic stage of the oocyte [36].

Slow cryopreservation has been used for a number of years; however, in some instances, it can be inconsistent and require expensive equipment. More recently, the process of vitrification has shown to be more successful for some cell and tissue types. Vitrification is a process that uses very high rates of cooling, so fast that water is solidified without crystallization, “like glass.” Some investigations of vitrification have shown that extremely high concentrations of cryoprotectants do not crystallize when cooled, even if

it is done slowly. This approach is attractive from a technical standpoint; unfortunately, cyroprotectant solutions are toxic to cells at very high concentrations. Solute toxicity is a major drawback of using vitrification for preservation, even with high cooling rates. To reduce toxicity, concentrations of cryoprotectants can be lowered as long as cooling is fast enough to preclude ice formation. For example, the cooling rate for embryos exposed to 8.5 M ethylene glycol must be at least 100,000!C/min for vitrification to occur [40]. To achieve an extremely rapid rate of cooling, a small volume of solution must be used. Recently, specialized storage devices designed to achieve rapid rates of cooling have been designed for the vitrification of eggs and embryos. They hold a very small volume of solution, usually less than 1 μl. These devices have various configurations; the ideal device is covered by a thin wall of plastic and can be submerged directly into liquid nitrogen, thus maximizing the cooling rate. Vitrification is more difficult for sizeable tissue samples that require larger amounts of cryosolution, simply due to the inability to be cooled so quickly.

Although many cells and tissues can be successfully cryopreserved using slow-freeze methods or vitrification methods without intracellular ice formation and can be stored in liquid nitrogen indefinitely, there is still a risk of ice formation during the thawing process if it is conducted improperly. If samples are thawed slowly, ice crystals can form and/or grow causing more damage; however, if samples are thawed rapidly enough, there is little time for ice nucleation and growth to occur [41, 42]. After thawing, there is further risk of damage during the course of removing cryoprotectants. If cells are immediately put into a significantly lower concentration of cryoprotectant, water will rapidly move into the cell and the cells can swell and burst. Therefore, it is usually advised that a series of decreasing concentrations of cryoprotectant is used to slowly remove the cryoprotectants and gently rehydrate cells. As an alternative, it can also be very effective to use a non-penetrating cryoprotectant such as sucrose to reduce osmotic shock during the step-down process [43].

Embryo and Oocyte Cryopreservation

During the course of the last 55 years, the science of reproductive biology, namely in vitro fertilization (IVF), has coincided with the ability to preserve embryos and oocytes [18]. The storage of gametes is no longer reserved for infertile couples; it now exists as an option for women who wish to preserve their fertility for various reasons. With advances in oncology and anti-cancer therapy, more reproductive age women are surviving cancer. Unfortunately, many anti-cancer regimens and treatments for other diseases, such as autoimmune disorders, are gonadotoxic [44, 45]. Depending on the diagnosis, some women are able to postpone treatment and proceed through ovarian stimulation in order to collect oocytes from preovulatory follicles for either oocyte cryopreservation or fertilization and subsequent embryo cryopreservation.

During the last 30 years, ovarian stimulation protocols have been perfected, sometimes generating numerous mature oocytes. Prior to the development and use of cryopreservation in the clinic, women were limited in the number of oocytes they were able to fertilize and had to discard excess gametes [46]. Today, a large number of

embryos are cryopreserved as standard-of-care in order to avoid unnecessary rounds of hormone stimulation. Cryopreservation of embryos and oocytes are the best options for pursuing a family for women undergoing fertility preservation, with or without a cancer diagnosis [47, 48]. The first successful embryo cryopreservation was performed with eight-cell mouse embryos in 1972 [49, 50], and it took less than a decade after the birth of Louise Brown [51] for the successful cryopreservation and re-implantation of a human eight-cell embryo [52–54]. Since then, protocols have been developed and proved successful for cryopreservation of human embryos of all developmental stages [52, 53]. The survival rate for slow-rate cryopreservation is approximately 75%, with a pregnancy rate between 20 and 30% per transfer [55]. Though vitrification of embryos has been reported to have a 90% survivability rate and a higher pregnancy rate than slow-freeze protocols, it has not yet replaced conventional methods of embryo cryopreservation [56, 57].

Although embryo cryopreservation has been successful, there are situations in which oocytes must be cryopreserved. Cryopreservation of oocytes not only avoids ethical issues surrounding the preservation and long-term storage of embryos but is ideal for women who do not have a male partner or sperm donor at the time of ovarian stimulation. However, cryopreservation of oocytes has been shown to be much more complicated than cryopreservation of embryos. Approximately 50–65% of oocytes survive slow-rate freezing, and they are usually damaged from intracellular ice formation due to their large cytoplasmic volume [58]. The majority of oocytes that are cryopreserved are mature, aspirated from large preovulatory follicles, but they are not fertilized in vitro. They are arrested at MII, at which point the oocyte contains a small spindle of microtubules that aligns the maternal chromosomes. Unfortunately, this spindle is extremely sensitive to changes in temperature, which results in the depolymerization of microtubule fibers. Upon the thawing process, microtubules attempt to repolymerize, resulting in abnormal spindles and misaligned chromosomes [33, 59, 60]. In the last few years, new protocols, including the development of vitrification methodologies, have increased the survivability of cryopreserved oocytes up to 80% [61, 62].

Another source of oocytes for gamete preservation is ovarian tissue removed for ovarian tissue cryopreservation. Large follicles !1 mm can easily be seen on the ovarian cortex and follicles greater than 5 mm may be aspirated to obtain immature cumulus-oocyte complexes. As the ovarian tissue is processed (discussed below), smaller antral follicles rupture releasing oocytes that fall to the bottom of the dish ranging in size and quality from incompetent denuded oocytes to larger cumulus

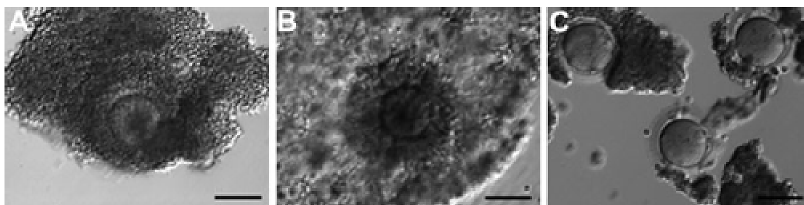

Fig. 3.2 Human cumulus–oocyte complexes isolated from large antral follicles during the tissue collection process at 0 h (**a**), 24 h (**b**), and 40 h (**c**) during the in vitro maturation process. *Black* measurement bar represents 100 µm

enclosed oocytes (Fig. 3.2a). Oocytes are collected from the bottom of every dish used and matured in vitro for up to 40 h. By 24 h in culture the cumulus granulosa, surrounding the oocyte, begins to mucify resulting in cumulus cell differentiation and expansion (Fig. 3.2b). By 40 h, oocytes are stripped of cumulus cells and are examined for meiotic stage (Fig. 3.2c); the resulting MII oocytes are vitrified for potential future use. Although there have been numerous reported pregnancies with in vivo matured cryopreserved oocytes [63–66], there have been few pregnancies using vitrified in vitro matured oocytes and no pregnancies have been reported with in vitro matured oocytes preserved by slow-rate cryopreservation [63, 64, 67].

Experimental Options for Gamete Preservation

During the last few years, there has been great progress in the field of oncofertility. Much research has been done in this field with the hope of broadening the range of fertility preservation options and for women facing potentially gonadotoxic treatments.

Ovarian Tissue Cryopreservation and Transplantation

Not all women are able to delay anti-cancer regimens for the 2–5 weeks needed to undergo hormonal stimulation to preserve mature oocytes or embryos. Other options have been developed to preserve gametes in order to restore fertility in women after treatment. Ovarian tissue cryopreservation and subsequent tissue transplant is still an experimental procedure that involves the removal of an ovary or piece of cortical tissue (Fig. 3.3a), which is cut into small pieces (Fig. 3.3b) that are cryopreserved until a woman chooses to restore her fertility. It is currently the only methodology that is feasible for fertility preservation in young girls [67, 68]. As mentioned previously, the cortex of a normal ovary is filled with arrested, immature, primordial follicles; there may be hundreds of primordial follicles in a 1-mm3 piece of tissue [69]. Analysis of human ovarian cortical tissue has shown that the number of primordial follicles varies in the ovarian cortex and is directly correlated with

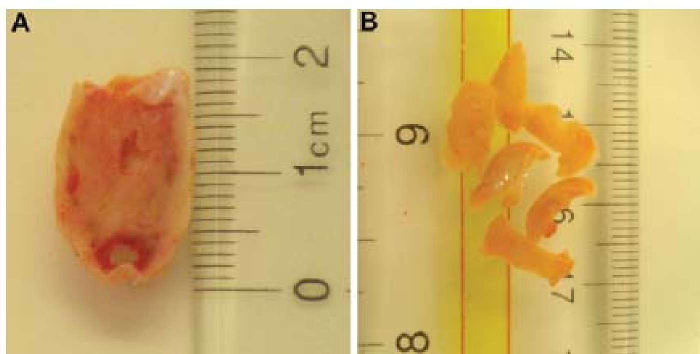

Fig. 3.3 Bisected human ovary showing the thick outer cortex and the inner vascular medulla (**a**). Human ovarian cortex cut into strips prior to cryopreservation (**b**)

a woman's age [70]. Unlike freezing embryos and mature oocytes, which contain large cytoplasmic volumes, the primordial follicles in cortical tissue contain small oocytes that easily survive the freezing process when the tissue is cut into small strips of 1–2 mm × 1–2 mm × 10 mm (Fig. 3.3b). Ovarian tissue transplant following tissue cryopreservation was first successfully completed in mouse [71, 72] and has since been successful in sheep [73] and primates, whose ovaries more resemble those of humans [74]. In the last few years, more than 30 cases have described the transplantation of cryopreserved or vitrified tissue to heterotopic sites such as the forearm [75], as well orthotopic sites such as the abdomen [76] or back to the residual ovary [77, 78]. It was found, on average, that hormone cyclicity resumed within 3 and 5 months of the ovarian tissue transplant, which represents the time it takes for follicle recruitment and subsequent growth [77, 79, 80]. Ovarian tissue transplantation has resulted in the birth of six children to date [77, 78, 81]. Although several groups have reported high levels of follicle-stimulating hormone (FSH) after transplant, which is associated with a low follicular reserve [79, 82], it is still too early to tell how long these transplants will remain functional. It is hypothesized that a single transplant could last several years depending on the age at which the tissue was removed as well as tissue exposure to gonadotoxic treatment prior to removal [83, 84]. For an excellent, in-depth review of ovarian tissue transplant see Demeestere et al. [85].

In Vitro Follicle Cryopreservation and Maturation

There are situations in which women diagnosed with hematologic or ovarian diseases or who have been diagnosed with BRCA1/2 positivity are unable to transplant their stored ovarian cortical tissue due to the possibility that the tissue may contain microscopic lesions [86, 87]. In addition, options for fertility preservation are only available for mature oocytes, embryos, and tissue containing primordial follicles, completely ignoring the vast number of growing yet immature gametes contained within the ovary in primary and early secondary follicles. Experimental studies of individual follicle cryopreservation and subsequent in vitro follicle maturation tackle this last remaining population of female gametes for use in fertility preservation.

As cortical tissue is isolated from the ovary, it can be cut into thin strips and cryopreserved as mentioned above; however, due to their increased oocyte size, primary and secondary follicles fail to survive the in situ freezing process [19, 88]. It is hypothesized that individual follicle isolation allows for better penetration of cryoprotectants, thus helping to stabilize physical connections between the follicle cells and the oocyte [89]. Therefore, a portion of cortical tissue can be cut into smaller pieces (2 mm3) and treated with enzymes such as liberase or collagenase that will break down stromal tissue to aid in the release of small follicles (Fig. 3.4) [90–92], which then can be cryopreserved for later use. Successful slow-rate cryopreservation of small secondary follicles has been shown in mice [89, 93], as well as in non-human primates and humans [89].

After thawing, individual follicles can be encapsulated into a 3D matrix such as alginate, a hydrogel made from seaweed, which supports free passage of amino acids and secreted

hormones and also serves as a scaffold for follicular development [94]. It has been shown that fresh isolated follicles from prepubescent mice are capable of follicle growth from 150 to 350 μm within 8 days of culture in alginate [95]. These follicles secrete estradiol and progesterone at elevated levels upon antrum formation, which correlates with an increase in *Star* and *Cyp19a1* gene expression necessary for steroid production and is characteristic of healthy follicles [96]. Within 8–10 days of follicle growth in the 3D matrix, follicles can be stimulated with FSH and LH to resume meiotic maturation and cumulus expansion, and the oocyte can then be fertilized, giving rise to healthy offspring [92]. Thus far, cryopreserved mouse follicles are capable of full follicle development in

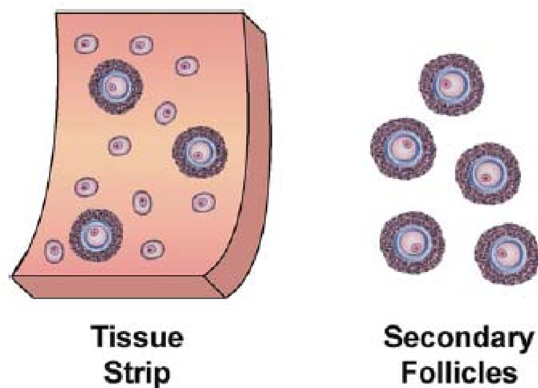

Fig. 3.4 The ovary is dissected into tissue pieces that are either cryopreserved or treated with collagenase for the dissection of secondary follicles used for in vitro follicle growth

the 3D alginate matrix, producing healthy oocytes that can be fertilized and support embryonic development up to the blastocyst stage [89].

Recently, mouse protocols have been adapted for long-term culture of nonhuman primate and human follicles. It is thought that since a primate follicle takes much longer than a mouse follicle to grow from primordial follicle to a preovulatory follicle (>90 days), the same would be true in in vitro culture [97]. Thus far, 30-day cultures have succeeded for both rhesus macaque [98] and human [99] secondary follicles encapsulated in alginate hydrogel. Primate follicles isolated from either the follicular phase or the luteal phase of the menstrual cycle were able to grow during a 14-day culture period; however, follicular phase follicle survival (78%) was much higher than that of luteal phase follicles (42%), which could be attributed to the larger size of follicles isolated in the follicular phase [98]. Encapsulated human secondary follicles isolated from ovarian tissue donated from cancer patients have also been successfully cultured for up to 30 days, with follicles forming large antral cavities and reaching sizes greater than 1 mm [99]. Follicle peptide and steroid hormone production appears to mimic that of antral follicles in vivo, with a characteristic increase in inhibin A and anti-Müllerian hormone (AMH) during follicle growth. Follicles that continued to grow showed a decrease in inhibin B, which correlates with dominant, preovulatory follicle selection. Thus far, 30-day human follicle cultures

are capable of supporting the growth of full-sized oocytes that are competent for fertilization (i.e., MII stage) [99].

Primate and human secondary follicle growth in culture has also been tested following individual follicle cryopreservation [89]. Although primate and human secondary follicles are able to survive the cryopreservation process and grow from an average 175–250 μm (primate) and 125–200 μm (human) during a 6-day culture period, the length of the culture period must be extended in order to see the full effects of cryopreservation on oocyte development [89]. Isolation and cryopreservation of individual secondary follicles expands option for women and children who cannot immediately seek fertility treatments and are not good candidates for other fertility sparing options such as ovarian tissue cryopreservation.

Conclusion

There have been great advances in gamete preservation both for infertile couples and for women and young girls who are faced with life-threatening diseases. Perfecting oocyte and embryo cryopreservation as well as developing new procedures, such as ovarian tissue cryopreservation/transplant and follicle cryopreservation followed by in vitro follicle maturation, is expected to generate more options for fertility preservation.

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