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CASE REPORT

Cytogenetic analysis of unfertilized oocytes following intracytoplasmic sperm injection using spermatozoa from a globozoospermic man

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A man with globozoospermia was treated in our in-vitro fertilization–intracytoplasmic sperm injection (ICSI) programme. In the treatment cycle, 24 oocytes were collected from his wife. All the oocytes were at metaphase II stage. The semen sample produced on the day had a normal sperm count, good motility, but with 100% globozoospermia. All oocytes were injected with randomly selected spermatozoa and of these, two oocytes showed two pronuclei and another contained a single pronucleus. The remainder were unfertilized. The normally fertilized oocytes (two pronuclear) cleaved to the four-cell stage and were transferred to the patient. At 48 h after ICSI, the 21 unfertilized oocytes were processed for cytogenetic analysis. All oocytes contained a haploid chromosome set. The only abnormality seen was a chromosome fragment in one metaphase. Eighteen oocytes contained decondensed sperm nuclei and of these, 14 nuclei were beginning to show signs of premature chromatin condensation (PCC) and the other four showed strong signs of PCC. Thus it appears that in some forms of globozoospermia, arrest of nuclear decondensation and/or PCC are another cause of fertilization failure. The most likely cause for this is the absence or down-regulation of spermatozoa associated activating factor in round-headed spermatozoa.

Key words: globozoospermia/ICSI/unfertilized oocytes

Introduction

With the introduction of intracytoplasmic sperm injection (ICSI), the majority of male factor infertility cases can be treated (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993). Pregnancies have been reported even using spermatozoa from men with a rare condition called globozoospermia (Liu *et al.*, 1995; Trokoudes *et al.*, 1995). In this inherited condition, the spermatozoa show severe gross and ultrastructural abnormalities, including the complete lack of acrosome, abnormal nuclear membrane and mid-piece defects. Due to the severity of the defects, the spermatozoa exhibit no binding to the zona pellucida (Aitken *et al.*, 1990) or fusion with the oolemma, causing failures in IVF and in subzonal sperm injection (Dale *et al.*, 1994). Even though fertilization has been achieved with globozoospermia using direct injection of spermatozoa into the ooplasm in ICSI, fertilization rates still remain poor. Furthermore, Liu *et al.* (1995) showed that four out of the seven men treated in their investigation had total fertilization failure.

In order to gain further knowledge on globozoospermia and the behaviour of these spermatozoa following injection into oocytes, we have studied the cytogenetics of failed ICSI oocytes obtained from a couple whose infertility was due to globozoospermia.

Case report

A 35 year old man and his 30 year old wife were referred to our clinic for infertility. The couple had had several treatment attempts at other in-vitro fertilization (IVF) centres and the diagnosis for the cause of infertility was determined as the 'round-headed' spermatozoa or the globozoospermia. At genetic counselling, it was revealed that his brother suffered from the same condition and that his parents were first cousins, which raised the possibility that this condition is an autosomal recessive disorder. There was no other feature in the family history to suggest that the globozoospermia was associated with any other abnormality. Karyotypes of both partners were normal.

Medical examination of the husband revealed normal findings, except that the prostate gland was found to be small. Semen analysis indicated normal count and motility, with morphology showing 100% round-headed spermatozoa. The wife had had an elective termination at 8 weeks of pregnancy in her previous marriage. Her laparoscopy and dye injection revealed that the left oviduct was blocked. The ultrasound findings of the pelvis were normal. For the last 5 years her menstrual cycles had been irregular, lasting 28–60 days. Previously they had been normal. She had gained some 27 kg in weight over the past 5 years and developed galactorrhoea approximately 3 years ago. There was no evidence of hypothyroidism. Her physical examination findings were normal with no sign of breast discharge, but she was overweight (107.5 kg).

The couple consented to an IVF–ICSI attempt. Successful follicular growth was achieved with a Lucrin (Leuprolide acetate; Abbott, Sydney, Australia) flare regimen in combination with three ampoules per day of follicle stimulating hormone (Metrodin; Serono, Sydney, Australia). A trigger injection of 10 000 IU human chorionic gonadotrophin (HCG, Pregnyl; Serono) was administered and after 35 h, transvaginal

oocyte aspiration was performed. Twenty-four mature oocytes were collected.

The semen sample produced on the day gave a total sperm count of 31×10^6/ml and a motile count of 14×10^6/ml, with 70% of the spermatozoa showing linear progression. The motile sperm fraction for the ICSI procedure was isolated using a 95:47.5% Percoll gradient. Following two washes in bicarbonate buffered human tubal fluid medium (HTFM) containing 10% heat inactivated patient's serum, the sperm count was adjusted and placed in culture droplets of HEPES-buffered HTFM just before the ICSI procedure.

Five hours after collection, the oocytes were placed in hyaluronidase solution (80 IU/ml) in order to remove the cumulus cells, washed and assessed for maturity. All 24 oocytes were at the metaphase II stage and were injected with randomly selected spermatozoa. The ICSI procedure was performed as described previously (Edirisinghe *et al.*, 1997a). The micromanipulation set-up consisted of a Nikon diaphot inverted microscope (Tokyo, Japan) with left and right Narishige micromanipulators (Model MN-108 and MO-108, Tokyo, Japan). The spermatozoa were immobilized by touching the sperm tails with the injection needle at right angles at the bottom of the dish without placing them in PVP.

The oocytes were examined for fertilization 18–20 h post-ICSI. Two pronuclei were visible in two oocytes and one oocyte contained only one pronucleus. On the following day, two good quality four-cell embryos scoring 2/4 and 3/4 (scored on a scale of 0–4, 4 being the best quality) were transferred into the uterus. The one-pronuclear oocyte remained uncleaved. The patient did not achieve a pregnancy in this attempt.

All 21 unfertilized oocytes were processed as described previously (Edirisinghe *et al.*, 1997b) for chromosomal analysis 48 h after ICSI. In brief, the oocytes were exposed to a hypotonic solution (1% sodium citrate) for 10 min and fixed onto glass microscope slides using a fixative consisting of 3 parts methanol:1 part glacial acetic acid. The slides were stained in 10% giemsa solution and examined under an oil immersion objective at $\times1000$ magnification.

All oocytes contained a haploid set of chromosomes. Nine metaphases were accurately countable (42.9%) and the only abnormality detected was a small chromosome fragment in one metaphase. Eighteen oocytes (85.7%) contained decondensed sperm nuclei and of these 14 appeared to show signs of premature chromatin condensation (PCC) and the other four showed strong signs of PCC. The three remaining oocytes did not appear to contain any part of a sperm head in the cytoplasm. It was observed that out of the 18 sperm nuclei, 12 were away from the oocyte chromosomes, two were near and two were amongst the oocyte chromosomes. Figure 1 shows, within the ooplasm, decondensed sperm heads which appear 'lumpy' as though PCC is beginning to occur. The sperm heads have indistinct edges with fine strands of what appear to be G1 chromosomes extending out. The location of the sperm nucleus relative to the oocyte chromosomes was either distant (Figure 1a), very close (Figure 1b) or amongst the oocyte chromosomes (Figure 1c).

As a control, the results obtained with round headed spermatozoa were compared with the cytogenetic data obtained from a control group of 50 oocytes which failed to fertilize in the ICSI programme after injection with normal ejaculated spermatozoa. In both groups, the sperm samples were prepared in an identical manner using Percoll gradient separation.

Of the 50 unfertilized control oocytes used from 14 patients, 28 were analysable and 17 (60.7%) had a single haploid set of chromosomes. The remaining 11 analysable oocytes all showed abnormalities. Five (17.9%) oocyte metaphases showed hypohaploidy, two (7.1%) were diploid and seven (25%) had one or more chromosome fragments. Sperm nuclei were found in 19/28, (67.9%) of the unfertilized oocytes and these were condensed (15.8%), decondensed (52.6%), metaphase stage (10.5%) or showed evidence of PCC (21.1%). Of the four sperm nuclei with PCC, one was a condensed chromatin mass with a partly decondensed region at the periphery similar to that of the 14 nuclei observed in the globozoospermia study. The other three PCC showed either decondensed chromatin with single stranded chromatids extending out ($n = 2$) or pulverization ($n = 1$). None of the sperm nuclei was found near the oocyte chromosomes.

Discussion

In this globozoospermia case, we achieved a fertilization rate of 8.3% with ICSI where 100% of the spermatozoa used for injection showed round-headed sperm morphology. It is evident from the limited number of globozoospermic patients treated in ICSI programmes, that the fertilization rate achieved is variable (Liu *et al.*, 1995; Trokoudes *et al.*, 1995) and that it is much lower than for any other abnormal sperm group, i.e. severe oligozoospermia, severe asthenozoospermia and other abnormalities (Van Steirteghem *et al.*, 1993). Total fertilization failure has been observed in some cases (Liu *et al.*, 1995). The variability in fertilization rates may result from the varying percentages of spermatozoa with globozoospermia seen in the ejaculate of different men with the problem (Syms *et al.*, 1984). Thus careful selection of spermatozoa at ICSI, especially selecting for the acrosome-carrying spermatozoa may improve fertilization rates for globozoospermic men (Al-Hasani *et al.*, 1996).

The haploid chromosomal complement in all the oocytes, except for a small chromosomal fragment seen in one metaphase, appeared intact and undisturbed. In our previous cytogenetic study of unfertilized ICSI oocytes, 37.3% of the oocytes showed some form of abnormality (Edirisinghe *et al.*, 1997b). The very low incidence of chromosomal abnormality seen in the present case may be due to: (i) the patient variation as we have observed in our previous study (Edirisinghe *et al.*, 1997b), (ii) improved ICSI technique, as no oocyte damage was seen with 86% of oocytes showing spermatozoa in the ooplasm, or (iii) the lack of possible adverse factors released due to the failure to complete sperm decondensation. It is of value to assess whether the majority of abnormalities normally seen in the oocyte chromosomal complement, especially the structural defects in unfertilized oocytes, are associated with sperm decondensation process and/or formation of metaphase chromosomes.

The appearance of the sperm heads in these oocytes may

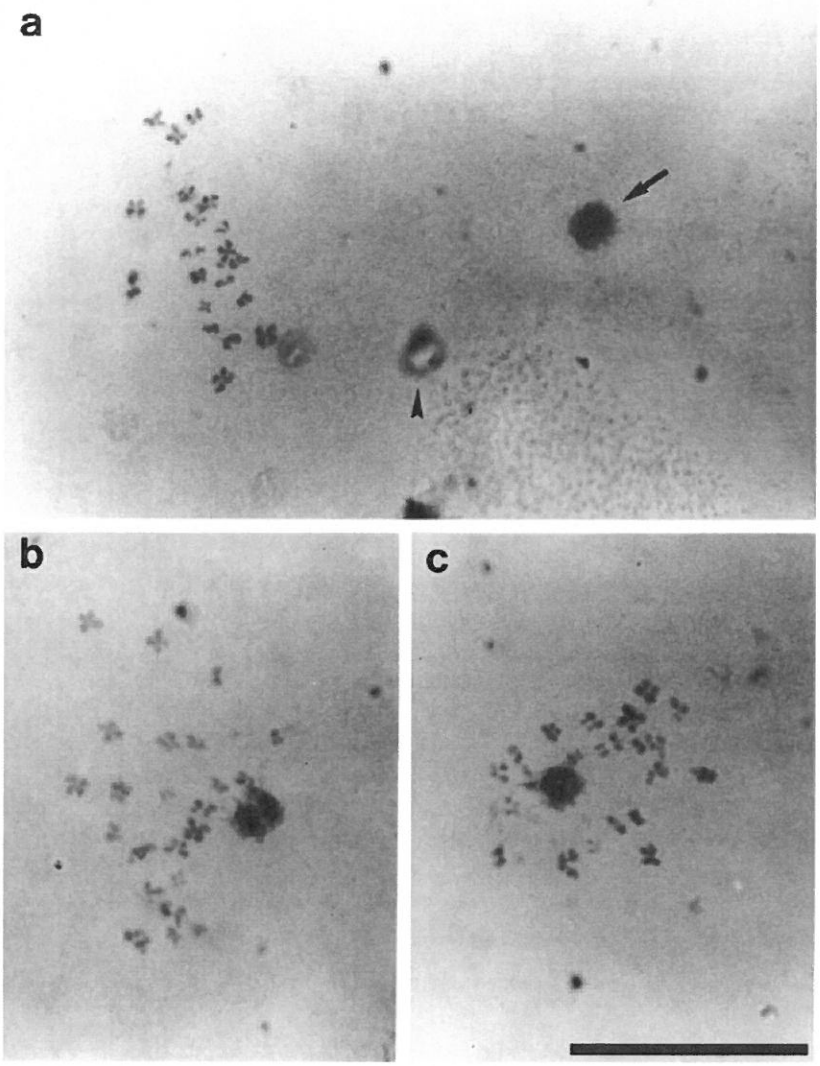

Figure 1. The intact haploid chromosome set of the oocyte and the decondensed sperm head, showing signs of premature chromosome condensation (PCC) (**a**) The sperm nucleus (arrow) appears 'lumpy', as though PCC is beginning to occur which lies away from the intact oocyte chromosomes. The indistinct ring between the sperm head and the oocyte chromosomes (arrowhead) is a non-staining inclusion in the cytoplasm with an accumulation of stain around it. (**b**) Sperm nucleus showing indistinct edges with fine strands of chromosomes (probably G1 chromosomes) extending out from it and lying very close to the oocyte chromosomes. (**c**) The sperm nucleus showing signs of PCC amongst the oocyte chromosomes. Bar = 10 μm.

be due to swelling and incomplete breakdown of nuclear membrane due to structural defects, as observed in the majority of spermatozoa in globozoospermia (Dale *et al.*, 1994). Ultrastructural investigations of round-headed spermatozoa show a lack of acrosome and defects in various membranes, the mid piece and the nucleus (Pederson and Rebbe, 1974). Nuclei of the majority of round-headed spermatozoa appear granular, suggesting disturbances in the nuclear condensation process (Lalonde *et al.*, 1988). An electrophoretic analysis of nuclear proteins has shown that round-headed spermatozoa contain more histones and intermediate proteins and fewer protamines than normal spermatozoa (Blanchard *et al.*, 1990). This finding further supports the view that round-headed spermatozoa have chromatin packaging defects. Poor chromatin packaging and/or damaged DNA may contribute to the failure of sperm decondensation after ICSI and thus failure of fertilization (Sakkas *et al.*, 1996). However, earlier work has shown normal nuclear chromatin decondensation following exposure of round-headed spermatozoa to appropriate chemicals *in vitro* (Lalonde *et al.*, 1988) or crushed hamster ova (Syms *et al.*, 1984). It was suggested, following these findings, that the spermatozoa from globozoospermia could proceed normally with fertilization once the barrier for sperm penetration is overcome (Syms *et al.*, 1984) as in ICSI. However, in the present case study, while all the spermatozoa from the globozoospermic sample decondensed after ICSI, the majority of oocytes injected with them (except the two normally fertilized and one with one pronucleus) were not activated and did not complete the second meiotic division. Golan *et al.* (1997) have recently reported a method for evaluation of chromatin

condensation in human spermatozoa, and it could be instructive to use this method to assess further the state of condensation of round-headed spermatozoa.

Another possible explanation for the fertilization failure could be the ageing of oocytes *in vitro* prior to ICSI which may lead to chromosomal aberrations (Macas *et al.*, 1996) or disturbances of the second meiotic spindle (Eichenlaub-Ritter *et al.*, 1988). However, this possibility is an unlikely explanation in the present case study as the oocytes were injected 4–6 h after oocyte collection and the majority of the metaphases showed intact haploid sets of chromosomes even at the time of analysis, 48 h after ICSI. Furthermore, in the unfertilized oocytes from the globozoospermia case, the sperm nuclei were observed near or among the oocyte chromosomes and a similar observation has been made for some oocytes following ICSI by others (Dozortsev *et al.*, 1994; Flaherty *et al.*, 1995a,b). This finding indicates the possible damage to the second meiotic spindle due to the injection of spermatozoa into the spindle region that could arrest the oocyte at metaphase II. Every precaution is taken to avoid the spindle region by placing the polar body either at the12 o'clock or 6 o'clock position and piercing the oocyte with the ICSI needle at the 3 o'clock position for sperm injection. However, it is possible for the polar body to be dislodged and move elsewhere when manipulating oocytes during hyaluronidase treatment (Flaherty *et al.*, 1995a,b). This may give a false indication of the location of the spindle. As none of the control oocytes showed the presence of sperm nuclei close to the spindle in the present study, it is unlikely that the spermatozoa were injected into the spindle region of six oocytes of the globozoospermia case. In some oocytes, the sperm nuclei or oocyte chromosomes could have moved subsequent to ICSI within the cytoplasm. No structures of the sperm head were visible in three oocytes, probably as a result of failed injection or ejection of the spermatozoa through the injection hole into the perivitelline space (Flaherty *et al.*, 1995a,b). The ejected spermatozoa are usually lost during the fixation of oocytes for analysis.

The most distinctive finding in this investigation was the very high rate of G1-PCC in the sperm nuclei when compared to unfertilized ICSI ova injected with normal spermatozoa. The PCC observed in this globozoospermia case is not the typical G1-PCC and may represent the early stages of this phenomenon or degenerative changes of the decondensed or swollen sperm nucleus (Dozortsev *et al.*, 1994). The arrest of sperm heads at a decondensed and early PCC stage may be associated with some degree of oocyte immaturity (Calefell *et al.*, 1991), where the presence of oocyte chromosome condensation factors may cause the sperm nucleus to undergo chromatin condensation prematurely (Schmiady and Kentenich, 1989). However, it is unlikely that all the oocytes collected from the wife could be immature, as they were all at metaphase II and the ICSI was performed 4–6 h after the oocyte recovery which allowed adequate time for complete cytoplasmic maturation to occur. Recently, Schmiady *et al.* (1996) studied the incidence of PCC in oocytes that remained unfertilized after ICSI. Their findings indicated a high incidence of PCC in failed ICSI oocytes (~28%) compared to the failed IVF oocytes (~10%). The sperm nuclei at G1-PCC showed a remarkable variation in the degree of condensation where degree I (highly condensed) and II (slightly condensed) were more common than degree III (decondensed) following ICSI. The G1-PCC nuclear condensation patterns observed for the round-headed spermatozoa in the present study are comparable to the degrees I and II described by Schmiady *et al.* (1996) for normal spermatozoa. One sperm nucleus in the control group showed a similar appearance, and sperm nuclei exhibiting such G1-PCC have also been observed in failed ICSI oocytes by other researchers (Dozortsev *et al.*, 1994; Flaherty *et al.*, 1995a,b). The reason for the high incidence of degrees I and II of sperm nuclear condensation following ICSI may be due to the degree of sperm membrane damage caused during the sperm immobilization, as discussed by Schmiady *et al.* (1996). On this basis, the extreme level of PCC observed in the present case suggests that round headed spermatozoa may be more susceptible to this form of damage than normal spermatozoa.

It has been suggested that with globozoospermia, oocyte-independent swelling of the sperm head occurs normally and that the oocyte activation dependent chromatin decondensation does not occur due to the lack of release of oocyte-activating sperm factor (Dozortsev *et al.*, 1995a,b). Recent work showed that round-headed spermatozoa failed to activate mouse oocytes following ICSI (Rybouchkin *et al.*, 1996), and it was concluded that these spermatozoa are deficient in the oocyte activation, either due to the absence or down-regulation of the spermatozoa associated oocyte activating factor. Failure of activation and the consequent failure of the oocyte to complete meiosis II is likely to contribute to the high rate of PCC seen in the present case.

Investigation of the cytogenetics of failed fertilization in other cases of globozoospermia are needed to determine whether the high rate of PCC is generally related to globospermia or was specific to this case. If so, then further studies using globozoospermia may help to clarify the molecular events associated with sperm nuclear decondensation and fertilization following ICSI.

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