Derleme Makalesi / Review Article

Jinekoloji - Obstetrik ve Neonatoloji Tıp Dergisi
The Journal of Gynecology - Obstetrics and Neonatology

DOI: 10.38136/jgon.775326

Yardımcı Üreme ve Fertilitenin Korunmasında Kriyoprezervasyon

Cryopreservation in Assisted Reproduction and Fertility Preservation

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ÖZ

Kriyoprezervasyon, canlı hücre veya dokuların metabolik ve biyokimyasal aktivitelerinin durdurulduğu çok düşük derecelerde saklanmasıdır. Kriyoprezervasyon teknikleri oldukça ilgi çekici olup dünya çapında gittikçe artan oranlarda kullanılmaktadır. Sperm, oosit ve embriyoların dondurularak saklanması, yardımcı üreme teknikleri tedavilerinde potansiyel birçok kullanım alanı bulmuş olup başarı artışında önemli rol oynamaktadır. Embriyo kriyoprezervasyonu, özellikle freze-all stratejisine olanak sağlayarak ovaryan hiperstimülasyon sendromu gibi yardımcı üreme teknikleri tedavi komplikasyonlarının önlenmesi, preimplantasyon genetik tanıya olanak sağlaması, tek embriyo transferi yapılıp diğer embriyoların saklanmasına olanak sağlayarak çoğul gebeliklerin önüne geçilebilmesi gibi pek çok nedenden ötürü her geçen gün kullanımı artan bir prosedür haline gelmiştir. Sperm ve oosit dondurulması ise fertilite kaybı ile yüz yüze kalan hastalar için uzun dönem kendi gamet hücrelerinin saklanabilmesine olanak sağlar. Over ve testis dokusu dondurulması ise prepubertal dönemdeki kız ve erkeklerde fertilite prezervasyonu için tek tedavi seçeneğidir. Diğerleri ile kıyaslandığında daha yeni teknikler olsalar da, gelecek vadeden ve yakın gelecekte kullanımının daha artacağı düşünülen tedavi seçenekleri olarak değerlendirilmektedirler.

Anahtar Kelimeler: Kriyoprezervasyon, oosit, sperm, embriyo, testis, over.

ABSTRACT

Cryopreservation is the technique of keeping living cells or tissues at ultralow temperature that no metabolic or biochemical activity can ocur. The use of cryopreservation techniques is highly attractive and has been increasingly applied worldwide. Cryopreservation of sperm, oocytes and embryos have been central to improvements in the assisted reproduction treatment success with many potential applications. Embryo cryopreservation is an established procedure and has been increasingly used due to novel indications as freeze-all strategy to reduce complications of assisted reproduction as ovarian hyperstimulation syndrome, pre-implantation genetic screening or single embryo transfer and cryopreservation of the remaining embryos to minimize the risk of multiple pregnancies. Sperm and oocyte cryopreservation has permitted the long term storage of gametes for patients with anticipated fertility decline. Ovarian and testicular tissue cryopreservation is the treatment options for prepubertal girls and boys requiring fertility preservation. Although they are relatively new techniques compared to gamete or embryo cryopreservation, they are both very promising and expected to be more widely implemented into the clinic in the near future.

Keywords: Cryopreservation, oocyte, sperm, embryo, testis, ovary.

INTRODUCTION

Cryopreservation, is a state of suspended animation of the tissues or cells, which holds them at a temperature between -140 and -200 °C that no biological activity can occur. Cryopreserved cells and tissues can endure storage for over extended periods of time with almost no change in functionality or genetic information. These features make the method highly attractive and attribute a key role in reproductive medicine (1-3). It has provided solutions to a number of clinical as well as technical problems of conventional in vitro fertilization (IVF) cycles. First, cryopreservation supports the fertility preservation of both male and female who are at risk of infertility due to gonadotoxic treatments. Additionally, this technique allows the postponement of embryo transfer during a treatment cycle to prevent ovarian hyperstimulation syndrome. Single embryo transfer and cryopreservation of surplus embryos is also an effective policy that diminishes the multiple pregnancy rates and enhances the cumulative pregnancy rates (2,4). Cryopreservation has facilitated the introduction of elective deferred embryo transfers in cases which necessitate pre-implantation genetic diagnosis or screening (5). By cooling of cells and tissues to subzero temperatures, it is intended to stop all their physiological processes taking place, so they can be stored almost indefinitely (6). Normally, cooling to such temperatures is not a physiological condition, causing stress and compromising survival of cells.

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Başvuru tarihi :29.07.2020
Kabul tarihi : 17.08.2020

INTRODUCTION

Mechanical damage by ice crystal formation, osmotic stress, membrane permeability and cytoskeletal structural changes due to the cold shock are the major problems that may cause damage to the cells (4,6). Biological systems involved lack of efficient internal defense mechanisms against chilling and ice formation, but intracellular structures and membranes could be protected from cryoinjuries and could be stabilized by cryoprotectants (CPAs).

CPAs are grouped into two major categories as permeating (P-CPAs) (that penetrate through the cell membranes) and nonpermeating (NP-CPAs) agents. P-CPA's are generally small, nonionic compounds which can diffuse through cellular membranes and replace the bulk of intracellular water without overdehydrating the cell. They solidify at lower temperatures than water, thus subsequently reduce the amount of intracellular ice formation at a given temperature (7). They also help to stabilize the cell membranes and protect the cytoskeleton (6). NP-CPAs are generally long chain polymers. As they are too large to pass through the cell membrane, they increase osmolarity of the medium in extracellular space, helping cellular dehydration (6,7).

In assisted reproductive technology (ART), known P-CPAs are glycerol, ethylene glycol (EG), dimethylsulphoxide (DMSO), propylene glycol or 1,2 propanediol (PROH), acetamide and glycerol. The most frequently used NP-CPAs are trehalose and sucrose, although glucose, galactose, and mannitol have also been proposed (4).

Although these agents are used in order to is to prevent damage during freezing, their incorrect use (time of exposure, concentration, temperature of exposure) may result in cytotoxicity (4,7). As an example, exposure of bovine blastocysts to ethylene glycol concentrations greater than 5.4 mol/l for 10 minutes was shown to be detrimental to continued development. Also DMSO and PROH can also cause depolymerization of oocyte tubulin, and meiotic spindle malformation (7). Additionally, DMSO leads to premature cortical granule release and zona pellucida hardening in oocytes, which can affect fertilization. As an example to concentration and temperature of exposure, it was reported that exposure to 1.5 mol/l DMSO at 37°C perturbed microtubule actin microfilament arrangement in mouse oocytes. However it was less apparent at lower temperatures (7).

Techniques

Cryopreservation processes can generally be grouped as slow freezing and vitrification.

a) Slow Freezing

Slow freezing achieves gradual stepwise cell dehydration, with substitution of the cytoplasmic water with low concentrations of CPAs (8). Generally, cells are cooled slowly using a controlled rate freezing machine, which allows samples to be cooled at various rates depending on the cell type (6-8). The main factors that determine the cooling rate include the surface area to volume ratio and membrane permeability, which changes at different temperatures (6). Too fast freezing rates will cause insufficient water loss from the cells. On the other hand, if it's too slow, the cells will be exposed to adverse conditions for longer than it is necessary.

Briefly, cells or tissues are equilibrated in an solutions containing low concentrations of P-CPAs, usually combined with exposure to NP-CPAs in the final step. Once loaded into specially designed straws, samples are cooled to around -6°C/ -8°C (at 2°C/min) followed by a manual seeding to induce nucletion (4,9,10). Then, a consistent slow cooling rate of 0.3–1.0°C/min is applied to reach a temperature at around −40 to −70°C, before the freezing device is plunged into liquid nitrogen (11). The thawing process for tissue that has undergone slow freezing begins with rapid warming in air or in a warm water bath. The tissue is then rehydrated by the application of decreasing concentrations of cryoprotectants, as reverse of freezing (12).

Main disadvantages of slow freezing are the requirement of a computer-controlled cryo-machine, which is expensive and relatively long duration of the procedure. Besides, the procedure has a high risk of freeze injury due to the formation of extracellular ice (8). But the procedure is simple to learn and does not require high manipulation skills (6,7).

b) Vitrification

As an alternative to the slow-freezing technique, vitrification is a process by which cell suspensions are transformed directly from the aqueous phase to a glass state after direct exposure to liquid nitrogen. Compared to slow freezing, vitrification is an ultrarapid technique that requires a high concentration of CPAs (4,8). In order to avoid toxicity of CPAs, the exposure time is drastically reduced (12). Thats why this technique requires good manipulation skills (8).

Briefly, after a pre-equilibration step at relatively low P-CPA concentrations, the samples are exposed to higher CPA concentrations in the final vitrification solution. After they are loaded to a carrier system, they are then cooled ultrarapidly to -196°C (>10,000°C/min) (4). Carrier systems are classified as closed and open systems. In closed systems the sample is physically separated from liquid nitrogen during the entire procedure, on the other hand direct contact between the sample and liquid nitrogen is allowed in open systems. The advantage of open system is the extremely high cooling rate, however it may have an increased risk of cross-contamination (12).

Major advantage of vitrification is the low risk of freeze injury, thereby providing a sufficiently high cell survival rate. At present, no evidence is found demonstrating an increased risk of subcellular disorganization, altered embryonic metabolic profiles and adverse perinatal and obstetric outcomes. So, vitrification is now considered as the method of choice to preserve both gametes and embryos nowadays (13).

Sperm Cryopreservation Techniques

Since its introduction and first reported success, human sperm cryopreservation has been regarded as a useful procedure for treatment of male infertility. The small structure and relative abundance create an advantage when compared to oocyte or embryos in development of effective freezing protocols, as smaller structures are proved to be less susceptible to damage by ice-crystal formation during the cryopreservation (5,14).

Traditionally, slow-freezing and vitrification protocols were considered comparably acceptable in the clinical setting (5). Slow-freezing protocols were the first method used to successfully freeze spermatozoa and remain the most commonly used technique worldwide. However, detailed investigations about this procedure revealed that it induces severe structural and functional damage to spermatozoa and impair cell membranes. High degree of fluidity in sperm membranes are due their high proportion of ether-linked fatty acids and phospholipidic hexanoic acid side chain contents. Freezing procedure induces a reduction in membrane fluidity which results in low sperm survival and motility (14). But the high numbers of spermatozoa obtained per sample made this lower survival rates acceptable (5). Pre-freezing semen quality parameters, such as sperm motility and the abstinence period of sperm donors, can also affect the cryosurvival rates of post-thaw spermatozoa (14).

Rapid freezing, which is another method, is the exposure of spermatozoa, which is mixed with CPAs and loaded to straws, to liquid nitrogen vapour at a distance of 15–20 cm for at least 10 min (14). The straws are submerged in the liquid nitrogen at -196 °C for final storage (4). Studies comparing slow and rapid freezing reported a significantly greater rate of chromatin deterioration

with rapid cooling compared with slow freezing (14).

Sperm vitrification is a novel technique that has been proposed as an alternative after the efforts in past years. It's a fast and simple (direct plunging the sperm sample to liquid nitrogen) method to cryopreserve spermatozoa. However, it requires a very high concentrations of P-CPAs when compared with slow freezing, which represents the main limitation of the procedure due to cytotoxic effects of the cryoprotectants (4). But more recent refinements in vitrification protocols specific to spermatozoa have eliminated the need for cryoprotectants and improved the post-thaw vitality, motility and DNA fragmentation (5).

In most circumstances, the sperm samples required for cryopreservation are obtained via masturbation. Samples can also be obtained by surgical procedures in cases of azoospermia or patients who are unable to produce a sample due to the physical or psychosocial reasons (5). Sperm washing is often performed before cryopreservation to remove decapacitation factors, cell debris, and reactive oxygen species that are potentially detrimental to sperm viability (4).

Sperm cryopreservation is an important fertility preservation option for patients undergoing gonadotoxic radiotherapy/chemotherapy with unavoidable spermatogenesis damage, or surgical removal of gonads. Non-oncologic conditions such as autoimmune diseases, hematopoietic stem cell transplantation, Klinefelter's syndrome, testicular damage, gender reassignment procedures also may require fertility preservation (15). Moreover, in azoospermic patients, to avoid repeated biopsies or aspirations, surgical testicular or epididymal sperm extractions may be frozen for subsequent use.

Despite the developments of the cryopreservation protocols over the years, it seems that cryopreservation may induce detrimental effects on sperm structure, particularly sperm motility. There is also conflicting results regarding the effect on sperm DNA integrity (16).

It has previously shown by many authors that the use of cryopreserved sperm samples does not compromise reproductive outcomes, yielding similar results regarding fertilization rate, embryo quality, implantation, and pregnancy rates when compared with those obtained with fresh spermatozoa both from ejaculate or testicular origin (17). Meta-analysis also indicate that there is no statistical difference in clinical pregnancy rates or fertilization rates in couples undergoing intracytoplasmic sperm injection (ICSI) or IVF using cryopreserved spermatozoa, when compared to fresh samples (18). It was also demonstrated that no statistically significant increase in adverse perinatal outcomes, congenital or chromosomal abnormalities was observed when cryopreserved samples were compared to fresh (5).

Testicular tissue cryopreservation

Cryopreservation of testicular tissue before gonadotoxic treatment is increasingly used for fertility preservation in prepubertal boys, in whom spermatogonia are the only germ cell type present (19).

Controlled slow-freezing protocols are most commonly used for immature human tissue and are associated with spermatogonial survival. Uncontrolled slow rate freezing has also resulted in spermatogonial survival in immature human testis tissues. Two studies have also shown spermatogonial survival using immature human testis with proliferation of spermatogonia in vitrified testis tissues after xenografting (20).

However, this approach is still regarded as an experimental procedure and for the majority of centres it is conducted as a part of an ethically approved clinical research study (19,20).

Oocyte cryopreservation

Since the first live birth from oocyte cryopreservation three decades ago, oocyte cryopreservation has become an important component of ART and many advances have been made in this field (22).

Oocytes are unique cells, which are much more bigger than that of spermatozoa and have low surface area to volume ratio. These characteristics eventually make them sensitive to cryopreservation and highly susceptible to intracellular ice formation. Studies revealed the negative effects of cryopreservation on the stability of microtubules and microfilaments of mammalian oocytes that are vital for normal chromosomal segregation. Zona hardening that results in low fertilization rates is also another reported difficulty associated with cryopreservation (22).

There are two basic techniques applied to the cryopreservation of human oocytes; controlled slow freezing and ultrarapid cooling by vitrification. During slow freezing, oocytes are first cooled to a temperature of -5 C to -7 C, at which equilibration and seeding take place. Then they are cooled at a slow rate of 0.3 – 0.5 C/minute, until a temperature of between -30 C and -65 C has been reached, before being added to liquid nitrogen for storage (23). Since its introduction, slow freezing has been extensively studied and modified to improve outcomes. Nevertheless, there are still concerns regarding the clinical efficiency of this technique. Comparisons between success rates using slow frozen and fresh oocytes, have suggested poorer outcomes with frozen/thawed oocytes (22).

The introduction of vitrification as an alternative method with first live birth was achieved in 1999. The procedure requires higher concentrations of CPAs, lowering the risk of ice nucleation and crystallization, and ultrarapid cooling takes place before submersion into liquid nitrogen (23). Vitrification has been reported to reduce the damage to internal structures leading to superior success rates.

The results of many randomized controlled trials suggested that in vitro fertilization using vitrified/warmed oocytes could produce similar fertilization and pregnancy rates to in vitro fertilization with fresh oocytes (22). A recent meta-analysis of five studies found that the rates of fertilization, embryo cleavage, high quality embryos and ongoing pregnancy did not differ between vitrification and fresh oocyte groups (24). Oocyte survival rates were reported to be over 84% (22).

With the improved outcomes, this strategy has been adopted by many centers worldwide and has replaced the slow freezing approach (4). In the light of these developments, the American Society for Reproductive Medicine (ASRM) lifted the experimental label applied to oocyte freezing in 2013 (25).

As previously mentioned there are two main classes of vitrification protocol; open and closed vitrification. In open systems the sample loaded onto its support and directly contacts with the liquid nitrogen, while in closed systems these entities are physically separated from the start to the end (4). Despite their proven proficiency, concerns have been raised over the sterility of open systems due to potential cross-contamination between the vitrification sample and liquid nitrogen. However, the use of closed systems raises new concerns about the efficiency of oocyte vitrification, due to their decreased cooling rates. Some studies report reduced fertilization, cleavage, and clinical pregnancy rates in the closed systems, while others suggested excellent clinical outcomes (22).

Oocyte cryopreservation is an important fertility preservation option for cancer patient before gonadotoxic treatments. It may also be needed in case of medical conditions such as endometriosis surgery which has the potential to reduce ovarian reserve postsurgery, early ovarian failure, or autoimmune diseases. Fertility preservation for nonmedical intentions, also called as "social freezing" has recently become a hot topic and considered by some authors as acceptable for age related fertility decline (4).

Ovarian tissue cryopreservation

While still considered an experimental procedure in most countries, ovarian tissue cryopreservation is the only realistic fertility preservation option for prepubertal girls, young adolescent girls and those requiring urgent treatment (3). It has been increasingly applied worldwide to restore fertility in patients with malignant and non-malignant pathologies with risk of premature ovarian insufficiency (26).

Conceptually, cryopreservation of ovarian tissue is more complex than that of gametes or embryos, requiring preservation of multiple cell types, which vary in volume and water permeability. Essentially, ovarian tissue cryopreservation is more similar to organ cryopreservation than to that of gametes or embryos.

Cortical region of the ovary which contains 90% of a follicular reserve is used for cryopreservation. Four to five pieces of ovarian cortex tissue measuring 10x5x1 mm in size are taken by a surgical procedure. As the primordial follicles are generally located 0.8 mm from the mesothelium, ovarian cortex grafts are recommended to be 1-1.5 mm in thickness. Cortical size is also important fort he permeation of CPAs as different types of cells and extracellular matrix compose the tissue (27). There is no consensus regarding the optimal procedure to cryopreserve ovarian tissue. Although slow-freezing technique is more widely used currently, an increasing number of groups have been adopting vitrification procedures.

It has yielded more than 130 live births up to now and almost all transplanted patients recovered their ovarian function (26). Also, two births have been reported from vitrified ovarian cortex (28). A recent meta-analysis reported live birth and ongoing pregnancy rate of 37.7% for ovarian tissue cryopreservation (29).

The concern regarding the potential of reintroducing malignant cells to the patient with ovarian tissue reimplantation, especially for the patients with leukemia, neuroblastoma, and Burkitt lymphoma who have the highest risk of ovarian metastasis, should always be kept in mind while evaluating the patients who are candidates for this procedure (26).

Embryo cryopreservation

Cryopreservation of embryos is an essential aspect of assisted reproduction treatments (11). Since the first reports of a successful pregnancy following embryo cryopreservation emerged in 1983, more than half a million live births have been achieved through such methods. The use of embryo cryopreservation has been increasing due to novel indications as freeze-all strategy to reduce complications as ovarian hyperstimulation syndrome, pre-implantation genetic screening at the blastocyst stage or single embryo transfer and cryopreservation of the remaining embryos to minimize the risk of multiple pregnancies (5,22). European Society of Human Reproduction and Embryology (ESHRE) revealed a 24.1% increase in 2014 over the previous year, evidencing the increasingly central role that embryo cryopreservation in clinical practice (30).

Two principal approaches for cryopreservation have been adopted; slow-freezing and vitrification (11). As large, multicellular structures, embryos are more resistant to freeze-thaw damage, and therefore early attempts to preserve them at −196 °C using slow freezing were largely successful. However, there was a need to develop a newer technique to avoid ice crystal formation and its associated damage by slow freezing. Vitrification has become a favoured procedure over the last decade (9,11). With vitrification, a post-thaw embryo viability has been reported between 78% and 100%, comparing to the 60% obtained with the slow-freezing method. This advantage is also reflected by the improvement in clinical pregnancy and live birth rates per embryo transfer (11). Recent data suggested that embryo freezing may be associated with increased risk of large-gestational-age babies and hypertensive disorders of pregnancy (4).

CONCLUSION

Since the early introduction of sperm cryopreservation, there has been tremendous improvements in the field of cryopreservation technology. Both gamete and embryo cryopreservation have been widely implemented in ART treatments. Moreover significant progress have been made in cryopreservation of ovarian and testicular tissue. Although regarded as experimental procedures now, with the collection of more data, they will be adopted by more clinicians and become widely used in very near future.

Conflict of Interest

The authors declare no conflict of interest.

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