CRYOBIOLGY AND FERTILITY PRESERVATION: A PERSPECTIVE ON PAST, CURRENT AND FUTURE STUDIES

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Abstract

Cryopreservation has been used over many decades for the maintenance of viable biological specimens. Its expansion into the area of fertility preservation has been a natural outcome of the increased risks to human fertility from diseases, such as cancer and its treatment protocols, including radiation and chemotherapy, and the general lifestyle trend to later marriages. The use of assisted reproductive techniques (ART) in preserving fertility have benefitted significantly from new scientific approaches, such as cryostorage, in which live cells and tissues are stored at low temperatures and revived when necessary. This review focuses on “cryopreservation science monitoring in reproductive biomedicine” to evaluate knowledge, trends, driving forces, impetus, and emerging technologies in order to draw a future roadmap for this field. Our analysis of the field of cryobiology emphasises the significance of strategic planning of cryobiology research to support more its extensive use in therapeutics in the future. The Royan Institute (Tehran, Iran) recognises this need and has developed a strategic plan to engage in multidisciplinary research on the application of cryobiology, including cryobioengineering, in disease mitigation. We hope that this study can help improve the quality and quantity of public discourse and expert awareness of the role for cryopreservation in fertility preservation within ART.

Keywords: cryobiology; cryopreservation; fertility preservation; reproduction
INTRODUCTION

The aim of this study is to explain the current options and outline the future development for human fertility preservation, through an examination of current knowledge and an assessment of the driving forces for innovative emerging technologies. The importance for improvements in human fertility preservation has both medical and nonmedical reasons.

The incidence rate of cancer in Iran is significantly lower compared to many other countries. According to the global reference report *Cancer Registration in Five Continents* (1), the cancer incidence rate in Iran is 150 per 100,000 people, compared to an average annual incidence of cancer in the world (excluding skin cancer) of 182 per 100,000 people. In addition, the average incidence of cancer in European countries is 267 per 100,000 and in the United States are 318 per 100,000, showing a lower incidence of cancer in Iran compared to European and American averages. However, statistics provided by Iranian Cancer Association reveal an alarming rise in cancer rates every year. The prevalence of cancer among women is four times higher than that of the world standard. Thirty years ago, the number of Iranian women with cancer under 30 years of age was twice as much as that of non-Iranian women. In addition to the impact of rising cancer risk factors, population growth, and aging on cancer incidence in developing countries (including 82% of the world’s population), the establishment of accurate and regular cancer incidence after the formation of the National Cancer Registration Network in Iran means that the cancer rate seems to be higher than before (2).

Breast cancer is now the most prevalent form of cancer in women of childbearing age. An estimated 15% of this type of cancer appears to occur in women under 40 years of age. Cervical cancer is another common type of cancer in women of reproductive age. Cancer has, thus, always been the focus of attention of medical researchers and considerable effort has been expended in treating the disease. Recent advances in cancer diagnosis and treatment during childhood and adolescence have increased the population of cancer survivors, but on the downside, treatments such as radiotherapy and chemotherapy reduce fertility in these patients who experience life-long infertility. There is, currently, scant information regarding their chances of fertility after cancer treatment (3).

In addition to the above forms of cancer and cancer treatments, patients with systemic diseases (like systemic lupus erythematosus, acute glomerulonephritis, behcet’s disease) as well as patients affected with different types of cancer, including muscle-skeletal cancers (such as Ewing’s sarcoma), osteosarcoma, hematopoietic cancers (e.g., leukemia and lymphomas), neuroblastomas and Wilms tumor, risk losing fertility during treatment (4).

The preservation of fertility is now considered crucial in patients with a variety of malignancies and other diseases as well as those with premature ovarian failure (POF). Special programs have been set up all over the world, to preserve fertility in patients undergoing chemotherapy/radiation treatments (5) (Figure 1).

![Figure 1. The necessity to preserve reproductive capacity.](image)

There are also many nonmedical reasons that impact human fertility. Due to social conditions and financial bottlenecks, and new lifestyle norms, men and women marry at later ages. One of the most important causes of infertility and fetal malformations is increased maternal age (6). This is caused by low quality of female gametes (egg) with increase in age of
the woman, which makes it necessary to preserve fertility and healthy gametes produced before the age of 35. At present, such procedures that preserve the fertility of younger women for childbearing is called "social freezing" and involves the freezing of oocytes, in vitro fertilized (IVF) embryos or ovarian tissue. Accordingly, it seems that the use of cryopreservation will be widely used in reproductive science in the coming years since the number of applicants with non-medical reasons for fertility preservation will rise, and consequently the increase in commercial appeal of freezing is inevitable (7, 8).

The promotion of public awareness on existing methods for fertility preservation as well as the expansion of knowledge of gynecologists, oncologists and other medical staff associated with infertility have resulted in development of new treatments and growing demand for services in this area (Figure 2).

**Figure 2.** Different ways to select the best strategy in reproductive cells and tissues cryopreservation.

**DIFFERENT METHODS FOR HUMAN FERTILITY PRESERVATION**

There are various methods to preserve human fertility, some of which are now regarded as ‘usual’ methods (9):

a) Hormonal support: pharmaceutical factors have similar structures in comparison to natural hormones. The aim of hormonal support is to suppress or inhibit the growth of gametes in order to help the protection of reproductive gonads during invasive treatments.

b) Oophoropexy: in this situation, ovaries are transposed within the pelvic to a position outside the radiation field. This method is only applicable to preserve damage caused by radiotherapy, not chemotherapy and is not always feasible.

c) Gonadal tissue cryopreservation: in this method, the physiological activities of gonads are significantly reduced and testicular or ovarian tissues resume their functions after thawing. Freezing of testis and ovarian tissues can inhibit the loss of gonadal reserve resulting from cancer treatments, congenital anomalies and premature ovarian failure.

As mentioned earlier, both of the slow freezing and vitrification protocols are used for freezing of the biological samples. In slow freezing, samples are slowly cooled in a programmable freezer, whereas in the later approach, samples are rapidly vitrified to prevent ice-crystal formation. Nowadays, both methods are used in infertility clinic centers to freeze embryos, eggs, sperm, testicular and ovarian tissues (9).

**CRYOBIOLOGICAL PRESERVATION STRATEGIES**

Cryobiology deals with the effect of low temperatures on different cell and tissue functions and the application of low temperatures for freeze-storage of biological samples (10). The preservation of cells, tissues, organs, or any other biological constructs, inside cryoprotective solutions and at −196°C is known as cryopreservation. Such low temperature allows the long-term preservation of cells or tissues in a constant and unchangeable physiological condition as long as devitrification is avoided (11, 12).

Cryobiology, as an applied science, is primarily concerned with preserving biomaterials at low temperatures. In such low temperatures, biological activities, even the apoptosis pathway, reach their lowest possible degree (13). Hypothermic storage is typically above 0°C, and the normal body temperature of mammals fluctuates from 32 to 37°C. The principle of hypothermic storage is that every 10°C reduction in temperature is accompanied by a 50% decrease in oxygen consumption. In hibernating animals, inhibition of the sodium pump activity is a mechanism to prevent acidosis associated with hypothermia. Therefore,
to preserve organs and tissues in in-vitro condition, special solutions are used to counter acidosis and increase intracellular calcium.

The freezing methods typically consist of three steps: placing the samples in cryoprotectant solution (dehydration), plunging the sample into the liquid nitrogen (freezing or cooling) and returning the sample to natural temperature (rehydration; thawing/warming). The two existing freezing methods slow freezing and vitrification, despite having the same three stages, can give varying cryopreservation results for different samples (12).

**CRYOPRESERVATION STRATEGIES IN THE FIELD OF REPRODUCTION**

**Ovarian, oocyte and embryo banking**

Although, egg freezing is a commonly used fertility preservation method in cases of single women, embryo freezing is often the choice method of fertility preservation in cases of women with a partner (14).

The freezing of embryos assumed importance after micro injection and in vitro fertilization came to be used in infertility treatments. In IVF, the hormone pill induces ovulation and enables women to release up to 30 eggs. The eggs and sperm are retrieved and combined in the laboratory and then the fertilized eggs are placed in special culture medium in incubators with CO₂ under special conditions. After 48 to 72 h, embryos are examined and their growth and quality are recorded. On the basis of embryo quality, mother's age and history of previous embryo transfer, a few embryos are chosen and transferred to the uterus. Earlier the IVF procedure was costly and time-consuming because there were no methods to store surplus oocytes or embryos. With the advent of freezing methods, the remaining embryos can be stored for future attempts, in case of failure of the current effort, and thus increase the chance of pregnancy (15).

The freezing technique can result in reductions in costs and mental and physical strain associated with repeated forced new stimulation cycles. With a variety of available cryopreservation methods, the number of embryos transferred to the uterus can be kept small, which reduces risks of multiple births, and more eggs and embryos can be made available for subsequent treatments. In addition, a limited number of embryos can be implanted in the woman at the right time and under appropriate circumstances. Consequently, embryos can be likely transferred rather than forcing new stimulation cycles at various times (15).

Numerous researches have been conducted on the safety of cryostorage methods. The results have shown the success and safety of existing embryo cryopreservation methods to a large extent. The first successful cryopreservation of oocytes in mice was carried out in 1977, and the first baby was born through in vitro fertilization in 1978 (16). The first pregnancy and live birth resulting from a frozen human embryo in 1983, was in Australia, as reported by Trounson and Mohr (17), and drew widespread attention. Following this, the rate of using frozen-thawed embryos gradually increased. In 1986, because of the ethics related issues associated with embryo cryopreservation, human oocyte cryopreservation was introduced into clinical practice based on a few studies on mouse oocytes (14). Both freezing techniques were approved as cryopreservation options for maintaining fertility in female cancer patients in 2006. After that, embryo and oocyte freezing have resulted in > 5 million and > 500,000 live births, respectively, worldwide. Oocytes can be cryopreserved in both mature and immature stages. The clinical results of oocyte cryopreservation depend on several factors, some of which are associated with health and quality of oocytes, and they may be affected by the process of cancer treatment (14). To preserve fertility, oocyte cryopreservation is mostly used in adult cancer patients and especially for young women who wish to delay pregnancy. Oocyte cryopreservation can be applied either for postponed pregnancy in young women or oocyte donation instead of embryos because of ethics related issues in some communities or absence of the husband (8).

Mature oocytes can now be easily frozen with different methods in embryology laboratories and many reports of live births have been recorded (18). However, the freezing of immature oocytes has not yet reached a satisfactory state, and the rate of live birth using this method is still very low. The use of new techniques such as vitrification has, to some extent, been able to increase oocyte survival after freezing. The main concern is the in vitro maturation of oocytes after freezing and thawing with respect to the time-consuming process of
oocyte retrieval and cryopreservation. Another matter is probable inefficiency of oocyte maturation medium. Consequently, immature oocyte cryopreservation has not yet been found to have suitable efficacy and needs more development and optimization studies (14). In general, immature oocyte cryopreservation in young single women and prepubertal girls with cancer is not recommended and must be accompanied by freezing pieces of ovarian cortical tissues (19).

When there is enough time for stimulated ovulation, embryo cryopreservation would be the best and most effective option in women with a partner, but oocyte cryopreservation is usually recommended for single patients. In case of ethics barriers, absence of ovulation induction, the urgency of cancer treatments or immaturity of the patient, cryopreserving ovarian tissues is offered as an appropriate strategy to help with the preservation of fertility (20). In cryopreserving ovarian tissues, the ovarian tissue which is a reservoir of follicles (in various stages of growth and development) is stored without any delay in the process of cancer treatment. Although nowadays cryopreservation of ovarian tissue is introduced as an experimentally new method to ART clinics, scientists witness reports of successful live births, thanks to transplantation of frozen/thawed ovarian tissue (21). After the first live birth from ovarian tissue cryopreservation and transplantation was reported in 2004 by a Belgium group (Donnez and colleagues), ovarian tissue freezing was recommended as a fertility preservation technique in 2018 for female cancer patients. So far more than 160 live births have been reported worldwide (22). Silber declares, “We now find that the procedure of frozen ovary transplant is very robust. All patients have returned to hormonal function and cycling, and over 75 percent get pregnant without IVF, and currently we have 23 healthy babies. It is even safe for leukemia affected patients if the ovary freeze is done after the first chemotherapy and certainly prior to the bone marrow transplant” (23). Therefore, ovarian freezing is considered as an appropriate method of fertility preservation in this type of cancer involvement.

**Sperm banking**

Sperm number, motility and morphology are important factors in the reproductive ability of men. The sensitivity of male germ cells to infection, trauma, environmental factors such as work-related pollution as well as to drugs, specifically chemotherapy and radiation treatments, are significant since they can damage spermatogenesis and lead to the complete cessation of sperm production (24). Chemo- and radiation therapies, surgery or combination of them used in cancer treatment can result in sterility in men, based on the type, amount and duration of the exposure. Dramatic improvements in advanced techniques of IVF have reduced the adverse effects of cancer treatment and cessation of spermatogenesis. Sperm cryopreservation and storage in sperm banks represent a practical and acceptable method to preserve fertility in cancerous men or those at risk of impaired spermatogenesis before any therapies. Cryopreserving sperm samples in liquid nitrogen at -196°C and applying advanced IVF, reduces the devastating influences of invasive chemotherapy and radiotherapy on male germ cells. However, this approach depends on the presence of sperm and is not used for immature boys. In such cases, testicular tissue preservation is helpful to preserve fertility (25).

**Testicular bank establishment**

Many drugs and therapies cause defects in testes, which leads to loss of testicular stem cells and subsequently results in infertility. Thus, preserving testicular tissue prior to any drug therapy or radiation therapy can be considered as a fertility preservation approach. Cryopreserving testicular tissue of children who are to be treated with chemotherapy and radiation may allow them to have babies after puberty (24).

Testicular tissue preservation is important in the patients with Klinefelter syndrome, Cryptorchidism, many benign tumors (such as Myelodysplastic syndrome, anemia and thalassemia, autoimmune diseases and lupus erythematosus), and especially cancer in immature males. With the development of cancer treatment techniques, the affected patients are expected to have longer lives. Fertility is reduced by about 46% in male survivors of childhood cancer, and it is reported that only 33% of these survivors have semen with normal quality (26). Germinal epithelium in testis is very sensitive to chemotherapy, especially to alkylating agents. These drugs cause direct damage to DNA and RNA and induce apoptosis. Somatic cells of the testis (Sertoli and Leydig cells) are less susceptible to
chemotherapy drugs. Germinal epithelium is also very sensitive to the effects of radiation in addition to chemotherapy since it has an impact on both somatic and germ cells. Following the toxic effects of chemotherapy and radiotherapy on gonads, researchers are seeking to apply effective methods of fertility preservation on these patients. However, this approach depends on the presence of sperm and is not used for immature boys (27).

Before any treatment for the diseases mentioned above, a biopsy of testicular tissue is obtained and cryopreserved to be used when necessary. In general, there are three strategies to reinstate fertility in young boys following testicular tissue cryopreservation:

1. isolation and freezing germ cells and transplanting to the testis after gonadotoxic treatments;
2. in vitro spermatozoa differentiation from isolated spermatogonial stem cells;
3. freezing testicular tissue and re-implanting to the native site after treatment.

Several studies have been carried out on testicular tissue cryopreservation in animals and with more limitations on human. Considering stem cells of testicular tissue, spermatogonial cells of human testis were isolated and cultured, and for the first time in 2002 the resulting colonies were stored for 6 months (28). Generally, few studies have been conducted on human testicular tissue due to constraints, including the lack of sufficient samples (25).

Experimental reproductive techniques such as cryopreservation and transplantation of ovarian tissue or cryopreservation of testicular tissue in prepubertal boys should be centralized but only partial implementation is possible in some regions and countries. There are significant differences in these centers network structures based on their size and number of facilities. The centers network can vary in size depending on the location from a very large national network, such as Oncofertility Consortium (www.oncofertility.northwestern.edu), to small national networks such as the Danish Network (www.rigshospitalet.dk). The establishment of national or international networks can help to bring together professionals, scientists and students in the field of fertility preservation, continuing education and research, collecting high quality and quantity data, and the holding of national and international congresses (29).

**HISTORY OF CRYOBIOLOGY**

The survival of living organisms, such as psychrophile bacteria and polar plants at temperatures below zero, the seasonal movement of flounders in cold water, and cold-hibernation of many organisms such as tardigrades, provide evidence that biological cells can survive in temperatures below freezing without changes in their fundamental structure and functions. Due to the slow-down of vital processes at low temperatures, cells are protected from degradation by microorganisms and enzymes and can be inactively stored for years, resuming their normal function upon rewarming. Cryobiology is the study of biological material or systems at temperatures below those normally experienced. Applied materials or systems of interest include proteins, cells, tissues, organs, or whole organisms. Therefore, the implementation of cryobiology science relies on different approaches, e.g., to cell and tissue preservation and to protection (30).

Cryobiology has been applied for microorganisms, plants, cells, tissues, and gametes and embryos of animals and humans. Long-term storage, organ perseveration, cryosurgery, unhealthy tissue destruction approaches, food and pharmaceutical freeze drying, and biological super-cooling are areas in cryobiology that have been investigated extensively in recent times (11).

The history of applied cryobiology dates back to ancient times. Low temperature was used in medicine in Egypt in 2500 BC (12). Hippocrates applied cold to prevent bleeding and swelling. With the advent of modern science, Robert Boyle (1627-1691) studied the effect of temperature on animals and published the first scientific document on freezing. The scientific use of cryobiology to preserve biomaterials started about 70 years ago (31).

In the 19th and early 20th century several studies were conducted on freezing, cold hardiness and frost resistance in plants. In the 1890’s Hans Molisch examined the effect of composition and concentration of substances in plant cell cytoplasm to detect what is essential for survival or death after freezing (32). Sugars were recognized by Maximov as a cryoprotectant in the early 1900’s (33). Over the following years, scientists studied the effects of rapid cooling and the biochemistry of cold hardiness on plant epidermal cells and insects.
Peter Mazur showed in 1963 that to avoid lethal intracellular freezing, cooling should be slow enough so that sufficient water has time to leave the cell during the extra-cellular freeze dehydration process (34). In 1949, bull semen was cryopreserved for the first time by a team of scientists led by Christopher Polge with the addition of a glycerol to protect against freezing damage (35). Afterwards, other solutes that had high aqueous solubility were investigated as protective chemicals for freezing. These additive chemicals were called ‘cryophylatic agents/solute moderators’ (36). In 1965, the Society for Cryobiology gave the current designation of ‘cryoprotectants’ for these additive chemicals. Since then, the term of ‘cryoprotectant’ has been used to refer to substances that protect living tissues from damages following low temperature (12).

In 1953, artificial insemination using frozen human sperm led to three pregnancies (37). Human sperm, eggs, and embryos are normally stored for research and infertility treatment purposes. The establishment of controlled-rate and slow-rate freezing methods in the 1970s resulted in the birth of a child conceived using the first frozen human embryo in 1983 (17). These studies provided a better understanding of cryopreservation for storage of organs, tissues, and valuable cells at low temperatures which lead to the first ovarian tissue cryopreservation in 1996 by Hovatta et al (38). In the same year the first pup birth from mouse ovarian tissue cryopreservation and transplantation was also achieved (39). In 2004, Donnez and his colleagues achieved the first live birth after orthotopic transplantation of cryopreserved ovarian tissue in cancer patient (40).

Afterwards, Silber et al., opened a new window in ovarian transplantation between monozygotic twins for premature ovarian failure, POF, and reported the first live birth in 2005 (23).

Programmable devices are being extensively used in recent years, for freezing biological human and animal blood products, eggs, embryos, sperm, stem cells and tissues in the hospitals and veterinary centers, as well as research laboratories. The storage and transport of large organs such as heart for grafting must be performed immediately after donation and only at low temperatures (but not freezing). The potentiality of tissue preservation for transplantation was primarily identified in 1970 by the work on liver transplantation of surgeon Thomas Starzl (41).

**OPTIMIZATION OF CRYOPRESERVATION TECHNIQUES**

Although cryopreservation preserves the structure of the living cells and tissue, it can lead to harm and lethality (42). The freezing damage is due to the formation of ice crystals that pierce the cell, or the effects of concentration changes of the composition in the liquid phase (43). In addition to the intracellular region, ice crystals are formed in the extracellular area of the cell in the controlled slow cooling process. The extracellular ice crystals can be harmful which provides the most significant disadvantage in this process (44). In contrast, the major disadvantage of vitrification is the cryoprotectant’s toxicity and high cost. In the vitrification method, high concentrations of cryoprotectants, which can cause osmotic injury, should be used to prevent intracellular ice crystal formation. Nevertheless, it should be considered that cryoprotectants are used in both cryopreservation methods to reduce ice crystal formation by the increment of total solute concentration (45). Of course the effects of these cryoprotectants depend on the sample type, volume, viscosity, and the cooling and warming/thawing rates (46).

Studies have suggested that embryo cryopreservation through vitrification gains advantages over the slow-freezing process. By way of that slow-freezing decreases the embryo metabolism and impairs embryo morphology, survival, and pregnancy rates. In addition, the vitrification approach can increase human embryo survival rates and produce fewer harmful effects (47, 48).

In contrast studies have indicated that in human ovarian tissue cryopreservation, slow freezing has better efficiency and is more practical. Cryopreservation of human ovarian tissue through vitrification activates the expression of apoptotic pathway-related genes in oocytes. Furthermore, the follicle oocyte’s morphology is poorer than the oocytes in ovarian tissue preserved by slow-freezing (49). Moreover, there are conflicting reports on oocyte cryopreservation by the slow-freezing or vitrification methods. Some studies have suggested a higher rate of fertilization and blastocyst formation for oocytes cryopreserved...
by slow-freezing (50, 29), although others disagree (51, 52, 53).

Ultra-structural and sub-lethal damages have been reported in the sperm after cryopreservation. These damages trigger oxidative and osmotic stresses, which subsequently alter lipid and protein arrangement, cause injury to the spermatozoa tail, and increase sperm DNA fragmentation (3, 19). Cryopreservation may also cause structural damages to mitochondria that change the ATP production processes and decreases spermatozoa viability and motility, ultimately leading to a failure in cryopreserved sperm quality (21).

As mentioned earlier, the modifications of general lifestyle in recent years which delay marriage and childbearing leads to the aging of gametes. Old gametes can be more susceptible to damage during the freezing process. In addition, although cryopreservation has benefits for fertility and childbearing, evidence has shown that live birth and pregnancy rates are greater when fresh compared to cryopreserved embryos are transferred (54).

After decades of using cryopreservation methods in fertility clinics, there is little information concerning their effects on the physiology, cellular, and molecular levels (genomics, proteomics, and epigenetics) in embryos, germ cells, and gonadal tissues (40). Despite the lack of knowledge in this field, some findings exist. Molecular damages associated with metabolism, epigenetics, DNA integrity, cell death, and cell architecture can be induced by cryopreservation in the embryo, oocyte, and sperm. Reactive oxygen species are often increased after cryopreservation and cell viability decreased. Membrane impairment is induced in both germ cell types but cytoskeleton impairment only occurs in the sperm. DNA fragmentation, apoptosis, epigenetic modifications, and mitochondrial damage are observed in the oocyte, sperm, and embryo. Additionally, lipidic profile changes and Ca²⁺ homeostasis impairment only occur in the oocyte.

Therefore, obtaining a healthy sample (with the ability to grow in the lab or body) after thawing is considered a very significant step and the outcome of a desired cryopreservation process is a livebirth. Numerous researchers are now seeking to discover or invent applicable materials or devices used in cryopreservation. In order to optimize the cryopreservation process, two aspects must be taken into consideration: a) the freezing medium and its components; b) necessary devices to immerse and store samples in nitrogen. Even though the use of strategies such as the application of different cryoprotectant components, proteins, and freeze-drying have secondary importance, they are critical in the cryobiology research field (42).

**NEW EMERGING TECHNOLOGIES**

In the last three decades, the availability of ART has grown at a significant pace compared to other fields of science and have resulted in numerous developments and innovations. The highest point of these developments is the emergence in infertility treatment centers of various ART, such as IVF, ovarian stimulation, cryopreservation, and storage of male and female sex gametes, embryos, and reproductive tissues. These progresses, as a result of their applications in infertility diagnosis and treatment, are revolutionary in the health care of infertile couples. Reproductive biotechnology owes current developments to all biological sciences in the fields of research, clinical studies, and cellular/molecular and developmental aspects. The recent achievements have generated other capabilities beyond the science itself. The establishment of sperm, egg, embryo, and reproductive tissue banks is one of the outcomes of the developments in cryopreservation science in the field of reproduction.

Assisted field cryobiology (AFC) is a new area of cryobiology science involving the cryopreservation of vital cells and tissues under magnetic field (43, 44). This approach has led to interesting results in the low temperature preservation of fertility especially when it is integrated with the use of nanoparticles (45, 55).

Despite the widespread use of cryopreservation to preserve fertility in patients or those who are interested in delaying childbirth due to medical or non-medical reasons, other efficient methods have also emerged with remarkable outcomes in the last decade.

The application of ovarian stem cells or other stem cell sources to produce male and female germ cells has assumed high priority in the field of infertility treatment (56). The use of embryonic stem cells derived mesenchymal cells in addition to mesenchymal stem cells obtained from body sources such as bone marrow etc., is being studied (57). Their direct injection into the
ovarian tissue or blood flow is one of the applicable approaches to restore reproductive activities among various patients, particularly those with premature ovarian failure, POF (58). Adult stem cells derived from fat (46), peripheral (47), or menstruation blood (48), as well as endometrium or other tissues related to the reproductive system or fetus structures, such as the amniotic membrane (50), umbilical cord (52), and peritoneum (57) can differentiate into male and female germ cells (sperm and eggs) in vitro. Although these options are still in the experimental phase, they can be good alternatives to invasive methods of fertility preservation in patients (53). For example, if stem cells differentiate into germ cells and can be applicable/applied in clinical practice, they may be an alternative to ovarian tissue cryopreservation and/or ovarian follicle isolation methods that require surgery and are considered invasive procedures (51).

Tissue engineering has become attractive in recent years and it has become acceptable to use “artificial gonads” (regenerated or stimulated in vitro ovaries and testes) as a perfect framework for male and female gamete production (sperm and egg). It seems that these processes can have profound implications in infertility treatment protocols in the future (49). Recent achievements on reconstruction of mouse primordial follicles in the case of integrated mouse and human artificial ovary xenotransplantation provides new opportunities for researchers interested in ovarian tissue engineering (59) (Figure 3).

**Figure 3.** The new emerging techniques in the field of reproductive cryobiology.

**PERSPECTIVE ON THE FUTURE OF REPRODUCTIVE CRYOBIOLGY**

Whilst a range of fertility preservation approaches by tissue cryopreservation has been successfully implemented at well-known centers around the world (Table 1), we anticipate an interesting and impressive future for cryobiology in the field of reproduction science. A detailed analysis of the progress to date in the field of cryopreservation as communicated through the latest scientific literature, at conferences and in other fora indicates significant opportunities for future developments. We suggest that the most effective indicators of progress will include:

1) Improvements in invasive methods of chemotherapy and radiotherapy can prevent collateral damages leading to both male and female fertility failures. These undesired effects can be circumvented through the use of fertility preservation and cryopreservation techniques (54).

2) Developments in short-term protocols to stimulate ovaries in order to obtain mature oocytes from the ovary.

3) Ovarian transposition in the body and out of the radiation field in cancer patients with radiotherapy treatments.

4) The use of hormonal drugs to lower ovarian reproductive activity for the preservation of active organs exposed to toxic and hazardous chemotherapy drugs. In the other words, this method reduces the gametes (eggs) production in the ovary so that it can be protected from damage during treatments (4).

5) The use of lyophilization to reduce the initial costs of samples prepared for freezing and probable transportation to other places, and to reduce the extra costs of long-term storage in liquid nitrogen.

Serious threats to reproductive fertility are caused by diseases such as cancer, their treatment methods and the increase in the age at which many people around the world intend to start a family. This paper discussed the techniques and approaches that have been applied for the preservation of fertility, with special reference to low temperature storage of reproductive biological samples. The significance of proper and well-organized planning and a highly documented strategic plan for the future of this science was further recognized in the Royan Institute (Tehran, Iran) following the present study. It is hoped that this
Table 1. Examples worldwide of ovarian and testis cryopreservation centers.

<table>
<thead>
<tr>
<th>Cryopreservation center</th>
<th>Country</th>
<th>Ovarian cryo</th>
<th>Testis cryo</th>
<th>Live births</th>
<th>*Cryo method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>National Ovarian and Testicular Tissue Transport and Cryopreservation Service (NOTTCS)</td>
<td>Australia &amp; New Zealand</td>
<td>✓</td>
<td>✓</td>
<td>Five livebirths after grafting of ovarian tissue</td>
<td>SF</td>
<td>Fertility Society of Australia (FSA)</td>
</tr>
<tr>
<td>The Centre for Reproductive Medicine at UZ Brussel</td>
<td>Belgium</td>
<td>✓</td>
<td>✓</td>
<td>&gt; 30 livebirths after grafting of ovarian tissue</td>
<td>SF</td>
<td>The Centrum voor Reproductieve Geneeskunde (CRG)</td>
</tr>
<tr>
<td>Laboratory of Reproductive Biology (LRB) Danish Network</td>
<td>Denmark</td>
<td>✓</td>
<td>✓</td>
<td>15 livebirths after grafting of ovarian tissue</td>
<td>SF</td>
<td><a href="http://www.rigshospitalet.dk">www.rigshospitalet.dk</a></td>
</tr>
<tr>
<td>German-Austrian-Swiss network FertiPROTEKT Consortium</td>
<td>Germany, Austria, and Switzerland</td>
<td>✓</td>
<td>✓</td>
<td>17 deliveries generated after 95 ovarian tissue transplantations</td>
<td>SF</td>
<td>(60)</td>
</tr>
<tr>
<td>Royan Human Ovarian Tissue Bank</td>
<td>Iran</td>
<td>✓</td>
<td></td>
<td>Two unsuccessful ovarian tissue transplantations</td>
<td>V</td>
<td>(61)</td>
</tr>
<tr>
<td>Ankara University Medical Faculty, Center for Assisted Reproduction and Infertility</td>
<td>Turkey</td>
<td>✓</td>
<td>✓</td>
<td>One live birth following ovarian tissue transplantation</td>
<td>SF</td>
<td>(62)</td>
</tr>
</tbody>
</table>

*SF, slow freezing; V, vitrification

study can help towards improving the quality and quantity of public health and will be useful for all related research groups.

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**REFERENCES**