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CASE REPORT

Achievement of pregnancy in globozoospermia with Y chromosome microdeletion after ICSI

Hulusi Bulent Zeyneloglu[1,4], Volkan Baltaci[2], Hakan Eyup Duran[1], Esra Erdemli[3] and Sertac Batioglu[1]

Department of [1]Obstetrics and Gynaecology, Division of Reproductive Medicine, [2]Medical Genetics, Faculty of Medicine, Baskent University Kubilay Sok, 36 Maktepo, Ankara 06570 and [3]Department of Histology and Embryology, Faculty of Medicine, Ankara University, Shihhye Ankara 06100, Turkey

[4]To whom correspondence should be addressed at: Mesa Koru Sitesi Manolya Blok 24, Cayyolu, Ankara, 06530 Turkey. E-mail: hnzeynelog@e-kolay.net

Pregnancy achieved with sperm from a patient with globozoospermia is rare, even after ICSI, since the activation of the oocyte may not occur in this disorder. Therefore, activation of the oocytes by piezoelectricity or calcium ionophores has been suggested, although spontaneous activation of the oocyte after ICSI has been reported in some cases. We report a successful pregnancy in a couple in which the male partner had globozoospermia with microdeletions in the Y chromosome with no further assisted activation after ICSI. During the diagnostic study of the husband, increased numerical chromosome abnormalities after fluorescent in-situ hybridization (FISH) and microdeletions in AZFa; sY86 and AZFb; sY 131 were detected. Out of the 13 oocytes injected, four fertilized and a twin pregnancy was obtained after replacement of four embryos. Healthy twin girls were delivered after a term pregnancy. Some patients with globozoospermia may also have Y chromosome microdeletions, which subsequently may be inherited by the male offspring in cases of achievement of pregnancy.

Key words: globozoospermia/ICSI/infertility/microdeletion

Introduction

Globozoospermia (100% round headed sperm, without acrosomal cap) is an uncommon disorder of sperm morphology associated with severe male infertility. Before the ICSI era, patients with this type of disorder were considered sterile. Major morphological defects described so far with this disorder are the absence of an acrosomal cap and an abnormal perinuclear cytoskeleton. Although the main reason for fertilization failure by IVF is the lack of acrosome reaction to penetrate the zona pellucida, it is still rare to achieve pregnancy even after ICSI. It is yet unclear whether some other defects co-exist, which may have further impact on sperm function and ultimately the fertilization process. Since this entity is universally rare, only single case reports or case-series have been reported. Fertilization and pregnancy rates in these reports have always been low, despite the application of ICSI. The first live birth in a couple with globozoospermia was reported in 1994, by ICSI (Lundin *et al.*, 1994). Some others failed to achieve pregnancy despite having a considerably high fertilization rate (~75%) after oocyte activation with calcium ionophore (Battaglia *et al.*, 1997). In this report, we present a couple with male infertility due to globozoospermia and related diagnostic analyses revealing genetic and structural abnormalities in the sperm, thereby making a contribution to the description of this morphological abnormality.

Case report

A couple with a history of infertility for 9 years due to globozoospermia is presented. The couple underwent an ICSI cycle 2 years ago, when nine oocytes were collected. Three embryos resulted and were transferred; however, pregnancy did not ensue.

The husband was 35 years old at the time of admission and his sperm revealed a count of 45×10^6/ml, 55% motility (progressively motile sperm count of 15×10^6/ml) and 0% morphology using strict criteria, with all sperm being round without an acrosomal cap. The patient's history revealed no abnormalities and his physical examination was also normal.

Karyotyping of the patient was obtained from his peripheral blood culture in a standard protocol (Rooney and Czepulkowski, 1992). G and Q-banding analyses were done at an ~400 band per haploid level. We detected a normal chromosomal constitution (46XY) from the patient.

We performed fluorescent in-situ hybridization (FISH)

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analysis on the sperm and microdeletion analysis on the peripheral blood specimen.

FISH

The sperm samples were obtained by masturbation. The methods of sperm preparation and FISH procedures were described previously elsewhere (Zeyneloglu *et al.*, 2000). Slides were examined using aqua, orange, green and FITC filters of 330–380 nm, 510–560 nm, 450–490 nm in wavelength respectively (Nikon, Tokyo, Japan). Gold filter (Vysis, Downer's Grove, IL, USA) allowed the simultaneous visualization of orange, green, yellow, aqua and blue fluorophores. Images were captured by Applied Imaging System with Cytovision® software (Applied Imaging System International, Newcastle-upon-Tyne, UK). Hybridization efficiency was 95% in both globozoospermic and normal groups of sperm. Autosomal probes (for chromosomes 13, 18, 21 probes) served as internal controls. Only intact cells and cells which did not overlap were scored. Two independent investigators scored slides blindly. In each slide 500 cells were counted, making a total of 1000.

The results of the FISH study revealed 2% numerical abnormalities of the chromosomes 13, 18, 21, X and Y. Meanwhile, a sperm sample from an age-matched fertile donor served as a control, whose results showed 0.8% numerical abnormalities of the corresponding chromosomes. The results of the control were within the normal range (Zeyneloglu *et al.*, 2000).

Microdeletion analysis

Genomic DNA was extracted from 5 ml peripheral blood samples of the patient. Following proteinase-K digestion and phenol/chloroform extraction, precipitation with ethanol was done (Maniatis *et al.*, 1982). Precipitated DNA was suspended in 50 µl TE (Tris-EDTA) and 2 µl of this suspension was used for each amplification.

Polymerase chain reaction (PCR) was used in order to screen for microdeletions in the AZF region of the Y chromosome. The genomic DNA was analysed for the presence of six sequence tagged sites (STS), namely the AZFa, AZFb and AZFc regions respectively (Vogt *et al.*, 1996). However, it should be kept in mind that there is still no consensus on the selection of the sites to analyse for microdeletions in the AZF regions.

The STS probes used were sY86 and DYS273 (AZFa), sY131 and DYS218 (AZFb) (Reijo *et al.*, 1995; Vogt *et al.*, 1996), sY254 and sY255 (AZFc) (Reijo *et al.*, 1995) and DYS14 (positive control). Products of PCR were analysed on a 2% agarose gel. We only used PCR assays that gave a reliable result in the control sample and we did not record a deletion from a patient unless at least two successive PCR amplifications of two blood samples taken separately yielded negative results.

PCR analysis showed failure of amplification of two STS: sY86 (in AZFa) and sY131 (in AZFb), whereas the other four STS, including the control, were amplified (Figure 1).

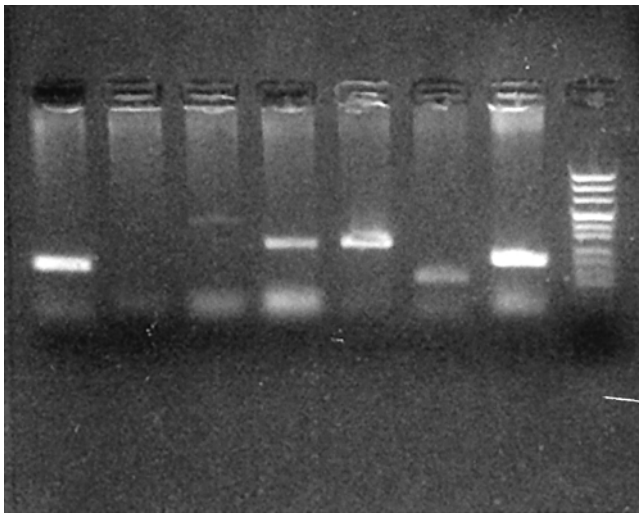

Figure 1. Polymerase chain reaction (PCR) products analysed on the 2% agarose gel electrophoresis show deletion of two different sequence tagged sites, in the AZFa; sY86 and AZFb; sY131 in columns 2 and 3 respectively. The columns (1–8) from left to right respectively: DYS 14 (+ control), 198 bp; sY86 (deleted), 320 bp; sY131 (deleted), 143 bp; SY 254 (normal), 300 bp; DYS 273 (normal), 326 bp; SY 255 (normal), 170 bp; DYS 218 (normal), 274bp; marker 100 bp.

Controlled ovarian stimulation, ICSI and embryo transfer procedure

After utilizing a long luteal down-regulation with leuprolide acetate (Lucrin™; Abbott, Istanbul, Turkey) of 1 mg/day, the wife was induced for controlled ovarian stimulation using recombinant FSH (Gonal-F™; Serono, Istanbul, Turkey) 300 IU/day starting on the third day of the menses. After 11 days of ovarian stimulation, when two follicles were ⩾18 mm, eight follicles were ⩾14 mm and six follicles were between 12 and 14 mm, HCG of 10 000 IU (Profasi™; Serono) was given. Fourteen oocytes were retrieved by transvaginal follicle aspiration under ultrasonographic monitoring, 36 h after HCG injection. All but one were MII oocytes, and were processed for ICSI. Since all sperm were globozoospermic, those appearing to have the best motility were selected for injection. Next day, four two-pronuclear oocytes were obtained (fertilization rate = 31%). At the time of transfer, which was 72 h after the oocyte retrieval, three embryos with six, six and five cells respectively, and one compact embryo with cell numbers more than 12 (morula stage), were observed. These four embryos were placed into the uterine cavity. A transfer of four rather than three embryos was preferred, as the couple had a previous IVF failure and low fertilization rate. Besides, the regulations in our country do not limit the number of transferred embryos to three. Luteal phase was supported by vaginal suppositories of micronized progesterone (Progestan™; Kocak, Istanbul, Turkey). The β-HCG value was 173 mIU/ml 12 days after embryo transfer. At 7 weeks since last period of menstruation, the transvaginal ultrasonography revealed two embryos with cardiac activity, in two separate gestational sacs. Targeting ultrasonography at 16 weeks showed two normal female fetuses. The couple refused to undergo genetic amniocentesis. The pregnancy was uneventful and followed by delivery of two female newborns weighing 3100 and 2900 g by Caesarean section at the 39th gestational week. Post-natal cytogenetic analysis of the newborns manifested normal chromosomal constitution (46,XX).

Discussion

This report confirms the previous reports of pregnancy in couples with globozoospermia without use of any methods to activate oocytes (Lundin *et al.*, 1994; Liu *et al.*, 1995; Trokoudes *et al.*, 1995; Kilani *et al.*, 1998; Stone *et al.*, 2000). Lundin *et al*. were the first to publish the occurrence of a twin pregnancy in a couple with globozoospermia at the second ICSI cycle, in which only 43% fertilization could be achieved (Lundin *et al.*, 1994). On the other hand, calcium chloride and ionophore were also used for ICSI to achieve fertilization and pregnancies in couples with a similar condition (Battaglia *et al.*, 1997; Rybouchkin *et al.*, 1997; Kim *et al.*, 2001). The common features of all these reports including the present one are: (i) globozoospermia with complete lack of acrosome; (ii) utilization of ICSI; and (iii) low fertilization rate. In addition, it appears that cleavage rates are lower than expected in some cases, despite the 100% cleavage rate after a fertilization rate of 31% in our case. Neither the aetiology of the globozoospermia nor the co-occurrence of other defects in addition to the absence of an acrosome are understood.

Since the first description of globozoospermia in 1971, many aspects of this rare syndrome have been described. Such sperm were reported to undergo nuclear decondensation when incubated with crushed hamster ovaries (Syms *et al.*, 1984). Decondensation and pronuclear formation was observed after direct injection of the globozoospermia into hamster oocytes (Lanzendorf *et al.*, 1988). On the other hand, a case report described that unfertilized oocytes of a couple with globozoospermia after ICSI had haploid chromosome sets, and most of these oocytes contained premature chromatin condensation of the sperm (Edirisinghe *et al.*, 1998). Premature condensation of the sperm occurs if the oocyte cannot be activated and remains arrested at metaphase II after the sperm nucleus is introduced into the oocyte with ICSI and is able to act with chromatin condensing factors (Schmiady *et al.*, 1996). Rybouchkin *et al.* also reported deficient oocyte activation capacity in globozoospermia, possibly secondary to the absence or down-regulation of the sperm associated oocyte-activating factor (Rybouchkin *et al.*, 1997). All the evidence indicates that a partial or total deficiency of oocyte-activating capacity of the sperm may be present in this disorder, although with individual variation.

Despite the suggestion of a familial linkage, the mode of the inheritance of globozoospermia is still unclear (Stone *et al.*, 2000). The mode of inheritance has been proposed as autosomal recessive; autosomal dominant; sex-restricted dominant/X linked (Stanislavov and Ganev, 1998); or polygenic (Carrell *et al.*, 1999). Carrell *et al.* have reported that parameters of basic semen analyses of two brothers with globozoospermia showed profound variations (Carrell *et al.*, 1999). One brother had 100% complete absence of acrosome, whereas the other had up to 4% acrosome-bearing sperm in the samples. Both brothers had increased chromatin decondensation after in-vitro exposure to heparin as compared with controls. However, aneuploidy rates were significantly increased in sibling 1 compared with sibling 2 in terms of both sex and 13th and 21st chromosomes (Carrell *et al.*, 1999). Recently, Viville *et al.* reported comparable aneuploidy rates (Viville *et al.*, 2000). FISH analysis of the semen sample of this case patient showed an increased aneuploidy rate when compared with the control, supporting the findings of others (Carrell *et al.*, 1999).

Three distinct regions, termed as azoospermia factor, AZF (AZFa, AZFb, AZFc), in proximal through distal Yq are required for normal spermatogenesis (Tiepolo and Zuffardi, 1976; Vogt *et al.*, 1996; Vogt *et al.*, 1997). The incidence of microdeletions is 15–20% in azoospermic men, falling to 7–10% in oligospermic men (Vogt *et al.*, 1996). Microdeletions of AZFa, AZFb and AZFc have been associated with Sertoli-cell only syndrome, spermatogenic arrest, and a variable phenotype respectively (Vogt *et al.*, 1996). The type of deletion has been suggested as a prognostic factor for sperm retrieval in men. Deletions including the AZFc region are associated with total absence of sperm (Silber *et al.*, 1998), although mature sperm have been found in 50% of azoospermic patients with AZFc deletions (Mulhall *et al.*, 1997). The presence of an AZFb deletion is a significantly adverse prognostic finding for testicular sperm extraction. The extent of the deletions is also important, e.g. complete deletion of a region (sY113-sY143) reflects spermatogenic arrest (Vogt *et al.*, 1996). Partial AZFb deletions have been associated with oligospermia. Progressive decrease of sperm number over several months has been described in some men with AZFc deletions (Girardi *et al.*, 1997). The AZF deletions are detected by the PCR screening of the STS. Although the frequency of microdeletions found is independent of the number of STS used, the higher number of STS would protect against inaccuracy (Simoni *et al.*, 1999). However, it has been suggested (Van Landuyt *et al.*, 2000) that only four STS markers representing the three AZF regions and a more distal region in AZFc might be sufficient to detect most Yq deletions.

The presence of Y chromosome microdeletions cannot be predicted on the basis of clinical findings or even the results of the semen analysis; moreover, there is no clear association between the localization of the deletions (AZF regions a, b and, c) and the clinical manifestations (Pryor *et al.*, 1997; Foresta *et al.*, 2001). Moreover, the patients who have microdeletions in the same region may have variations in terms of both spermiogram and clinical findings (Mulhall *et al.*, 1997). Microdeletions in the AZF region are not limited to azoospermic men, these deletions have also been detected in men with severe oligospermia. In addition, the phenotypic variations of the microdeletions may result from mosaicism of the Y-chromosome microdeletion which may remain undetected by the PCR analysis (Reijo *et al.*, 1996); oligozoospermic men without microdeletions (tested by PCR analysis) fathered 'ICSI sons' with microdeletions (Kent-First *et al.*, 1996).

The presence of a microdeletion in the globozoospermic patients has not previously been described in the literature, probably due to the rarity of this condition. Van Landuyt *et al*. described eight patients with microdeletion who had obtainable sperm in the ejaculates; one patient had microdeletion in AZFc region, severe oligozoospermia and abnormal morphology in

the semen analysis: small and absent acrosomes and amorphous heads (Van Landuyt *et al.*, 2000), indicating that microdeletions may co-exist with head anomalies.

Increased FISH abnormalities of the sperm and genetic linkage may imply a genetic mechanism for globozoospermia which is yet to be defined. One such mechanism has recently been described in mice, where a single gene defect caused sterility and globozoospermia (Kang-Decker *et al.*, 2001). Male mice lacking the Hrb gene (also called Rab or hRip), which encodes a protein that plays role in vesicle trafficking, docking and fusion, produced sperm without acrosome, which were incapable of zona attachment and fertilization. Whether a similar pathophysiology is present in men with globozoospermia is not known. In order to explain genetic mechanisms of the globozoospermia, the presence of polymorphism or other gene mutations yet unknown must be described. While likelihood of polymorphism cannot be excluded in this case report where two microdeletion sites have been detected, any of two microdeletion sites, if not both, may be related to the globozoospermia.

This report is the first to show microdeletions in a man with globozoospermia in AZFa and AZFb regions of the Y chromosome. Although it is difficult to reach a conclusion on the basis of just one case with globozoospermia, which is in itself a very heterogeneous disorder, further studies are required to confirm any possible associations between microdeletions and globozoospermia.

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