Cryopreservation and strategies for increasing of *in vitro* fertilization rate using assisted reproductive technologies; review of sperm morphology

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ABSTRACT

At present, we are not able to offer our patients a selection technique whereby spermatozoa used for fertilization are preventively tested for DNA integrity, the assumption that vacuolization of the sperm nucleus may reflect some underlying DNA defects, which could undermine male fertility potential, is a promising perspective. Consequently, motile sperm organelle morphology examination (MSOME) evaluation coupled with conventional ICSI gave rise to a new micromanipulation technique called intracytoplasmic morphologically selected sperm injection (IMSI), which is currently one of the most debated issues in the assisted reproductive technologies (ART) field. Vitrification is an alternative method of freezing based on the rapid cooling of water to a glassy state through extreme elevation of viscosity without intracellular ice crystallization. Vitrification removed numerous issues pertaining to the slow freezing of liquid water to an ice phase, particularly extensive rehydration and osmotic impairment, the increased ionic strength of concentrated eutectic solutions, shifts in pH, and high electric field. Despite those concerns, it is a fact that the implementation of this new method has rekindled interest in the role morphology would have to find functionally competent sperm with the highest fertility potentials.

KEY WORDS: Infertility, In Vitro Fertilization, assisted reproductive technologies, Morphology.

1. INTRODUCTION

The mature spermatozoon is an actively motile, free swimming cell consisting of a head, which contains a nucleus endowed with a genetic traits a father can transmit to his offspring, and a tail or flagellum, which provides the motility that assists in transport of the sperm to the site of fertilization and ensures that it is appropriately oriented for penetration of the coatings of the ovum (Fawcett and Bloom, 1994). The human sperm head is 4 to 5 µm in length and 2.5 to 3.5 µm in width. The greater part of its bulk consists of the nucleus, whose chromatin has become greatly condensed to diminish its volume for greater mobility and to protect its genome from damage in transit to the egg (Fawcett and Bloom, 1994). Most of the nucleus is covered by the acrosomal cap−an organelle containing enzymes that have an important role in sperm penetration during fertilization. The mammalian sperm head varies greatly in size and shape from species to species (Fawcett and Bloom, 1994; Solati and LoBat, 2016). The sperm tail is about 55 µm long and varies in thickness from about 1 µm near the base to 0.1 µm near its tip. It presents four segments along its length recognizable with the light microscope by slight differences in thickness and in the nature of their sheaths (Fawcett and Bloom, 1994). From proximal to distal, these regions are the neck, the middle piece, the principal piece, and the end piece. There are significant differences in the internal structure of these segments. These cannot be clearly resolved in fresh preparations but require special cytological techniques, or electron microscopy, for their demonstration. The description of spermatozoon structure that follows is based largely on electron microscopic studies (Fawcett and Bloom, 1994; Soleymani, 2008). In the 19th and the first half of the 20th century, scientists elucidated some aspects of the mechanisms of cold adaptation of living matter, particularly those utilized by plants and fungi. The most significant primary share in this field were made by Father Luyet, who has been the pioneer of the science of cryobiology. He found that ice damage must be avoided, and that vitrification could be a method for protection of cell viability (Fawcett and Bloom, 1994; Luyet, 1937). In the 1990s, vitrification was used in new areas such as oocytes and ovarian tissues. But, the sensitivity of germ cells to cryoprotective agents made this approach prohibitive, despite the fact that vitrification can be simple, fast, and cheap. A novel innovation came in 2002 when Nawroth, vitrified human sperm excluding conventional cryoprotective agents **(**CPAs) by using a simply constructed cryonic copper loop to present the sperm in a film, or a pellicle. The key was very fast cooling up to hundreds of thousands˚ C/min. It has developed that the rate of warming is even more important to survival under these conditions than is cooling. Thus, it seems that intracellular vitrification could be attained at fairly low rates of cooling minus the use of CPAs and preliminary dehydration. Most cells would not survive cryopreservation without CPAs, which can minimize cryoinjury of cells. CPAs are low molecular weight chemicals that aim to protect spermatozoa from freezing damage or ice crystallization by declining the freezing point of materials. There are two categories of CPAs, and they differ in their ability to enter the plasma membrane (Di Santo, 2011). Initially, permeating CPAs including dimethyl acetaldehyde; dimethyl sulfoxide, glycerol, glycol, ethylene and methanol, stabilize cell plasma membrane proteins and reduce concentrations of electrolytes. In contrast, no permeating CPAs such as albumins, dextran's, egg yolk citrate, hydroxyethyl, polyethylene glycols, polyvinyl pyrollidone and sucrose, minimize intracellular crystallization by increasing viscosity of the sample. This is seemingly enabled by the low

intracellular water content. All of the methods stated above depend on storage at an ultralow temperature (196˚C in liquid nitrogen) ice-free vilification (Di Santo, 2011).

History of human spermatozoa cryopreservation: Remarkably, the first reference of empirical sperm freezing dates as far back as the late 16th century, but it was only with the discovery in 1937 by Bernstein and Petropavlovski that glycerol can aid spermatozoa in surviving long term freezing, that sperm cryopreservation became practical (Bernstein and Petropavlovski, 1937). Development of artificial insemination for the dairy industry led to further important research in the field of cryobiology (Isachenko, 2003). Briefly after conducting the experiment on the animals, the first pregnancies were reported in humans after insemination with frozen spermatozoa. The next milestone was the discovery of the possibility to store human spermatozoa in liquid nitrogen (LN) at -196°C, resulting in superior recovery rates compared to storage at higher temperatures between -20 and -75°C. After the era of empirical freezing, cryobiology matured to its fundamental stage, aiming on the biophysical and biochemical principals of cryopreservation, further advancing the field (Isachenko, 2003).

Biological aspects of freezing: Living cells have an isotonic condition with a melting point of their intracellular water of approximately -0.6°C. When cells are cooled below this standard freezing point, super cooling takes place and remains in a metastable state up to -5°C (Katkov, 2006). Water crystallization and ice formation begin between -5 and -15°C, beginning with the formation of an ice nucleus (seed crystal) in the extracellular water. This 'nucleation' can be induced at a higher temperature by the application of external facilitation of ice formation, often referred to as "seeding". Before that stage, water stay unfrozen inside the cell as the membrane prevents ice crystals from intracellular penetration (Isachenko, 2003). Solutes are excluded from ice formation. This makes the concentrations of solutes in the extracellular water to increase. Permeability of plasma makes the chemical imbalance created diffusion of the solutes in the cell, which creates a push to repel water into the extracellular area. Cells thus undergo excessive dehydration, losing up to 95% of their intracellular water content. This increases the intracellular concentration of solutes, resulting in denaturation of proteins, pH shifts and potential cell death (Braga, 2011). Since, speed of cooling is critical, inaccurate cooling rates can adversely influence the viability of the sperm, motility, plasma membrane integrity and mitochondrial function. In the slow cooling protocol, there is enough time for intracellular water efflux and balanced dehydration. If cooling is too slow, damage may occur due to exposure of cells to high concentrations of intracellular solutes. Extreme cellular dehydration leads to shrinkage of cells below the minimum cell volume necessary to maintain its cytoskeleton, genome related structures, and ultimately cellular viability (Mazur, 1972). Instead, if cooling rates becomes very quick, exterior ice can induce intracellular ice creation and potential separation of the plasma membrane and impaired intracellular organelles. Furthermore, mechanical damage of cells is likely because of extracellular ice density and close proximity of frozen cells can result in cellular deformation and membrane damage. On the other side, with ultra-rapid cooling, the amount of ice creation is unimportant and the entire cell suspension undergoes vitrification. In this level water transitions, ice formation slows, molecular diffusion and aging stops, and liquids turn into a glass-like condition (Mazur, 1972). Notwithstanding the comparative insensitivity of human sperm to freezing, optimal cooling rates are needed to ensure appropriate sperm recovery. Interchangeably, controlled rate freezers can be used to cryopreserve human semen (Mazur, 1972). Most of these protocols utilize a "no seeding" option where samples are cooled from room temperature to -4°C at the rate of 2°C/min, followed by an increase of the cooling rate to 10°C/min until -100°C is reached, and finally plunging into LN2. Contrary to these sluggish freezing techniques, single step ultra-rapid cooling is utilized for the vitrification technique (Mazur, 1972).

Modalities of assisted reproductive technologies (ART): Sperm cryopreservation is extensively utilized in combination with assisted reproductive technologies (ART) such as intrauterine insemination (IUI), In Vitro Fertilization (IVF) and Intra Cytoplasmic Sperm Injection (ICSI). Despite the finding of new CPAs, significant numbers of spermatozoa still do not survive cryopreservation. Both freezing and thawing can inflict irreversible injury on a proportion of human spermatozoa, marked by a significant increase in some apoptosis markers. Lipid peroxidation can lead to a decrease in sperm velocity, motility, viability, and mitochondrial activity (O'connell, 2002). The recovery rates of intact spermatozoa are extremely dependent on the pre-freezing sample (Schael, 2006). Low quality semen may have more tendency to DNA damage and cell expiry after cryopreservation than normal semen samples, thus having lower fertilizing capacity. It has been shown that reactive oxygen species (ROS) production impacts membrane fluidity and the recovery of motile, viable spermatozoa after cryopreservation. Too, semen samples covering leukocytes may have higher DNA fragmentation. Furthermore, the cryopreservation course can reduce the antioxidant action of the semen fluid making spermatozoa more susceptible to ROS-induced damage (Gosalvez, 2009). The occurrence of sperm DNA damage may also be associated with the thawing process. A rapid upsurge in post thaw sperm DNA fragmentation over time has been practical, with the highest rate of fragmentation occurring during the first four hours after thawing (Gosalvez, 2009). Normozoospermic semen samples seem to be more resilient to damage induced by freezing and thawing in comparison to oligozoospermic or asthenozoospermic samples. Previous studies indicated that motile spermatozoa can be recovered after five refreezing and thawing

rounds in normozoospermic samples, but only after two rounds in cases of oligozoospermia. Spermatozoa of infertile men were resistant to a lesser extent in relation to the damage during cryopreservation in comparison to the spermatozoa from fertile men. Optimization of both CPAs concentrations and cryopreservation protocols will maximize survival of spermatozoa and thus improve ART outcome (Gosalvez, 2009).

Vitrification of spermatozoa: Vitrification is an alternative method of freezing based on the rapid cooling of water to a glassy state through extreme elevation of viscosity without intracellular ice crystallization (Katkov, 2006). In recent studies, vitrification of spermatozoa was ineffective which was possibly because of increased concentrations of permeable CPAs (30-50% compared to 5-7% with slow freezing) and low tolerance of spermatozoa to permeable agents. Transitory contact to an increased concentration of CPAs can cause toxic and osmotic shock and would be deadly for spermatozoa (O'connell, 2002).

One likely plan to inferior the concentration of CPAs could be to growth the speed of cooling and warming temperatures as higher rates of cooling and warming, require lower concentrations of CPAs; these conditions can help eliminate intracellular ice crystallization, and facilitate the formation of a glassy state (Katkov, 2006). Another option is to add non-permeable CPAs–such as carbohydrates– to permeable CPAs to minimize osmotic shock by reducing osmotic pressure and stabilizing the nuclear membrane. Because the intracellular matrix of human spermatozoa covers big quantities of proteins and sugars, they can be effectively frozen in the lack of permeable CPAs using protein- and sugar-rich non-permeable agents (Koshimoto, 2000). Successful vitrification of human spermatozoa was first reported by the Isaschenkos' group (Nawroth, 2002). The high viscosity of the intracellular milieu due to large amounts of proteins, nucleotides and sugars and low water in human spermatozoa content determines the aptitude of human sperm to be vitrified at comparatively low cooling rates. It was noted that human spermatozoon is one of the smallest germ cells among mammals, has almost no residual histones and has very compacted DNA, which indirectly confirms this hypothesis. The major advance in effective vitrification of human spermatozoa deprived of the utilization of permeable CPAs was stated only recently by the Isachenko group (Isachenko, 2004), who actually re-invented the work of the "pioneers" in the 1930-40s mentioned above. The combination of enormously high rates of cooling/warming and utilization of vitrification media containing proteins and polysaccharides made it feasible to avoid de-vitrification during warming without use of toxic CPAs (8, 23). The same group compared viability, survival rate and sperm DNA damage between slow freezing and vitrification and found that DNA integrity was independent from the mode of cooling and the presence of cryoprotectants in thawed spermatozoa (Isachenko, 2004). The group reported that changes in the mitochondrial membrane potentials relate to the type of vitrification media with the best achieved results when both sugar and albumin were added to the media. To achieve high cooling rates, the vitrification specimen volume needs to be kept to a minimum. Specially designed freezing carriers, such as cryoloops and electron microscope copper grids have been suggested for vitrification of human spermatozoa (Isachenko, 2004). However, placing drops of semen directly into LN_2 raises the issue of the potential risk of microbial or viral cross contamination during freezing and storage. The ultra-high freezing rates utilized for vitrification, via direct plunging of specimens into LN2, leads to solidification of a solution by an intense increase in viscosity during cooling which avoids water crystallization and damaging ice formation (Katkov, 2006). Vitrified spermatozoa were successfully utilized in ICSI treatment with clinical pregnancy resulting in delivery of healthy twins (Isachenko, 2004). While only a small volume 0.2 to 40 µl of sample suspension was frozen in the past, recently larger amounts of spermatozoa (100 µl) were successfully vitrified using newly developed straw packaging system (SPS) made from cut in half 0.25 ml plastic straw (Isachenko, 2004). A first live birth was reported following intrauterine insemination of semen vitrified without permeable cryoprotectants from patient with oligoasthenozoospermia making this freezing technique even more attractive in clinical practice (Isachenko, 2004).

Strategies for evaluation of sperm morphology: Sperm morphology has become an area of intense interest in the evaluation of male infertility. To expand the diagnosis and treatment of male infertility, several methods have been proposed to objectively assess sperm morphology: light microscopy, Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). In the last decade, a novel method including real-time high-magnification (up to ×6000) morphological analysis of unstained isolated motile spermatozoa, motile sperm organelle morphology examination (MSOME), has been introduced (Bartoov, 2001). MSOME can identify not only conventional morphological sperm alterations with a definition close to that of SEM, but also more specifically sperm head vacuoles, considered by Bartoov, (2001) as nuclear defects. The MSOME method has been applied to sperm injection, giving rise to Intracytoplasmic Morphologically Selected Sperm Injection (IMSI). The first publications demonstrated an upsurge in the pregnancy rate utilising IMSI compared with ICSI (Bartoov, 2001). The influence of normal nuclear morphology in sperm on ICSI attainment has been emphasized, while the impact of sperm vacuoles observed with MSOME in ARTs has been question. The variable presentation of sperm vacuoles (size, number, localization and frequency), their mode of occurrence, their biological significance and their impact on the quality and fertilization ability of human spermatozoa have been described (Bartoov, 2001). Several studies have tried to characterize sperm vacuoles, determine the sperm irregularities associated with the incidence of vacuoles, test the diagnostic value of MSOME for male infertility or question the usages of IMSI. Since its introduction, ICSI has

revolutionized the approach to male infertility by offering treatment to couples who were previously excluded from conventional IVF (Bendikson, 2008). Poor-quality samples with severe impairment of sperm count, motility, and morphology turned to be unexpectedly suitable to achieve pregnancy and deliver healthy babies given that fertilization can occur even in cases of total teratozoospermia, globozoospermia, and megalozoospermia (Vanderzwalmen, 2014). On the other hand, fertilization, pregnancy, implantation, embryo quality, and blastocyst formation rates are negatively affected by severe morphological anomalies. Even if there is a tendency among embryologists to select for injection into the ooplasm only the most good-looking sperm and discard the most distorted ones (i.e., round, large, or tapered),visual assessment under 200-400× magnification can actually identify mostly rough alterations in sperm shape and size. However, it overlooks a variety of head defects, which could be indicative of impaired sperm function and DNA integrity, as is significantly frequent in cases of oligo-astheno-teratozoospermia (OAT). Moreover, ICSI involves the use of a spermatozoon that would never be able to penetrate the ZP, because of structural defects whose severity seems to be related to the incidence of chromosome aneuploidies. Hence, its introduction into clinical practice has increased the likelihood that a genetically abnormal sperm may be selected for fertilization and participates in the embryo development. In this respect, several negative-impact factors, both genetic and epigenetic in origin, have been recognized in embryos following an ICSI procedure, and there is considerable concern regarding the increased risk of chromosomal irregularities in infants conceived through ICSI. As a result, their highly predictive value for male fertility potential was demonstrated. However, analysis of the sperm organellar morphological characteristics involved high costs since it had to be carried out only on fixed and stained sperm cells from selected cases of unexplained infertility and repeated ART failures. For that reason, a few years later sperm functional morphology criteria based on real-time observation of individual motile sperm cells under high magnification were developed. On the basis of historical data, MSOME, being able to detect subtle sperm morphological malformations, which might remain unnoticed during standard microinjection, and allow the identification of spermatozoa with the best morphology, was introduced to improve the ICSI success rates. Particularly in the form of large nuclear vacuoles that were proposed to reflect damage in the nuclear DNA content and organization (Antinori, 2011).

DISCUSSION

Vitrification eliminated many of the issues pertaining to the slow freezing of liquid water to an ice phase, principally broad rehydration and osmotic damage, the augmented ionic strength of concentrated eutectic solutions, shifts in pH, high electric field, etc. Though, vitrification in high concentrations of vitrificants introduced its own set of problems. Notable amongst them were osmotic impairment during addition and removal of vitrificants, CPA toxicity, mechanical cracking of glasses, and devitrification due to inadvertent thermal cycling during storage. While there are many risk factors associated with freezing of cells, thawing can also dramatically affect survival rates of spermatozoa (Braga, 2011). When frozen samples go back to the ambient temperature, a setback of the freezing process happens. Cells that were frozen by the slow method are more exposed to rapid thawing, due to the fast entry of water into cells causing uncontrollable swelling and osmotic shock. If cells were frozen fast, intracellular ice crystals could re-crystallize and form greater crystals during a slow melting. To diminish toxic effects, CPAs have to be promptly detached from the cell suspension by washing samples in isotonic solution. So, the thawing procedure and CPAs elimination method utilized need to consider the primary technique that was used for freezing. Vitrification can elude lethal intracellular ice formation and the harmful effects of high salt concentrations during freezing and rewarming (thawing). The conventional method of vitrification used to reserve large cells (embryos, oocytes), tissues, or organs requires high CPA concentrations with the consequent lethal. Most of the methods presently used to assess sub-lethal damage to spermatozoa are invasive and sometimes require the use of sophisticated techniques that are not routinely available in clinical laboratories. Methods involving the use of fluorescent probes and/or fixation prevent the subsequent use of the spermatozoa in ART (8, 30). Hence, most labs evaluate the entire population of frozen-thawed sperm, rather than the motile post thaw sperm to be used for ART (Berkovitz, 2006). It would be useful to identify negative effects of cryopreservation that might appear in motile post–thaw spermatozoa. Hence, we aimed to examine the potential worth of motile sperm organelle morphology examination (MSOME) of frozen-thawed sperm. Indeed, it was suggested that nuclear abnormalities could be detected by MSOME under high magnification. At present, we are not able to offer our patients a selection technique whereby spermatozoa used for fertilization are preventively tested for DNA integrity, the assumption that vacuolization of the sperm nucleus may reflect some underlying DNA defects, which could undermine male fertility potential, is a promising perspective. Despite those concerns, it is a fact that the implementation of this new method has rekindled interest in the role morphology would have to find functionally competent sperm with the highest fertility potentials (Isachenko, 2004).

2. CONCLUSION

The MSOME may be useful for evaluating frozen-thawed sperm before ART protocols. Lastly, MSOME could be of use in research (e.g. the comparison of various cryopreservation methods). Vitrification had adverse effects on sperm parameters of motility and normal morphology. However, this technique did not increase the rate of vacuolization of sperm head or severe alteration in sperm fertility potential. Since, the majority of human

spermatozoa contained vacuolization in head region, it is highly recommended to use MSOME technology for assessment of sperm fine morphology during clinical microinjection procedure.

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