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Reliable single sperm cryopreservation in Cell Sleepers for azoospermia management

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Summary

Conventional sperm freezing methods perform best when freezing sperm samples containing at least hundreds of spermatozoa. In this severe male factor infertility case series, we examined the reproductive outcomes in 12 intracytoplasmic sperm injection cases where spermatozoa used were frozen in Cell Sleepers. Cell Sleepers are novel devices in which individual spermatozoa can be frozen in microdroplets. The case series included five men with obstructive azoospermia, six with nonobstructive azoospermia and one with cryptozoospermia, in whom microscopic sperm retrievals from testicular sperm extraction (TESE), micro-TESE extracts and a centrifugation procedure resulted in less than 50 spermatozoa. A total of 304 microscopically retrieved spermatozoa were frozen in 20 Cell Sleepers using a rapid manual cryopreservation method. A total of 179 mature oocytes were injected with recovered thawed spermatozoa, resulting in a fertilisation rate of 65.9% (118 of 179), with no total fertilisation failures. In 10 cases, an embryo transfer was performed, three on day 3 and seven on day 5, resulting in a per cycle pregnancy rate of 58.3% (seven of 12). Four of the pregnancies have progressed past 20 gestation weeks. The recovery and use of spermatozoa that were frozen in Cell Sleepers was uncomplicated and effective and eliminated the need to perform any microscopic sperm retrieval procedures on the day of oocyte collection. Modification of the routine sperm cryopreservation methodology to include the use of Cell Sleepers increases the range of sperm samples that can be effectively cryopreserved, to include men with severe male factor fertility.

Introduction

The development of intracytoplasmic sperm injection (ICSI) technology has extended the treatment capacity of human assisted reproduction technology (ART), to include men with azoospermia. ICSI has therefore provided these men, for whom it was previously not possible, the chance to father a biological child. Studies on the use of ICSI in the treatment of severe male factor infertility have shown that while fertilisation rates may differ according to the aetiology of the male factor, the pregnancy rates do not differ (Friedler *et al.*, 2001; Wald *et al.*, 2006; Borges *et al.*, 2007; Ishikawa *et al.*, 2009; Di Santo *et al.*, 2012). Azoospermia currently affects approximately 1% of the male population and up to 20% of men in the infertile population. This incidence, according to current speculation, may increase in the future. Albeit controversial, it is believed that semen quality has declined over time and that it will continue to decline (Rolland *et al.*, 2013).

In cases of azoospermia, spermatozoa can be obtained directly from the testes for use in ICSI with the use of surgical sperm retrieval (SSR) procedures. If large numbers of spermatozoa are retrieved from the testicular tissue or epididymal fluid, the fresh spermatozoa can be used for oocyte injections and all the superfluous spermatozoa frozen using conventional cryopreservation methods. The cryopreservation of superfluous spermatozoa in multiple devices is an important consideration in the management of azoospermia, as it avoids the need to repeat SSR procedures when multiple ICSI treatments are required to produce a viable pregnancy. Repeat procedures not only increase the cumulative costs of treatment, but may also adversely affect the physiology (i.e. deterioration of spermatogenesis,

inflammation, irreversible atrophy and partial testicular devascularisation) of testes and, therefore, their continued capacity to produce spermatozoa (Cohen *et al.*, 1997; Hafez *et al.*, 2009). However, often no superfluous spermatozoa are cryopreserved, as conventional sperm cryopreservation methods are not ideal for the cryopreservation of small sperm number samples (Hafez *et al.*, 2009). Testicular tissue processing results in relatively large sperm extraction volumes that require centrifugation before cryopreservation, a procedure that may have a detrimental effect on the quality of testicular spermatozoa. In addition, the post-thaw recovery of spermatozoa is complicated by the need to reprocess the sperm sample to decrease the sample volume and remove the cryoprotectant, when using conventional large volume sperm freezing devices (Desai *et al.*, 2004).

The development of an effective method to freeze single spermatozoa would further reduce the need to perform repeat SSR procedures. In the past, repeat sperm retrieval procedures were only avoided when large numbers of spermatozoa were retrieved and sperm could be cryopreserved conventionally in multiple sperm freezing devices. An effective single sperm cryopreservation method would mean that even men with extremely low numbers of spermatozoa would gain the chance to have multiple ICSI treatments from a single SSR procedure. In all cases, the knowledge that spermatozoa were available on the day of oocyte collection reduces the stress for both the couple receiving treatment and the clinic providing the treatment. In a review of methods that have been used to cryopreserve small numbers of spermatozoa in the past, Hafez *et al.* (2009) found that nine different carriers have been trialled, seven nonbiological and two biological carriers. This review included the study of Cohen *et al.* (1997), which reported the first successful attempt to cryopreserve individual spermatozoa, using cell-free human, mouse and hamster zona pellucida. The full list of carrier types trialled were cell-free zona pellucida (human and animal), straws, ministraws, open-pulled straws, sperm microdroplets, ICSI pipettes, Volvox globator spheres, alginate beads, 5-mm copper loop, cryoloops and agarose microspheres. The sperm recovery rates reported, calculated as the ratio of the number of spermatozoa recovered after thawing over the number of spermatozoa initially frozen, ranged between 59% and 100%, and the fertilisation rates between 18% and 67% (Hafez *et al.*, 2009). However, to date, there have been no large-scale studies reporting on reproductive outcomes that include pregnancy and live birth outcomes for any of the novel methods listed.

Operation efficiency in the laboratory requires that the method chosen for cryopreservation of single spermatozoa is not only reliable, but also complimentary to the existing routine micromanipulation procedures used. The Cell Sleeper (Nipro, Japan) is a nonbiological, commercially available, ready-to-use device, which has been shown to have the potential to be a device well suited for the routine cryopreservation of single spermatozoa (Endo *et al.*, 2012a,b). In their first study, Endo *et al.* (2012a) investigated the most effective cryopreservation methodologies for Cell Sleeper sperm freezing. In their second study, Endo *et al.* (2012b) examined the clinical outcomes from ICSIs performed with the use of frozen-thawed Cell Sleeper sperm in severe male fertility patients. In our study, we examine sperm recovery rates, fertilisation rates, embryo quality and pregnancy outcomes in 12 ICSI cases in which frozen-thawed spermatozoa cryopreserved in Cell Sleepers were used.

Materials and methods

Patients

This case series study was performed at a private ART clinic, Antalya IVF, between November 2012 and August 2014. Twelve consecutive cases of ICSI treatments in which the spermatozoa used for the injection of oocytes were recovered from Cell Sleepers have been included in the study. Cell Sleeper cryopreservation was only used for sperm cryopreservation in cases where low numbers of spermatozoa were retrieved from processing procedures to obtain sperm. In one case of cryptozoospermia, spermatozoa were retrieved from the pellet after centrifuging the entire ejaculate, and in the cases of azoospermia, spermatozoa were retrieved from the seminiferous tubules extracted using SSR procedures. The diagnostic details of the male and female partners in each of the 12 cases, as well as their fertility histories, are summarised in Table 1. Patients gave written consent before both the SSR procedures and the ICSI procedures for the use of nonidentifiable data, and our institutional ethics committee gave approval for the study (ethics committee study approval no. 455).

Media

Cook IVF Media (Cook Medical, Limerick, Ireland) was used in all manipulation and culture procedures described as specified by the manufacturer (i.e. gamete manipulations, embryo cultures and embryo transfers). A solution for the cryopreservation (cryopreservation solution) of spermatozoa was prepared by diluting SpermFreeze solution (Vitrolife, Goteborg, Sweden) 1 : 1 with human serum albumin supplemented (10 mg ml^{-1}, by manufacturer) Sydney IVF Gamete Buffer (Cook Medical).

Table 1 Demographic details of the male and female partners in the 12 cases

Case No.	Male diagnosis	Male age (years)	Female diagnosis	Female age (years)	Infertility (years)	Previous ICSI[a]	G:P[b]
1	NOA: FSH 36.7 IU l^{-1}, 46XY	35.1	DOR[c] (AFC[d] = 4)	31.4	0.83	1	0 : 0
2	OA: 46XY	36.3	DOR (AFC = 5)	36.2	14.0	2	0 : 0
3	OA: 46XY	41.1	Obesity and age	40.1	20.0	4	0 : 0
4	OA: 46XY	32.0	Normal	28.5	5.0	0	0 : 0
5	NOA: hypogonadotropic hypogonadism (LH = 0.55 IU l^{-1})	34.7	Normal	26.9	9.0	0	0 : 0
6	OA: 46XY	49.1	Normal	35.3	12.0	1	1 : 1
7	NOA: Klinefelter (47XXY)	34.2	Normal	32.3	5.0	0	0 : 0
8	NOA: hypogonadotropic hypogonadism (LH = 0.71 IU l^{-1})	46.5	Normal	34.4	8.0	0	0 : 0
9	NOA: Undescended testes	30.7	Normal	31.7	0.83	0	0 : 0
10	Cryptozoospermia: Sertoli-cell–only	43.3	Normal	35.3	18.0	0	0 : 0
11	OA: 46XY	32.9	Normal	30.7	10.0	0	0 : 0
12	NOA: FSH 23.2 IU l^{-1}, 46XY	25.7	Smoking	20.7	0.83	0	0 : 0
	Mean (STD)range	36.8 (6.87) 25.7–46.5		32.0 (5.04) 20.7–40.1	8.62 (6.53) 0.83–20.0		

Note: male diagnosis; nonobstructive azoospermia (NOA) and obstructive azoospermia (OA).
[a]Previous ICSI treatments in which Cell Sleeper spermatozoa were not used.
[b]gravidity and parity.
[c]DOR; decreased ovarian reserve.
[d]AFC; antral follicle count.

Sperm preparation

Men with azoospermia underwent testicular sperm extraction (TESE) procedures to retrieve spermatozoa. If no spermatozoa were retrieved from the initial TESE tissue extractions, micro-TESE procedures were performed. Transverse incisions were made through the outer scrotal capsules to expose the tunica albuginea. In TESE procedures, incisions were made in the albuginea, and by applying manual pressure, small fragments of protruding testicular tissue were excised, without optical magnification. In micro-TESE procedures, larger incisions were made through the albuginea and enlarged seminiferous tubules were selectively excised under optical magnification (SOM 62; KAPS, Asslar/Wetzlar, Germany). Excised testicular tissue or seminiferous tubules were processed in media containing culture dishes (60 × 15 and 35 × 10 mm; BD Falcon, Franklin Lakes, NJ, USA). The tubules were processed using forceps, surgical blades and 1-ml insulin syringes with needles bent to an angle suitable for tissue manipulation, under optical magnification (SZ51; Shinjuku-ku, Tokyo, Japan). The processing media was aspirated, spread on the bottoms of the large culture dishes and covered with culture oil. The man with cryptozoospermia provided a semen ejaculate prior to undergoing a SSR procedure. The semen ejaculate was mixed 1 : 1 with media and centrifuged at 500 ***g*** for 5–10 min, the supernatant removed, and the resultant pellet resuspended with 200 μl of media. The re-suspended pellet was aspirated, spread on the bottoms of large culture dishes and covered with culture oil. All aspirates and resuspensions were examined for spermatozoa on the heated stage (set to read 37 °C in microdroplets; Fujinomiya-shi Shizouka-ken, Japan) of an inverted microscope (IX71; Olympus), with an injection pipet (ICSI, Origio Inc, Charlottesville,VA, USA) set-up. All motile spermatozoa located were aspirated using an injection pipet and ejected into storage microdroplets covered with culture oil for storage until cryopreservation.

Cell sleeper method

The Cell Sleeper (Nojicho, Kusatsu, Japan) used consisted of an outer vial, an inner tray and screwcap. The inner tray was removed from the vial and placed in the lid of a large culture dish, and a 2.0-μl droplet of cryopreserva-

tion solution was pipetted into the tray, in a central position. All the motile spermatozoa placed in the storage microdroplets were reaspirated and ejected into the droplet on the tray – making a note of the precise number of spermatozoa ejected. Immediately after spermatozoa were ejected into the droplet on the tray, the tray was returned to the vial and the vial closed with the screwcap. The vial was secured onto a cryopreservation cane held in a horizontal position and the cane suspended horizontally 4–5 cm above the surface of liquid nitrogen (LN2; pre-calibrated minus 115–130 °C; using a thermocouple; Cole-Parmer Instrument Company, Vernon Hills, IL, USA) for 2 min. After 2 min, the vial was submerged in LN2 for at least 1 min and the cane with the attached vial was stored submerged in liquid nitrogen (MVE BioMedical, Garfield Heights, Ohio, USA).

On the day of the oocyte pickup (OPU), the cane with the vial was removed from the cryotank; the vial was detached from the cane and left to warm at room temperature (RT) for 1 min. The inner tray was removed from the vial placed in the lid of a large culture dish and the spermatozoa containing droplet was covered with warmed (37 °C) culture oil. The lid with the tray was placed on the heated stage (37 °C) of an inverted microscope with an injection pipet set-up. The relocated spermatozoa were aspirated, using the injection pipet, washed and ejected into the 'sperm' microdroplet of a dish set-up for ICSI. In trials prior to the start of the study, 105 spermatozoa were frozen in five Cell Sleepers (5, 10, 15, 25 and 50 spermatozoa). After thawing, 93 spermatozoa (88.6%) were recovered from the Cell Sleepers (5, 10, 13, 22 and 43 spermatozoa).

ART treatment

Controlled ovarian stimulation (COS) for multifollicular development was performed using a GnRH antagonist (Cetrotide; 0.25 mg; Merck Serono, Istanbul, Turkey) control and a combination of rFSH (Gonal-F; Merck Serono) and hMG (Menopur; Ferring pharmaceuticals, Mumbai, İndia) for stimulation. Ovulation was induced (Ovidrel 250 μg/0.05 ml; Merck Serono and/or Gonapeptyl® 0.1 mg; Ferring pharmaceuticals) when at least three follicles reached 17 mm. Transvaginal ultrasound-guided follicular aspiration was performed 36 h later. Oocytes that were collected from the follicular aspirates were washed, denuded, pipetted into microdroplets covered with culture oil and placed into an incubator. Culture conditions were set at 6% CO_2, 5% O_2 and 37.0 °C (K-Systems; Kivex Biotec ltd, Birkerod, Denmark). All and only metaphase II oocytes were injected, 2–4 h after oocyte collection, using only spermatozoa recovered from Cell Sleepers. After injection, oocytes were activated by exposure to calcium ionophore A23187 (10 mmol l^{-1}; Sigma, St Louis, MO, USA) for 15 min. Oocytes were checked for fertilisation (2PN) 16–18 h post-injection. All embryos from normally fertilised zygotes were cultured with the intention to develop blastocysts (days 5 and 6). Blastocysts that resulted were cryopreserved by vitrification. Vitrification and warming of blastocysts were performed using the Cryotop methodology, unmodified, as described by the manufacturer (Kitazato, BioPharma Co. Ltd, Fuji, Shizuoka, Japan). Fresh (embryos and blastocysts), vitrified and warmed (blastocysts) embryo transfers were performed using a Hamilton syringe (50 μl; Hamilton syringes, Sigma-Aldrich, St Louis, MO, USA) attached to an embryo transfer catheter (Sure View; Wallace; Smiths Medical International Ltd, Kent, UK) and transabdominal ultrasound guidance. Frozen embryo transfers (FET) were performed after endometrial preparation using oestrogen (Estrofem, Novo Nordisk Pharmaceuticals Pty Ltd, Baulkham Hills, NSW, Australia) supplementation and endometrium:embryo synchronisation using progesterone (Crinone, Merck Serono) supplementation.

Embryo and blastocyst grading

Embryos were scored according to blastomere number, blastomere size and regularity, and the percentage of fragmentation. Good quality embryos, embryos regarded as having the highest quality, were those embryos with equal-sized and spherical blastomeres, and with <10% fragmentation. Embryos were scored on a numeric scale of 1–3 to indicate good (i), fair (ii) and poor (iii) quality embryos (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Blastocysts were scored using a numeric (1–6, early blastocyst to hatched blastocyst) and alphabetical scale (A–C, good, fair and poor). Blastocyst expansion was scored on the numerical scale, and inner cell mass (ICM) quality (according to the number and degree of compaction of the cells), and trophectoderm (TE) quality (according to the number, size and contiguous arrangement of the trophectoderm cells), on the alphabetical scale (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Outcomes

Pregnancy outcomes were defined as follows: a day 14 βhCG value of >30 IU ml^{-1} was defined as a pregnancy, an ultrasound visible foetal heart beat (FHB) after the 7th gestational week was defined as a clinical pregnancy, and normal foetal development (NFD) past the 20th gestational week was defined as an ongoing pregnancy.

Results

The 12 male partners in the case series were all initially diagnosed with azoospermia, five men with obstructive azoospermia (OA) and seven men with nonobstructive azoospermia (NOA) (Table 1). The man diagnosed with Sertoli-cell-only syndrome (originally classified as NOA) had his diagnosis changed to cryptozoospermia, as 42 motile spermatozoa were retrieved from the semen ejaculate he produced on the day of his TESE procedure. Eight of the 12 women in the case series were diagnosed with normal fertility, two were diagnosed having DOR (decreased ovarian reserve), and two were diagnosed with poor fertility based on weight (39.0 kg m^{-2}) and age (40.1 years) and smoking (≈20 cigarettes/day) (Table 1). The patient demographics of age, duration of fertility, and previous treatment and pregnancy history are summarised in Table 1. The couples in four of the cases had previous ICSI treatments. The couple in case 6 had a live delivery of a normal infant in 2003, resulting from an ICSI pregnancy in which fresh TESE spermatozoa were used for oocyte injections. The couples in cases 2 and 3 had previous unsuccessful ICSI treatments.

Three hundred and four motile spermatozoa were retrieved from the sperm processing procedures performed in the study, 210 motile spermatozoa from the NOA men and 94 motile spermatozoa from the OA men (Table 2). In seven of the 11 men who had SSR procedures, the spermatozoa were obtained by micro-TESE, as the initial TESE procedure failed to deliver spermatozoa. The sperm freeze–thaw data in the 12 cases are summarised in Table 2. Cell Sleepers were removed from cryostorage only once, the number of metaphase II oocytes retrieved was known, and all unused motile spermatozoa were refrozen in Cell Sleepers. In 11 cases, the number of spermatozoa held in frozen storage was greater than the number of available metaphase II oocytes. In the only exception, case 4, 14 mature oocytes were collected, but only seven spermatozoa were in frozen storage. Ninety four per cent (249 of 265) of spermatozoa frozen in Cell Sleepers were recovered after thawing. In seven of the cases, all the spermatozoa recovered had retained their motility, and in five of the cases, all spermatozoa recovered showed no obvious signs of motility. The overall post-thaw motility (sperm survival) averaged 55.8% (139 of 249), and the mean per case post-thaw motility (sperm survival) was 58.3 (700/12). Although some of the spermatozoa had progressive motility after placing them in the 'sperm holding' microdroplets of ICSI dishes, most of the spermatozoa (61.5%, 56 of 91) only had nonprogressive or 'twitching' movement. In case 6, a single straw containing testicular material was cryopreserved by conventional means from the TESE procedure which provided the fresh spermatozoa for the couple's successful ICSI treatment. The straw was thawed prior to the couple's next ICSI treatment cycle, as only low numbers of spermatozoa were observed prior to cryopreservation. The thawed testicular material was processed, and seven motile spermatozoa were retrieved and refrozen in a Cell Sleeper.

One hundred and seventy-nine oocytes of the 187 mature oocytes retrieved at OPU were injected. In the

Table 2 Sperm retrieval and sperm freeze–thaw outcomes using Cell Sleepers

Case No.	Sperm origin	Cell Sleeper:sperm frozen[a]	No. required[b]	No. thawed[c]	No. ecovered	No. motile (%)
1	TESE	1:5	5	5	5	0 (0)
2	micro-TESE	1:12 and 2:7	8	12	12	12 (100)
3	TESE	1:14 and 2:15	4	14	12	12 (100)
	micro-TESE	1:7	14	7	7	0 (0)
5	micro-TESE	1:11 and 2:18	15	29	27	0 (0)
6	TESE	1:7	6	7	7	7 (100)
7	micro-TESE	1:16	10	16	12	12 (100)
8	TESE	1:15 and 2:16	27	31	31	0 (0)
9	micro-TESE	1:21 and 2:22	35	43	40	0 (0)
10	Ejaculate	1:16 and 2:26	26	42	40	40 (100)
11	micro-TESE	1:15 and 2:17	5	15	14	14 (100)
12	micro-TESE	1:27 and 2:17	32	44	42	42 (100)
	Total	304	187	265	249	139 (55.8)
	Retrieval rate				94.0%	

[a]Cell Sleeper:sperm frozen: the number of sperm frozen in an individual Cell Sleeper (Case No. 1; Cell Sleeper number 1 contains 5 spermatozoa).

[b]No. spermatozoa required: the number equals the number of metaphase II oocytes obtained at the OPU.

[c]All motile spermatozoa retrieved and not used for oocyte injection were refrozen.

seven cases with motile spermatozoa, 91 mature oocytes were injected, and in the five cases with nonmotile spermatozoa only, 88 mature oocytes were injected (Table 3). The fertilisation, transfer, embryo/blastocyst cryopreservation and pregnancy outcome data are summarised in Table 3. The overall per oocyte fertilisation rate was 65.9% (118 of 179), and the mean per case fertilisation rate was 61.6% (range, 25.0–86.7%). The fertilisation rate for the oocytes injected with nonmotile spermatozoa was 76.1% (67 of 88). In none of the cases did total fertilisation failure occur.

In all the treatment cycles, embryo culture was set up with the intention to transfer embryos on day 5, as blastocysts. The transfer days were, however, changed according to the number and the quality of embryos developing in culture. The treatment cycles in cases 3 and 10 were cancelled, as no viable embryos were obtained. In both these cases, only motile spermatozoa were used for oocyte injection. In cases 1, 2 and 6, the embryo transfer day was changed to day 3. In cases 4, 5, 7, 8, 9, 11 and 12, embryo cultures were continued through to day 5, from which a 24.7% (24 of 97) useable blastocyst rate of was obtained. Fresh blastocyst transfers were performed in cases 4, 5, 7 and 11, and frozen blastocyst transfers were performed in cases 8, 9 and 12. The fresh transfers in the latter three cases were cancelled, because the patients were assessed as being at increased risk of developing OHSS. In these three cases, all viable blastocysts were cryopreserved, and blastocyst transfers performed in subsequent FET cycles. In total, 10 embryo/blastocyst transfers were performed from the initial 12 OPUs, resulting in a 58.3% (day 14 βhCG >30 IU l^{-1}, seven of 12) pregnancy rate per OPU and a 70.0% (seven of 10) rate per transfer. Six of the pregnancies have been confirmed as clinical pregnancies (normal foetal heart at >7 weeks), with four pregnancies confirmed as ongoing pregnancies (normal foetal growth >20 weeks).

Discussion

Over the years, a number of devices (i.e. biological and nonbiological) have been trialled to find the one most suitable to use when needing to cryopreserve small numbers of spermatozoa. All the methods reported on in peer-reviewed publications have demonstrated a reasonable measure of success, but with varying degrees of technical difficulty (Hafez *et al.*, 2009). The Cell Sleeper cryopreservation method reported on in our study is a relatively simple method, requiring a limited number of manipulations, and using a rapid freezing method very similar to that used for conventional manual sperm freezing. A Cell Sleeper is also a closed system in the form of a standard cryovial that can be stored in unrestricted liquid nitrogen storage tanks, without the need to modify the internal storage systems. In this study, we only investigated the use of Cell Sleepers to cryopreserve sperm in

Table 3 Embryology and pregnancy outcomes following the use of Cell Sleeper frozen spermatozoa

Case No.	ICSI[a]	Motile spermatozoa	No. Fertilised (%)	Blastocysts vitrified		Embryos or Blastocysts transferred		Pregnancy outcome[d]
				Freeze-all[b]	Supernumerary[c]	Fresh ET	FET	
1	5	No	2 (40.0)			6 (2), 3 (3)		OP (29 weeks)
2	8	Yes	4 (50.0)			7 (2), 6 (2)		NP
3	4	Yes	1 (25.0)					Cycle cancelled
4	6	No	4 (66.7)		4AA, 2BB	4AB, 1A		OP (22 weeks)
5	15	No	13 (86.7)			4AB		OP (22 weeks)
6	6	Yes	3 (50.9)			8 (1), 6 (2)		OP (22 weeks)
7	10	Yes	8 (80.0)			4AB, 4AB		CP (14 weeks)
8	27	No	22 (81.5)	3BB, 2BB, 4AB, 4AB, 2BB			3BB, 2BB	P (βhCG = 287 IU l^{-1})
9	35	No	26 (74.3)	4AA, 2BB, 3BB, 3BB, 1B			4AA, 3BB	NP
10	26	Yes	11 (42.3)					Cycle cancelled
11	5	Yes	4 (80.0)			4AB, 4AB		CP (7 weeks)
12	32	Yes	20 (62.5)	3BB, 1B, 3BB, 2BB, 2BB			3BB, 1B	NP

[a]The number of metaphase II oocytes injected.

[b]Freeze-all: cycles in which all blastocysts were vitrified, due to an increased OHSS (ovarian hyperstimulation syndrome) risk.

[c]Supernumary: blastocysts vitrified supernumary to the blastocysts transferred.

[d]P: pregnant (day 14 βhCG >30 IU l^{-1}), NP: not pregnant, CP: clinical pregnancy (FHB >7 weeks), OP: ongoing pregnancy (NFD >20 weeks).

Note: embryo description; '6 (2)' a 6 cell with a grade 2 for quality, blastocyst description; '3BB and 1B'a grade 3 expanded blastocyst with a B-quality inner cell mass and a B-quality trophectoderm, a grade 1 expanded blastocyst with a grade B overall quality. Transfer description: Fresh ET (fresh embryo/blastocyst transfer) and FET (frozen embryo/blastocyst transfer).

cases where small numbers of spermatozoa were retrieved from sperm processing procedures (i.e. testicular tissues and semen ejaculates). However, we believe the method to have broader application potential in ART. For example, the method can be used when there is a therapeutic need to microscopically select and store spermatozoa with specific motility (i.e. sperm progression pattern) and/or morphological features (i.e. normal acrosomes and normal morphology).

When thawing a Cell Sleeper, the frozen sample (sperm microdroplet) does not have to be delivered to a new 'recovery' microdroplet or medium containing well, or retrieved from a secondary carrier. The microdroplet in which the spermatozoon was placed at cryopreservation is the microdroplet from which the spermatozoa will be recovered after thawing. These technical elements all contribute to reducing the risks of losing spermatozoa in the recovery process – effectively protecting which in many cases is an extremely limited resource. Searching for the number of spermatozoa needed to inject all mature oocytes obtained can be a time-consuming task. If the time taken is prolonged, it can affect the operational efficiency of the laboratory, as well as embryo developmental outcomes, if oocyte injection takes place outside, which is regarded as the optimal post-OPU time for insemination. There has been some evidence to suggest that oocytes may have an optimal time for insemination, coinciding with specific nuclear and cytoplasmic maturational events (Yu *et al.*, 2011). In their case study, Endo *et al.* (2012b) reported that most spermatozoa were recovered within 30 min after starting the search for sperm. Although no exact time measurements were taken in our study, on recollection, none of the searches in the 12 cases went beyond 40 min.

Compared with other cell types, sperm cells are less sensitive to cryodamage, because of the high fluidity of their membranes and the low intracellular water content (Benson *et al.*, 2012). Notwithstanding this relative resistance to changes and damage, sperm cryopreservation processes have the potential to affect sperm motility, sperm fertilising capacity and spermatozoa-dependent embryo developments (Benson *et al.*, 2012; Di Santo *et al.*, 2012; Ohlander *et al.*, 2014). It has also been shown that spermatozoa from severe male factor patients were more susceptible to changes and damage, because of existing testicular dysfunction (Ohlander *et al.*, 2014), and have therefore been observed to lose their motility more readily through the freeze–thaw process (Endo *et al.*, 2012b; Ohlander *et al.*, 2014). This link between sperm pathophysiology and the capacity to preserve motility may have been the reason for the 'all or nothing' post-thaw motility outcomes observed in our study. In 5 of our 12 cases, all the spermatozoa recovered for injection had lost their motility after thawing. Motility, however, may not be an absolute indicator of sperm viability. Fresh and frozen immotile spermatozoa used to inject oocytes have resulted in normal fertilisations, albeit at reduced rates (Ohlander *et al.*, 2014). The use of nonmotile spermatozoa in our case series resulted in a fertilisation rate of 76.1% and in four pregnancies from five embryo transfers. In our study, the use of nonmotile spermatozoa for oocyte injection therefore did not result in reduced fertilisation. The reason for this outcome could possibly be related to the fact that all spermatozoa frozen were motile prior to the cryopreservation process, and while their motility was not preserved, their viability was. Endo *et al.* (2012b), in the discussion of their results, also speculated on this phenomenon.

In only the one other published study to report on the embryology and pregnancy outcomes of oocytes injected with spermatozoa recovered from Cell Sleepers, Endo *et al.* (2012b) reported the delivery of a healthy infant from the transfer of a single day 5 expanded blastocyst. In our study, we were able to report on the ICSI reproductive outcomes of 12 cases. In another study analysing the reproductive outcomes of conventionally frozen-thawed TESE spermatozoa in ICSI, from both OA and NOA men, a total fertilisation rate of 61.2% and a pregnancy rate of 35.8% was obtained (Ishikawa *et al.*, 2009). In our study, using Cell Sleeper frozen-thawed TESE spermatozoa from OA and NOA men, we obtained an overall fertilisation rate of 65.9% and per OPU pregnancy rate of 58.3%. The fertilisation outcomes also revealed that where motility was confirmed before cryopreservation, the use of thawed nonmotile spermatozoa to inject oocytes had no adverse effect on fertilisation or pregnancy outcomes. At the time of writing, six of the pregnancies achieved were confirmed as clinical pregnancies (FHB >7 weeks), with four of the six pregnancies confirmed as ongoing pregnancies (NFD >20 weeks).

The major drawbacks to our study are the small number of cycles accumulated in the 2 years and the lack of an appropriate control group; however, the study does reflect the value of Cell Sleepers in routine male factor fertility treatments. From the fertilisation and pregnancy outcomes obtained in our severe male factor infertility case series, we believe that Cell Sleepers with the sperm freezing methodology used were compatible with normal reproductive outcomes. The recovery of spermatozoa from the Cell Sleepers was also found to be remarkably uncomplicated and efficient, and the retrieval and use of the recovered spermatozoa was also found to easily integrate into our routine ICSI programme. The Cell Sleeper methodology described here has now eliminated our need to perform SSR procedures on the day of the OPU, as the cryopreservation of spermatozoa is no longer

dependent on the number of spermatozoa retrieved from the microscopic sperm processing procedures.

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