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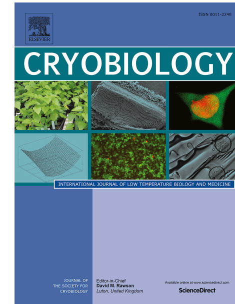

First birth of a healthy infant following intra-cytoplasmic sperm injection using a new permeable cryoprotectant-free sperm vitrification protocol

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1 TITLE: First birth of a healthy infant following Intra-cytoplasmic Sperm Injection using a new
2 permeable cryoprotectant-free sperm vitrification protocol

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11 ABSTRACT

12 PURPOSE: The purpose of this study is to present the first birth of healthy infant born following
13 ICSI using the new permeable cryoprotectant-free sperm vitrification protocol Easy-Sperm®.

14 PRINCIPAL RESULTS: A 39 years old woman and his 40 years old partner underwent egg
15 donation treatment at IVF-Spain Alicante (Spain). Half of the mature oocytes obtained from a
16 young and healthy donor were fertilized by ICSI, using slow-frozen spermatozoa and the other
17 half with vitrified spermatozoa. A total of 5 blastocysts were obtained on day 5 (3 resulting from
18 vitrified spermatozoa and 2 from frozen sperm). The best embryo, with AA quality (derived from
19 one of the oocytes fertilized with vitrified sperm) was transferred. The woman conceived and,
20 following a normal pregnancy, delivered a healthy boy.

21 CONCLUSIONS: To the best of our knowledge, this is the first case report of a successful
22 pregnancy and delivery of a healthy infant from ICSI with permeable vitrified spermatozoa in an
23 oocyte donation program with transfer on blastocyst stage.

24 KEY WORDS: sperm vitrification, sperm cryopreservation, oocyte donation, blastocyst, live birth.

25 Sperm cryopreservation is an essential procedure of Assisted Reproduction Technologies (ART)
26 important to preserve male fertility. Currently, the most commonly used technique for sperm

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27 cryopreservation is slow freezing [7]. Nevertheless, this technique has been shown to produce
28 dramatic changes in sperm quality at both structural and functional levels [2,4]

29 During the last years, vitrification has been proposed as a better alternative to conventional
30 freezing in many cell types [8]. This technique is based on the ultra-rapid descent and rise of
31 temperatures avoiding crystal formation and its associated effects [5]. Vitrification has shown
32 improved cell survival rates and reduced cell damage. This technique has been widely used in
33 oocyte and embryos but it has been hardly applied in human sperm.

34 Recently, the development of a new permeable cryoprotectant-free vitrification protocol
35 (Easysperm®, iGLS S.L., Alicante, Spain) for human spermatozoa provided an enhanced and
36 reliable alternative for sperm cryopreservation [1]. This novel method has been tested in
37 normozoospermic samples rendering better preservation of sperm quality on all the parameters
38 studied (motility, vitality, DNA structure, acrosome reaction, morphology and cytoskeleton)
39 compared to slow freezing [1]. At our centre (IVF-Spain Alicante, Spain) we have successfully
40 used this protocol to cryopreserve and use for fertilisation a sperm sample from a couple whose
41 female partner (39 years old) had ovarian failure and was recommended egg donation
42 treatment with his partner´s (40 years old) sperm. The cycle treatment was initiated in 2016.
43 Sperm sample analysis before cryopreservation showed normal volume (1.7ml), concentration
44 (>150x 10^6/ml), motility (47% progressive motility and 14% non-progressive motility) and
45 10.67% of sperm DNA fragmentation, as assessed by Sperm Chromatin Structure Assay (SCSA).
46 Sperm cryopreservation was performed using two techniques: conventional slow freezing and
47 the new sperm vitrification kit EasySperm® (iGLS, Alicante, Spain). In brief, for slow freezing
48 GM501 SpermStore® (Gynemed, Lensahn, Germany) was used. Following manufacturer´s
49 instructions, the sperm sample was mixed with the medium (proportion 1:0.7). The mixture was
50 equilibrated during 10min at RT and then introduced into a straw and sealed. Straws were
51 frozen just above the level of the liquid nitrogen during 15 min and then stored in a LN_2 tank.
52 For sperm vitrification, the sperm sample was diluted with EasySperm® vitrification medium
53 (V1)1:1. The mix was maintained at 37°C during 5min before the cooling procedure was
54 performed. Vitrification was carried out in a sterile container filled with 75-100ml of LN_2.
55 Around 20µl aliquots of spermatozoa suspension were dropped directly into liquid nitrogen
56 (LN_2). Upon contact with the LN_2, a sphere is immediately formed. The spheres were retrieved
57 using a special metal strainer, transferred to 1.8ml cryotubes (Nunc, Roskilde, Denmark) and
58 stored in LN_2 tank.

59 Ovarian stimulation of the selected oocyte donor was achieved using the GnRH antagonist
60 protocol. On day 3 of the cycle, the donor was stimulated by recombinant follicle-stimulating
61 hormone (225 IU.Puregon®; MSD, Ballerup, Denmark). A daily GnRH-antagonist (Orgalutran®;
62 MSD, Ballerup, Denmark) dose of 0.25mg was used starting on stimulation day 6 and was
63 administered until de day of ovulation induction. Procrin 0.2 was used to induce final oocyte
64 maturation when 3 follicles ≥19mm were present. Estradiol and progesterone levels on the day
65 of trigger were 6056pg/ml and 2.10ng/ml, respectively. The egg retrieval was performed 36h
66 after trigger.

67 The cycle previous to the egg retrieval, the patient injected Decapeptyl® 3.75 (Ipsen Pharma
68 S.A, Signes, France). Endometrial preparation for embryo transfer was performed using
69 estrogens 6mg (Provamés® 2mg ; Sanofi Aventis, Osny, France) from the third day of her cycle
70 until the day of egg retrieval, where progesterone 800mg (Utrogest®; Dr. Kade, Besins Pharma
71 GmbH, Berlin, Germany) was added until the 12th week of pregnancy.

72 On the egg retrieval day, the man was not present, so the previously cryopreserved sperm
73 samples were used for fertilization. The vitrified sperm sample was warmed following Easy-
74 Sperm® manufacturer's instructions using 2ml of the previously warmed (37°C) V2 solution
75 provided in the kit. After warming, the sample was maintained at 37°C for 5 min before sperm
76 preparation. Concentration after warming was 120mill/ml and progressive motility (A+B) 40%.
77 Frozen sperm sample was thawed at room temperature. Concentration after thawing was
78 150mill/ml and progressive motility (A+B) 35%.

79 Both, vitrified/warmed and frozen/thawed samples were prepared by density gradient (40/80;
80 centrifugation 300g x 12min; PureSperm®, Gynemed Lensahn, Germany) and then washed using
81 MHM-C (Multipurpose Handling Medium®-Complete, Irvine Scientific, California, USA;
82 centrifugation 600g x 8min). Supernatant of both samples was removed and sample was
83 capacitated by swim-up using MHM-c at RT. After 15 min, spermatozoa on the surface were
84 collected yielding the following: vitrified/warmed spermatozoa preparation contained $0.3x10^6$
85 spermatozoa per ml and 92% progressive motility; the prepared frozen/thawed sample
86 contained $0.6x10^6$spz/ml and 93% progressive motility.

87 From the egg-donor, 15 cumulus-oocyte complex (COC) were collected. After combined GM501
88 Hyaluronidase (Gynemed, Lensahn, Germany) and mechanical stripping, a total of 12 MII
89 oocytes were identified. Six mature oocytes were fertilized via Intra-cytoplasmic Sperm Injection
90 (ICSI) with slow-frozen/thawed spermatozoa and the remaining 6 were injected with

91 vitrified/warmed spermatozoa. A total of 10 zygotes were obtained (6 derived from vitrified
92 spermatozoa fertilized oocytes and 4 from frozen spermatozoa injected oocytes). All embryos
93 were cultured in a benchtop incubator (Geri®, GeneaBiomedx, Sydney, Australia) using CSC
94 (Continuous Single Culture with HSA, Irvine Scientific, California, USA). The embryos were
95 imaged by Geri® system and High, Medium and Low scores were calculated [10]. Five
96 blastocysts were produced on day 5; two from ICSI using frozen sperm (blastocyst grade 1 and
97 Medium score and one blastocyst 4AC [3] and Low score and three resulting from oocyte
98 injection of vitrified spermatozoa (5AA, 4AA, 4AA [3] all of them High score). The best
99 morphological embryo (5AA) [3] was transferred on day 5 into the uterine cavity. The remaining
100 embryos were vitrified by Kitazato® (BioPharma Co., Shizuoka, Japan) on the same day. Ten days
101 after embryo transfer, beta-HCG in blood plasma was 457UI/ml. In August 2017, a healthy boy
102 was born after 36 weeks of pregnancy with normal height (49 cm) and weight (2500g) at birth.

103 Despite not being a routine procedure in most IVF clinics, sperm vitrification represents a viable
104 and efficient tool for sperm storage in ART patients. Sperm storage has been traditionally
105 performed in most centres worldwide by slow freezing [7] . Although widely used, slow freezing
106 protocols have been shown to produce dramatic changes in sperm quality at both structural
107 and functional levels as a result of the formation of ice crystals and the toxic effects caused by
108 the use of cryoprotectants [2, 4]. Moreover, the method is labor and time-consuming as it
109 requires cooling at very slow rates. Due to these disadvantages, new alternative methods have
110 been developed [7]. One of the most innovative of these methods is vitrification; this technique
111 is based on the ultra-rapid increase and decrease of temperatures with or without the use of
112 non-permeable cryoprotectants. The improvement of vitrification has already been widely
113 demonstrated in oocytes and embryos; this technique has been shown to be a successful
114 alternative to slow freezing in these type of cells confirmed by the birth of numerous healthy
115 babies after its use [8].

116 Nonetheless, vitrification of spermatozoa is still a rather unexplored methodology, with limited
117 studies showing its efficacy in male gametes. Recently published results from the use of the
118 permeable cryoprotectant-free vitrification protocol (Easy-Sperm®, iGLS S.L., Alicante, Spain) on
119 normozoospermic sperm samples have shown improved preservation of sperm structure and
120 function compared to conventional slow freezing. Sperm motility, vitality, DNA integrity,
121 acrosome and cytoskeleton structure were significantly better preserved in vitrified samples [1].

122 Using this protocol, we here describe new data regarding the performance of vitrified
123 spermatozoa on fertilization and developmental ability of the embryos produced, reporting

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124 what it is, to our knowledge, the first clinical case of successful embryo development to day 5,
125 pregnancy and birth of a healthy neonate through egg donation treatment using this new
126 protocol. Other successful cases resulting from the application of different vitrification
127 techniques had already been published. In 2012 and 2013 the first births using human sperm
128 vitrified in the absence of permeable cryoprotectants and using 0.25µl straw were published [6,
129 9]. In contrast to the present report, in both cases, sperm was packaged into 0.25µl straw and
130 embryos were not cultured to the blastocyst state, so the quality of embryos could not be
131 studied and only low volumes of samples could be stored. In the present case, long culture and
132 timelapse imaging was carried out so we could observe that the qualities and the kynetics of the
133 embryos produced were better than the embryos obtained using slow freezing.

134 This successful clinical case shows that sperm cryopreservation using the permeable
135 cryoprotectant-free vitrification protocol EasySperm® is a safe approach for sperm storage,
136 offering easy performance and improved sperm quality preservation at both structural and
137 functional levels [1] and can be successfully used for intracytoplasmic sperm injection to
138 achieve normal pregnancy and birth. Vitrification of human spermatozoa becomes also a
139 promising alternative for sperm cryopreservation enabling the performance of fertilization
140 regardless of the availability of the male partner and guaranteeing good embryo development
141 and healthy offspring.

142 Further studies and clinical trials must be developed for comparison of slow freezing and
143 vitrification on IVF cycles outcomes. In spite of the promising results obtained in the present
144 study, the spread of sperm vitrification requires more detailed studies with larger number of
145 cases and future monitoring of the baby's health.

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148 AUTHOR'S ROLES:

149 L.M. performed sperm vitrification, embryo culture and transfer and drafted the manuscript.
150 M.E drafted the manuscript. MJ.G-T participated in the revision of the manuscript. J.A
151 performed medical ART treatment, performed embryo transfer and supervised the manuscript
152 preparation.

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155 **CONFLICT OF INTEREST:** None declared

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