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CASE REPORT

WHAT DO WE KNOW ABOUT THE GENETIC BACKGROUND OF PRIMARY CILIARY DYSKINESIA (PCD) AND THE REPRODUCTIVE APPROACH?

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Abstract:
Introduction: Primary ciliary dyskinesia (PCD; MIM 244400) is a heterogeneous autosomal recessive genetic disorder associated with infertility due to impaired sperm motility in men. We describe two such cases and give a literature review on the genetic background and reproductive outcome.
Materials and methods: Two primary infertile couples were referred to our clinic. Native semen, analyzed according to the WHO manual (2010), showed that male partners have completely immotile sperm with a negative pentoxifylline test. Sample vitality was compared according to different semen preparation methods (gradient density and washing method only). Genetic testing was done by Whole Exome next-generation Sequencing (WES) analysis. For the IVF/ICSI procedure, semen was prepared using the gradient density method, and prior to the ICSI procedure itself, a hypo-osmotic swelling test (HOST) was done.
Results: Semen analysis showed oligoasthenozoospermia in Patient 1 and oligoasthenoteratozoospermia in Patient 2. Blood draw for hormones and karyotype showed no irregularities in either case. Patient 1 was previously diagnosed with PCD, while Patient 2 was not. Parallel vitality testing did not show any differences between the two semen preparation methods in either of the two cases. Genetic testing in Patient 2 showed a pathogenic apparently homozygous CCDC40:c.2440C>T variant in exon 14 of the CCDC40 gene (MIM 613799). Fertilization rate after HOST/ICSI in both patients was 100%, and the final outcome for both patients was the birth of a healthy child.
Conclusion: Although PCD has diverse etiology, assisted reproduction techniques such as HOST give these couples a good chance for parenthood. Advances in testing and strict adherence to advised procedures are to be credited for such outcome improvement. Additionally, our recommendation for PCD patients and patients with immotile sperm is to do genetic testing and counselling prior to the IVF/ICSI procedure.

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Submitted: June, 2019
Accepted: August, 2019

Key words: primary ciliary dyskinesia (PCD), male infertility, CCDC40, hypo-osmotic swelling test (HOST), ICSI

INTRODUCTION

Primary ciliary dyskinesia (PCD; MIM 244400) is heterogeneous autosomal recessive genetic disorder involving permanent ubiquitous structural and/or functional ciliary abnormalities. If PCD is accompanied with dextrocardia/situs inversus, this condition is then called Kartagener syndrome.[1]
Cilia have a complex structure. The central part of a cilium is its cytoskeletal structure called the axoneme. It consists of 9 outer microtubule doublets, which are accompanied with 2 additional central singlet microtubules[2] in case of motile cilia, such as respiratory and fallopian tube cilia and sperm flagella. Those microtubules are associated with many proteins responsible for cilia movement. Inner (IDA) and outer (ODA) dynein arms are motor complexes required for providing the motor force for structure movement. The radial spokes provide physical support by building structures between peripheral microtubules and the central pair. They are also responsible for governing ciliary beat and waveform by transducing signals between dynein arms and the center of the axoneme. Nexin-dynein regulatory complexes regulate and orchestrate dynein activity by generating coordinated microtubule sliding in motile axonemes.[3]
More than 30 genes have been implicated in the PCD condition (Chodhari et al, 2004), and most of them

encode for axonemal dyneins (*DNAI1*, *DNAI2*, *DNAH5*, *DNAH11*, *DNAAF2* and *LRRC50*).[4]

Gene mutations can be reflected in ultrastructure changes detectable by transmission electron microscopy (TEM). Around 90% of individuals with PCD have ultrastructural defects affecting ODA and/or IDA protein(s).[5]

Recognized ODA defects have been associated with the following genes: *DNAH5*, *DNAI1*, *DNAI2*, *DNAL1*, *NME8*, *CCDC114*, *CCDC151*, *ARMC4* and *TTC25*. In 38% of the cases, mutations are located on *DNAI1* and *DNAH5*.[5]

ODA+IDA defects are known to occur due to mutations in multiple genes that encode for proteins needed for pre-assembly of ciliary axonemes: *DNAAF1*, *DNAAF2*, *DNAAF3*, *LRRC6*, *C21ORF59*, *HEART2*, *ZMYND10*, *DYX1C1*, *SPAG1*, *CCDC103* and *PIH1D3*.[6]

IDA defects are associated with MTD (microtubule disorganization) in less than 50% of cases. These defects are reflected in ciliar structural changes such as missing inner dynein arms. Mutation in *CCDC39* and *CCDC40* is the major cause of PCD with axonemal disorganization and absent inner dynein arms.[7, 8]

Although gene mutations have a strong impact on sperm motility, they are not always visible as ultrastructure changes detectable with TEM. This is the case with mutations in *DNAH11* and *HYDIN*.[6]

Except for the abovementioned gene mutations which have an impact on ODA and IDA, there are also mutations in genes that encode for central microtubule structures (*RSPH4A* and *RSPH9*), nexin links and dynein regulatory complex (*CDCC164* and *GAS8*), thioredoxin (*TXNDC3*), and X-linked gene (*RPGR*) associated with retinis pigmentosa.[9]

Symptoms of absent cilia motility are chronic sinus infections, chronic middle ear infections and hearing loss, recurrent respiratory infections and infertility. Many PCD patients are still undiagnosed or misdiagnosed, and only a limited number of centers have extensive experience in the diagnosis and management of PCD. Therefore, the European Respiratory Society brought consensus recommendations for diagnosis, monitoring and management of PCD.[10]

The main consequence of impaired sperm motility in male PCD patients is infertility.[11] The aim of this paper was to present literature data on reproductive outcomes following intracytoplasmic sperm injection (ICSI) with completely immotile ejaculated sperm in patients with PCD and to improve the understanding of the genetic background of this condition.

MATERIAL AND METHODS

We treated two couples with primary infertility caused by male partners with completely immotile sperm. Clinical and biological data of the two reported PCD patients are presented in Table 1.

Semen analysis

Native semen concentration, motility and morphology parameters were analyzed according to the WHO manual (2010).[12] Additionally, both samples were tested with pentoxifylline in order to enhance motility, but test results were negative.

Vitality testing after different semen preparation methods

Semen was aliquoted and processed using the gradient density method and the sperm washing method.

Table 1. Clinical and biological data on two reported PCD patients

	Case 1	Case 2
Age of couple	30 (female) and 32 (male)	34 (female) and 32 (male)
Semen analysis	volume: 2.5ml concentration: $14x10^6$/ml vitality 82% and morphology 11.5%	volume: 1ml concentration: $1.5x10^6$/ml vitality 40.5% and morphology 1.5%
Hormonal status (male)	FSH 3.6 IU/l, LH 5.8 IU/l, testosterone 4.7 ug/l	FSH 4.7 IU/l, LH 6.9 IU/l, testosterone 4.5 ug/l
PCD recognised previously	No	Yes
Karyotype	Normal in both partners	Normal in both partners
Genetic testing	No	A pathogenic apparently homozygous CCDC40:c.2440C>T variant was identified in exon 14 of the CCDC40 gene (ref seq NM_017950.3)
Vitality % gradient density method vs. sperm wash method	71% vs 68%	38% vs 35%
Previous IVF attempts	No	Yes (semen donation program in another clinic)
Ovarian stimulation	Clomiphene citrate/HMG/hCG	rFSH/GnRHa/hCG
Fertilisation rate	2 embryos of 2 MII oocytes	9 embryos of 9 MII oocytes (1 zygote was 3PN)
Embryo transfer	Fresh cycle: 2 embryos on day 3	Fresh cycle: „freeze all“ FET: 1 embryo
IVF outcome	live birth at 38+2 weeks, female: length 47cm/weight 2440g	live birth at 40 weeks, male: length 51cm/weight 3700g

Gradient density method: the ejaculate was centrifuged at 80% / 40% density gradient (PureCeption 80% Lower Phase Gradient / PureCeption 40% Upper Phase Gradient, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA) for 15 minutes at 300g, then washed twice with a washing medium (Quinns Advantage® Sperm Washing Medium, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA) for 7 minutes at 600g. The obtained sperm pellet was resuspended in 0.5 ml of sperm washing media.

Washing method: the ejaculate was centrifuged in a sperm wash medium for 10 minutes at 600g twice. The obtained sperm pellet was resuspended in 0.5 ml of sperm washing media.

After the semen samples were processed, they were analyzed using the Eosin vitality test (Figure 1) preformed according to the WHO manual (2010).

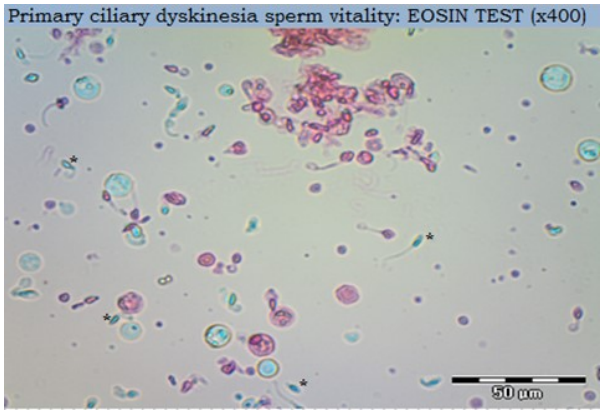

Figure 1. Vitality distinguish by eosin staining
(vital sperm were transparent (*); non-vital sperm were dyed red)

Genetic testing

Patient genomic DNA (gDNA) was extracted from peripheral blood. Whole exome sequencing (WES) was performed in the genetic testing service facility using next generation sequencing (NGS).

IVF/ICSI procedure

Gametes and embryos were cultivated in incubators adjusted to 6% CO_2 and 5% O_2. All culture media were prepared a day earlier; a culture was prepared in drops of oil-covered medium. The equipment used in the procedures was as follows: disposable plastic material for the IVF procedures (Nunc Art IVF Product Line, Thermo Scientific™, USA), 17G needles (Cook Medical, William A. Cook Australia Pity.Ltd., Australia), micromanipulation pipettes (Research Instruments Ltd, UK) and catheters for embryo transfer (ET) (Embryo Transfer Catheter, Cook Medical, USA).

The ejaculate was prepared using the gradient density method as already described above. The obtained sperm pellet was resuspended in 0.2 ml of fertilization medium (Quinns Advantage® Fertilization Medium, SAGE In-vitro Fertilization Inc., CooperSurgical, USA) and placed into an incubator while waiting for insemination. After retrieval from follicular fluid, oocytes were washed in the fertilization medium, granulosa cells were mechanically removed up to corona radiata, after which oocytes were placed into a Petri dish with 40 µl culture medium droplets covered with oil (Oil for Tissue Culture, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA).

After a 3-hour period of pre-incubation, the ICSI procedure was performed. In the Petri dish with the oil-covered stabilized fertilization medium, oocytes were first enzymatically denuded (ICSI Cumulase, Origio, Denmark), washed through a series of 40 µl drops and placed into 10 µl drops for the ICSI procedure. Sperm was placed into one drop of 40 µl fertilization medium. From a semen pool, the sperm with best morphologic features were chosen and placed into a hypoosmotic solution (HOS) prepared according to Sallam et al.[13] Sperm were tested one by one. After the appearance of tail loop (Figure 2), a characteristic trait of sperm vitality, the sperm was transferred into the washing medium as soon as possible to recover. Following that, the sperm was placed into polyvinylpyrrolidone (PVP 7%, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA) and the tail was mechanically drilled prior to the ICSI procedure itself. After 20 hours, fertilization was checked, culture medium was changed, and the culture continued in a sequential medium (Quinns Advantage® Cleavage Medium, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA). After 48 hours another quality grading of embryonic development was done.

For extended cultivation, the medium (Quinns Advantage® Blastocyst Medium, SAGE In-vitro Fertilization, Inc., CooperSurgical, USA) was changed on day 3. Embryonic development was checked and assessed on day 4 and day 5.

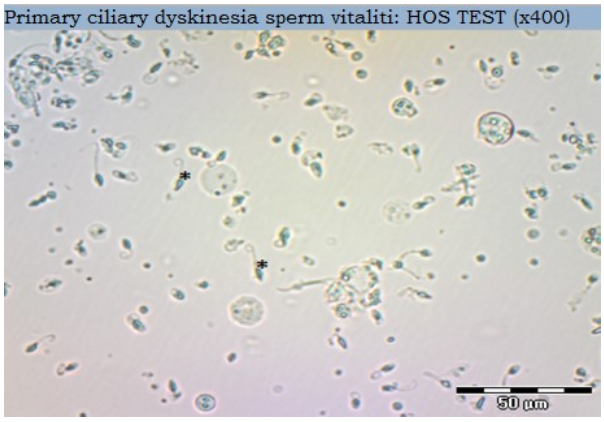

Figure 2. Hypo osmotic swelling test
(vital sperm with tail loop (*))

Vitrification

On day 5, blastocysts of sufficient grading quality, according to Istanbul Consensus (2011), were vitrified (Vitrification Media, Kitazato Corp., Japan) using an

open system carrier (Cryotop, Kitazato Corp., Japan) and cryopreserved in liquid nitrogen, as per the manufacturer's instructions.

Thawing

Thawing of embryos was performed using thawing solutions (Vitrification Media, Kitazato Corp., Japan), as per the manufacturer's instructions. After thawing, embryos were cultured in droplets of blastocyst medium covered with oil, for 2 hours prior to ET.

Embryo transfer

Embryos were put in one well dish with the preincubated medium without oil, and transferred using the embryo transfer catheter.

RESULTS

Clinical data and IVF/ICSI outcome of two PCD patients are presented in Table 1.
Peripheral blood hormone values for both male patients were normal. Karyotyping of peripheral blood lymphocytes has shown a normal male karyotype for both patients. Both patients have suffered from chronic sinus infections, recurrent respiratory infections and infertility, but only in Patient 2 were the symptoms recognized as PCD in childhood. This patient's genetic testing has shown that he carries the homozygous CCDC40:c.2440C>T variant identified in exon 14 of the CCDC40 gene (ref seq NM_017950.3).

Vitality test according to semen preparation method

A comparison between the two different methods of sperm preparation suggested that there was no difference in vital sperm percentage after sperm preparation with the gradient density or the sperm washing method.

IVF procedure and outcome

After the ICSI procedure, fertilization rate in both cases was 100%. In Case 2, one zygote was 3PN.
In Case 1, embryo transfer was performed in a fresh cycle on day 3. One embryo had eight cells, and the other one had two cells (Figure 3).

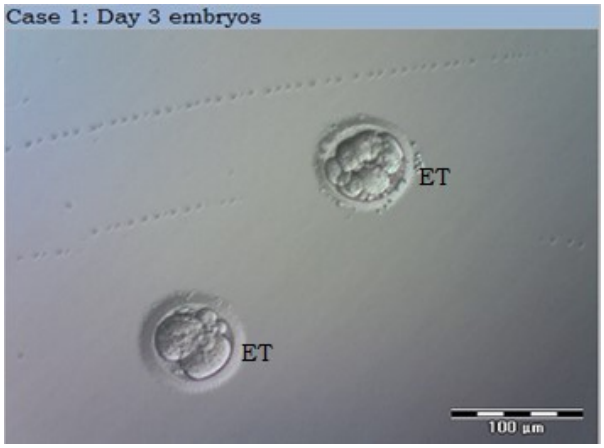

Figure 3. Hypo osmotic swelling test
(ET: transferred embryos)

In Case 2, there were eight embryos on day 3 (Figure 4A). All embryos were frozen in a fresh cycle, and three blastocysts were cryopreserved, each separately, on day 5 (Figure 4B).
In Case 2, pregnancy was achieved from the frozen/thaw cycle (Figure 5).

DISCUSSION

Literature data (Table 2) on PCD patients with exclusively immotile sperm have shown good reproductive prognosis after IVF/ICSI procedures with

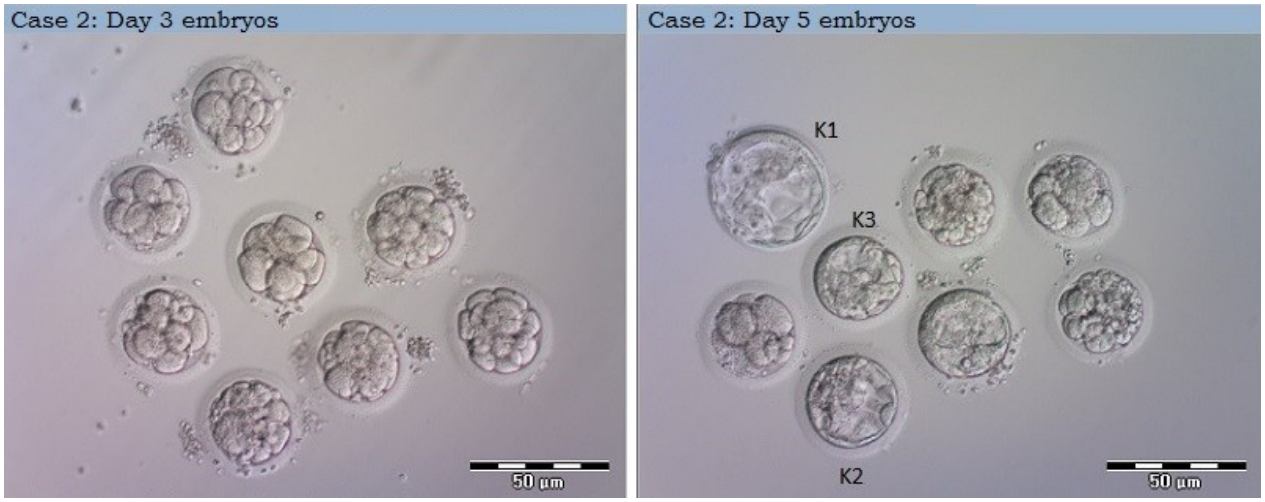

Figure 4. Case 2 embryos on day 3 (A) and day 5 (B) Cryopreserved embryos (K1, K2, K3)

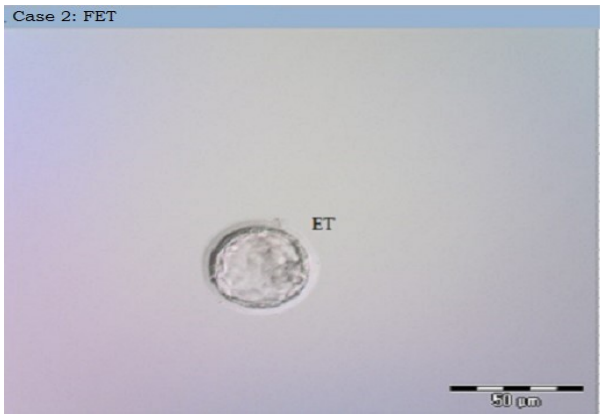

Figure 5. Case 2 embryo from frozen/thaw cycle
(ET: embryo transferred)

ejaculated sperm: fertilization rate was 55%, pregnancy rate 45% and live birth rate 36% [14]. When using HOST, as we described previously, our fertilization and success rate were even higher.

The *CCDC40* (MIM 613799) gene, located on chromosome 17q25, encodes a protein that is necessary for motile cilia function. In their study, Becker-Heck et al[8] showed using TEM analysis that PCD patients with mutated *CCDC40* have cilia with a variety of defects in several axonemal structures. Some of them have absent or eccentric central pairs, displacement of outer doublets and reductions in the mean number of inner dynein arms. It is interesting that outer dynein arms suffered no malformations. Following that, the authors concluded that *CCDC40* might have several functions, such as physical interaction with the other axonemal components, serving as a part of the axoneme structural scaffold, and possibly having a role as dynein regulatory complex component. This gene has 20 exons.[8] Mutations in this gene occur most frequently in exons 3 and 10, but they are also described in most of the other exons and even some introns. A person might have homozygous mutation or compound heterozygous mutations, depending on the case.[7, 25]

Our Patient 2 male patient has been diagnosed with PCD type 15 (MIM 613808), which means he has a mutation in the *CCDC40* gene present in exon 14 in the homozygous form (CCDC40:c.2440C>T variant). This mutation caused protein truncation because Arginine was replaced with a premature stop codon. There are two citations in literature describing this specific mutation. The identical mutation was described in a patient with North European origin.[7] The other one was described in a Yugoslavian patient, but in combination with another mutation in exon 7 on the second allele.[8]

CONCLUSION

Although PCD has diverse etiology, assisted reproduction techniques in combination with HOST give these couples a good chance for parenthood. Advances in testing and strict adherence to procedures advised are to be credited for such outcome improvement. Additionally, our recommendation for PCD patients and patients with immotile sperm is to do a genetic testing and counselling prior IVF/ICSI procedure.

Table 2. Reproductive outcomes following ICSI with completely immotile ejaculated sperm in patients with PCD

Semen quality	Fertilization technique (ICSI)	IVF outcome Fertilisation rate (F); live birth (LB)	Reference
Patient 1: sperm concentration 75x10^6/mL Patient 2: sperm concentration 210x10^6/mL	random sperm pick	F: 66%; LB: twins F: 50%; LB: singleton	[15]
Patient 1: sperm concentration 58x10^6/mL	random sperm pick	no fertilization	[16]
Patient 1: severe oligozoospermia Patient 2: normal sperm count; normal morphology; vitality 63%	HOST 1) 50% ejaculated with HOST 2) 50% TESE with HOST	no fertilization F: 44%; F: 55%; LB: singleton (from TESE)	[17]
Patient 1: sperm concentration 1,4x10^6/mL; morphology 3%	HOST	F: 66%; LB: singleton	[18]
Multiple cases with total immotility (not exclusively PCD)	1) random sperm pick 2) tail laser shot	take home baby rate 16.7% take home baby rate 28%	[19]
Patient 1: sperm concentration 24-40x10^6/mL; morphology 4%; vitality 40%	HOST	F: 83%; LB: twins	[20]
Patient 1: sperm concentration 0,9x10^6/mL; vitality 54%	pentoxifylline-activated sperm	F: 58%; LB: singleton	[21]
Patient 1: sperm concentration 43x10^6/mL	HOST	F: 50%; LB: twins	[22]
Patient 1: sperm concentration 1,8 x10^6/mL; vitality 32%	1) HOST 2) HOST with oocyte activation	no fertilisation F: 66%; LB: twins	[23]
Patient 1: sperm concentration 5x10^6/mL; morphology 0%; vitality 54%	laser assisted viability assessment (LAVA)	F: 45%; LB: triplets	[24]

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