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RESEARCH COMMUNICATION

Vitrification of a small number of spermatozoa in normozoospermic and severely oligozoospermic samples

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Abstract

Despite broad utilization of sperm cryopreservation, little progress has been made to modify freezing protocols or to improve rates of sperm survival. Vitrification is an alternative method for freezing human spermatozoa without toxic permeable cryoprotectants (CPAs). The purpose of our study was to optimize the vitrification and post thaw recovery of a small number of spermatozoa using only nonpermeating CPAs in a closed straw system in normozoospermic and severely oligozoospermic samples. Individual motile spermatozoa ($n = 295$) were selected from semen samples of 15 normozoospermic and 10 severe oligozoospermia patients. Overall sperm recovery after vitrification was 80% ($n = 236$) with 80% ($n = 189$) viability and 41.5% ($n = 98$) retained post-warming motility. Two different loading techniques were compared to transfer selected spermatozoa into straws in preparation for vitrification: by spontaneous capillary action (CA) and with the aid of a polar body biopsy (PBB) pipette. There was evidence that the PBB loading technique increases the odds of spermatozoa recovery in both subsets ($p = 0.01$ and $p = 0.04$) in the normal and abnormal subsamples, respectively.

Abbreviations: CA: capillary action; CBS: Cryo Bio System; CPAs: cryoprotectants; DNA: deoxyribonucleic acid; HOST: hypo-osmotic swelling test; ICSI: intracytoplasmic sperm injection; IUI: intrauterine insemination; IVF: *in vitro* fertilization; OPS: open-pulled straw; PBB: polar body biopsy; ROS: reactive oxygen species; WHO: World Health Organization

Keywords

Cryopreservation, oligozoospermia, sperm vitrification

History

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Introduction

Autologous cryopreservation of human spermatozoa is a common procedure applicable in variety of circumstances ranging from fertility preservation for cancer patients to the clinical management of male infertility. Semen cryopreservation also allows proper screening and quarantine of sperm donors to eliminate the risk of the transmission of infectious pathogens to a recipient.

Conventional slow cryopreservation has been in place for many years with little to no changes in the freezing protocol and methodology [Woods et al. 2004]. Slow cryopreservation involves the use of cryoprotectants (CPAs) to minimize cryoinjury of cells and to avoid intracellular ice formation, but can be toxic [Ozkavukcu et al. 2008]. Most slow freezing protocols rely on the use of permeating CPAs, such as dimethylacetaldehyde, dimethyl sulfoxide, glycerol, glycol, and ethylene that stabilize the plasma membrane and reduce intracellular concentrations of electrolytes but could induce osmotic damage of cells. In contrast, nonpermeating CPAs: albumins, egg yolk, hydroxyethyl containing compounds, and sucrose, minimize intracellular ice crystallization by increasing viscosity with minimum cytotoxic effect, but might not be as efficient as permeating CPAs [Henry et al. 1993].

The velocity of cooling and thawing are crucial to the survival of spermatozoa. Suboptimal cooling rates may either lead to cell dehydration and damage to cells' cytoskeleton or intracellular ice formation with damage of plasma membrane and intracellular organelles [Henry et al. 1993; Morris et al. 2012].

Despite the wide practice of sperm cryopreservation, little progress has been made to modify freezing protocols or to improve rates of sperm survival in the past decade [Woods et al. 2004]. Vitrification has been explored by several groups as an alternative method for freezing human spermatozoa [Desai et al. 2004; Endo et al. 2011; Isachenko et al. 2004; Nawroth et al. 2002]. It has been shown that human spermatozoa can be vitrified without toxic permeable CPAs using only nonpermeable CPAs such as sucrose and serum albumin in an open or a closed system [Isachenko et al. 2004; Isachenko et al. 2012; Sanchez et al. 2012]. During sperm vitrification, ultra rapid cooling rates can be achieved by freezing very small volumes. Rapid cooling prevents the formation of intracellular ice by allowing solidification of the

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Table 1. Comparison of two loading techniques and spermatozoa quality.

	Normozoospermic ($n = 15$)			Oligozoospermic ($n = 10$)		
Variable	Capillary action	PBB pipette	p Value	Capillary action	PBB pipette	p Value
Patient age*	36.2 (±4.9)	40.7 (±5.7)	0.12	38.8 (±6.5)	40.2 (±3.6)	0.69
Initial sperm concentration*	52 (±23.5)	62 (±31.9)	0.43	1.8 (±1.3)	6.2 (±3.5)	0.76
Initial sperm motility (%)	62 (±5.2)	61 (±9.4)	0.98	24 (±10.7)	35 (±26.4)	0.19
Post wash concentration*	35 (±11.5)	45 (±25.1)	0.28	0.6 (±0.6)	2.2 (±2.5)	0.88
Post wash motility (%)	89 (±8.1)	94 (±3.6)	0.68	51 (±24.1)	69 (±29.1)	0.09
Number vitrified	103	89	–	49	54	–
Post warming recovery**	76 (73.8%)	77 (86.5%)	0.01	35 (71.4%)	48 (88.9%)	0.04
Post warming motility**	31 (40.7%)	34 (44.2%)	0.68	12 (34.3%)	21 (43.7%)	0.44
Post warming viability**	60 78.9%)	66 (85.7%)	0.10	27 (76.9%)	36 (75%)	0.69

PBB: polar body biopsy. Numbers are mean ± SD, unless stated otherwise. *10^6 spermatozoa per milliliter; **Binary data n (%).

extracellular medium to a glass-like state [Fahy 1986; Morris et al. 2012]. Our group has also previously shown that large numbers of human spermatozoa from normozoospermic samples can be successfully vitrified in small volumes [Moskovtsev et al. 2012].

In addition to the method utilized for cryopreservation and thawing, the survival of spermatozoa depends on the initial quality of semen. It has been demonstrated that semen from patients with oligozoospermia and/or asthenozoospermia have a tendency toward reduced cryosurvival after using standard freezing techniques [Schuster et al. 2003]. However, in cases with severe oligozoospermia, cryptozoospermia, epididymal or testicular samples, both the conventional method of cryopreservation or present vitrification techniques are less suitable. While there are some publications on the concept of cryopreservation of a small number or single spermatozoa available, the concern remains about the technical difficulties of such procedures, the toxic effect of permeable CPAs or risk of cross-contamination in an open freezing system [Cohen et al. 1997; Desai et al. 2004; Endo et al. 2011]. An additional difficulty achieving post thawing/warming recovery of a very small number of spermatozoa is apparent due to adherence of spermatozoa to the walls of the straws or the loss of valuable spermatozoa during the subsequent thawing sperm wash procedures. The purpose of this study was to optimize vitrification and post thaw recovery of a small number of spermatozoa using nonpermeating CPAs in a closed straw system for both normozoospermic patients and men with severe oligozoospermia.

Results

Individual motile spermatozoa were selected from semen samples of 15 normozoospermic patients and 10 patients with severe oligozoospermia. A small number of motile spermatozoa, 5 to 20 per straw (mean 11.8) were preselected to micro droplets and loaded to the open-pulled straw (OPS). In total 295 spermatozoa were vitrified in 25 straws from two groups of patients followed by warming, recovery, and reassessment. Overall sperm recovery after vitrification was 80% ($n = 236$) with 80% ($n = 189$) viability and 41.5% ($n = 98$) retained post-warming motility.

Semen samples from both groups of patients were randomly assigned to compare two different loading techniques to transfer selected spermatozoa into the OPS in preparation for vitrification: by spontaneous capillary action (CA) and with the aid of the polar body biopsy (PBB) pipette. No significant differences were observed in the following variables: patient age, initial sperm concentration, motility, as well as post-wash parameters (sperm concentration and motility) between two loading techniques in both groups of patients as summarized in Table 1. The use of the PBB pipette allowed loading of all selected spermatozoa in each case, in comparison to the CA technique, where between 10 to 25% of preselected spermatozoa remained in the micro droplets after the loadings were completed. Overall, 152 spermatozoa were loaded to the OPS by CA and 111 spermatozoa were recovered (73%), in comparison to the loading of 143 spermatozoa with the aid of the PBB pipette with the recovery of 125 (87%), $p = 0.002$. The use of the PBB pipette was more efficient for recovery of spermatozoa after vitrification in cases of normozoospermic and oligozoospermic samples, Table 1. No significant differences were observed between recoveries of spermatozoa from normozoospermic versus severe oligozoospermic samples when the same loading technique was used. Post-warming viability and motility of spermatozoa were similar for both loading techniques for two types of samples (Table 1). Individual data stratified by groups and loading technique is displayed in Figure 1. The viability of vitrified immotile spermatozoa was also assessed. The percentages of immotile but viable spermatozoa ranged between 31 and 42% (mean 37%) per sample and were similar between normozoospermic and oligozoospermic samples.

Discussion

Cryopreservation of a small number or single spermatozoa remains challenging. While a limited number of studies have addressed this subject, novel cryopreservation initiatives are required to improve sperm survival and recovery. Individual spermatozoa have been successfully frozen in several types of biological carriers, including empty human or hamster zona pellucida [Walmsley et al. 1998] or injected into algae prior to freezing [Just et al. 2004]. Contamination of human spermatozoa with animal or algae genetic material is the major concern for this application. Some non-biological cryopreservation carriers were also suggested, including mini-

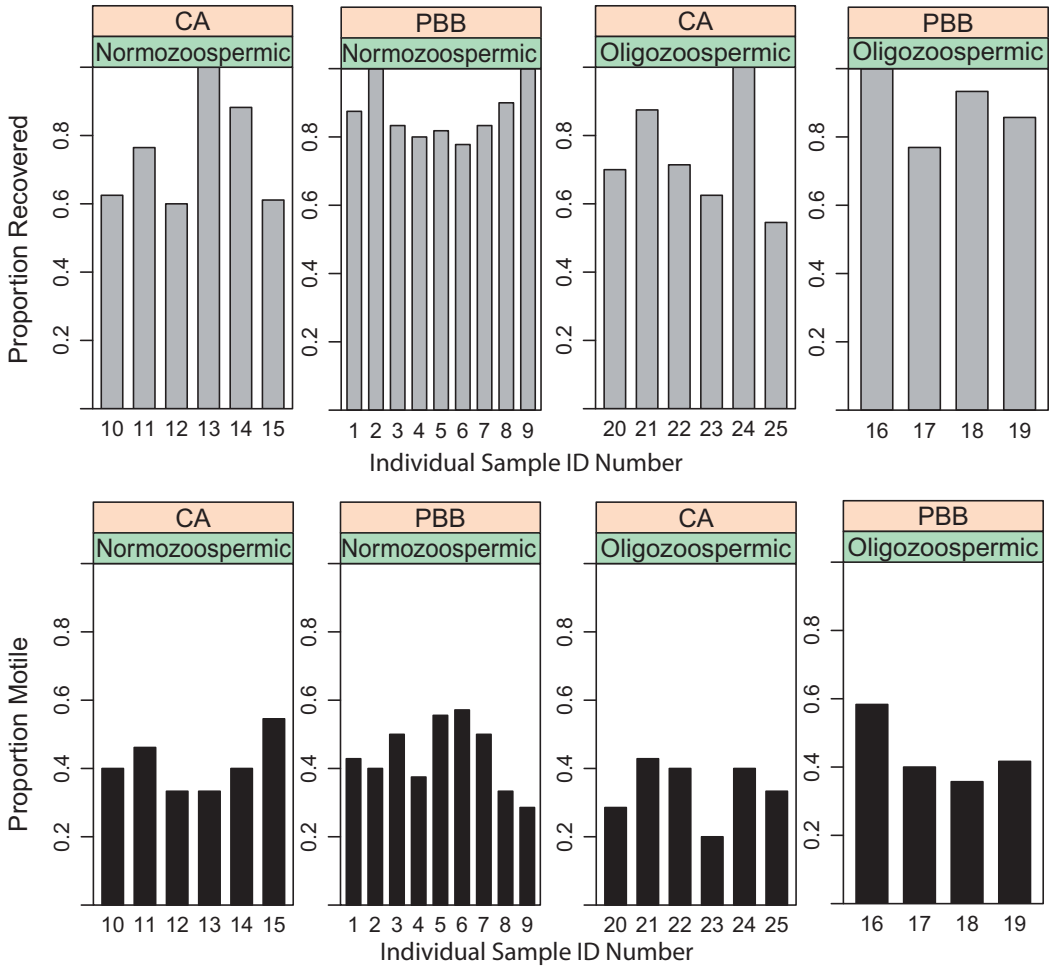

Figure 1. Recovery of sperm numbers and motility after vitrification. Preselected motile spermatozoa from normozoospermic and oligozoospermic semen samples were loaded into open-pulled straws using either spontaneous capillary action (CA) or with the aid of a polar body biopsy (PBB) pipette prior to vitrification. The graphs show recovery post warming of sperm numbers and motility stratified by loading technique and specimen type.

straws made of embryo storage strawscut into smaller sections [Desai et al. 2004], the OPS [Isachenko et al. 2005], intracytoplasmic sperm injection (ICSI) pipettes [Sohn et al. 2003], nylon cryoloops [Desai et al. 2004; Isachenko et al. 2004; Nawroth et al. 2002; Schuster et al. 2003], as well as designed devices like 'Cell Sleeper' and 'CryoTop' [Endo et al. 2011], and even microspheres of gelatinized agarose [Herrler et al. 2006; Isaev et al. 2007]. Reported sperm recovery ranged from 59 to 100% with survival rates of 8 to 85% and varied considerably among the studies due to the diversity of techniques and type of the frozen samples [AbdelHafez et al. 2009].

Slow freezing is the primary recognized method of sperm cryopreservation. However spermatozoa can also be frozen by an alternative method of vitrification that is based on the rapid cooling of water to a glassy state without intracellular ice formation. Successful vitrification of human spermatozoa without the inclusion of permeable CPAs was reported by several groups with good sperm survival [Isachenko et al. 2004; Nawroth et al. 2002]. In contrast, lower viability (8%) was reported after ultra rapid freezing in comparison to slow freezing [Sohn et al. 2003]. Safety of sperm vitrification was validated by the reports of the live births achieved after utilization of vitrified sperm for both intrauterine insemination (IUI) and ICSI [Desai et al. 2012; Endo et al. 2012; Isachenko et al. 2012; Sanchez al. 2012].

Our group has also demonstrated successful vitrification of spermatozoa from normozoospermic patients in small volume (10 μL) in the closed system [Moskovtsev et al. 2012]. Vitrification resulted in similar levels of sperm deoxyribonucleic acid (DNA) damage as conventional sperm cryopreservation. However, recovery of sperm motility was significantly higher in the vitrified samples [Moskovtsev et al. 2011]. In the present study we took the next step towards justifying vitrification for a small number of spermatozoa (5–20 spermatozoa per straw) in the closed system for both normozoospermic patients and patients with severe oligozoospermia. In addition, two different loading techniques were compared to transfer selected spermatozoa into the OPS in preparation for vitrification by spontaneous CA and with the aid of the PBB pipette equipped micromanipulator. We recognize that ideally the same number of patients would be randomized to both groups, however, sampling constraints prevented this from being possible.

The loading of selected spermatozoa aided by the PBB pipettes was somewhat more time-consuming, however it allowed precise loading of all selected spermatozoa on the first attempt. Moreover, the use of the PBB pipette was more

efficient for recovery of spermatozoa after vitrification in both normozoospermic and oligozoospermic samples. In contrast, loading by spontaneous CA resulted in loading an uncontrolled number of spermatozoa that sometimes left up to one quarter of preselected spermatozoa in the micro droplets. Several attempts of capillarity flow were required to load all preselected spermatozoa, undermining the time saving effort in the first place.

The recovery rates of viable spermatozoa depend on the quality of semen before cryopreservation [Borges et al. 2007; Schuster et al. 2003]. Spermatozoa from fertile men have been shown to be more resistant to cryodamage than spermatozoa of infertile individuals when conventional sperm cryopreservation was utilized [Donnelly et al. 2001]. The extensive production of reactive oxygen species (ROS) impacting the impacting the quality of sperm DNA and membrane fluidity has been suggested to affect the recovery of viable spermatozoa after cryopreservation in infertile men [de Paula et al. 2006]. Nevertheless, our results show that similar recovery rates can be obtained from normozoospermic and severely oligozoospermic samples when preselected motile spermatozoa are vitrified. We suggest that vitrification with nonpermeable CPAs avoids the use of more toxic cryoprotectants, and negates the subsequent thawing sperm wash procedures.

A micromanipulator equipped with the PBB pipette is an effective method for simultaneous loading of 5 to 20 motile spermatozoa into each OPS, allowing freezing of multiple small aliquots of spermatozoa from the same sample. Each straw could contain a sufficient number of spermatozoa for a single ICSI cycle to avoid additional cryodamage caused by refreezing and thawing rounds of auxiliary spermatozoa. The technique could be of potential benefit to patients with highly compromised semen such as patients with severe oligozoospermia and cryptozoospermia, and perhaps for freezing of epididymal and testicular spermatozoa.

Materials and Methods

Selection of Subjects

The study was approved by the Institutional Research Ethics Board. Semen samples were obtained with informed consent from 15 normozoospermic patients and 10 patients with severe oligozoospermia undergoing IVF at CReATe Fertility Centre. Semen samples were collected by masturbation after 2 to 5 d of sexual abstinence. A routine semen analysis was performed according to WHO [2010] guidelines. Once IVF treatment has been completed, remaining semen was utilized for vitrification of a small number of spermatozoa using nonpermeating conditions in a closed straw system. Motile spermatozoa were separated by density gradient centrifugation. Briefly, semen was placed on the top of two layers of PureSperm 40/80% gradient (Nidacon Itnl., Mölndal, Sweden) and centrifuged at 400 x g for 15 min allowing separation of motile spermatozoa on the bottom of a 15 mL centrifuge tube. The pellet was re-suspended in 1 mL of PureSperm Wash (Nidacon) medium and centrifuged at 500 x g for 10 min. In cases of cryptozoospermia, ejaculates were washed once with PureSperm Wash medium at 500 x g for 10 min to remove seminal plasma. The final pellets obtained from both types of samples were re-suspended in 0.3 mL of vitrification medium based on InVitroCare HTF Hepes medium (InVitroCare INC, Frederick, MD, USA) supplemented with 0.25 M sucrose (Sigma Aldrich, Oakville, ON, Canada), and 20% LifeGlobal® Protein Supplement (IVF Online, Guelph, ON, Canada). Samples were spread on a bottom Falcon™ Petri dish (Fisher Scientific, Ottawa, ON, Canada), overlaid with mineral oil (Vitrolife, Göteborg, Sweden), and assessed under a dissecting microscope. Individual motile spermatozoa were picked up using the PBB pipette (Sunlight Medical, Jacksonville, FL, USA) equipped with a micromanipulator and transferred to a 5 μL micro droplet of vitrification medium at 37 °C without any surrounding debris.

Two different loading techniques were used to transfer selected motile spermatozoa from semen samples of normozoospermic ($n = 15$) and severely oligozoospermic patients ($n = 10$). The spontaneous CA loading technique was randomly assigned to 6 normozoospermic and 6 severely oligozoospermic samples, where the 0.130–0.133 mm micromanipulation pipette (Vitrolife) was placed into 5 μL droplet of vitrification medium allowing spermatozoa to flow by the CA into pipette following release of spermatozoa into the narrow end of the 0.5 mL OPS (Minitüb, Tiefenbach, Germany). In the second loading method, selected spermatozoa from 9 normozoospermic and 4 severely oligozoospermic samples were collected under a microscope with the aid of the PBB pipette equipped with a micromanipulator. The PBB pipette was placed into the narrow end of the OPS prefilled with 5 μL of vitrification medium and spermatozoa were released. The micro droplet was reassessed for the presence of any remaining spermatozoa after each loading was completed and the number of spermatozoa left behind were recorded. The OPS was inserted into a 0.5 ml high security CBS straw (Cryo Bio System, Rambouillet, France). The CBS straws were hermetically closed from both sides using a CBS sealer and immediately plunged into liquid nitrogen and stored for at least 24 h before warming.

For warming, the end of the CBS straw was cut with scissors and the OPS was removed with forceps. The open narrow end of the OPS with vitrified spermatozoa was immediately immersed vertically into InVitroCare medium supplemented with 20% protein (LifeGlobal) at 37 °C for 10 s. The capillary flow of the warm medium allowed a melting of vitrified sample. Subsequently, the contents of the OPS was expelled with the help of a syringe into a droplet of media and covered with some mineral oil. Each sample was assessed to confirm the number of recovered spermatozoa and sperm motility under inverted light microscope equipped with Hoffman optics. Vitality was assessed with a modified hypo-osmotic swelling test (HOST) as previously described [Verheyen et al. 1997]. Spermatozoa with no changes in a straight tail were considered to be non-viable, while live spermatozoa had controlled swelling visualized by the curling of its tail.

Statistical analysis

The data in Table 1 was reported as mean ± SD, unless stated otherwise. For binary variables we reported count and percentage. Logistic regression was used to evaluate the

effect of loading techniques on 3 endpoints: recovery of spermatozoa, motility of spermatozoa, and viability of spermatozoa. All models were adjusted for the effect of donor age and were conducted separately by spermatozoa quality (abnormal and normal). All p values were two-sided, and statistical significance was defined as $p < 0.05$. Data were analyzed in the R statistical software (Version 3.1.0).

Declaration of interest

The material contained in the manuscript is original, has not been published, has not been submitted or is not being submitted elsewhere. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author contributions

Conceived and designed the experiments: VK, SIM, MC, AG-ML, CLL; Performed the experiments: VK, MC; Analyzed the data: VK, SIM, AG-ML, CLL; Contributed reagents/materials/analysis tools: SIM, MC, AG-ML; Wrote the manuscript: VK, SIM, CLL.

References

AbdelHafez, F., Bedaiwy, M., El-Nashar, S.A., Sabanegh, E. and Desai N. (2009) Techniques for cryopreservation of individual or small numbers of human spermatozoa: A systematic review. Hum Reprod Update **15**:153–64.

Borges Jr. E., Rossi, L.M., Locambo de Freitas, C.V., Guilherme, P., Bonetti, T.C., Iaconelli, A., et al (2007) Fertilization and pregnancy outcome after intracytoplasmic injection with fresh or cryopreserved ejaculated spermatozoa. Fertil Steril **87**:316–20.

Cohen, J., Garrisi, G.J., Congedo-Ferrara, T.A., Kieck, K.A., Schimmel, T.W., and Scott, R.T. (1997) Cryopreservation of single human spermatozoa. Hum Reprod **12**:994–1001.

de Paula, T.S., Bertolla, R.P., Spaine, D.M., Cunha, M.A., Schor, N. and Cedenho, A.P. (2006) Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. Fertil Steril **86**:597–600.

Desai, N., Blackmon, H., and Goldfarb, J. (2004) Single sperm cryopreservation on cryoloops: An alternative to hamster zona for freezing individual spermatozoa. Reprod Biomed Online **9**:47–53.

Desai, N., Goldberg, J., Austin, C., Sabanegh, E. and Falcone, T. (2012) Cryopreservation of individually selected sperm: Methodology and case report of a clinical pregnancy. J Assist Reprod Genet **29**:375–9.

Donnelly, E.T., Steele, E.K., McClure, N. and Lewis, S.E. (2001) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod **16**:1191–9.

Endo, Y., Fujii, Y., Kurotsuchi, S., Motoyama, H., and Funahashi, H. (2012) Successful delivery derived from vitrified-warmed spermatozoa from a patient with nonobstructive azoospermia. Fertil Steril **98**:1423–7.

Endo, Y., Fujii, Y., Shintani, K., Seo, M., Motoyama, H., and Funahashi, H. (2011) Single spermatozoon freezing using Cryotop. J Mamm Ova Res **28**:47–52.

Fahy, G.M. (1986) The relevance of cryoprotectant ''toxicity'' to cryobiology. Cryobiology **23**:1–13.

Henry, M.A., Noiles, E.E., Gao, D., Mazur, P., and Critser, J.K. (1993) Cryopreservation of human spermatozoa. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. Fertil Steril **60**:911–18.

Herrler, A., Eisner, S., Bach, V., Weissenborn, U. and Beier, H.M. (2006) Cryopreservation of spermatozoa in alginic acid. Fertil Steril **85**:208–13.

Isachenko, V., Isachenko, E., Katkov, I.I., Montag, M., Dessole, S., Nawroth, F., et al (2004) Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod **71**:1167–73.

Isachenko, V., Isachenko, E., Montag, M., Zaeva, V., Krivokharchenko, I., Nawroth, F., et al (2005) Clean technique for cryoprotectant-free vitrification of human spermatozoa. Reprod Biomed Online **10**:350–4.

Isachenko, V., Isachenko, E., Petrunkina, A.M. and Sánchez, R. (2012) Human spermatozoa vitrified in the absence of permeable cryoprotectants: Birth of two healthy babies. Reprod Fertil Dev **24**:323–6.

Isaev, D.A., Zaletov, S.Y., Zaeva, V.V., Zakharova, E.E., Shafei, R.A., and Krivokharchenko, I.S. (2007) Artificial microcontainers for cryopreservation of solitary spermatozoa. Hum Reprod **22**:i154–5.

Just, A., Gruber, I., Wober, M., Lahodny, J., Obruca, A., and Strohmer, H. (2004) Novel method for the cryopreservation of testicular sperm and ejaculated spermatozoa from patients with severe oligospermia: A pilot study. Fertil Steril **82**:445–7.

Morris, G.J., Acton, E., Murray, B.J. and Fonseca, F. (2012) Freezing injury: The special case of the sperm cell. Cryobiology **64**:71–80.

Moskovtsev, S.I., Kuznyetsov, V., Spiridonov, S., Lulat, A., Crowe, M., and Librach, C.L. (2011) Comparison of vitrification and slow vapor protocols for cryopreservation of human spermatozoa. In: Proceeding of Annual Canadian Fertility and Andrology Society Meeting: p. 119.

Moskovtsev, S.I., Lulat, A.G.-M., and Librach, C.L. (2012) Cryopreservation of human spermatozoa by vitrification vs. slow freezing: Canadian experience. In: Cryopreservation Book 1. Katkov, I, ed. Rijeka, Croatia: Tech - Open Access Publisher, pp. 84–7.

Nawroth, F., Isachenko, V., Dessole, S., Rahimi, G., Farina, M., Vargiu, N., et al (2002) Vitrification of human spermatozoa without cryoprotectants. CryoLetters **23**:93–102.

Ozkavukcu, S., Erdemli, E., Isik, A., Oztuna, D., and Karahuseyinoglu, S. (2008) Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. J Assist Reprod Genet **25**:403–11.

Sanchez, R., Isachenko, V., Petrunkina, A.M., Risopatron, J., Schulz, M. and Isachenko, E. (2012) Live birth after intrauterine insemination with spermatozoa from an oligoasthenozoospermic patient vitrified without permeable cryoprotectants. J Androl **33**:559–62.

Schuster, T.G., Keller, L.M., Dunn, R.L., Ohl, D.A., and Smith, G.D. (2003) Ultra-rapid freezing of very low numbers of sperm using cryoloops. Hum Reprod **18**:788–95.

Sohn, J.O., Jun, S.H., Park, L.S., Kim, E.K., Chung, T.G., and Lee D.R. (2003) Comparison of recovery and viability of sperm in ICSI pipette after ultra rapid freezing or slow freezing. Fertil Steril **80**:128.

Verheyen, G., Joris, H., Crits, K., Nagy, Z., Tournaye, H., and Van Steirteghem, A. (1997) Comparison of different hypo-osmotic swelling solutions to select viable immotile spermatozoa for potential use in intracytoplasmic sperm injection. Hum Reprod Update **3**:195–203.

Walmsley, R., Cohen, J., Ferrara-Congedo, T., Reing, A. and Garrisi J. (1998) The first births and ongoing pregnancies associated with sperm cryopreservation within evacuated egg zonae. Hum Reprod **13**:61–70.

WHO (2010) WHO laboratory manual for the examination and processing of human semen, 5th ed. World Health Organization, WHO Press, Geneva, Switzerland.

Woods, E.J., Benson, J.D., Agca, Y., and Critser, J.K. (2004) Fundamental cryobiology of reproductive cells and tissues. Cryobiology **48**:146–56.