Human Reproduction, pp. 1–10, 2020
doi:10.1093/humrep/deaa072

human reproduction

ORIGINAL ARTICLE *Andrology*

Modified permeable cryoprotectant-free vitrification method for three or fewer ejaculated spermatozoa from cryptozoospermic men and 7-year follow-up study of 14 children born from this method

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Submitted on November 2, 2019; resubmitted on January 25, 2020; editorial decision on March 17, 2020

STUDY QUESTION: What technique can be used to successfully cryopreserve three or fewer ejaculated spermatozoa from cryptozoospermic men and is the physical and cognitive development of children born after this technique normal?

SUMMARY ANSWER: The modified cryopreservation method for three or fewer human spermatozoa from cryptozoospermic men showed a recovery rate above 95% and a survival rate just under 90%, and the physical and cognitive abilities of the children born after ICSI were comparable to those born after natural conception.

WHAT IS KNOWN ALREADY: Clinical outcomes of ICSI using cryptozoospermic men's ejaculated spermatozoa are considered to be inferior to that using testicular spermatozoa from microsurgical testicular sperm extraction (Micro-TESE), possibly because the DNA fragmentation rate is higher in ejaculated spermatozoa than in testicular spermatozoa from Micro-TESE.

STUDY DESIGN, SIZE, DURATION: Evaluation of the efficiency of cryopreservation of three or fewer spermatozoa was conducted retrospectively at St. Mother Clinic. The physical and cognitive development of children born after this method was studied between 2011 and 2018.

PARTICIPANTS/MATERIALS, SETTING, METHODS: This study included 28 cryptozoospermic men who had three or fewer morphologically normal and motile spermatozoa in their ejaculate after centrifugation and who preferred using cryopreserved spermatozoa to Micro-TESE. Control subjects were 31 cryptozoospermic patients using fresh spermatozoa from their ejaculates and 20 non-obstructive azoospermic patients with fewer than 10 spermatozoa obtained by TESE and vitrified. Clinical outcomes among three groups, vitrified spermatozoa from the ejaculate, fresh spermatozoa from the ejaculate and vitrified spermatozoa from the testis, were statistically analysed. For the 7-year follow up study of the 14 children born after ICSI using the ejaculated vitrified spermatozoa, the Japanese government-issued Boshi Kenko Techo (Mother-Child Handbook) and Kinder Infant Development Scale (KIDS scale) were used to determine whether their physical and cognitive development was comparable to that of naturally conceived children.

MAIN RESULTS AND THE ROLE OF CHANCE: Recovery and survival rates were 97.8% (510/521) and 87.1% (444/510) for vitrified spermatozoa from the ejaculate and 92.7% (152/164) and 60.5% (92/152) for vitrified spermatozoa from the testis. Clinical pregnancies (%), miscarriages (%) and live birth rates (%), respectively, among the three groups were as follows: vitrified spermatozoa from the ejaculate: 15(25.0), 2(13.3), 13(21.7); fresh spermatozoa from the ejaculate: 26(24.3), 5(19.2), 20(18.7); and vitrified spermatozoa from the testis: 3(16.7), 0(0.0), 3(16.7). Among the groups, there were no statistically significant differences except for the sperm survival rate and the oocyte fertilisation

rate, which were lower for vitrified spermatozoa from the testis compared with vitrified spermatozoa from the ejaculate. The 7-year follow-up study showed that the physical and cognitive development of 14 children born after ICSI using vitrified ejaculated spermatozoa from the ejaculate was comparable to that of naturally conceived children.

LIMITATIONS, REASONS FOR CAUTIONS: The maximum number of spermatozoa to which this method can be applied successfully is about 10. When the number of aspirated spermatozoa is over 10, some of them change direction after colliding with each other inside the aspiration pipette and reach the mineral oil, and once this happens, they cannot be expelled out of the pipette. Even though we did not find evidence of DNA fragmentation, further studies with larger participant numbers and longer time periods are necessary.

WIDER IMPLICATIONS OF THE FINDINGS: This technique is very useful for the cryopreservation of very small numbers of testicular spermatozoa (fewer than 10) in order to avoid or reduce Micro-TESE interventions.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was received to undertake this study. There are no competing interests.

TRIAL REGISTRATION NUMBER: N/A

Key words: physical and cognitive development / vitrified spermatozoon / cryptozoospermia / Cryotop / tail first aspiration

Introduction

Cryptozoospermia is one of the most severe male infertility conditions, defined by the WHO as the situation when spermatozoa cannot be observed in a fresh ejaculate. However, some sperm can be found after an extended centrifugation separation (World Health Organization, 2010). When the number of spermatozoa number is fewer than three, the patients are almost always diagnosed with azoospermia without doing centrifugation or an additional careful microscopic observation (Bendikson *et al*., 2008; Hauser *et al*., 2011). Conventionally, Micro-TESE is performed on these patients. When the number of morphologically normal and motile spermatozoa found in the ejaculate or after Micro-TESE is fewer than three, vitrification of the collected spermatozoa is considered. Recent reviews have reported the advantages of using spermatozoa collected from Micro-TESE over fresh or cryopreserved spermatozoa from the ejaculate of cryptozoospermic men and have suggested that the clinical outcomes using frozen spermatozoa are lower than that of using fresh testicular sperm from Micro-TESE. Concerns about the detrimental effect on DNA integrity of ejaculated spermatozoa have also been raised (Aitken and Krausz, 2001; Suganuma *et al*., 2005; Bendikson *et al*., 2008; Hauser *et al*., 2011; Ku *et al*., 2018; Amirjannati *et al*., 2012; Ben-Ami *et al*., 2013; Cui *et al*., 2016; Ketabchi, 2016; Abhyankar *et al*., 2016; Kang *et al*., 2018). However, the cryopreservation of three or fewer ejaculated spermatozoa from cryptozoospermic men should be given more consideration. There are many patients who, when given a choice, want to have their babies using ejaculated spermatozoa in spite of the necessary frequent sperm collections for cryopreservation and the assumed risks of DNA fragmentation.

Endo *et al*. (2011, 2012) reported three healthy babies born after using vitrified spermatozoa from patients with severe male infertility (one with cryptozoospermia and two with testicular spermatozoa from Micro-TESE). However, the survival rate they obtained after warming was only 40%. So, we modified their method by attempting a permeable cryoprotectant-free vitrification associated with a tail first aspiration method in order to raise the sperm recovery and survival rates and reduce the surgical and financial burden of Micro-TESE on patients (Weissman *et al*., 2008; Abhyankar *et al*., 2016). In addition, the physical and cognitive ability development of 14 children born after applying the method was assessed to investigate concerns raised regarding the genetic and epigenetic risks of this method.

Materials and Methods

Subjects

In this study, the modified vitrification method for three or fewer spermatozoa was used for ICSI treatment for 28 cryptozoospermic patients (83 cycles) who chose the cryopreservation of spermatozoa from their ejaculate over Micro-TESE.

Furthermore, we compared the clinical outcome with two other groups in our clinic to demonstrate the viability of the cryopreserved ejaculated spermatozoa from cryptozoospermic men (vitrified ejaculated spermatozoa: VES). One control group included ICSI patients using fresh spermatozoa when sufficient numbers could be found in the ejaculate at the day of oocyte retrieval (fresh ejaculated spermatozoa: FES). The control other group included cases when fewer than 10 testicular normal spermatozoa could be found in Micro-TESE, and these had been cryopreserved using the same method (vitrified testicular spermatozoa: VTS) as VES (Fig. 1).

Vitrification of three or fewer spermatozoa

Our vitrification and warming method for three or less spermatozoa is based on a previously published protocol (Endo *et al*., 2011, 2012) but instead of using glycerol, we used only sucrose, an impermeable cryoprotectant. We also modified the aspiration method by aspirating the spermatozoa tail first.

Whenever no spermatozoon was found in the first semen analysis, the semen was diluted with three to five times the volume of medium (HTF, Kitazato, BioPharma, Tokyo, Japan) solution containing 0.3% human serum albumin (HSA, Irvine Scientific, Santa Ana, USA) and then centrifuged at 320*g* for 10 min. After removal of the supernatant, the spermatozoa pellet was dissolved in a droplet media of HTF with 10% SPS (Serum Protein Substitute, Cooper Surgical, Inc., Trumbull, USA) that was placed and covered with mineral oil (Ovoil, Vitrolife, Göteborg, Sweden) in a 60-mm culture petri dish (FALCON353652, Corning, New York, USA) under an inverted microscope. When motile and morphologically normal spermatozoa could be found in the droplet

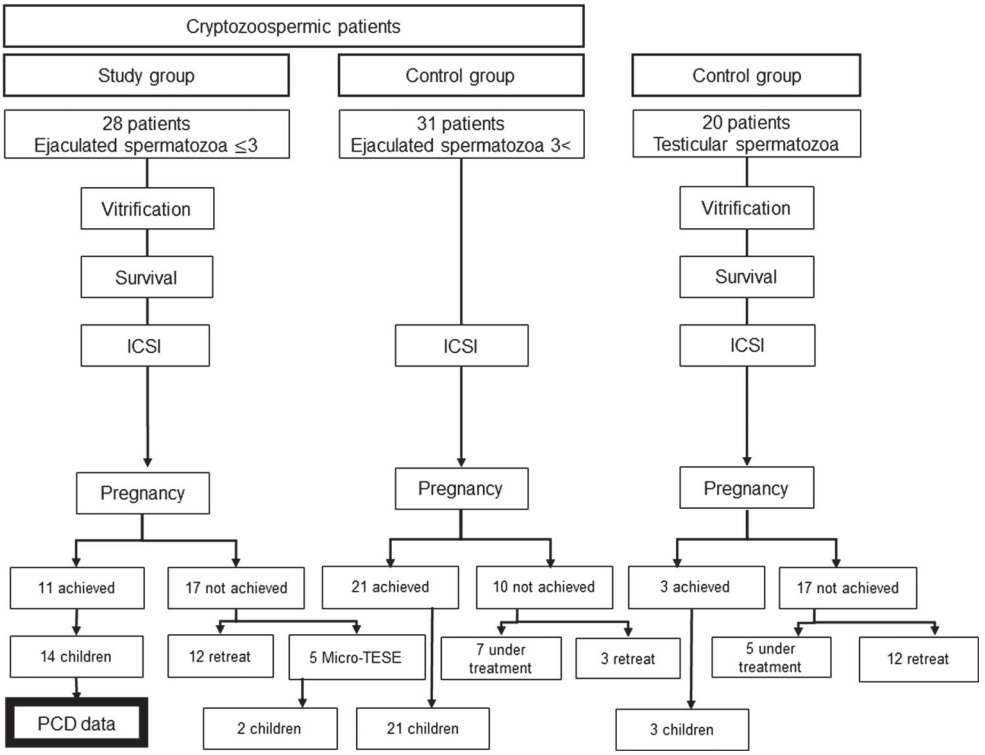

Figure 1 Flowchart of this study. Clinical outcomes using vitrified ejaculated spermatozoa (VES) were compared with two control groups (vitrified testicular spermatozoa (VTS) or fresh ejaculated spermatozoa (FES)).

media, they were aspirated carefully and moved to another HTF droplet media of 0.5 µl. We used a self-made glass pipette with a rounded tip and a wider diameter (10 µm), which was prepared with a glass puller (Sutter Instrument, Novato, USA) and a micro-forge (Narishige, Tokyo, Japan), to prevent damaging the spermatozoon (Fig. 2B). Then, each motile spermatozoon was carefully aspirated tail first into the pipette, and put into a 2-µl microdroplet media of the vitrification medium (HTF supplemented with 0.1 M sucrose and 10% SPS) near the tip of the Cryotop (Kitazato Corporation, Tokyo, Japan) (Fig. 2B–E) and then submerged in liquid nitrogen vapour for 2 min and plunged it in liquid nitrogen immediately (Fig. 3A and B). Semen collection, sperm pickup, vitrification and storage were repeated until enough spermatozoa (more than 10 spermatozoa) were accumulated for use in ICSI.

On the day of oocyte retrieval, the vitrified spermatozoa were warmed by dipping them into a droplet media of HTF with 10% SPS (2 µl) covered with mineral oil warmed at 37°C (Fig. 3C and D). Successfully recovered motile sperm were selected and used for ICSI (see the Supplementary Video).

Collection of oocytes

Oocytes were collected under the long protocol with GnRH agonist (Suprecure; Mochida, Tokyo, Japan) and FSH (Gonal-F, Merck, Darmstadt, Germany) plus HCG (HCG Mochida, Mochida, Tokyo, Japan). Oocytes were collected 37 h after the injection of hCG 5000 iU (HCG Mochida, Mochida, Tokyo, Japan) and preincubated for 4 h in medium (G-IVF, Vitrolife, Göteborg, Sweden) until ICSI. Embryos were cultured in one step medium (SAGE 1-Step, Origio, Yokohama, Japan) at 37°C in 6% CO_2, 5% O_2 and 89% N_2 and followed with Time Lapse (EmbryoScope$^+$, Vitrolife, Göteborg, Sweden) video. Embryos were transferred on the 3rd to 5th days after the oocyte retrieval.

Follow-up study of physical and cognitive development of children

Data from 14 children who were born after the vitrification of ejaculated spermatozoa at St. Mother Obstetrics and Gynecology Clinic from September 2011 to December 2018 were gathered for 7 years and analysed. We evaluated the physical and cognitive development (PCD) of the children by using the Japanese government issued Boshi Kenko Techo (birth to 3 years) (Nakamura, 2010; Yoshida *et al*., 2014; Hashimoto *et al*., 2016) and KIDS Scales (3 to 7 years) (Hashimoto *et al*., 2013).

In Japan, all mothers receive a Boshi Kenko Techo, which is distributed through local governments. This handbook, given to all pregnant women free of charge when they register their pregnancy, instructs parents to keep records of the mothers during pregnancy, delivery and post-delivery period as well as the physical and cognitive states of infants until they are 3 years old. Information recorded by parents in the handbook is properly checked and verified by attending paediatricians. The value of this book for the objective screening and early detection of children's disorders and abnormalities by paediatricians has already been established.

Data on children from 3 to 7 years was also analysed using the KIDS scale. The Kinder Infant Development Scale (KIDS) was developed

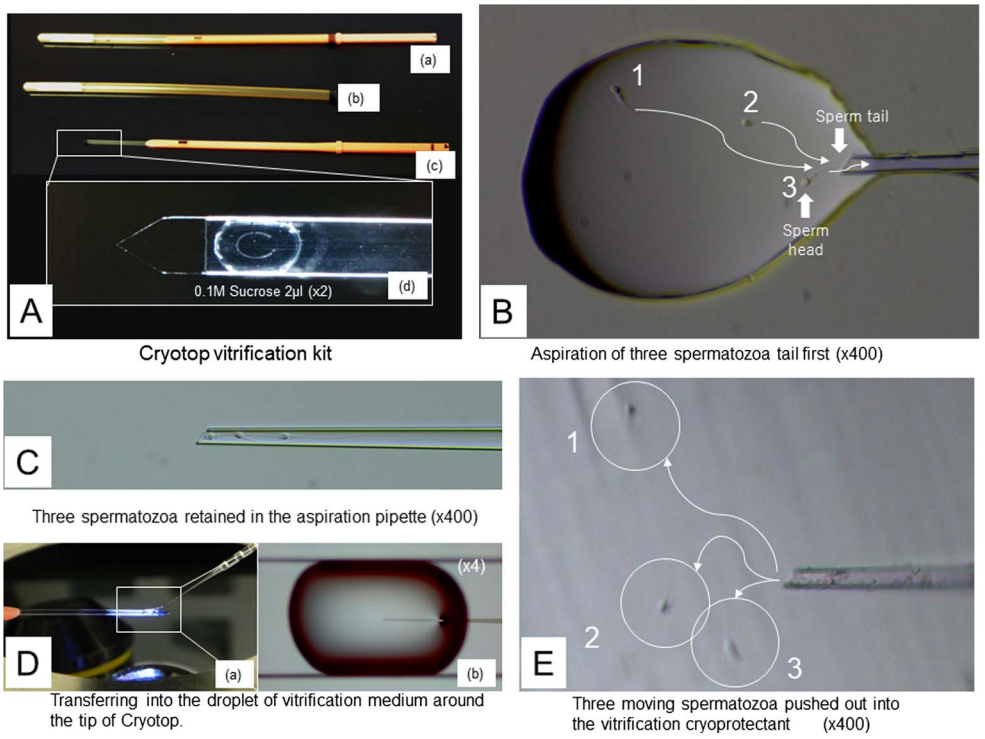

Figure 2 Preparation of the small number of spermatozoa for cryopreservation Cryotop vitrification kit. (**A**-a), a cover straw (A-b), consists of a propylene tip (10 mm × 1 mm × 120 μm) with a plastic handle (A-c) and magnified microdroplet media of cryoprotectant near the tip of cryotop (×2) (A-d). Three spermatozoa that were collected in the droplet media of HTF solution with 10% SPS covered with mineral oil are aspirated into 0.5-μl microdroplet media of HTF into the glass pipette tail first (**B**). The three spermatozoa aspirated tail first are retained near the tip of the aspiration pipette (×400) (**C**). Transfer of spermatozoa into the microdroplet media of 2 μl of cryoprotectant from the dish to Cryotop (**D**-a, b). The three moving spermatozoa were pushed out from the pipette and successfully transferred into the droplet media of vitrification medium (HTF solution with 0.1 M sucrose and 10% SPS) (**E**).

by the Center of Developmental Education and Research in Japan to assess the total development of children according to age (Gabel *et al*., 1986). KIDS scale consists of a list of questions about the child's behaviour, language ability, emotions and activities in kindergarten and in the first year of elementary school (3 to 7 years old).

Developmental age (DA) was calculated by the conversion table comprised of six items covering exercise, handling of objects, language comprehension and expression and social skills. The development quotient (DQ) is calculated by dividing the subject's aggregated DA by the chronological age (CA) and multiplying it by 100 (DQ = DA/CA × 100).

Ethical aspects

Vitrification of small number of human spermatozoa was conducted at the Saint Mother Obstetrics and Gynecology Clinic with the informed consent of all participating patients. The Institutional Review Board of the Saint Mother Obstetrics and Gynecology Clinic approved single human spermatozoon vitrification on 20 December 2010.

Statistical evaluation

Data were evaluated by chi-squared test, and the difference was considered significant at the $P = 0.05$ level.

Results

We report 14 babies born after the modified vitrification (VES) of three or fewer spermatozoa from cryptozoospermic men. We compared data with two control groups (VTS, FES) to clarify the effectiveness of this method and conducted a follow up study on the babies from birth to up to 7 years of age.

In Table I, we show the hormonal level (LH, FSH and testosterone) and testicular size of patients in each group of VES, VTS and FES.

Table II presents the total number of patients, no. of transfer cycles, sperm recovery rate after warming (%), sperm survival rate after warming (%), fertilisation rate (%), no. of embryos transferred and no. of embryos cryopreserved among the three groups. In these results, there were statistically significant differences in the sperm survival rate after warming and fertilisation rate between VES and VTS. The results indicate that testicular spermatozoa are vulnerable to vitrification and that VES showed a significantly higher fertilisation rate than VTS (Table II).

Table III summarises the total number of patients, age of patients and cumulative number of transfer cycles, pregnancy rate (%), miscarriage rate (%) live birth rates (%) and cumulative number of live offspring, among the three groups.

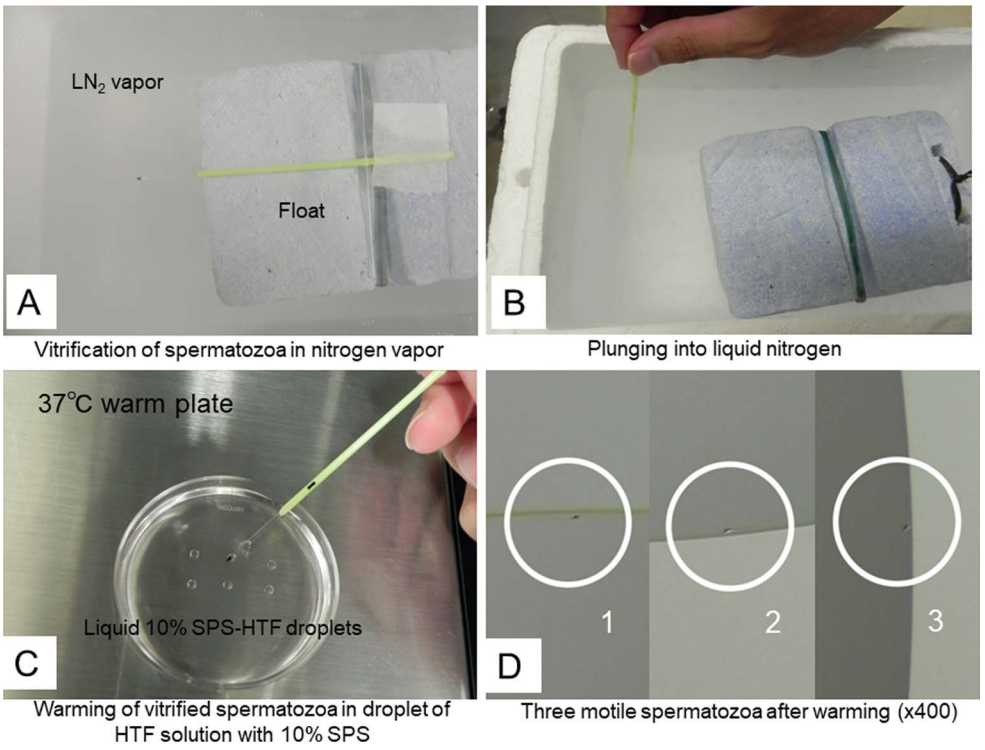

Figure 3 Vitrification and warming operations of spermatozoa placed on the Cryotop. The Cryotop was left in nitrogen vapour for 2 min (**A**). Then, it was plunged into liquid nitrogen and stored (**B**). Warming was performed by immediately inserting the tip of the Cryotop into HTF solution with 10% SPS (**C**). All the spermatozoa that had been cryopreserved in liquid nitrogen were moving well after warming (**D**).

Table I Hormonal levels and testicular size in the three groups.

Spermatozoa	No. of patients	Age of patients	Mean level of hormone			Testicular size (ml)
			LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)	
Vitrified ejaculated spermatozoa (VES)	28	33.7 ± 4.9	7.75 ± 3.77	17.98 ± 10.05	4.30 ± 1.91	7.46 ± 2.50
Vitrified testicular spermatozoa (VTS)	20	28.4 ± 5.8	11.85 ± 7.12	26.09 ± 13.78	3.79 ± 1.72	4.40 ± 2.97
Fresh ejaculated spermatozoa (FES)	31	34.6 ± 4.8	6.10 ± 3.20	14.46 ± 9.11	4.78 ± 1.87	9.62 ± 1.61

The pregnancy and delivery rates did not differ significantly statistically among the three groups. In the vitrified ejaculated spermatozoa group, the ICSI treatment was successful in 11 (39.3%) of the 28 cryptozoospermic patients and 14 babies were born (Fig. 1, Table III). Of the remaining 17 cases, 5 patients who performed five rounds of unsuccessful ICSI treatment with vitrified ejaculated spermatozoa decided to be treated with Micro-TESE. Three cases in which normal spermatozoa were found resulted in pregnancies by ICSI, and two of the cases had healthy babies (Cases 4 and 5). The remaining 12 couples chose to retreat from treatment (Fig. 1, Table IV).

Physical and congenital development (PCD) of the children born from the vitrified ejaculated spermatozoa group, obtained from Boshi Kenko Techo (birth to 3 years) and KIDS scale (3 to 7 years), are summarised in Table V. As of today, all children born after the present vitrification method are growing well without any physical or mental problems. No evidence of Prader–Willi syndrome, Angelman syndrome, Wiskott–Aldrich syndrome or any other physical or mental abnormalities has been found. In 13 out of 14 children (in one case the baby was less than 2 years) DA calculated by KIDS scales was higher than the CA, resulting in higher DQ score as compared with the average score (105) in the naturally born normal child population in Japan (Table V). Patients' information, developmental stages of embryos at the time of transfer, karyotypes and birth rates are summarised in Table VI.

Table II Embryological outcome of ICSI with the vitrified ejaculated, vitrified testicular spermatozoa and fresh ejaculated spermatozoa.

Spermatozoa	No. of patients	No. of transfer cycles	Sperm recovery rate after warming (%)	Sperm survival rate after warming (%)	Fertilisation rate (%) [fert/total]	No. of embryos transferred mean (range)	No. of embryos cryopreserved mean (range)
Vitrified ejaculated spermatozoa (VES)	28	60	97.8 [510/521]	87.1[a] [444/510]	52.7[b] [224/425]	1.52 (1–2)	0.72 (0–1)
Vitrified testicular spermatozoa (VTS)	20	18	92.7 [152/164]	60.5[a′] [92/152]	37.2[b′] [29/78]	1.73 (1–2)	0.53 (0–1)
Fresh ejaculated spermatozoa (FES)	31	107	Not applicable	Not applicable	52.2 [302/579]	1.41 (1–2)	1.83 (0–4)

a-a′ and b-b′: $P < 0.05$ (chi-squared test)

Table III Clinical outcome of ICSI with vitrified ejaculated, vitrified testicular spermatozoa and fresh ejaculated spermatozoa.

Spermatozoa	No. of patients	Age of patients		No. of transfer cycles	Pregnancy rate (%)	Miscarriage rate (%)	Live birth rate (%)	No. of live offspring
		Husband	Wife					
Vitrified ejaculated spermatozoa (VES)	28	33.7 ± 4.9	33.7 ± 4.1	60	15 (25.0)	2 (13.3)	13 (21.7)	14 (DZT*x1)
Vitrified testicular spermatozoa (VTS)	20	28.4 ± 5.8	32.0 ± 3.7	18	3 (16.7)	0 (0.0)	3 (16.7)	3
Fresh ejaculated spermatozoa (FES)	31	34.6 ± 4.8	35.8 ± 5.3	107	26 (24.3)	5 (19.2)	20 (18.7)	21 (DZT*x1)

*DZT, dizygotic twins

Table IV Clinical outcome of Micro-TESE-ICSI in cryptozoospermic patients who had no successful outcome with vitrified ejaculated spermatozoa.

Case	No. of sperm collected (motility and morphology)	Cryopreservation	Treatment	Pregnancy	Final outcome
1	0	Not done	–	–	–
2	6 (immotile with pinhead)	Not done	–	–	–
3	20 (weak moving)	+	TESE-ICSI	+	–
4	8 (normal)	+	TESE-ICSI	+	Birth
5	200 (normal)	+	TESE-ICSI	+	Birth

Discussion

We believe the cryopreservation of three or fewer spermatozoa in ejaculate from cryptozoospermic men should be given more consideration despite the fact that it has been widely accepted that Micro-TESE is the conventional treatment for cryptozoospermic men. We should consider that there are many patients who, when given a choice, want to have their babies using ejaculated spermatozoa in spite of the necessary frequent sperm collections for freezing and the assumed risks of DNA fragmentation.

There seem to be two main problems with the conventional methods of cryopreserving low numbers of ejaculated spermatozoa. The first one is the low motility rate after warming. We believe that might be caused by the use of glycerol as cryoprotectant.

Vitrification without permeable cryoprotectants was applied to human spermatozoa first by Isachenko *et al.* (2003, 2012). In the method, the sperm suspension mixed with sucrose is enclosed in plastic capillaries and plunged directly into liquid nitrogen. The method is used widely in the world for cryopreservation of a considerable number of spermatozoa and is superior to the conventional slow cooling with permeable cryoprotectants (Said *et al.*, 2010; Di Santo *et al.*, 2012) in terms of simplicity and ease of the preparation (Hammadeh *et al.*, 2001; Tongdee *et al.*, 2015). On the other hand, Endo *et al.* (2011, 2012) reported single spermatozoon cryopreservation using ultra-rapid vitrification with glycerol and sucrose. The method is more suitable than that by Isachenko *et al.* (2012) for the preparation of small number of spermatozoa. However, the motility of the vitrified ejaculated spermatozoa from asthenozoospermia patients in the

Table V Summarised data of physical and cognitive development of 14 infants.

Case	Baby	Data of physical and cognitive development by Boshi Kenko Techo (birth∼3 years)															Data of physical and cognitive development According to KIDS scale (3∼7 years)		
		1 month			6∼7 months			9∼10 months			1.5 years			3 years			Chronological age (CA)	Developmental age (DA)	Developmental quotient (DQ)*
		BW (g)	BH (cm)	PCD	BW (g)	BH (cm)	PCD	BW (g)	BH (cm)	PCD	BW (g)	BH (cm)	PCD	BW (g)	BH (cm)	PCD			
1	1	3540	53.5	N	6500	64.5	N	7570	68.8	N	9120	77.2	N	12 650	95.0	N	6Y11M	7Y4M	106
2	2	5354	52.5	N	9305	66.5	N	10 400	70.2	N	12 500	81.4	N	16 500	98.4	N	6Y7M	7Y1M	108
	3	4916	52.5	N	10 180	67.0	N	10 650	70.0	N	12 100	80.2	N		n-A		2Y0M	n-A	n-A
3	4	4105	52.6	N	8505	69.8	N	9750	75.0	N	10 750	79.6	N	15 155	97.4	N	5Y9M	6Y4M	110
4	5	4120	56.0	N	8360	71.5	N	8880	74.5	N	9700	81.0	N	14 200	99.0	N	5Y0M	6Y9M	135
	6	3765	53.8	N	7440	67.0	N	9020	74.7	N	10 400	84.3	N	15 300	99.5	N	3Y0M	5Y3M	170
5	7	3450	48.6	N	6250	63.5	N	7220	67.2	N	9250	76.5	N	12 250	90.2	N	4Y7M	5Y5M	118
6	8	3840	52.0	N	7390	67.2	N	8525	69.3	N	9600	78.6	N	14 300	98.2	N	4Y2M	4Y11M	118
7	9	4065	52.2	N	7820	68.4	N	9170	71.3	N	9500	78.9	N	12 700	91.7	N	4Y1M	4Y7M	112
8	10	3207	51.8	N	7520	65.2	N	8960	70.5	N	11 500	80.9	N	13 150	92.0	N	3Y10M	4Y2M	109
	11	2912	49.8	N	6850	63.5	N	8580	67.2	N	10 800	79.5	N	12 920	90.6	N	3Y10M	4Y2M	109
9	12	3520	42.8	N	7380	64.3	N	8035	66.5	N	10 079	77.4	N	13 200	90.3	N	3Y7M	4Y8M	130
10	13	4000	53.5	N	n-D	n-D	n-D	8350	68.4	N	10 000	79.2	N	13 300	89.9	N	3Y3M	3Y11M	121
11	14	3400	52.5	N	7860	68.5	N	8570	71.5	N	9800	79.0	N	14 100	92.5	N	3Y1M	4Y1M	132

BW, body weight (g); BH, body height (cm); N, normal; n-D, no data; PCD, physical and cognitive development; n-A, not applicable

*The procedure to calculate DQ is to divide the total of the subject's developmental age (DA) across domains by the chronological age (CA) and multiply it by 100 (DQ = DA/CA × 100).

Table VI Information about patients.

Case	Baby	Parents		Hormone level of husbands			Testicular size (ml) (rt/lt)	Data from ICSI to embryo transfer				Data of birth		
		Age (years)						% oocytes fertilised [fert/total]	Developmental stage × no. of embryos transferred	Day of ET	Karyo type of foetus	No. of embryos cryopreserved	No. of live offspring	Gestational weeks	BW (g)
		Husband at ICSI	Wife at embryo transfer	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)									
1	1	31	29	8.8	19.4	1.9	5/5	28.6 [4/14]	Blastocyst x1	5	46XX	1	1	40	3154
2*	2	37	35	5.3	7.4	8.1	12/12	50.0 [6/12]	Morula x1 + Blastocyst x1	5	46XY	0	1	39	3658
	3	42	40					60.0 [3/5]	10cell x1 + 14cell x1	3	46XY		1	40	3538
3	4	38	37	2.3	11.8	6.7	9/9	85.7 [6/7]	Blastocyst x2	5	46XY	1	1	40	3366
4*	5	43	28	5	10.6	7.37	8/8	42.9 [3/7]	Blastocyst x1	5	46XY	0	1	38	2890
	6	45	30					71.4 [5/7]	Blastocyst x1	5	46XX		1	38	2944
5	7	41	40	8.9	13.5	4.4	8/8	100 [2/2]	3cell x1 + 6cell x1	2	46XY	0	1	39	2686
6	8	30	30	5.5	13.9	5.3	10/10	57.1 [4/7]	Blastocyst x1	5	46XX	1	1	39	3172
7	9	40	36	8.5	10.1	2.6	10/10	50.0 [1/2]	12cell x1	4	46XX	0	1	40	3324
8**	10	32	35	9.5	13.7	4.0	8/8	100 [5/5]	Blastocyst x2	5	46XY	1	2 (DZT)	37	2664
	11										46XX				2024
9	12	37	34	4.9	20.1	4.18	8/8	36.0 [9/25]	Blastocyst x1	5	46XX	2	1	37	2625
10	13	32	32	4.9	7	4.5	9/9	71.4 [10/14]	Blastocyst x1	5	46XX	2	1	40	3130
11	14	36	35	4.2	8.5	3.5	10/10	55.6 [5/9]	10cell x2	3	46XX	0	1	38	2762

*Case 2 and 4: two infants
**Case 8: DZT (dizygotic twin)

study was 44.4% and all of the vitrified ejaculated spermatozoa from oligozoospermic patients became immotile after warming (Endo *et al*., 2012). Similar to the previous studies, Berkovitz *et al*. (2018) attempted vitrification with a commercial cryoprotectant containing glycerol and sucrose for small numbers of spermatozoa and the average motile spermatozoa rate was 33%. In some cases all the vitrified spermatozoa lost their motility. Watanabe *et al*. (2017) recently demonstrated how DNA and membrane damage occur in cryopreserved human spermatozoa and suggest that potential damage of the plasma membrane caused by freezing allows rapid permeabilisation of water and ions, followed by DNA breaks in the sperm warming process. Glycerol may be responsible for DNA damage along with plasma membrane disintegration. We started to use the ultra-rapid direct-vitrification procedure with a permeable cryoprotectant-free medium with sucrose to improve the motility rate for spermatozoa in 2010 (unpublished).

The second problem with the conventional method is the low recovery rate after warming of originally motile spermatozoa in the ejaculate before vitrification. We began to look for a new method to be able to recover all frozen spermatozoa. In the conventional method, a very small number of spermatozoa are aspirated into the micro pipette head-first. This aspiration method is very easy, but a head-first aspirated spermatozoon moves toward the mineral oil filling the glass needle and if the spermatozoon attaches to the mineral oil, it can never be expelled. So, we changed the aspiration method from head-first to tail-first (Fig. 2B). We found that the new method was successful in retaining the three or fewer spermatozoa aspirated into an aspiration pipette at the lower part of it and preventing them from moving forward up to the border between the medium and mineral oil (Fig. 2E). Using this tail first aspiration method, the recovery rate reached almost 100% and the survival rate was just under 90%. This is the first time this tail-first aspiration technique for vitrification has been reported in the world.

Another reason why we believe cryopreservation needs to be considered as a choice for the patients is the fact that all of the 14 children born after ICSI using the VSE found in cryptozoospermic patients following our method showed comparable development to naturally conceived children in terms of physical and cognitive abilities according to the standards set by the Boshi Kenko Techo and the questionnaires that define the KIDS scale. Other authors have also reported births of healthy babies after using vitrified spermatozoa (Endo *et al*., 2012; Isachenko *et al*., 2012).

There are cases of Micro-TESE in which only a very few spermatozoa are found in the biopsied tissues. Without cryopreservation, repeated Micro-TESEs are necessary. Our vitrification technique was also applicable and efficient for cryopreservation of the testicular spermatozoa in such patients (Tables II and III). The motility was well maintained in the testicular spermatozoa after warming (60.5%). The reported motility of the vitrified testicular spermatozoa in previous studies was around 40% or completely lost (Endo *et al*., 2011 and 2012; Berkovitz *et al*., 2018; Spis *et al*., 2019). We believe our method can be an option for ICSI treatment with very small numbers of spermatozoa collected by Micro-TESE.

The ICSI treatment was successful in 13 out of 28 cycles (46.4%) for 11 cryptozoospermic patients and 14 babies were born (Table III). In addition, the fertilisation, pregnancy and live birth rates after ICSI were similar between vitrified or fresh ejaculated spermatozoa from cryptozoospermic men. The results suggest that DNA damage is not significantly increased in the transferred embryos which had been fertilised with motile spermatozoa successfully recovered after vitrification. The clinical pregnancy rate per transfer cycle with normal spermatozoa is about 20% in Japan (JSOG: https://plaza.umin.ac.jp/~jsog-art/). Therefore, the effectiveness and risk of the embryo transfer with vitrified ejaculated spermatozoa from cryptozoospermic men appear to be equal to that in the conventional ICSI (Perez-Cerezales *et al*., 2011).

Furthermore, the absence of risk with the modified vitrification method is confirmed by the analysis of the children with both the Boshi Kenko Techo and the KIDS scale. These results suggest that there is no significant DNA damage in the ejaculated spermatozoa from cryptozoospermic men. Nonetheless we make it a rule to explain the risks to couples who want to receive this treatment before starting it.

We recommend patients proceed to Micro-TESE after five rounds of unsuccessful trials. In this study, birth rates with VES and VTS were 21.7% (13/60) and 16.7% (3/18) with no statistically significant differences. We think Micro-TESE should be indicated after five unsuccessful rounds of ICSI using VES.

In conclusion, we had 14 babies born after cryopreservation of 1 to 3 spermatozoa from the ejaculates from cryptozoospermic men using the combination of permeable cryoprotectant-free vitrification method and Cryotop device. The children were found to have developed normally in terms of physical and cognitive ability. We believe this treatment is beneficial for cryptozoospermic men who wish to use the fresh spermatozoa in their ejaculates despite the extremely small number available. However, the length of our study is just 7 years so further long-term follow up study for offspring born after this method is necessary.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We thank the late Dr Hiroshi Kusunoki and Dr Chiemi Toyama, for their devotion to the development of this cryopreservation method, and Mr Roberto Rodriguez, for his advice in the preparation of this manuscript.

Authors' roles

M.O., A.T. and A.I. designed the research; M.O., Y.T. and S.W. performed the research; M.O., A.T., M.N. and T.Y. analysed the data; Y.T., I.T. and M.O. contributed reagents or analytic tools; M.O., A.T. and S.W. wrote the paper.

Funding

No external funding was received to undertake this study.

Conflict of interest

None declared.

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