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A novel solution for freezing small numbers of spermatozoa using a sperm vitrification device

Arie Berkovitz[1,2,3], Netanella Miller[1,2,*†], Michal Silberman[4,5], Michael Belenky[3], and Pavel Itsykson[3]

[1]Department of Gynecology and Obstetrics, Meir Medical Center, 59 Tchernichovsky St, Kfar Saba, Israel [2]Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel [3]Department of Gynecology and Obstetrics, Assuta Medical Center, 13 Eliezer mazal st., Rishon Lezion, Israel [4]Department of Gynecology and Obstetrics, Bnai Zion Medical Center, 47 Golomb St., Haifa, Israel [5]Department of Gynecology and Obstetrics, The Technion – Israel Institute of Technology, Haifa, Israel

*Correspondence address. Department of Gynecology and Obstetrics, Meir Medical Center, 59 Tshernichovsky St., Kfar Saba, Israel. Tel/fax: +972524763324; E-mail: millerne@me.com

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STUDY QUESTION: Does a novel sperm vitrification device (SpermVD) provide an efficient method for freezing a small number of human spermatozoa from men suffering from non-obstructive azoospermia?

SUMMARY ANSWER: The novel SpermVD is an efficient and simple carrier method for freezing a small number of spermatozoa in low-volume droplets, reducing post-thaw search time from hours to minutes, allowing a 96% recovery rate and leading to successful use of sperm for fertilization.

WHAT IS KNOWN ALREADY: Previous methods for cryopreservation of small numbers of human spermatozoa (e.g. mini-straws, ICSI pipette, alginate beads, cryoloop) have been proposed as a solution for cases of severe male infertility. Many drawbacks have prevented their widespread use, including cumbersome preparation and sperm retrieval procedures, and the fact that the thawed spermatozoa are not immediately available for micromanipulation and required additional treatment which posed excess risk of harm.

STUDY DESIGN, SIZE, DURATION: We conducted a feasibility experiment of the novel SpermVD and a prospective cohort study of ICSI cycles in men suffering from non-obstructive azoospermia in two outpatient fertility IVF clinics, from 2015 through 2017.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All patients underwent extended ejaculate search prior to the day of oocyte retrieval, and any single motile spermatozoa found was transferred to 0.8 µl droplets of 1:1 washing medium/cryoprotectant on the SpermVD, then plunged into liquid nitrogen for cryopreservation. In patients with non-obstructive azoospermia who underwent surgical TESE, both the motile and immotile spermatozoa found underwent cryopreservation using the SpermVD. On the day of oocyte retrieval, the SpermVD was thawed, directly transfered to the ICSI plate and retrieved spermatozoa were used for the ICSI procedure.

MAIN RESULTS AND THE ROLE OF CHANCE: The prospective cohort included 44 cases. We used the SpermVD to vitrify 631 spermatozoa, of which 540 (86%) were motile. The average number of frozen spermatozoa per patient was 14.3 ± 9.3. After thawing, we retrieved 607 spermatozoa, producing a recovery rate of 96%. The average number of thawed spermatozoa was 13.8 ± 9.2. The recovery of 180 thawed motile sperm accounted for 33% of all frozen motile spermatozoa. The fertilization rate was 59%. Of 44 oocyte retrieval procedures, 24 (55%) clinical pregnancies were achieved. The delivery rate (not including three ongoing pregnancies) was 32% and the miscarriage rate was 29%.

LIMITATIONS, REASONS FOR CAUTION: Although we presented the SpermVD on 44 cases, a larger cohort would provide more information. Moreover, we cryopreserved only motile sperm from the ejaculates and not immotile sperm, thus limiting the knowledge regarding the efficacy of the VD for immotile sperm from this source.

WIDER IMPLICATIONS OF THE FINDINGS: The novel SpermVD is a simple efficient carrier, optimizing the protocol for freezing a small number of spermatozoa. It may allow for the routine use of frozen spermatozoa after TESE for men suffering from non-obstructive

†The authors consider that the first two authors should be regarded as joint First Authors.

azoospermia and thus avoid repeated TESE surgeries. Furthermore, in cases of non-obstructive azoospermia, routine cryopreservation of the retrieved spermatozoa prior to the IVF cycle may avoid the risk of cycle cancelation and thus decrease the number of unnecessary oocyte retrieval procedures.

STUDY FUNDING/COMPETING INTEREST(S): There was no external funding. There are no competing interests.

TRIAL REGISTRATION NUMBER: IRB no 00119-16-ASMC.

Key words: sperm vitrification / small number of spermatozoa / non-obstructive azoospermia / vitrification device / intracytoplasmic sperm injection / fertilization rate

Introduction

Male factor contributes to 23–25% of infertile cases (Hull *et al.*, 1985; Templeton, 1995). Azoospermia, defined as the absence of sperm in the ejaculate, is identified in ~10–15% of infertile males (Jarow *et al.*, 1989). However, in some cases, after an extended sperm search, there is the occasional presence of spermatozoa in the ejaculate of men diagnosed with azoospermia (Tournaye *et al.*, 1995). In order to achieve paternity, men suffering from azoospermia are usually referred for intracytoplasmic sperm injection in which the presence of one spermatozoon per oocyte can achieve pregnancy (Palermo *et al.*, 1992). Sperm retrieval for the ICSI procedure can be extracted from semen ejaculate or by surgical testicular sperm extraction (TESE).

For men suffering from azoospermia or men with the occasional presence of spermatozoa after an extended search, cryopreservation of the retrieved spermatozoa, prior to the IVF cycle, may avoid the risk of cycle cancelation when no suitable spermatozoa for injection are detected on the day of oocyte retrieval. Moreover, cryopreserved, retrieved spermatozoa can be used for subsequent ICSI cycles. In our previous publication (Miller *et al.*, 2017), we found that sperm can be retrieved in 78% of men suffering from azoospermia, following an extended search of ejaculated spermatozoa on the day of oocyte retrieval. This suggests that there are still 22% of cases where no spermatozoa can be found on the day of oocyte retrieval and a solution for these men should be found.

Efficient cryopreservation of a small number of retrieved spermatozoa from men suffering from non-obstructive azoospermia can also avoid repeated TESE surgeries in cases of failed treatment, thus reducing the risks entailed in this procedure, i.e. damage to the testes, epididymal fibrosis, testicular atrophy and degradation of spermatogenic function (Schlegel and Su, 1997; Andersson *et al.*, 2004).

Various methods for cryopreservation of small numbers of human spermatozoa have been proposed. Cohen *et al.* (1997) suggested an empty zona pellucida procedure. Just *et al.* (2004) developed spherical Volvox globator algae as a cryopreservation vehicle. Additional methods, such as agarose microspheres (Isaev *et al.*, 2007), straws (Isachenko *et al.*, 2005), ICSI pipettes (Gvakharia and Adamson, 2001), cryoloops (Desai *et al.*, 2004), and cell sleepers (Endo *et al.*, 2012; Coetzee *et al.*, 2016) have also been tested.

A recent review which included 30 reports on all the previous methods and techniques for cryopreservation of individual or small numbers of human spermatozoa concluded that the ideal container or vessel/platform that could be used universally has yet to be developed. Novel cryopreservation technology specifically designed to handle small numbers of spermatozoa needed to be further explored (Abdelhafez *et al.*, 2009).

The purpose of this study was to describe a new device and method for cryopreservation of a small number of spermatozoa, which optimizes fertility preservation.

In order to validate efficacy of the sperm vitrification device, the recovery rate and motility percentage after thawing were first measured in a feasibility experiment. The clinical efficacy of the sperm vitrification device (SpermVD) was then estimated in a prospective cohort study that also recorded ICSI outcomes in terms of fertilization rate, pregnancy rate and miscarriage rate.

Materials and Methods

Validation of the method – a feasibility experiment

In order to validate the efficacy of the SpermVD before clinical use in an IVF procedure, we used the SpermVD to freeze motile spermatozoa from three men with from severe oligoteratoasthenoazoospermia (OTA). All men signed an informed consent form. After each of these men underwent ICSI, the discarded spermatozoa from the same ejaculate were divided into two samples. One sample was tested in the SpermVD and the second was tested in a standard freezing tube.

We defined satisfactory results to permit the clinical use of the SpermVD as follows: recovery rate (number of spermatozoa found after thawing) of at least 90% and post-thaw motility rate equal to or greater than the survival rate. The survival rate was calculated based on the sample that was evaluated on the standard freezing tube and the post-thaw motility rate was defined as post-thaw motility/original motility ×100.

Sperm vitrification

A 0.8–1 µl droplet of a 50/50 v/v mixture of Quinn's Advantage Sperm Freezing Medium (Sage In-Vitro Fertilization Inc. Trumbull, CT, USA) and Quinn's Sperm Washing Medium was placed onto each well of the sperm vitrification device (VD) (Rafimed Inc., Yavne, Israel) (see Fig. 1). The SpermVD was submerged into oil on the search plate and spermatozoa were transferred from the collection droplets to the droplets on the SpermVD wells (Fig. 2A). The SpermVD was then removed from the plate and placed inside a labeled 3.6 ml cryovial (Danyel Biotech Inc., Rehovot, Israel). The vial containing the SpermVD was immediately loaded onto a labeled aluminum holder and submerged into liquid nitrogen.

Sperm thawing

The following were placed on a sterile glass-bottom 50 mm plate (World Precision Instruments Inc., Sarasota, FL, USA): several 0.6 µl sorting

droplets of Quinn's Sperm Washing Medium; several 4 µl droplets of Quinn's Sperm Washing Medium for oocyte placement and a 4 µl droplet of 10% PVP for filling the microcapillary, with ample space left for placing the SpermVD (Fig. 2B). The plate was then covered with paraffin. The cryovial was removed from the liquid nitrogen, opened and left to thaw at room temperature until complete liquification of the oil covering the droplets. The SpermVD was then placed under oil on the plate and examined under ×200 magnification using an inverted phase contrast microscope. Spermatozoa were located in the SpermVD droplets, transferred to the collection droplet and subsequently used for ICSI (Fig. 2B). The recovery of spermatozoa motility was determined by carefully observing the spermatozoa in the SpermVD droplet immediately following thawing. Motility was defined as any movement of the sperm head or tail, separately, to account for Brownian motion. The motility recovery rate was defined as the percentage of spermatozoa demonstrating motility after thawing. If spermatozoa could not be found after thawing, they were counted as lost spermatozoa. See Fig. 3 for the entire procedure.

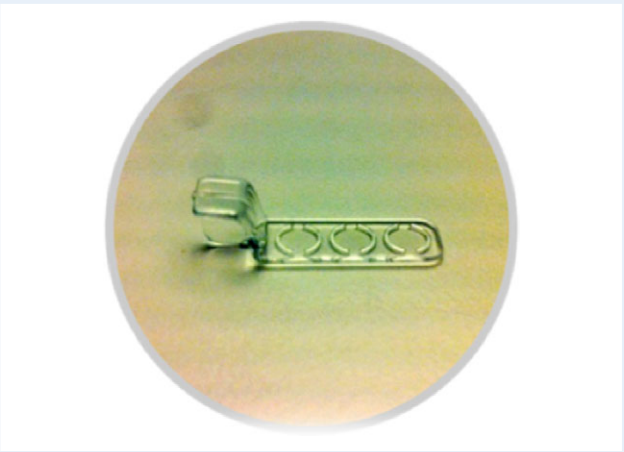

Figure 1 The sperm vitrification device.

Study design – the clinical study

A prospective cohort study of ICSI cycles was achieved by extended search of ejaculated spermatozoa or by micro-TESE for men suffering from non-obstructive azoospermia from 2015 through 2017.

Patients

Data were collected from IVF clinic records of two outpatient centers (Assuta Medical Center, Rishon Letzion, Israel and IVF department of Bnai Zion Medical Center, Haifa, Israel). All couples whose male partner was diagnosed with non-obstructive azoospermia were included in the study. All patients underwent extended ejaculate search prior to the day of oocyte retrieval, and any single motile spermatozoa found was transferred to the SpermVD for cryopreservation.

These spermatozoa were used as backup if less than four motile spermatozoa were found by additional extended ejaculate search conducted on the day of oocyte retrieval. For patients with non-obstructive azoospermia who underwent surgical TESE, the motile and immotile spermatozoa found underwent cryopreservation using the SpermVD.

On the day of oocyte retrieval, both motile and immotile spermatozoa from the SpermVD were thawed and used for the ICSI procedure. All embryos were transferred on Day 3.

Search for rare spermatozoa

Sperm samples were obtained from patients with non-obstructive azoospermia via masturbation or by using a spermicide-free polyurethane condom after 10–15 days of abstinence. After being allowed to liquefy at room temperature for 20 min, the samples were loaded on PureCeption Sperm Separation Media (Sage In-Vitro Fertilization Inc., Trumbull, CT, USA) with a gradient of 1 ml 40% v/v (upper phase) and 0.5 ml 80% v/v (lower phase). This consisted of a sterile colloidal suspension of silane-coated silica particles in HEPES-buffered human tubal fluid (HTF) containing 10 mg/l gentamicin. It was centrifuged for 20 min at 300 rpm at room temperature. The upper liquid was then removed and the pellet was resuspended in Quinn's Sperm Washing Medium (Sage In-Vitro Fertilization) and centrifuged again for 10 min at 600 rpm at room temperature. After this procedure was performed twice, most of the liquid was removed and the remaining 100 µl were mixed and distributed into 10 µl droplets on a 90 mm sterile plastic Petri dish (De-Groot, Rosh Ha'Ayin, Israel). A 4 µl

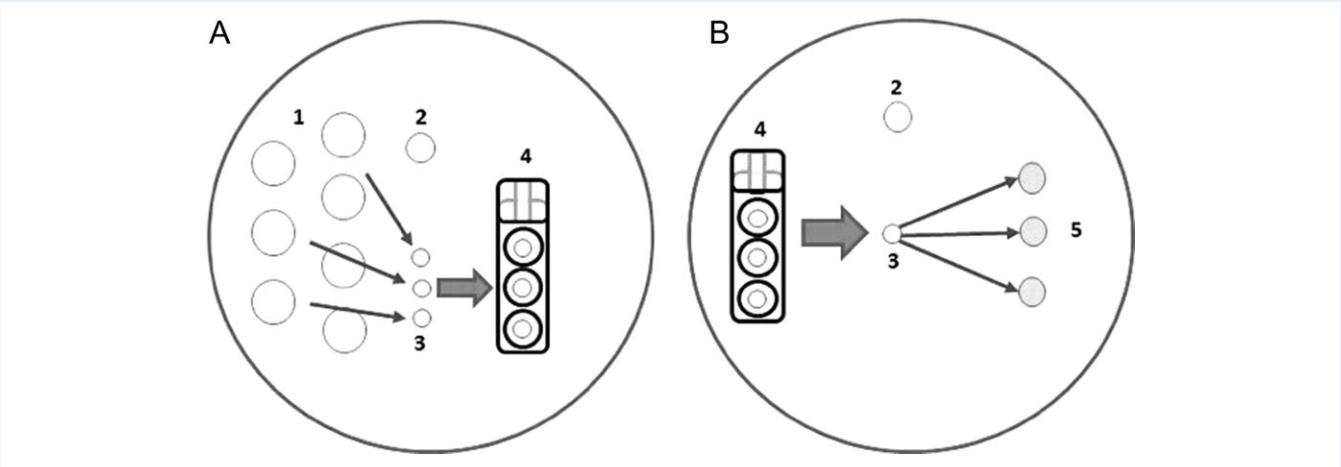

Figure 2 Plate layout for rare spermatozoa search and retrieval. **A**. Plate layout for sperm search. **B**. Plate layout for sperm retrieval from sperm vitrification device (SpermVD). 1 – sample droplets; 2 – PVP 10%; 3 – collection droplets; 4 – sperm VD; 5 – fertilization droplets.

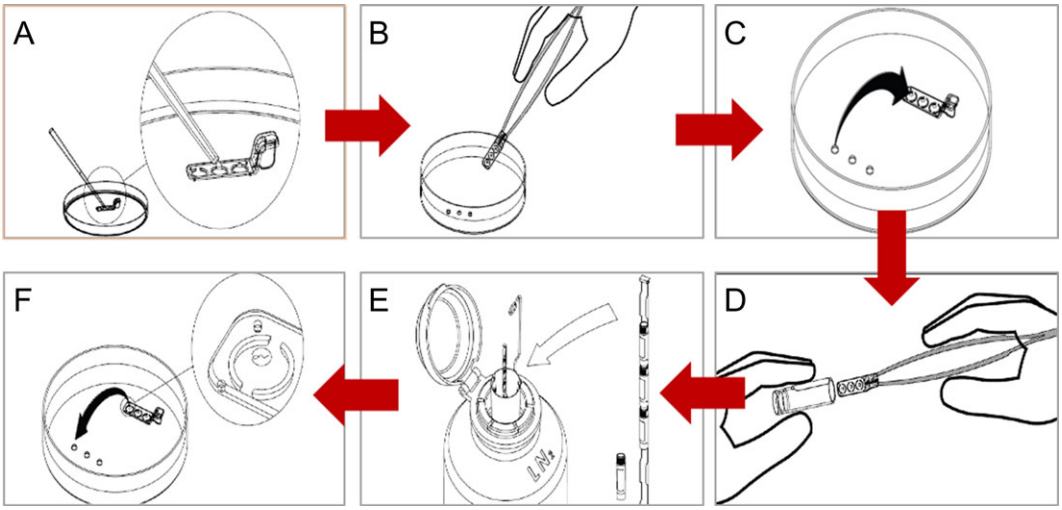

Figure 3 The Sperm Vitrification Process on the Vitrification Device (SpermVD). **A**. Place drops of cryoprotectant on the SpermVD. **B**. Place the SpermVD into the plate with sperm. **C**. Transfer the sperm cell onto the SpermVD. **D**. Transfer the SpermVD into a cryovial. **E**. Put the cryovial with the SpermVD into liquid nitrogen. **F**. Take the SpermVD out of the liquid nitrogen and transfer the cells from the SpermVD to sperm washing medium.

droplet of polyvinyl pyrrolidone (PVP) 10% solution (Sage In-Vitro Fertilization) for filling the microcapillary and several collection droplets of 0.6 µl of Quinn's Sperm Washing Medium were added to the plate. The plate was then covered with light paraffin oil (Sage In-Vitro Fertilization). The 10 µl droplets were thoroughly searched for the presence of spermatozoa under ×200 magnification using an inverted phase contrast microscope Nikon Eclipse Ti (Nikon Instruments Inc. Melville, NY, USA) with an Invenio 3SII camera and DeltaPix software (DeltaPix, Smorum, Denmark). Any spermatozoa found were transferred to a collection droplet and if present, sperm exhibiting progressive motility were given priority. This procedure was performed using the micromanipulation system TransferMan NK2 with CellTram Oil pump (Eppendorf, Hamburg, Germany), equipped with a sterilized, nonangulated, glass microcapillary with a 12 µm diameter tip (TPC, Thebarton, Australia).

MicroTESE

The surgery is performed in an operating room sharing a window to an embryologic laboratory. After usual positioning and patient preparation and induction of general anesthesia, the scrotal median raphe is cut. Testicular envelopes of the larger/firmer testicle are first sharply cut. The tunica vaginalis is clamped on each side, and the testicle is brought outside it and balanced using gauze pads. A Vicryl® 5/0 stay suture is placed at the far side of an imaginary line of the equator plane of the testicle. A thin sharp blade is used for a delicate and long equatorial cut of the tunica albuginea, starting near the stay suture and encompassing 2/3 to 3/4 of the equatorial diameter. The edges of the tunica cut surface are grasped with fine clamps, which stop sub-tunical bleeding and help retract the testicular upper and lower poles apart. The operating microscope is brought into the operative field to begin careful and meticulous screening of testicular seminiferous tubules, at both parts of the testicle. A ×20 magnification is used to find thicker or more opaque individual tubules or groups of tubules, in which the chance of finding sperm is greater. These tiny tissue fragments are transferred to the embryologic lab for further mechanical and enzymatic disintegration followed by a meticulous sperm search by at least two embryologists using advanced micromanipulators. In case sperm are not found during the initial tissue processing at the lab, systematic multiple micro-biopsies are taken from the open testicle, encompassing both upper and lower cut surfaces, as well as deeper area. After fine bipolar diathermic hemostasis, the operating microscope is taken out of the operative field and the tunica albuginea edges are closed with running Vicryl® 5/0 from the stay suture. The tunica vaginalis is closed using running Vicryl® 4/0 suture, and the testicle is placed back inside its hemiscrotum. Testicular envelopes are approximated with interrupted Vicryl® 4/0 suture. In case sperm have not yet been found in the embryologic lab, the same procedure is performed on the contralateral testicle. A 0.25% Bupivacaine solution is injected at the surgical cut and the skin edges are approximated with interrupted Vicryl Rapide® 4/0 suture.

Cord block is performed for the operated side(s) with 10 ml of 0.25% Bupivacaine solution.

Ethical issues

The Institutional Review Board approved the study design, IRB no 00119-16-ASMC and all patients gave informed consent for inclusion in the study.

Statistical analysis

The analysis included demographic characteristics, recovery rate after thawing (number of thawed spermatozoa among all spermatozoa that went through vitrification), percentage motile sperm after thawing (motile sperm after thawing among motile sperm frozen), fertilization rate (number of fertilized oocytes among all sperm injected oocytes), pregnancy rate (number of clinical pregnancies among oocyte retrieval procedures), delivery rate (live births among oocyte retrieval procedures) and miscarriage rate (number of miscarriages among number of pregnancies). Statistical analysis was performed using SPSS ® 20.0 package for windows (SPSS Inc.). Data are expressed as percentages.

Results

Validation of the method

The validation of the method was conducted at the Assuta Medical Center, Rishon Letzion clinic. Three semen samples were frozen on

Table I Results of validation of the method on men suffering from oligoteratoasthenoazoospermia (OTA).

No. of samples	No. of devices tested	No. of frozen sperm	No. of sperm retrieved	Retrieval rate	Motility rate	Standard survival rate*
3	39	1184	1116	94.3%	41%	29%

*Standard survival rate refers to samples processed with the standard freezing method.

39 SpermVDs. A total of 1184 spermatozoa were frozen using the device, with a mean of 30.4 spermatozoa for each device.

Among all of the vitrified spermatozoa, we retrieved 1116 spermatozoa (94.3%). Of these spermatozoa, 457 were motile (41%) (Table I). The survival rate based on the spermatozoa that were processed with standard freezing method was 29%. Since the 94.3% retrieval rate with the VD was greater than 90% and the 41% post-thaw motility rate with the VD was greater that the survival rate (29%), we continued to the second phase of clinical testing of the SpermVD.

Recovery, motility and fertility outcomes of frozen–thawed spermatozoa

The cohort included 44 cases; 36 cases were conducted at the Assuta Medical Center and eight cases were at the Bnai Zion Medical Center. Among the 44 cases, eight cases had sperm retrieved from TESE and 36 had sperm from the ejaculate. Table II shows the data regarding the 44 patients of the cohort including demographics, number of oocytes and parameters of post-thaw spermatozoa from the SpermVD for each patient and the fertility outcomes. Table III shows a summary of this information.

The women were a mean age of 33.3 ± 7.4 years and the men were a mean age of 37.4 ± 7.5. Five women in the cohort used oocyte donation due to their age being beyond 41. The oocyte donor age ranged from 25 to 30 years old.

We conducted the vitrification using the SpermVD on 631 spermatozoa, of which 540 (86%) were motile. The immotile sperm were obtained only from the TESE procedure. The average number of frozen spermatozoa was 14.3 ± 9.3. After thawing, we retrieved 607 spermatozoa, with a recovery rate of 96%. The average number of thawed spermatozoa per patient was 13.8 ± 9.2. The recovery of 180 thawed motile spermatozoa accounted for 33% of all frozen motile spermatozoa. After thawing, we used both motile and immotile spermatozoa for the ICSI procedure. All embryos transferred were the result of thawed spermatozoa and were not from fresh spermatozoa.

The fertilization rate, calculated as the number of fertilized oocytes among the total number of mature oocytes reached 59%. The pregnancy rate was calculated as number of pregnancies resulting from oocyte retrieval procedures. In our cohort, 24/44 (55%) clinical pregnancies were achieved. Delivery rates were calculated with and without ongoing pregnancies (beyond 24 gestational weeks) as number of deliveries among oocyte retrieval procedures. The delivery rate was 39% including ongoing pregnancies and 32% without including ongoing pregnancies. The miscarriage rate was 29% based on the number of pregnancies.

In addition, 10 women had 1–2 surplus embryos for cryopreservation. Since most had not yet undergone embryo transfer, we did not add them to the cumulative results.

Discussion

The present study presents a novel and efficient technique demonstrating the feasibility of successfully cryopreserving small numbers of spermatozoa using the sperm vitrification device. To the best of our knowledge, this study has the largest patient cohort (44 cases) among similar studies conducted to date.

The study included more cases where sperm were retrieved from ejaculates (36) than from TESE (8), because all men were offered and consented to an extended search with use of the new SpermVD for freezing a small number of spermatozoa before surgical intervention.

In the current study, using the SpermVD, after thawing we found a recovery rate of 96% of retrieved spermatozoa and thawed motility rate of 33%. These findings are similar to previous reports with retrieval rates ranging from 59% to 100% (Abdelhafez *et al.*, 2009). These results have strong clinical significance because we tested the device on patients with non-obstructive azoospermia, who have very few spermatozoa.

As mentioned, even after an extended search in men suffering from non-obstructive azoospermia (Miller *et al.*, 2017), no spermatozoa were found on the day of oocyte retrieval in 22% of cases. A possible solution for these men is to cryopreserve a small number of sperm cells. Thus, optimizing a sperm freezing protocol for few spermatozoa is an important process for preserving male sperm, especially for men suffering from azoospermia. An efficient option, such as the SpermVD can increase the number of sperm retrieved before the day of oocyte retrieval, thus decreasing the need for repeated TESE.

There is an ongoing effort to achieve efficient technologies to cryopreserve a small number of spermatozoa. The first attempt used human or hamster zonae for storing one or a few spermatozoa (Cohen *et al.*, 1997). It failed to become mainstream because it depended on a biological carrier, with the potential risk of contamination and disease transmission.

Since then, additional methods for cryopreservation of small numbers of human spermatozoa have been proposed (e.g. agarose microsphere, ICSI pipette) (Gvakharia and Adamson, 2001; Isaev *et al.*, 2007). However, none of these methods were efficient enough and each had drawbacks which prevented their widespread use. In most methods, the thawed spermatozoa were not immediately available for micromanipulation and required additional treatment, which posed excess risk of harm to the thawed spermatozoa. Moreover, no live births have been reported using these methods (Abdelhafez *et al.*, 2009).

Table II Post-thaw motility recovery and thaw efficiency of sperms and fertility outcome for the entire cohort.

Patient no.	Female age	Female BMI	Female smoking	No. of oocytes	No. of frozen spermatozoa	No. motile frozen spermatozoa	No. thawed spermatozoa	% motile, thawed spermatozoa	Fertilization rate (%)	No. transfer embryos	Pregnancy	Source	Outcome	Frozen embryos
1	22	21	No	11	2	2	2	100%	100%	2	Yes	Ejaculate	Delivery	
2	32	21	No	7	7	7	6	17%	30%	2	Yes	Ejaculate	Delivery	
3	29	22	No	19	22	22	22	13.60%	7%	1	No	Ejaculate		
4*	27	22	No	5	15	15	14	45%	50%	2	Yes	Ejaculate	Delivery	
5	37	25.8	No	3	5	0	5	0	66%	2	No	TESE		
6	31	20	No	21	18	18	12	50%	57%	2	No	Ejaculate		
7	29	19.8	No	9	26	26	24	36%	50%	3	No	Ejaculate		
8	35	25	No	13	45	45	43	18%	40%	2	Yes	Ejaculate	Delivery	2
9	24	26.7	No	9	13	13	13	8%	0	0	No	Ejaculate		
10	34	23.7	No	8	22	22	22	50%	66%	2	No	Ejaculate		
11	23	29	No	6	8	8	8	50%	50%	2	No	Ejaculate		
12	23	29	No	10	15	15	15	0	60%	3	Yes	TESE	Delivery	
13[2]	50	23.5	No	6	29	29	28	22%	75%	2	Yes	Ejaculate	Delivery	
14[2]	49	23	No	6	26	26	26	27%	50%	3	Yes	Ejaculate	Miscarriage	
15*	45	20.4	No	6	25	25	25	16%	75%	2	Yes	Ejaculate	Delivery	1
16*[2]	41	25.3	No	6	18	18	18	50%	90%	2	Yes	Ejaculate	Delivery	
17*[2]	45	22.7	No	6	25	25	25	20%	100%	2	Yes	Ejaculate	Delivery	2
18	27	22.9	No	20	22	22	22	68%	41%	2	Yes	Ejaculate	Delivery	
19	39	21.3	No	14	4	4	4	75%	100%	2	Yes	Ejaculate	Delivery	1
20	30	22	No	4	4	0	4	0	33%	1	No	TESE		
21[2]	45	21	No	6	24	24	24	8%	41%	1	Yes	Ejaculate	Miscarriage	2
22	37	25.8	No	6	5	0	5	0	66%	2	No	TESE		
23	38	28.9	No	8	6	0	6	0	66%	2	No	TESE		
24	37	28.2	No	7	5	5	5	60%	66%	2	No	Ejaculate		
25	30	22	No	5	14	14	14	40%	50%	2	Yes	Ejaculate	Delivery	1
26	37	23	No	8	23	23	23	60%	28%	2	No	Ejaculate		
27*	25	19.8	No	13	5	5	4	100%	100%	2	Yes	Ejaculate	Delivery	2
28*	35	28.2	No	7	4	4	3	100%	100%	2	Yes	Ejaculate	Miscarriage	
29[1]	34	20	No	13	21	0	21	0	78%	1	Yes	Ejaculate	Miscarriage	2
30	31	20	No	13	15	15	15	60%	20%	2	No	Ejaculate		
31[1]	40	21	No	5	6	2	6	0%	100%	1	Yes	Ejaculate	Delivery	
32	30	26.2	No	8	5	5	5	60%	33%	2	Yes	Ejaculate	Miscarriage	2
33	26	21	No	11	7	7	5	20%	33%	2	No	Ejaculate		
34	27	17.7	No	13	4	0	4	0	75%	2	Yes	TESE	Ongoing	
35	31	19.2	Yes	8	12	2	12	0	17%	1	No	Ejaculate		

36	38	25.8	No	6	6	0	6	0	50%	3	Yes	TESE	Miscarriage	
37	25	21	No	8	15	15	15	0	55%		No	Ejaculate		
38	40	20.4	Yes	3	12	12	8	75%	66%	2	No	Ejaculate		
39[1]	34	22	No	1	12	1	12	100%	100%	1	Yes	Ejaculate	Miscarriage	
40*	22	21	No	8	29	29	29	35%	83%	2	Yes	Ejaculate	Ongoing	
41	38	23	No	8	12	12	9	40%	30%	2	No	Ejaculate		
42	33	21	No	10	8	8	3	38%	66%	2	No	Ejaculate		
43	25	19.1	No	6	15	0	15	0	25%	1	No	TESE		
44*	36	20.3	No	10	15	15	15	8%	1	100%	Yes	Ejaculate	Ongoing	2

*Bnai Zion Center.
[1]Loss of motility before freezing.
[2]Oocyte donor.

An additional technique was presented using the Cryoloop (Desai *et al.*, 2004). Its advantages included a high retrieval rate and the use of non-biological material. Although the Cryoloop was considered an effective sperm vehicle, its major drawback as a carrier is its open system, which has a potential risk of cross-contamination (Abdelhafez *et al.*, 2009).

The Cell Sleeper (Endo *et al.*, 2012; Coetzee *et al.*, 2016) was also evaluated for vitrification of a small number of spermatozoa. Recovery, motility and viability rates of vitrified-warmed spermatozoa were similar between the Cell Sleeper and the CryoTop, in a study that included semen from only 10 infertile men and did not evaluate fertilization, pregnancy and delivery rates.

In our experience, using the cell sleeper, we found that one possible disadvantage is that it contains an inner rigid tray, which makes it difficult for the micromanipulator to directly reach all spermatozoa, which can lead to loss of spermatozoa. Additional methods, such as hyaluronan microcapsules (Tomita *et al.*, 2016) and agarose capsules were used to cryopreserve small numbers of spermatozoa (Hatakeyama *et al.*, 2017). Yet, the potential for fertilization and embryo growth after using these methods has not been investigated.

Sun *et al.* (2017) described a platform for cryopreservation of few spermatozoa using a Cryopiece for four patients. They encountered a few issues that were resolved in our study using the SpermVD, including freezing lone spermatozoa in low-volume droplets, decreasing post-thaw sperm loss and successfully using immotile sperm for fertilization (Sun *et al.*, 2017).

Compared to the other methods, the main technological advantage of the SpermVD is that it overcomes two main obstacles related to cryopreservation. The previous method of bulk sample freezing were incompatible with small numbers of spermatozoa, because it requires another lengthy search of the thawed sample and there is an inevitable risk of cell loss due to the large sample volume and due to preliminary procedures, such as centrifugation and removal of the upper phase (Abdelhafez *et al.*, 2009). The SpermVD resolves these issues by creating an environment for the sperm similar to that used in bulk freezing (a 1:1 ratio of wash medium and cryoprotectant), while using a much smaller volume (0.8 µl). This reduces the post-thaw search time from hours to minutes and allows a nearly 100% recovery rate.

Moreover, the SpermVD allows direct transfer of the spermatozoa from the device to the ICSI plate without harming the frozen spermatozoa. The size of the SpermVD is suitable for standard vitrification test tubes used in fertility units, thus allowing for easy insertion and removal of the SpermVD without shaking the tube.

Regarding IVF outcomes, there is a paucity of cohort studies that have evaluated IVF outcomes following cryopreservation of spermatozoa and that report on fertilization, pregnancy and delivery rates.

Sereni *et al.* (2008) reported ICSI results using frozen–thawed testicular spermatozoa in six cases using a cell culture dish as carrier. The average fertilization rate was 17.6%. Embryo transfer in three cases resulted in one biochemical pregnancy. A study that evaluated the pregnancy and delivery rate using the Cell Sleeper showed that with three patients and 148 cryopreserved sperm, one delivery was achieved (Endo *et al.*, 2012). A recent device, the cryopiece, which was used for four patients, achieved a fertilization rate of 73%, with four babies born at term (Sun *et al.*, 2017).

Using the SpermVD, we found fertilization and pregnancy rates of 59% and 55%, respectively. Moreover, we found at least a 32%

Table III Post-thaw motility recovery and thaw efficiency of sperm and fertility outcomes.

Characteristic	Outcome
Cycles (*n*)	44
TESE/Ejaculate (*n*)	8/36
Female Age (Average, SD)	33.3 ± 7.4
Female BMI (Average, SD)	22.8 ± 3
Male age (Average, SD)	37.4 ± 7.5
Number of oocytes	8.43 ± 3.89
No. of spermatozoa frozen (Average ± SD)	14.3 ± 9.3
No. of motile frozen sperm (Average ± SD)	12.3 ± 10.8
No. of spermatozoa thawed (Average ± SD)	13.8 ± 9.2
Spermatozoa recovery rate % (SD, CI)	96% (10%, 94–97%)
Thawed sperm motility % (SD, CI)	33% (32%, 29–37%)
Fertilization Rate % (SD, CI)	59% (28%, 52–60%)
Pregnancy Rate % (CI)	55% (40–68%)
Delivery Rate without ongoing pregnancies % (CI)	32% (20–46%)
Delivery Rate with ongoing pregnancies % (CI)	39% (26–53%)
Miscarriage Rate % (CI)	29% (15–50%)

Data are presented as mean, SD and/or CI.

delivery rate which provides further promising data regarding the efficacy of the SpermVD.

Our patients had a 29% miscarriage rate. This high rate is probably related to the demographics of the study population. As the spermatozoa was obtained from men with azoospermia, it would be expected that the sperm quality is reduced, thus leading to a higher abortion rate.

The major strengths of our study are its prospective design and the relatively large sample size. It is also important to mention that the efficacy of the device was measured in two clinics that achieved similar results. In addition, the IVF outcome analysis, including pregnancy rate and miscarriage rate had good clinical results.

This study had some limitations. Although we presented the SpermVD on 44 cases, a larger cohort would provide more information. This limitation relates to the relative rarity of non-obstructive azoospermia in the overall population. Additionally, we cryopreserved only motile sperm from the ejaculate and not immotile sperm, thus limiting our knowledge regarding the efficacy of the VD for immotile sperm.

Yet, the results of the present study present new options when choosing the most efficient treatment for such cases; use of the VD method in additional centers may further elucidate its clinical efficacy.

In conclusion, the novel SpermVD is an efficient and simple carrier that optimizes the protocol for freezing a small number of spermatozoa in low-volume droplets. This significantly reduces the post-thaw search time and decreases post-thaw sperm loss, allowing a 96% recovery rate and successful use of the sperm for fertilization. In men suffering from non-obstructive azoospermia, routine cryopreservation of sperm retrieved from the ejaculate, prior to the IVF cycle may avoid the risk of cycle cancelation and decrease the number of unnecessary oocyte retrieval procedures. It also may allow for the routine use of frozen spermatozoa after TESE for men suffering from non-obstructive spermatozoa and avoid repeated TESE surgeries.

Authors' roles

A.B, M.B and P.I participated in the concept and design of the study, patient recruitment, use of the SpermVD and data collection. N.M. was responsible for data analysis and drafted the manuscript. M.S participated in patient recruitment, use of the SpermVD and data collection. A.B, M.B and P.I revised the drafts. All authors approved the final version of the manuscript.

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Conflict of interest

None declared.

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