Human sperm vitrification: state of the art

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Summary

The cryopreservation of human spermatozoa is routinely performed to preserve fertility prior to radiotherapy or chemotherapy in cancer patients or for reproductive medicine programmes. The gold standard method for sperm preservation is rapid freezing. An alternative to conventional cryopreservation protocols is vitrification, which to date has not been used for routine sperm cryopreservation. Sperm vitrification has some advantages: it renders the use of potentially toxic permeable cryoprotectants superfluous, it enables even a low number of spermatozoa to be preserved, and could have great potential in assisted reproduction. However, some doubts about this method still remain: not all carriers are standardized and certified for cryogenics. There is also a concern about the technical difficulties or risk of cross-contamination in an open freezing system. This mini-review summarizes the studies conducted on different sperm vitrification technologies, including cryoprotectant-free and non-permeable cryoprotectant systems and the different carriers used. It also discusses the literature data on live births from vitrified spermatozoa and compares vitrification against slow and rapid freezing, above all in terms of sperm motility, viability and DNA integrity.

KEY WORDS: sperm cryopreservation, slow freezing, rapid freezing, cryoprotectant, artificial reproductive techniques.

Introduction

The cryopreservation of human spermatozoa is routinely used to preserve fertility prior to radiotherapy or chemotherapy in cancer patients or for reproductive medicine programmes. The conventional sperm cryopreservation includes *slow and rapid freezing* methods. *Slow freezing* uses automatic equipment which takes cells from room temperature to the storage temperature at a controlled rate. The sample is first diluted with cryoprotectant and then taken from room temperature to 5° C at a rate of 0.5-1°C/min. The temperature is then lowered to -

80° C at 1-10° C/min, and finally the sample is plunged in liquid nitrogen at -196°C (1). On the other hand *rapid freezing* not require automatic equipment. The most common technique is vertical freezing, carried out in cryogenic containers of a suitable size. Semen samples are diluted with cryoprotectant and left to equilibrate at 37° C for 10 minutes. The suspension is aspirated with a vacuum pump directly into straws. The straws are sealed and placed in nitrogen vapour for 8 minutes, and then immersed in liquid nitrogen at -196° C (2). In both of these methods, the cells are exposed to cryoprotectant before freezing to protect them from cooling damage. There are two main classes of cryoprotectant. *Permeable cryoprotectants*, which penetrate the cell membrane. This class includes dimethyl sulfoxide (DMSO), glycerol, 1,2 propanediol (PROH) and ethylene glycol. These hydrophilic substances cross the membrane very easily, creating an osmotic gradient and causing water to leave the cell by lowering the freezing point even further. *Non-permeable cryoprotectants*, which do not cross the cell membrane. These include sucrose, fructose, glucose, dextrose, starch, lipoproteins and polyvinylpyrrolidone (PVP). These large molecules increase the concentration of extracellular solutes, thus generating an osmotic gradient that causes water to leave the cell, causing dehydration before freezing takes place (3). The gold standard method is rapid freezing and the most common cryoprotectant is glycerol with egg yolk, which reduces the incidence of osmotic stress within the spermatozoa (4). However, freezing with permeable cryoprotectants leads to mechanical cell injury due to intracellular and extracellular ice crystal formation and can cause chemical and physical damage to the sperm membrane, due to changes in lipid-phase transition and increased lipid peroxidation (5). An alternative to conventional cryopreservation protocols is vitrification, which to date has not been used for routine sperm cryopreservation. Vitrification is an "ice-free" cryopreservation method involving the solidification of a solution through an extreme elevation in its viscosity during cooling (6). To eliminate ice formation and create a glass-like state, small liquid suspensions must be frozen extremely rapidly. The standard method for the cryopreservation of oocytes or embryos involves the use of high concentrations of permeable cryoprotectants in combination with a rapid cooling rate, achieved by plunging the vitrification solution directly into liquid nitrogen (LN) (7). However, vitrification protocols involving highly concentrated cryoprotectants are not suitable for human spermatozoa, due to the lethal effect of osmotic shock. It is known that human spermatozoa contain large amounts of protein, sugars and other components that make the intracellular matrix highly viscous and may act as natural cryoprotectants. They can be successfully frozen even without permeable cryoprotectants, using only protein and sugar-rich extracellular non-permeable cryoprotectants (8). Carbohydrates are used to compensate the osmotic effects of permeable cryoprotectants and stabilize the sperm membrane. Sucrose can thus be considered a natural, non-toxic cryoprotectant. For this reason, the use of different concentrations of sucrose has been investigated during human sperm vitrification/warming (8-12). Sperm vitrification protocols involving a non-permeable cryoprotectant and directly plunging the sperm suspension into LN have been demonstrated to offer good sperm survival (13-16). This mini-review summarizes the studies conducted on different sperm vitrification technologies, investigating cryoprotectant-free and non-permeable cryoprotectant systems and the different carriers used as well as the impact of direct contact of spermatozoa with LN. It also discusses literature data on live births from vitrified sperm and compares vitrification against slow and rapid freezing, above all in terms of sperm motility, viability and DNA integrity.

History

Vitrification was first used in the early 1900s, but has only recently aroused interest as an alternative to routine cryopreservation methods. Although the ability of human spermatozoa to survive at temperatures below zero was discovered in 1897, the first studies investigating the possibility of freezing spermatozoa at very low

temperatures were conducted in the 1930s (17). In 1938 Jahnel discovered that some spermatozoa survive after freezing at -79 °C, at -196 °C (in LN) and at -269 °C (in liquid helium) (18). Various Authors attempted to reproduce these results in later years by freezing spermatozoa at the same temperatures in glass capillary tubes, but achieved limited success only in semen samples frozen at -79 °C (19). In 1942, Hoagland and Pincus tried to identify different devices to facilitate semen vitrification in LN, finding poor sperm survival in almost all their experiments (20). In 1945, Parkes conducted comparative tests of the vitrification of human semen samples in ethyl alcohol with solid CO_2 (-20 °C, -79 °C) and in LN (-196 °C) for different freezing times, finding a good percentage of motile sperm cells with some devices at -79 °C and at -196 °C. This study focussed on the freezing/thawing rate and on the different devices used for freezing (17). One of the major limitations of this method was, in fact, the lack of devices enabling the procedure to be performed more quickly.

Despite some encouraging results, work on the vitrification of human semen was abandoned due to growing interest in the use of cryoprotectants, as proposed by Polge et al. in 1949. These Authors found that glycerol could protect the seminal fluid of a number of species, including humans, against damage from freezing (21). This study marked the dawn of a new age in which semen cryopreservation became a routine procedure thanks to the use of permeable cryoprotectants. Their success led to a diminished interest in vitrification, which required very high concentrations of cryoprotectants that were toxic to spermatozoa.

Methods and carriers

A number of sperm vitrification protocols have been published in recent years, with different methods for vitrifying spermatozoa and different combinations of cryoprotectants, if any. Different devices have also been tested. Isachenko et al. recently highlighted the need to consider asepsis as a criterion for cryoprotectant-free sperm vitrification techniques (22). In fact many of the described protocols involve the direct contact of spermatozoa with LN, and hence a potential risk of microbial contamination.

Methods involving direct contact with liquid nitrogen

Cryoloop

The first important study investigated the effects of cryoprotectant-free vitrification on swim-up-prepared human spermatozoa in comparison with standard slow freezing and vitrification with permeable cryoprotectant. Cryoprotectant-free vitrification was obtained by direct plunging of spermatozoa loaded onto loops into LN. The loops were thawed by plunging them into 10 mL of medium at 37 °C and then placing them in a CO_2 incubator for 10 min at 37 °C. The spermatozoa were then concentrated by centrifugation and the pellet was resuspended in culture medium. This study concluded that cryoprotectant-free vitrification was effective for human spermatozoa (13).

Cryoloop was subsequently explored as a means to cryopreserve small volumes and numbers of spermatozoa using permeable cryoprotectant (23), as well as in protocols using an ultra-rapid freezing rate for cryoprotectant-free vitrification (24). In the latter study, the Authors have evaluated semen quality after cryoprotectant-free cryopreservation of human spermatozoa by freezing in LN vapour and by vitrification method plunging directly into LN. Ejaculates were obtained from 38 healthy men and were prepared by swim-up. Loops loaded with a sperm suspension were cooled for 3 min in LN vapour at -160 °C by holding them ~1.0 cm above the LN level in a foam box before immersion into LN (24).

No differences in the motility, DNA integrity and fertilization ability of the warmed spermatozoa was found among the two different vitrification regimes.

Spheres

In a study of 23 normozoospermic patients, Isachenko et al. demonstrated that the inclusion of 0.25 M sucrose in combination with 1%

human serum albumin (HSA) in the vitrification medium has a cryoprotective effect on mitochondrial membrane integrity, acrosomal status and sperm motility and viability in samples prepared by swim-up. In this study small droplets (30 µl) were dropped directly by micropipette into the LN with a strainer. Upon contact with the LN, a sphere immediately formed and solidified at the bottom of the strainer. The spheres were packed into cryovials. Warming was performed by submerging the spheres one by one into medium pre-warmed to 37 °C (16). The same vitrification and warming protocols were used by Agha-Rahimi et al. in a study that showed that vitrification in artificial seminal fluid can preserve spermatozoa comparably with vitrification in HSA and sucrose (25). Taherzadeh et al., carried out experiments on 20 normal ejaculates using spermatozoa prepared by swim up, with 0.5 M sucrose used for vitrification of the spheres in cryovials. The Authors investigated the influence of vitrification on the fine structure of human spermatozoa using motile sperm organelle morphology examination (MSOME) and fertility potential with zona binding assay. The study showed that vitrification had adverse effects on sperm parameters and increased vacuolization in the sperm head, which may interfere with the fertilization process (26). In a recent study Aizpurua et al. used a new sperm vitrification kit, evaluating the performance of a permeable cryoprotectant-free sperm vitrification protocol on native semen samples. After addition of vitrification medium and incubation, the samples were plunged drop by drop (20 µl) into LN to form solid spheres. Fifty spheres were stored in cryotubes for at least 10 days to enable the preservation of large volumes of samples. The spheres were warmed by placing them in 4 mL of warmed solution. After 5 min at 37 °C, the samples were centrifuged and then resuspended in wash medium (27).

Cryotop

The cryopreservation of a small number of human sperm can also be assessed by Cryotop, a special vitrification device consisting of a thin film strip attached to a hard plastic handle. This carrier was found to be suitable for the cryopreservation of human sperm even in the absence of cryoprotective agents. Chen et al., using swim-up from ejaculate sperm collected from 21 normozoospermic patients, reported that post-warming sperm cryopreserved without sucrose had a higher viability and lower sperm chromatin and DNA damage than those cryopreserved with 0.25 M sucrose (28).

Methods involving full isolation from liquid nitrogen

Isachenko et al. presented data demonstrating the extremely high cryo-resistance of viruses, bacteria, mycoplasma and fungi. Some bacteria are also highly resistant to ionizing radiation and ultraviolet light, and the colony-forming ability of ultraviolet irradiated mycoplasma cells completely recovered after 3 hours in the dark (22). These data were the grounds for the development of cryoprotectant-free vitrification techniques involving full sperm isolation from LN. Some cryovials and straws allow spermatozoa to be cryopreserved without direct contact with LN and hence with no risk of cross-contamination by different pathogens.

Open pulled straws and standard open straws

Some years ago Isachenko et al. tested sperm vitrification in ejaculates from 16 oligoasthenoteratozoospermic (OAT) patients. The aim of their study was to compare the motility and viability of sperm cryopreserved using four different methods and carriers: cryoloops, spheres, open-pulled straws and standard open straws. For the last two methods a 1 to 5 µl aliquot of sperm suspension was deposited inside the end of standard open 0.25 mL straws or drawn inside the end of open-pulled straws by capillary action; these straws were placed inside a 90 mm straw that was hermetically sealed using a hand-held sealer and then plunged into LN. No statistically significant difference in the analysed parameters was found among the different cryopreservation regimes and the Authors concluded that the open-pulled straw method was preferable because it allowed isolation of the spermatozoa from LN, thus minimising the potential risk of microbial contamination (15).

Kuznyetsov et al. showed that a micromanipulator equipped with polar body biopsy pipette is an effective method for vitrification of a small number of spermatozoa in an open-pulled straw inserted into a 0.5 mL high security straw. The straws were hermetically sealed at both ends (29). In another study a 50 µL plastic capillary was filled with 10 µL of sperm suspension by aspiration. The capillary was then inserted into a 0.25 mL straw, which was sealed at both ends using a heat sealer; it was concluded that the aseptic vitrification of human spermatozoa in capillaries can effectively preserve these cells from cryo-injury (10). In another study, large-volume sperm suspensions were cooled in 0.5 mL high security straws hermetically sealed at both ends using the flame of an alcohol burner and forceps. The straws were immediately immersed horizontally into LN. Suspensions of swim-up-prepared spermatozoa obtained from normozoospermic patients were diluted with 0.25 M of sucrose for aseptic vitrification finding good sperm quality after thawing (9).

Cell Sleeper

Cell Sleeper is a type of vial used as a cell cryopreservation container, which is equipped with an inner tray and sealed with a screw cap. It has been used to freeze small numbers of spermatozoa. Similar recovery, motility and viability rates of vitrified-warmed human spermatozoa were seen with the cell sleeper and the cryotop (30).

Comparison between vitrification and slow or rapid freezing

Vitrification compared with slow freezing

A number of studies in the literature have compared vitrification against slow freezing to establish which method is more efficient. One of the first, by Nawroth et al., compared the effects of vitrification (with and without cryoprotectants) against the slow freezing method (with and without cryoprotectants) in 30 semen samples from healthy subjects, each divided into native and swim-up samples. This study confirmed that both cryoprotectant-free slow freezing and vitrification with cryoprotectants drastically reduce the baseline motility of both the native sample and spermatozoa separated by swim-up. The best results were obtained with cryoprotectant-free vitrification of spermatozoa separated by swim-up. However, there was no difference between cryoprotectant-free vitrification and slow freezing in morphology, post-thaw viability, recovery of motile sperm (percentage of post-thaw motility x 100% divided by percentage of pre-freeze motility) or acrosomal reaction. The study also found that the embryo formation rate achieved with post-vitrification sperm was comparable to that of fresh sperm (13). The Authors therefore suggested the use of cryoprotectant-free sperm vitrification as a potential assisted reproduction technique. In 2004, the same researchers analysed 18 normozoospermic semen samples for the effects of cryopreservation on motility, morphology, viability and sperm chromatin integrity. The samples were separated by swim-up and underwent vitrification and slow freezing (both methods performed with and without cryoprotectants). There was no difference between cryoprotectant-free vitrification and slow freezing with cryoprotectants in post-thaw percentage of motile sperm or in chromatin integrity evaluated by Comet assay (14). Isachenko et al. conducted a number of studies with the aim of developing an aseptic method for the vitrification of human spermatozoa for use in intrauterine insemination (IUI) (9), intracytoplasmic sperm injection (ICSI) or fertilization *in vitro* with embryo transfer (FIVET) (10). Their 2011 study (9) was conducted on 52 samples from normozoospermic patients separated by swim-up and divided into three aliquots: non-cryopreserved control sample, sample frozen with conventional slow method using glycerol, and sample vitrified in the presence of sucrose. Both slow freezing and vitrification were performed using 0.5 mL straws sealed at both ends. The motility, viability and acrosomal membrane integrity in vitrified samples was significantly higher than in the slow-frozen samples. No significant difference in the percentage of capacitated sperm analysed by chlortetracycline staining was found between

the two methods. In 2012 (10), the same researchers analysed 68 OAT samples subjected to swim-up and divided into three aliquots as described above. Motility (1, 24 and 48 hours after thawing), viability, acrosomal integrity and capacitation were analysed in thawed samples. Permeable cryoprotectant-free vitrification showed a greater ability to protect the spermatozoa than the conventional method; one hour after thawing, the vitrified samples presented a significantly higher motility, viability and acrosomal integrity and a lower percentage of capacitated sperm than the slow-frozen samples. The two methods have also been compared by other groups, such as Moskovtsev et al. These Authors evaluated 11 samples from infertile patients, which were subjected to density gradient centrifugation and analysed by Computer Assisted Sperm Analysis (CASA). They found a significant decrease in post-thaw motility with both methods, but the results following vitrification were better than those following slow freezing. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis of DNA damage did not reveal any significant difference between the two methods (unpublished data) (31). Zhu et al. investigated the effects of vitrification with different sucrose concentrations in semen samples from 48 healthy donors and 10 infertile patients. The samples underwent swim-up and were divided into six aliquots: native, slow-frozen, and vitrification in cryogenic vials at different sucrose concentrations (0.15 M, 0.20 M, 0.25 M, 0.30 M). The optimal sucrose concentration for vitrification was found to be 0.20 M. This concentration enabled the recovery of higher quality spermatozoa with increased progressive motility, plasma membrane integrity and acrosomal integrity in comparison with slow-frozen samples (11). There were no significant differences between the various methods in chromatin integrity evaluated using the sperm chromatin dispersion (SCD) test. In 2015, Slabbert et al. studied vitrification (with non-permeable cryoprotectant) and slow freezing in 35 normozoospermic semen samples, finding no difference in post-thaw motility. However, they found a significantly higher mitochondrial membrane potential and a lower percentage of DNA fragmentation on TUNEL assay in the vitrified sperm than in slow-frozen sperm (8). Also in 2015, Ali Mohamed analysed 33 normo- and oligoasthenozoospermic semen samples treated with swim-up or double centrifugation and divided into three groups: fresh control, slow-frozen sperm and vitrified sperm (with non-permeable cryoprotectant). No significant differences were found between the two methods in the motility, viability or mitochondrial membrane potential of the post-thaw sperm (12). Finally, Aizpurua et al. recently conducted a study on 18 normozoospermic samples, each divided into three aliquots and treated with different methods: unfrozen, vitrified using a specific new kit developed for vitrification without permeable cryoprotectants, and slow-frozen. The results demonstrated that use of the kit led to a significantly higher percentage of viable sperm, progressive motility, and number of sperm with intact acrosome and lower DNA fragmentation than seen in slow-frozen samples (27) (Table 1).

Vitrification compared with rapid freezing

Few studies have compared vitrification against rapid freezing. Satirapod et al. investigated sperm motility, morphology, viability and DNA fragmentation in normozoospermic semen samples. No significant differences were seen between the two procedures for the first three parameters, but a decreased level of sperm DNA fragmentation was observed after vitrification (with permeable cryoprotectant). It should be stressed that the Authors did not describe the number of patients studied, so it is difficult to interpret this study from a statistical perspective (32). Agha-Rahimi et al. examined 30 swim-ups from normozoospermic semen samples subjected to rapid freezing and vitrification with permeable and non permeable cryoprotectant. The Authors investigated post-thaw sperm motility, viability, DNA fragmentation, and maturation, analysed by hyaluronic acid binding. They concluded that there were no statistically significant differences between the two methods in any of the parameters, and that vitrification was not superior to rapid freezing in terms of the recovery of motile sperm (33) (Table 2).

Table 1. Literature data on comparison between Vitrification *vs* Slow Freezing method.

Authors (year)(reference no.)	Caseload	Semen Preparation	Vitrification Cryoprotectant	Device	Sperm Motility Pre/Post Thawing (%) mean±SD	Viability Pre/Post Thawing (%) mean±SD	Sperm DNA damage Pre/Post Thawing (%) mean±SD
					PR	SW **	
Nawroth, et al. 2002 (13)	30 N	Raw Semen	PC	DC LN	SW (DC LN)	V (NC): 59.5±14.1^{ns}	/
		SW	NC	FI LN	V (NC): 87.3±7.9 / 49.5±12.5^{b}	SF(PC): 59.1 ±9.7	
					SF (PC): 87.3 ±7.9 / 37.9±14.4		
					PR		COMET*
Isachenko, et al. 2004 (14)	18 N	SW	PC	DC LN	V (NC): 89.5 ±7.1 / 51.5±4.5^{ns}	/	V (PC): 85.1 / 87.2^{ns}
			NC		SF (PC): 77.5±8.9 / 46.7±4.1		SF (PC): 85.1 / 84.6
							V (NC): 89.5 / 84.7^{ns}
							SF(NC):dal 89.5 / 83.5
					PR		
Isachenko, et al. 2011 (9)	52 N	SW	NPC	FI LN	V: 85.0±5.1 / 76.0±4.7^{b}	V: 98.2±0.5 / 54.0±5.0^{b}	/
					SF: 85.0 ±5.1 / 52.0±3.9	SF: 98.2±0.5 / 28.3±3.5	
					PR mean±SEM	mean±SEM	
Isachenko, et al. 2012 (10)	68 OAT	SW	NPC	FI LN	V: 35.0±9.5 / 28.0±6.0^{b}	V: 96.0±0.6 / 56.0±5.1^{b}	/
					SF: 35.0±9.5 / 18.0±9.2	SF: 96.0±0.6 / 22.0±3.5	
					TM		TUNEL
Moskovtsev, et al. 2012 (31)	11 infertile	DDG	NPC	FI LN	V: 68.0±10.8 / 25.4±13.6^{b}	/	V: 7.5±5.5 / 9.6±4.4^{ns}
					SF: 68.0 ±10.8/ 14.6±10.2		SF: 7.5±5.5 / 9.5±5.1
					PR (0.20 M Sucrose)	(0.20 M Sucrose)	SCD (0.20M Sucrose):
Zhu, et al. 2014 (11)	58 N	SW	NPC	FI LN	V: 92.2±3.8 / 47.5±6.8^{b}	V: 99.0±0.9 / 73.2±6.9^{b}	V: 93.0±3.3 / 90.1±2.8^{ns}
					SF: 92.2±3.8 / 36.4±8.7	SF: 99.0±0.9 / 63.9±6.3	SF: 93.0±3.3 / 87.2±4.7

					TM		TUNEL**
Slabbert, et al. 2015 (8)	35 N	DDG	NPC	FI LN	V: 74.5±9.02 / 20.5±7.4^{ns}	/	V: 2.8±1.0^{a}
					SF: 74.5±9.02 /23.9±12.6		SF: 3.9±1.4
	DC				PR (DC)	DC	
Ali Mohammed, et al. 2015 (12)	9 N +8 OAT	DC and SW	NPC	FI LN	V: 50.4±10.2 / 28.4±5.1^{ns}	V: 37.3±10.2/ 20.5±5.9 ^{ns}	
					SF: 50.4±10.2 / 26.0±3.9	SF: 37.3±10.2 / 18.3±5.3	/
	SW				PR (SW)	SW	
	8 N +8 OAT				V: 61.8±8.4 / 39.7±5.6^{ns}	V: 65.4±16.5 / 35.9±10.6^{ns}	
					SF: 61.8±8.4 / 36.3±5.3	SF: 65.4±16.5 / 32.3±7.9	
					PR mean±SEM	mean±SEM	TUNEL *
Aizpurua, et al. 2017 (27)	18 N	Raw Semen	NPC	DC LN	V: 47.7±4.1 / 18.2±2.7^{b}	V: 87.8±1.7 / 76.4±1.53 ^{b}	V: 12.6 / 20.0^{b}
			(kit)		SF: 47.7±4.1 / 11.3±2.7	SF: 87.8±1.7 / 55.1±4.8	SF: 12.6 / 27.4

Abbreviations

a p< 0.01 Vitrification vs. Slow Freezing
b p< 0.05 Vitrification vs. Slow Freezing
ns: no significant difference between Vitrification and Slow Freezing
* SD not available
**Pre-treatment data non available
ABBREVIATIONS
N, Normozoospermic
OAT, Oligoasthenoteratozoospermic
DC, Double centrifugation
DDG, Discontinuous Density Gradient
SW, Swim-up
NC, No Cryoprotectant
NPC, Non-permeable cryoprotectant
PC, Permeable cryoprotectant
DC LN, Direct Contact with Liquid Nitrogen
FI LN, Full Isolation from Liquid Nitrogen
PR, Progressive Motility
TM, Total Motility
SF, Slow Freezing
V, Vitrification

Table 2. Literature data on comparison between Vitrification *vs* Rapid Freezing method.

Authors (year) (reference no.)	Caseload	Semen Preparation	Vitrification Cryoprotectant	Device	Sperm Motility Pre/Post Thawing (%) mean±SD	Viability Pre/Post Thawing (%) mean±SD	Sperm DNA damage Pre/Post Thawing (%) mean±SD
					TM median (range)		TUNEL
Satirapod, et al. 2012 (32)	*	Raw Semen	PC	DC LN	V: 65.7(51-83) / 21.9(6-48)^{ns} RF: 65.7(51-83) / 20.5(6-47)	V: 80.9±7.8 / 24.3±12.4^{ns} RF: 80.9±7.8 / 25.4±13.4	V: 19.1±5.7 / 59.8±11.5^{a} RF: 19.1±5.7 / 64.9 ±13.9
					TM		TUNEL
Agha-Rahimi, et al. 2014 (33)	30 N	SW	PC NPC	DC LN	V(NPC): 96.2±2.5 / 53.9±9.5^{ns} RF: 96.2±2.5 / 48.6±14.2	V(NPC): 95.8±3.9 / 64.4±10.0 ^{ns} RF: 95.8±3.9 / 63.2±7.6	V(NPC): 11.6±4.5 / 15.7±4.4^{ns} RF: 11.6±4.5 / 16.6±5.6

Abbreviations

a $p < 0.05$ Vitrification vs. Rapid Freezing

ns: no significant difference between Vitrification and Rapid Freezing

*Caseload not available

ABBREVIATIONS

N, Normozoospermic

SW, Swim-up

NPC, Non-Permeable Cryoprotectant

PC, Permeable Cryoprotectant

DC LN, Direct Contact with Liquid Nitrogen

TM, Total Motility

RF, Rapid Freezing

V, Vitrification

Live birth from vitrified spermatozoa

To our knowledge, only four successful deliveries have been reported following the use of thawed vitrified spermatozoa. In fact, there are only three case reports in the literature. Isachenko et al. reported the case of a 39-year-old woman with endometriosis and her 39-year old husband with oligoasthenozoospermia who underwent ART. The patient's semen was treated by swim-up using a vitrification solution containing human tubal fluid (HTF) and 1% HSA, supplemented with sucrose 0.25 M. The spermatozoa were frozen using the standard cut straws closed system: 10 µL of sperm suspension was placed in a 0.25 mL cut straw, which was in turn placed in a handheld 0.5 mL straw, which was then hermetically sealed. The semen was then rapidly frozen in HTF+HSA 1% before being pre-warmed and used for ICSI, resulting in the birth of healthy twin girls (34). In the same year, Sanchez et al. described the case of a 39-year-old woman and 35-year-old man affected with primary fertility of 3 years' duration. The semen sample, which was oligoasthenozoospermic, was treated with swim-up and underwent vitrification with the same solution and carriers as in the previous study. The thawed semen was used for intrauterine insemination (IUI), leading to the birth of a healthy boy (35). Finally, Endo et al. reported on three couples undergoing ART. The first consisted of a 29-year-old woman and her 30-year-old partner with non-obstructive azoospermia (NOA), who underwent MicroTese; the second couple were both 34 years old, with the male partner suffering from oligozoospermia, and the third were both 37 years old, with the male partner suffering from NOA. The spermatozoa were vitrified with a freezing medium containing both glycerol and sucrose. The Authors used Cryotop and/or Cell Sleeper. Only the first couple achieved pregnancy; the spermatozoa recovered by MicroTese were vitrified in a Cell Sleeper and subsequently used for ICSI (36) (Table 3).

Table 3. Literature data on live births from vitrified spermatozoa.

Authors (year)(reference no.)	Caseload	Semen preparation	Vitrification Cryoprotectant	Device	ART Tecnique	Live birth
Isachenko, et al. 2012 (34)	♀ 39 yrs ♂ 39 yrs OAT	SW	NPC	FI LN	ICSI	twin girls
Sanchez, et al. 2012 (35)	♀ 39 yrs ♂ 35 yrs OA	SW	NPC	FI LN	IUI	one boy
	3 COUPLES:					
	1 - ♀ 29 yrs, ♂ 30yrs NOA	1- micro -TESE	PC	1 -FI LN	ICSI	1- one boy
Endo, et al. 2012 (36)	2- ♀ 34yrs, ♂ 34 yrs SO	2- WM		2 -FI LN and DC LN		
	3- ♀ 37 yrs, ♂ 37 yrs NOA	3- micro-TESE		3- DC LN		

Abbreviations
NOA, Non Obstructive Azoospermia
OA, Oligoasthenozoospermic
OAT, Oligoasthenoteratozoospermic
SO, Severe Oligozoospermia
SW, Swim-up
TESE, Testicular Sperm Extraction
WM, Semen only washed with medium by centrifugation
NPC, Non-Permeable Cryoprotectant
PC, Permeable Cryoprotectant
DC LN, Direct Contact with Liquid Nitrogen
FI LN, Full Isolation from Liquid Nitrogen

Conclusions

Vitrification is now an established, widely used, and standardised method for preserving oocytes. However, the published scientific evidence for its use for sperm preservation is scanty and conflicting. Vitrification has some advantages: it renders the use of potentially toxic permeable cryoprotectants superfluous, it enables even a low number of spermatozoa to be preserved (37) and can be considered a sperm cryopreservation technique with great potential in assisted reproduction. However, there are still a number of concerns with this method. For example, not all the carriers have been standardised and certified for cryogenics. There are also concerns about the technical difficulties or risk of cross-contamination by system in direct contact with liquid nitrogen. Furthermore, to date no prospective randomised trials have been conducted that demonstrate the superiority of vitrification over conventional freezing or of the use of one carrier over another. The studies published in the literature often have very small caseloads and apply the method mainly to normozoospermic samples. It should be interesting evaluate these protocols in large caseload and in low sperm quality samples. It would allow a better evaluation of possible damage of the vitrification method. For these reasons, semen vitrification is still an experimental method mainly used for research, not as a routine method to help men father children. The gold standard for semen cryopreservation is still rapid freezing, given the particular properties of the sperm cell that make this process much simpler than for larger cells such as oocytes and embryos. It also offers good post-thaw viability and reproductive outcome. The benefits and success of cryopreservation must be assessed in terms of the number of live births and the number of men who have become fathers thanks to cryopreservation. Spermatozoa cryopreserved with the conventional method lead to reported success rate of achieving parenthood between 33 and 72% (mean 54%) (38). These data were confirmed by a recent systematic review reporting 49% of patients who achieved parenthood using banked semen, 19% of live birth and 23% of clinical pregnancy (39). In contrast, there are still insufficient literature data on the numbers of live births following vitrification. Further studies are therefore needed to establish the efficiency of sperm vitrification. It does, however, remain a highly promising method.

References

1. Behrman SJ, Sawada Y. Heterologous and homologous insemination with human semen frozen and stored in a liquid-nitrogen refrigerator. Fertil Steril. 1966;17:457-466.
2. Sherman JF. Cryopreservation of human semen. In Handbook of the Laboratory Diagnosis and Treatment of Infertility (eds Keel BA, Webster BW). CRC Press Boca Raton Ann Arbor, Boston. 1990:229-260.
3. Paoli D, Lombardo F, Lenzi A, Gandini L. Sperm cryopreservation: effects on chromatin structure. Adv Exp Med Biol. 2014;791:137-150.
4. Agarwal A, Tvrda E. Chapter 5 Slow Freezing of Human Sperm. Methods Mol Biol. 2017;568:67-78.
5. Gao D, Mazur P and Critser J. Fundamental Cryobiology of mammalian spermatozoa. In Reproductive Tissue Banking (eds AM Karow and JK Critser). Academic press, London. 1997:263-328.
6. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. Theriogenology. 2007;67:81-89.
7. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, Vanderpoel S, Racowsky C. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. Hum Reprod Update. 2017;23:139-155.
8. Slabbert M, du Plessis SS, Huyser C. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. Andrologia. 2015;47:594-599.
9. Isachenko V, Maettner R, Petrunkina AM, Mallmann P, Rahimi G, Sterzik K, Sanchez R, Risopatron J, Damjanoski I, Isachenko E. Cryoprotectant-Free Vitrification of Human Spermatozoa in Large (up to 0.5 mL) Volume: a Novel Technology. Clin Lab. 2011;57:643-650.
10. Isachenko V, Maettner R, Petrunkina AM, Sterzik K, Mallmann P, Rahimi G, Sanchez R, Risopatron J, Damjanoski I, Isachenko E. Vitrification of human ICSI/IVF spermatozoa without cryoprotectants: new capillary technology. J Androl. 2012;33:462-468.
11. Zhu J, Jin RT, Wu LM, Johansson L, Guo TH, Liu YS, Tong XH. Cryoprotectant-free ultra-rapid freezing of human spermatozoa in cryogenic vials. Andrologia. 2014; 46:642-649.
12. Ali Mohamed MS. Slow cryopreservation is not superior to vitrification in human spermatozoa; an experimental controlled study. Iran J Reprod Med. 2015;13:633-644.
13. Nawroth F, Isachenko V, Dessole S, Rahimi G, Farina M, Vargiu N, Mallmann P, Dattena M, Capobianco G, Peters D, Orth I, Isachenko E. Vitrification of human spermato-

zoa without cryoprotectants. Cryo Letters. 2002;23:93-102.
14. Isachenko E, Isachenko V, Katkov II, Rahimi G, Schöndorf T, Mallmann P, Dessole S, Nawroth F. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Hum Reprod. 2004;19:932-939.
15. Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F, Dessole S, Katkov II, van der Ven H. Clean technique for cryoprotectant-free vitrification of human spermatozoa. Reprod Biomed Online. 2005;10:350-354.
16. Isachenko E, Isachenko V, Weiss JM, Kreienberg R, Katkov II, Schulz M, Lulat AG, Risopatrón MJ, Sánchez R. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction. 2008; 136:167-173.
17. Parkes AS. Preservation of human spermatozoa at low temperatures. Br Med J. 1945;2:212-213.
18. Jahnel F. Ueber die Widerstandsfehigkeit von menschlichen Spermatozoonen gegenueber starker Kaelte. Klin Wochenschr. 1938;17:1273-1274.
19. Shettles LB. The respiration of human spermatozoa and their response to various cases and low temperature. Am J Physiol. 1940;128:408-415.
20. Hoagland H, Pincus G. Revival of mammalian sperm after immersion in liquid nitrogen. J Gen Physiol. 1942; 25:337-344.
21. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 1949;164:666.
22. Isachenko V, Rahimi G, Mallmann P, Sanchez R, Isachenko E. Technologies of cryoprotectant-free vitrification of human spermatozoa: asepticity as criterion of effectiveness. Andrology. 2017;5:1055-1063.
23. Desai NN, Blackmon H, Goldfarb J. Single sperm cryopreservation on cryoloops: an alternative to hamster zona for freezing individual spermatozoa. Reprod Biomed Online. 2004;9:47-53.
24. Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, van der Ven H. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod. 2004;71:1167-1173.
25. Agha-Rahimi A, Khalili MA, Nottola SA, Miglietta S, Moradi A. Cryoprotectant-free vitrification of human spermatozoa in new artificial seminal fluid. Andrology. 2016;4:1037-1044.
26. Taherzadeh S, Khalili MA, Agha-Rahimi A, Anbari F, Ghazali S, Macchiarelli G. Vitrification Increased Vacuolization of Human Spematozoa: Application of MSOME Technology. J Reprod Infertil. 2017;18:225-230.
27. Aizpurua J, Medrano L, Enciso M, Sarasa J, Romero A, Fernández MA, Gómez-Torres MJ. New permeable cryoprotectant-free vitrification method for native human sperm. Hum Reprod. 2017;32:2007-2015.
28. Chen Y, Li L, Qian Y, Xu C, Zhu Y, Huang H, Jin F, Ye Y. Small-volume vitrification for human spermatozoa in the absence of cryoprotectants by using Cryotop. Andrologia. 2015;47:694-699.
29. Kuznyetsov V, Moskovtsev SI, Crowe M, Lulat AG, Librach CL. Vitrification of a small number of spermatozoa in normozoospermic and severely oligozoospermic samples. Syst Biol Reprod Med. 2015;61:13-17.
30. Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Simple vitrification for small numbers of human spermatozoa. Reprod Biomed Online. 2012;24:301-307.
31. Moskovtsev SI, et al. Cryopreservation of human spermatozoa by vitrification vs slow freezing: Canadian experience. Current Frontiers in Cryobiology. 2012;77-100.
32. Satirapod C, Treetampinich C, Weerakiet S, Wongkularb A, Rattanasiri S, Choktanasiri W. Comparison of cryopreserved human sperm from solid surface vitrification and standard vapor freezing method: on motility, morphology, vitality and DNA integrity. Andrologia. 2012;44:786-790.
33. Agha-Rahimi A, Khalili MA, nabi A, Ashourzadeh S. Vitrification is not superior to rapid freezing of normozoopsermic spermatozoa: effects on sperm parameters, DNA fragmentation and hyaluronan binding. Reprod Biomed Online. 2014;28:352-358.
34. Isachenko V, Isachenko E, Petrunkina AM, Sanchez R. Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies. Reprod Fertil Dev. 2012;24:323-326.
35. Sanchez R, Isachenko V, Petrunkina AM, Risopatron J, Schulz M, Isachenko E. Live birth after intrauterine insemination with spermatozoa from an oligoasthenozoospermic patient vitrified without permeable cryoprotectants. J Androl. 2012;33:559-562.
36. Endo Y, Fujii Y, Kurotsuchi S, Motoyama H, Funahashi H. Successful delivery derived from vitrified-warmed spermatozoa from a patient with nonobstructive azoospermia. Fertil Steril. 2012;98:1423-1427.
37. AbdelHafez F, Bedaiwy M, El-Nashar SA, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. Hum Reprod Update. 2009;15:153-164.
38. van Casteren NJ, van Santbrink EJ, van Inzen W, Romijn JC, Dohle GR. Use rate and assisted reproduction technologies outcome of cryopreserved semen from 629 cancer patients. Fertil Steril. 2008;90:2245-2250.
39. Ferrari S, Paffoni A, Filippi F, Busnelli A, Vegetti W, Somigliana E. Sperm cryopreservation and reproductive outcome in male cancer patients: a systematic review. Reprod Biomed Online. 2016;33:29-38.