Reduced oocyte activation and first cleavage rate after ICSI with spermatozoa from a sterile mouse chromosome mutant

E.B.Baart[1,5], G.W.van der Heijden[2], F.A.van der Hoeven[1,3], R.Bakker[3], T.G.Cooper[4] and P.de Boer[1,6]

[1]Laboratory of Genetics, Wageningen Institute of Animal Sciences, Wageningen University, 6701BH Wageningen, The Netherlands, [2]Department of Obstetrics and Gynaecology, University Medical Centre St Radboud, 6500HB Nijmegen, The Netherlands, [3]Experimental Animal Centre, 6703HD Wageningen University, The Netherlands and [4]Institute of Reproductive Medicine of the University of Münster, D-48129 Münster, Germany. [5]Current address: Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, Erasmus Medical Center, 3015 GD Rotterdam, The Netherlands. [6]Current address: Department of Obstetrics and Gynaecology, University Medical Centre St Radboud, 6500HB Nijmegen, The Netherlands. To whom correspondence should be addressed at: Department of Obstetrics and Gynaecology, UMCN St Radboud, 415 OBGYN, PO Box 9101, 6500HB Nijmegen, The Netherlands. E-mail: P.deBoer@Obgyn.UMCN.nl

BACKGROUND: Male mice, heterozygous for two semi-identical reciprocal translocations *T(1;13)70H* and *T(1;13)1Wa* are usually sterile. We have investigated this oligoasthenoteratozoospermic mouse model using ICSI. METHODS: B6D2F1 oocytes were injected with epididymal or testicular sperm from fertile or sterile translocation carriers and from chromosomally normal fertile controls. ICSI efficiency was determined by pronucleus formation and first cleavage rates. For arrested zygotes, cell cycle progression was evaluated by BrdU incorporation and incubation with okadaic acid. RESULTS: Epididymal sperm from infertile translocation carriers showed a slightly lower fertilization rate (70% vs. 92%, 95% and 95% for fertile translocation carriers and two groups of normal fertile control males, respectively) and a severely reduced cleavage rate (33% vs. 87%, 96% and 89% for the same control groups). However, the use of testicular sperm significantly improved the cleavage rate (62% vs. 83% for normal fertile controls). Development of arrested zygotes was delayed or blocked during S- and G2-phase. CONCLUSIONS: Whereas control testicular and epididymal sperm performed equally well, the use of testicular sperm from oligospermic *T/T′* males significantly increased first cleavage rates when compared to the low rates with epididymal sperm. Epididymal storage in oligospermics may negatively influence zygote division.

Key words: DNA damage/ICSI/male sterility/mouse/zygote

Introduction

Intracytoplasmic sperm injection (ICSI) has now been established as the method of choice to overcome male infertility in IVF clinics around the world. ICSI results in high fertilization and pregnancy rates when sperm from men with severe oligospermia is used (Van Steirteghem *et al.*, 1993; Nagy *et al.*, 1995). Nevertheless, concerns regarding the risks of the procedure for the offspring remain, as both mutation rates for numerical and structural chromosome abnormalities are increased compared to the general population (Bonduelle *et al.*, 2002).

Classically, the assessment of human sperm is carried out by WHO criteria for concentration, motility and morphology (WHO, 1999). When using sperm for ICSI, the concentration is low and there are strong indications that motility and morphology are not reliable criteria for the absence of DNA damage (human: Lopes *et al.*, 1998; Twigg *et al.*, 1998; mouse: Ahmadi

and Ng, 1999). If DNA damage is present in the spermatozoon, this may lead to *de novo* mutations and structural chromosome abnormalities in the developing embryo. Cytogenetic proof of this principle in human sperm at first cleavage division in heterologous fertilization systems has been obtained in a number of studies (Martin *et al.*, 1994; Lee *et al.*, 1996; Rybouchkin, 1997). For studying the genesis of chromosome mutations from sperm of men with oligoasthenoteratozoospermia (OAT), mouse models are needed.

To this purpose, we have investigated whether mice with chromosomal male sterility and an OAT phenotype produce sperm suitable for ICSI. The majority of males heterozygous for the two semi-identical reciprocal translocations *T(1;13)70H* and *T(1;13)1Wa*, are sterile (*T/T′*, De Boer *et al.*, 1986; Peters *et al.*, 1997). During first meiotic prophase in primary spermatocytes at the time of homologous chromosome pairing, two heteromorphic bivalents (a large one between the

13^{1}H, 13^{1}Wa and a small one between the 1^{13}H, 1^{13}Wa translocation chromosomes) are produced by synaptonemal complex formation. Subsequently, in the large 13^1 bivalent, an adaptive structurally normal synaptonemal complex is always formed, not revealing the interruption in homology. In the majority of primary spermatocytes of around 80% of *T/T′* males, this process fails for the small 1^{13} heteromorphic bivalent. In these infertile males, spermatocyte death commences during the second half of pachytene with a peak at first meiotic division, as described for other sterile reciprocal translocation heterozygotes (De Boer *et al*., 1986). Thereafter, spermiogenesis results in additional cell death and a large variation in sperm morphology and motility.

When synaptic adaptation occurs for the small 1^{13} heteromorphic bivalent as well, and a structurally normal synaptonemal complex is formed in over half of the pachytene spermatocytes, fertility is restored (De Boer *et al*., 1986; Peters *et al*., 1997). This is in keeping with the notion that for these spermatocytes, meiotic cell cycle checkpoints no longer result into apoptosis (Odorisio *et al*., 1998; De Vries *et al*., 1999). The obstructive effect of aberrant synaptic behaviour of unspecified chromosomes and chromosome segments during first meiotic prophase on gametogenesis, especially spermatogenesis, has been thoroughly documented in both man (Speed, 1989) and mouse (De Boer and De Jong, 1989).

We have used both epididymal and testicular spermatozoa from these infertile males for injection into mouse oocytes and compared fertilization rates by pronucleus formation and rates of cleavage to the two-cell stage to those of fertile controls carrying the same genetic background with or without the translocations.

Materials and methods

Reagents and media

All inorganic and organic reagents were purchased from Sigma-Aldrich Chemie Gmbh. (Steinheim, Germany), unless otherwise stated.

For oocyte collection and -injection MEM-alpha was used (Cat no. 22571, GIBCO Life Tech, Gaithersburg, MD), supplemented per 500 ml with 2.5 g Hepes, 684 mg 50% sodium lactate solution, 55 mg sodium pyruvate, 65 mg penicillin G (1596 U/mg) and 6% fetal calf serum (BioWhittaker Europe, Verviers, Belgium), hereafter referred to as MEM-alpha medium. After injection, oocytes were cultured in G1 (Barnes *et al*., 1995) under silicone oil (Aldrich, 14,615-3). During the course of the experiments however, the culture medium was changed to human tubal fluid HTF (BioWhittaker Europe, Verviers, Belgium), containing 10% GPO (pasteurized blood plasm-protein solution, CLB, Amsterdam, The Netherlands) under mineral oil (International Medical, Zutphen, The Netherlands). This did not influence fertilization or cleavage efficiencies (data not shown).

Mice

Four to eleven-month old *T(1;13)70H/T(1;13)1Wa* double heterozygous males (*T/T′*) on a random bred Swiss genetic background (*HsdCpb:SE*), served as sperm donors. They were generated in the experimental animal centre, Wageningen University, by crossing homozygous *T(1;13)70H* females with homozygous *T(1;13)1Wa* males (De Boer *et al*., 1986). *T/T′* males were tested for infertility by mating them twice with high-littersize NMRI (*HsdWin:NMRI*)

virgin females (De Boer *et al*., 1986). Absence of decidual reactions at day 13 of pregnancy indicated sterility. The spermiogram from the caput epididymis of infertile *T/T′* males resembles that of OAT *Ts(1^{13})70H* tertiary trisomic males (De Boer *et al*., 1976): sperm counts are low, and both motility and morphology are poor. As controls, fertile *T/T′* males were used, next to Swiss random bred wild-type males and NMRI random bred males.

B6D2 F1 females (Iffa-Credo, Someren, The Netherlands) were used as oocyte donors and were kept in a 10 h dark/14 h light schedule, with lights on at 11 am. Superovulation was induced by i.p. injection of 7.5 IU pregnant mare's serum gonadotrophin (PMSG) (Intervet, Boxmeer, The Netherlands) around 9 pm, followed by 7.5 IU hCG (Intervet, Boxmeer, The Netherlands) 48 h later.

Morphology of the epididymis of T/T′ males

From four *T/T′* males of proven sterility, one testis with attached caput, corpus, cauda epididymidis and vas deferens was fixed for 24 h in 3.7% formaldehyde in a phosphate buffer containing 46 mM Na$_2$HPO$_4$ and 33 mM NaH$_2$PO$_4$, washed and stored in 100% ethanol until histological investigation.

Preparation of epididymal and testicular sperm

Testes weights were recorded and sperm counts taken from the caput epididymidi (Searle and Beechey, 1974). Each cauda epididymidis was placed in silicone oil and the contents squeezed into the oil with forceps. For all fertile males, contents were transferred to HTF Hepes medium and left to disperse for 30 min at 37°C. The caudal contents from infertile *T/T′* males were placed in either Spermatocyte Isolation Medium (SIM) (Heyting and Dietrich, 1991) or Nucleus Isolation Medium (NIM) buffer (Kuretake *et al*., 1996) without phenylmethylsulphonyl fluride (PMSF), supplemented with 0.4% BSA at 37°C for 30 min. Samples were stored at room temperature (RT) and viability was checked with a live–dead sperm kit (Molecular Probes, Leiden, The Netherlands).

To obtain testicular sperm, tubuli seminiferi were isolated in NIM buffer, cut into small pieces and squeezed. Clumps of spermatogenic cells were dissolved in 100 µl NIM buffer, supplemented with 25 µg/ml DNase I and 2.6 mg/ml Collagenase IV. Cells were centrifuged for 5 min at 170 *g*, washed twice with NIM buffer and then resuspended in 50 µl NIM buffer at RT.

Collection of MII oocytes and intracytoplasmic sperm injection

Oocytes were freed from the oviducts 13 h after hCG, and stored without cumulus cells at 37°C for up to 4 h. Microinjection at 18°C was performed as previously described (Kimura and Yanagimachi, 1995), selecting normal looking and motile spermatozoa. A fresh sperm sample was transferred from medium to polyvinyl pyrrolidone (PVP) (360 kDa) for each injection round of eight oocytes (~30 min). Epididymal spermatozoa from infertile *T/T′* quickly lost motility once in 12% PVP (<10 min), so it cannot be excluded that initially immotile spermatozoa were used as well. Therefore, in one set of experiments, sperm from sterile *T/T′* males was specifically selected for motility or immotility directly after dispersion of the contents of two cauda epididymidi in 200 µl HTF Hepes medium with 3% BSA (Fraser and Quinn, 1981). Motile or immotile, normal looking sperm were transferred by holding pipette to the 12% PVP and injected. Testicular spermatozoa could only be selected by morphological appearance, because of inherent lack of motion.

After injection, oocytes were kept on the cool stage for 5 min, then gradually warmed to 37°C and placed in culture medium at 37°C, 5% CO$_2$ in air. Fertilization was determined 7–9 h after injection by the presence of a second polar body and two visible pronuclei. After 19–20 h, zygotes were scored for first cleavage. At

Table I. Fertility indices of four different sperm donors and resulting fertilization and cleavage rates of mouse oocytes after injection of epididymal spermatozoa

Sperm source	Testis weight (mg)	Sperm count[a]	Number of oocytes injected	Number (%) of surviving oocytes	Number (%) of fertilized oocytes	Number (%) of cleaved embryos
NMRI						
1	[b]	[b]	33	28 (85)	26 (93)	26 (100)
2	[b]	[b]	39	30 (77)	28 (93)	27 (96)
3	[b]	[b]	36	29 (81)	29 (100)	27 (93)
Total			108	87 (81)	83 (95)	80 (96)
Swiss						
1	136	400	20	16 (80)	15 (94)	12 (80)
2	113	584	19	19 (100)	17 (89)	15 (88)
3	109	454	28	24 (86)	24 (100)	23 (96)
Total			67	59 (88)	56 (95)	50 (89)
T/T′ fertile						
1	79	156	30	25 (83)	22 (88)	22 (100)
2	104	165	21	20 (95)	20 (100)	19 (95)
3	121	312	38	34 (89)	32 (94)	20 (63)
4	105	378	34	31 (91)	27 (87)	27 (100)
Total			123	110 (89)	101 (92)	88 (87)
T/T′ infertile						
1	60	3	19	17 (89)	10 (59)	3 (30)
2	50	4	36	23 (64)	20 (87)	6 (30)
3	57	11	35	25 (71)	14 (56)	2 (14)
4	[b]	15	24	20 (83)	17 (85)	5 (29)
5	61	17	28	22 (79)	17 (77)	7 (41)
6	60	22	29	24 (83)	19 (79)	4 (21)
7	50	26	13	11 (85)	8 (73)	2 (25)
8	57	37	31	21 (68)	9 (43)	1 (11)
9	59	56	36	25 (69)	14 (56)	2 (14)
10	90	110	36	33 (92)	27 (82)	19 (70)
Total			287	221 (77)	155 (70)*	51 (33)

*$P < 0.01$, compared to *T/T′* fertile.
[a]Actual haemocytometer counts are given.
[b]Not determined.

Statistics

The Mann–Whitney U test has been used to compare fertilization rates and first cleavage rates between males of the different experimental groups

this point, oocytes injected with spermatozoa from infertile *T/T′* males not showing signs of fertilization were given hypotonic treatment and chromosome preparations were made (Tarkowski, 1966). After staining with 4,6-diamidino-2-phenylindole (DAPI) (125 ng/ml), chromosomes were examined under a Zeiss Axiophot2 fluorescence microscope.

Treatment of zygotes from infertile T/T′ males with bromo-deoxyuridine or okadaic acid

In three independent injection sessions, all oocytes injected with epididymal spermatozoa from infertile *T/T′* males were cultured in medium containing 5 µg/ml 5-bromo-2′-deoxyuridine (BrdU) to demonstrate DNA replication. After 20 h of culture, fertilized oocytes that had failed to cleave to the two-cell stage and still possessed visible pronuclei, were fixed (Tarkowski, 1966). BrdU incorporation was detected with the RPN202 kit (Amersham Life Science, Alrington Heights, IL) in combination with a goat anti-mouse IgG conjugated with FITC (Jackson Immuno Research laboratories, PA, USA), as described previously (Baart *et al.*, 2000) and DNA was counterstained using DAPI.

Okadaic acid (OA), as a specific inhibitor of serine/threonine phosphoprotein phosphatases (PP) 1 and 2A was used to induce premature chromosome condensation (PCC), in a pattern dependent on the cell cycle stage of the pronucleus. (zygote G1-, S- or G2-phase; Dyban *et al.*, 1993). In three injection sessions with sperm of sterile T/T′ males, oocytes with visible pronuclei 20 h after injection, were incubated for 1 h in 10 µM OA, subsequently transferred to fresh medium and cultured for another 1–2 h, after which chromosome preparations were made and inspected with DAPI.

Results

Morphology of the epididymis in T/T′ sterile males

Epididymides with efferent ducts, initial segment, caput, corpus and cauda were of normal size and development, the secretions in the cauda, as judged from periodic acid-Schiff (PAS) staining, being normal as well. All segments had principal and basal cells, the initial segment had apical cells and the caput and cauda regions had clear cells. One male only presented with vacuoles in the caput region, a characteristic seen in mice of some genetic backgrounds.

Zygote development after injection of epididymal sperm

Oocytes were injected with epididymal sperm from four different types of donors and zygote development was monitored (Table I). Injection of sperm heads from *NMRI* males led to high fertilization rates: 95% showed two pronuclei and a second polar body 7 h after injection. Of fertilized oocytes, 96% showed normal cleavage to two-cell embryos after 20 h of culture. These results are comparable to those of others (Kimura and Yanagimachi, 1995) after injection of

Table II. The effect of sperm motility on number of cleaved zygotes in infertile T/T′ males

T/T′ infertile	Males (n)	Testis Weight (mg)	Sperm count[a]	Number of injected and surviving oocytes	Number (%) of cleaved embryos
Motile sperm of severe oligospermics	2	58/69	4/19	19	6 (32)
Immotile sperm of severe oligospermics	2	58/68	4/24	20	6 (30)
Immotile sperm of mild oligospermics	2	80/80	99/120	33	20 (61)

[a]Actual haemocytometer counts are given.

Table III. Fertility indices of two different sperm donors and resulting fertilization and cleavage rates of mouse oocytes after injection of testicular spermatozoa

Sperm source	Testis weight (mg)	Sperm count[a]	Number of oocytes injected	Number (%) of surviving oocytes	Number (%) of fertilized oocytes	Number (%) of cleaved embryos
Swiss						
1	105	>300	42	38 (90)	30 (79)	22 (73)
2	110	>300	22	20 (91)	19 (95)	19 (100)
3	120	>300	14	10 (71)	9 (90)	7 (77)
Total			78	68 (87)	58 (85)	48 (83)
T/T′ infertile						
1	45	0	12	10 (83)	9 (90)	8 (89)
2	60	8	25	23 (92)	18 (78)	6 (33)
3	55	11	33	29 (88)	18 (62)	14 (78)
4	60	36	10	10 (100)	7 (70)	4 (57)
Total			80	72 (90)	52 (72)	32 (62)*,**

*P<0.025, compared to *Swiss* + *T/T′* fertile of Table I.
**P<0.01, compared to *T/T′* fertile of Table I.
[a]Actual haemocytometer counts are given.

B6D2 F1 oocytes with *B6D2* F1 sperm (98% and 99%, respectively).

After injection of sperm heads from chromosomally normal Swiss males, the average fertilization rate of surviving oocytes was 95% and 89% of fertilized oocytes cleaved to the two-cell stage. For the fertile *T/T′* group these figures were 92% and 87%, respectively. Results obtained from the infertile *T/T′* group were more variable. An average fertilization rate of only 70% was observed (Mann–Whitney U test with fertile *T/T′*, $P < 0.01$). Furthermore, on average only 33% of fertilized oocytes developed to the two-cell stage after 20 h of culture, while most remained at the pronuclear stage. Some pronuclear oocytes still cleaved after 24 h, indicating no block at the pronuclear stage, but a severe delay in their development.

The influence of sperm motility in sterile males

Viability staining in sterile males ($n = 3$) showed 22–40% of live spermatozoa from the cauda epididymis directly after isolation, and this decreased to 18–31% after 5 h of incubation at RT. Table II gives the results of separating motile from non-motile spermatozoa prior to PVP incubation. In these small series, no effect of selection for motility or immotility was apparent when males were oligospermic.

Zygote development after injection of testicular sperm

Oocytes were injected with testicular spermatozoa from fertile Swiss and infertile *T/T′* males (Table III). Mostly, spermatozoa with normal head morphology, but still exhibiting a cytoplasmic droplet, were used. Injection of testicular sperm from Swiss control mice resulted in slightly lower fertilization and cleavage rates than after injection with epididymal sperm. Testicular sperm from infertile *T/T′* males produced variable results among different males with an average fertilization rate of 72% (Table III). This is comparable to epididymal sperm (Table I, *T/T′* infertile). However, the cleavage rate obtained with testicular sperm from males with very low epididymal sperm counts increased to 62% (Table III). Although this is lower than cleavage rates for epididymal control sperm (Mann–Whitney U test: *T/T′* infertile compared to Swiss and fertile *T/T′* males combined, $P < 0.025$), it is significantly higher than cleavage rates obtained with epididymal sperm from infertile *T/T′* males (Mann–Whitney U test: testicular sperm of *T/T′* infertile compared to epididymal sperm of *T/T′* infertile, $P < 0.01$).

Cytogenetic analysis of unfertilized oocytes

After injection with epididymal sperm from infertile *T/T′* males, 45 oocytes classified as unfertilized. In 9 of 45 oocytes, 20 metaphase II chromosomes with remnants of the first polar body only were found, indicating injection failure. In 18 oocytes, an intact or slightly decondensed sperm head was visible next to 20 metaphase II chromosomes. In a further 18 oocytes, premature chromosome condensation (PCC) of the sperm chromosomes was observed (Figure 1). In these oocytes, the sperm nucleus had transformed into discrete chromosomes. In 7 of these 18 oocytes, the prematurely condensed chromosomes were heavily fragmented and rearranged chromosomes could be seen (Figure 2).
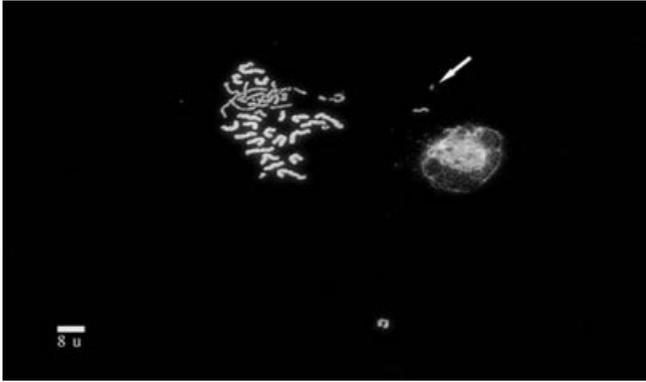
1143

Figure 1. Meiotic metaphase II chromosomes with two chromatids and single chromatid sperm chromosomes in an inactivated secondary oocyte after ICSI with an epididymal spermatozoon from a sterile *T/T'* male. A small chromatin fragment of likely male descent is visible (arrow).
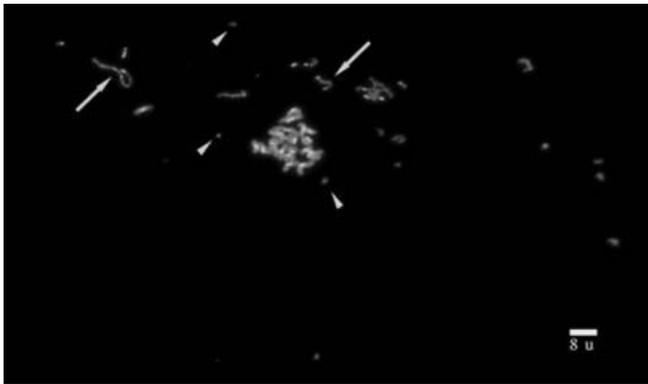

Figure 2. Premature chromosome condensation (PCC) in a secondary oocyte injected with an epididymal spermatozoon from a sterile *T/T'* male. Constitutive heterochromatin is discernible as brighter DAPI fluorescense. Many acentric fragments (arrowheads) and examples of rearranged chromosomes (arrows) are visible.

BrdU incorporation and OA condensation patterns in pronuclear zygotes

A total of 24 one-cell embryos with visible pronuclei after 20 h of culture were analysed for BrdU incorporation. BrdU was consistently detected in both pronuclei and the second polar body (Figure 3). Pronuclei must therefore have passed through G1-phase of the cell cycle and be in either S- and/or G2-phase. The pronuclear membranes in one zygote had broken down spontaneously in the time between selection of the pronuclear zygotes and fixation, and first metaphase chromosomes could be inspected (Figure 4). Several chromosome exchanges and (a)centric fragments were observed. In 13 of 27 zygotes treated with OA, both pronuclei were condensed into a G2-phase type chromosome condensation (Figure 5), resembling metaphase chromosomes at the first mitotic division. These nuclei both had completed S-phase, but did not make a timely transition into M-phase and subsequent cleavage. Karyotype analysis was not possible due to the spreading characteristics, especially of the male pronucleus. In nine other oocytes, the two pronuclei were asynchronous, with one pronucleus showing G2-type PCC and the other S-type PCC,
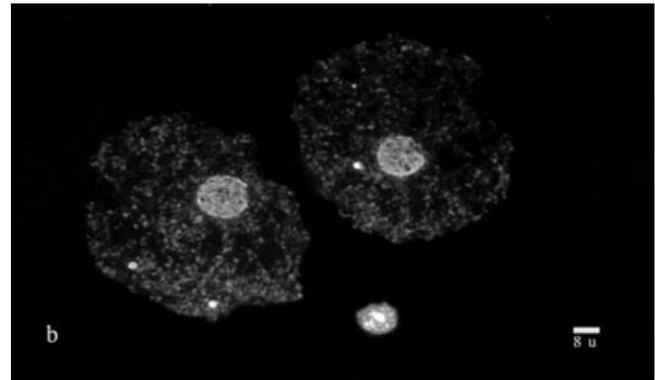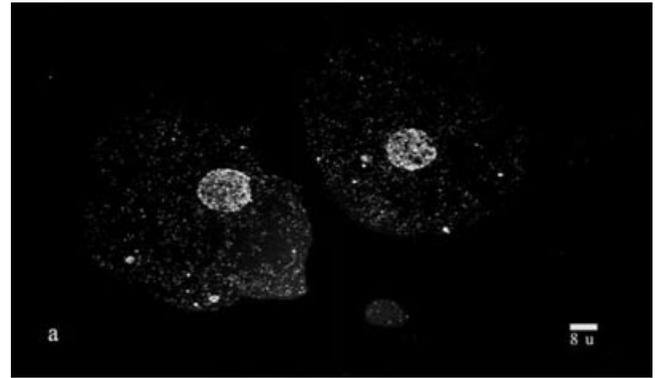
Figure 3. (A) Pronuclear zygote, around 20 h after ICSI with an epididymal spermatozoon from a sterile *T/T'* male. BrdU incorparation indicative for S and/or G2-phase is illustrated by (bright) fluorescence. (B) The same zygote as in (A), showing the DAPI DNA background.
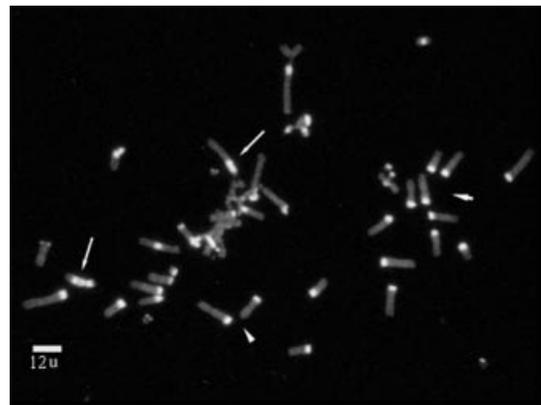

Figure 4. Spontaneous first cleavage metaphase of a delayed pronuclear zygote. Normal chromosomes most likely are of female origin (arrowheads), a set characterized by chromosome fragments and rearrangements often involving centric heterochromatin (arrows) of male origin.

characterized by a decompacted chromatin mass with a 'pulverized' appearance. In three more oocytes, two pronuclei had S-type PCC.

Discussion

In this study, we investigated the effect of poor quality mouse spermatozoa on the fertilization process and zygote develop-
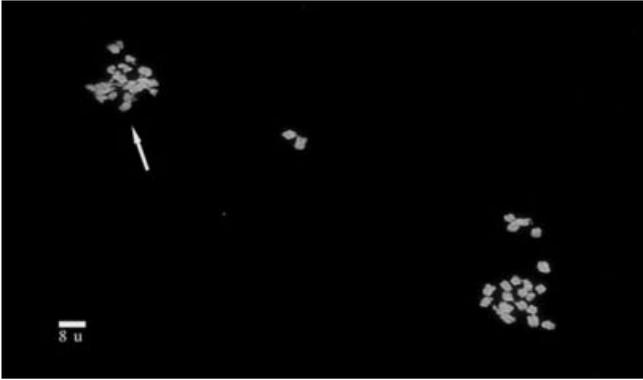
Figure 5. OA-induced G2 chromosome condensation in a pronuclear zygote from an epididymal spermatozoon of sterile *T/T′* descent. The most likely male chromosome complement is indicated by an arrow.

ment after ICSI. To do so, we used mice heterozygous for two different but semi-identical reciprocal translocations. Individual animals with this karyotype can produce up to normal sperm levels or can have an OAT phenotype, resulting in infertility (De Boer *et al.*, 1986; Peters *et al.*, 1997). The two main outcomes, using sperm from the cauda epididymis of infertile males, are: a) a small but significant decrease in oocyte activation rate; and b) a substantial decrease in the percentage of activated oocytes that develop to the two-cell stage in time. We show that the latter effect is due to arrest or delay at the S- or G2-phase of pronuclear development. Furthermore, we have obtained evidence that sperm chromosome damage accompanies this reduction in zygote development.

Origin of the DNA damage in epididymal sperm from sterile *T/T′* males

During first meiotic prophase in these males, an indication for the persistence of recombinational double-strand DNA breaks is found in those pachytene spermatocytes, that do not form a structurally normal small translocation chromosome 1^{13} synaptonemal complex (Mahadevaiah *et al.*, 2001). In these primary spermatocytes, death starts during the pachytene stage, as in comparable male sterile mouse models (De Boer *et al.*, 1986). Spermatocytes that fail to meet the prerequisites for meiotic recombination are removed from the germ line. Therefore, it is less likely that unrepaired meiotic DNA damage persists post meiosis in the epididymal spermatozoa of these males. Thus, the chromosome damage observed at the zygote stage more likely is of a post meiotic nature, when during sperm differentiation, DNA repair becomes inactive (Sega and Sotomayor, 1982).

In the mouse and rat, an increase in the presence of endogenous nicks in the DNA of spermatids undergoing elongation was demonstrated by *in situ* nick translation (McPherson and Longo, 1992, 1993; Sakkas *et al.*, 1995). These authors propose that the creation and ligation of DNA nicks in testicular spermatids play an important role in chromatin remodelling prior to protamination. It is not known whether in a situation of OAT the ligation step is

defective, thus leading to impaired chromatin stability and the presence of DNA damage.

Sperm transport through the epididymis has been timed in the mouse using radioisotopic labelling at premeiotic S-phase. Thus, in a normal male, transport takes 2–3 days each for respectively caput–corpus, corpus–cauda and cauda–vas deferens (Sega and Sotomayor, 1982). These experiments have to our knowledge never been repeated in mice with oligospermia. In humans, a reduced production of spermatozoa is highly correlated with longer transit times through the epididymis (Johnson and Varner, 1988). If we assume the same to hold true for mice, spermatozoa from our infertile translocation carriers would start to age in the epididymis. The improvement in first cleavage rate when using testicular sperm from sterile males (Table III) also implicates epididymal storage to play a role in the reduction of two-cell embryo yield with 'infertile' sperm.

One other mouse ICSI study used sperm from mice deficient in the protein phosphatase 1cγ gene that only expresses itself as male sterility. When malformed testicular sperm from *Pp1cγ* –/– males was used for ICSI in *B6D2* F1 oocytes, a detrimental effect on development *in vitro* between the 8-cell and blastocyst stage emerged. However, in contrast to the *T/T′* model, no effect was found up to the two-cell stage (Davies and Varmuza, 2003). Possibly, the testicular origin of sperm in *Pp1cγ* –/– males favours two-cell development. Furthermore, also in contrast to our *T/T′* model, Varmuza *et al.* (1999) report that in their male mutants, meiotic prophase was unaffected (by histology).

Reduced oocyte activation by epididymal sperm from sterile males

Injections with epididymal sperm of sterile *T/T′* males resulted into: a) elevated levels non-decondensed sperm nuclei; and b) premature chromosome condensation (PCC) in the presence of female second meiotic chromosomes: failure of oocyte activation by the sperm. PCC is known from the IVF clinic (Schmiady *et al.*, 1996), and has often been attributed to precocious fertilization of an 'unripe oocyte' (Zenzes *et al.*, 1990). This interpretation is strengthened by IVF results in the mouse (Kubiac, 1989). However, sperm decondensation problems were noted in the majority of activation failures after human ICSI (Lopes *et al.*, 1998) and a link is evident with deviant sperm chromatin compaction, as determined with CMA3 fluorescence and positive *in situ* nick translation (Sakkas *et al.*, 1998). Thus, activation of the oocyte by the sperm is likely hampered when chromatin compaction is incomplete. However, research into the heterogeneity of chromatin compaction in sterile *T/T′* males is needed to determine if this contributes to the observed reduction of oocyte activation. In the related OAT tertiary trisomic *Ts(1^{13})70H* mouse model, no evidence for underprotamination has been found as indicated by normal total thiol levels in the sperm head (De Boer *et al.*, 1990).

DNA damage checkpoints and repair

In Figures 2 and 4, male chromosome rearrangements are visible, which is evidence of double-strand DNA repair. Ligation of double-strand DNA breaks leading to chromosome

abnormalities at PCC (Figure 2) must have occurred before condensation of the chromatin, likely shortly after sperm penetration. With artificially inactivated mouse sperm to induce PCC after ICSI, an identical result was obtained (Perry *et al.*, 2000).

Zygotes which did not progress timely to the first cleavage after injection of T/T′ sperm consistently showed BrdU incorporation, indicating DNA synthesis to have started. Therefore, development could either be delayed during S-phase or at the subsequent G2-phase. OA stimulation of arrested pronuclear zygotes after ICSI showed the majority to have two pronuclei at G2-phase, followed by one pronucleus at S-phase and the other at G2. It was demonstrated earlier by heterologous ICSI using necrotic human sperm and mouse oocytes (Rybouchkin *et al.*, 1997), that the human paternal pronucleus was delayed in development. So the male pronucleus most likely is the one at S-phase in this study. The presence of a checkpoint in the mouse during the first mitotic cell cycle, after the pronuclei have reached competence to replicate DNA, has been described previously (Fulka *et al.*, 1999). Also, a p53-dependent S-phase damage checkpoint was found recently in mouse oocytes, using irradiated sperm and genetic manipulation of p53 status (Shimura *et al.*, 2002). Our results, combined with data on the processing by mouse zygotes of irradiation-induced DNA breaks in spermatozoa (Matsuda and Tobari, 1989), indicate that the zygotic cell cycle is not characterized by a G1–S checkpoint for DNA damage, so typical of somatic cells (Hartwell and Kastan, 1994). Nevertheless, commencement of DNA synthesis in zygotes is dependent on full male pronucleus development in mammals (Comizolli *et al.*, 2003; and references therein).

Mouse ICSI as a model system

The results obtained with OAT epididymal sperm in this study show a delay and/or interrupted development for the majority of fertilized embryos at the zygote stage, a phenomenon not observed with ejaculated OAT sperm in the human (Nagy *et al.*, 1995; Tarlatzis en Bili, 2000). A direct comparison between the propagating capacity of epididymal and testicular sperm within an oligospermic individual is not available. In the human, comparisons have been made between epididymal sperm in obstructive azoospermia (OA) vs. testicular sperm in non-obstructive azoospermia (NOA, Wennerholm *et al.*, 2000; Friedler *et al.*, 2002) and between testicular sperm in OA vs. testicular sperm in NOA (de Croo *et al.*, 2000; Vernaeve *et al.*, 2003). Summarizing these data, testicular sperm from NOA patients were on average somewhat less effective in fertilization and possibly cleavage rate, an effect smaller but similar to the one observed in Table III for sterile T/T′.

Using the alkaline Comet assay to assess DNA fragmentation, a difference in DNA integrity between testicular and epididymal sperm in human obstructive azoospermia was also noted (Steele *et al.*, 1999; O'Connell *et al.*, 2002). In agreement with our results, more damage was observed in epididymal spermatozoa. Thus, it appears that the use of poor quality semen with possible DNA damage negatively affects embryo development, but the response of the human embryo to the presence of DNA damage may be different in comparison to the mouse.

In conclusion, oligospermia in the T/T′ mouse model affects fertilization and first cleavage rates. This is most pronounced for epididymal sperm, but the same tendency is observed for testicular sperm. In normospermic controls, a difference between testicular and epididymal sperm was not apparent. Epididymal sperm from fertile T/T′ males did not differ from chromosomally normal fertile controls. Although epididymal sperm from sterile T/T′ males is not completely representative for ejaculated human gametes used for ICSI, this study offers interesting observations on chromosome instability when PCC is observed and when first cleavage is delayed. Also, it demonstrates a role for zygotic S-phase in detecting chromosomal instability or other aspects of chromatin structure. The T/T′ mouse model is a good candidate for a follow-up study of the fate of two-cell mouse embryos after TESE.

Acknowledgements

References

Ahmadi A and Ng S-C (1999) Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 284,696–704.

Baart EB, de Rooij DG, Keegan KS and de Boer, P. (2000) Distribution of Atr protein in primary spermatocytes of a mouse chromosomal mutant: a comparison of preparation techniques. Chromosoma 109,139–147.

Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C and Trounson AO (1995) Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod 10,3243–3247.

Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A and Liebaers I (2002) Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 17,2600–2614.

Comizolli P, Urner F, Sakkas D and Renard JP (2003) Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the S-phase in bovine zygotes. Biol Reprod 68,1934–1940.

Davies T and Varmuza S (2003) Development to blastocyst is impaired when intracytoplasmic sperm injection is performed with abnormal sperm from infertile mice harboring a mutation in the protein phosphatase 1c gamma gene. Biol Reprod 68,1470–1476.

DeBoer P, van der Hoeven FA and Chardon JAP (1976) The production, morphology, karyotypes and transport of spermatozoa from tertiary trisomic mice and the consequences for egg fertilization. J Reprod Fertil 48,249–256.

DeBoer P, Searle AG, Van der Hoeven FA, de Rooij DG and Beechey CV (1986) Male pachytene pairing in single and double translocation heterozygotes and spermatogenic impairment in the mouse. Chromosoma 93,326–336.

DeBoer P and de Jong JH (1989) Chromosome pairing and fertility in mice. In Gillies CB (ed) *Fertility and chromosome pairing: recent studies in plants and animals.* CRC Press, Boca Raton, Florida, pp. 37–76.

DeBoer P, Redi CA, Garagna S and Winking H (1990) Protamine amount and cross linking in mouse teratospermatozoa and aneuploid spermatozoa. Mol Reprod Dev 25,297–301.

DeCroo I, van der Elst J, Everaert K, De Sutter P and Dhont M (2000) Fertilization, pregnancy and embryo implantation rates after ICSI in cases of obstructive and non-obstructive azoospermia. Hum Reprod 15,1383–1388.

DeVries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P and te Riele H (1999) Mouse MutS-like protein Msh5 is required for proper chromosome synapsis in male and female meiosis. Genes Dev 13,523–531.

Dyban AP, De Sutter P and Verlinsky Y (1993) Okadaic acid induces premature chromosome condensation reflecting the cell cycle progression in one-cell stage mouse embryos. Mol Reprod Dev 34,402–415.

Fraser LR and Quinn PJ (1981) A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. J Reprod Fertil 61,25–35.

Friedler S, Raziel A, Strassburger D, Schachter M, Soffer Y and Ron-El R (2002) Factors influencing the outcome of ICSI in patients with obstructive and non-obstructive azoospermia: a comparative study. Hum Reprod 17,3114–3121.

Fulka J Jr, First NL, Fulka J and Moor RM (1999) Checkpoint control of the G2/M phase transition during the first mitotic cell cycle in mammalian eggs. Hum Reprod 14,1582–1587.

Hartwell LH and Kastan MB (1994) Cell cycle control and cancer. Science 266,1821–1828.

Heyting C and Dietrich AJJ (1991) Meiotic chromosome preparation and protein labelling. Methods Cell Biol 35,177–202.

Johnson L and Varner DD (1988) Effect of daily spermatozoan production but not age on transit time of spermatozoa through the human epididymis. Biol Reprod 39,812–817.

Kimura Y and Yanagimachi R (1995) Intracytoplasmic sperm injection in the mouse. Biol Reprod 52,709–720.

Kubiac JZ (1989) Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. Dev Biol 136,537–545.

Kuretake S, Kimura Y, Hoshi K and Yanagimachi R (1996) Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol Reprod 57,789–795.

Lee JD, Kamiguchi Y and Yanagimachi R (1996) Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphology after injection into mouse oocytes. Hum Reprod 11,1942–1946.

Lopes S, Jurisicova A and Casper RF (1998) Gamete-specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. Hum Reprod 13,703–708.

Mahadevaiah SK, Turner JMA, Baudat F, Rogakou EP, De Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM and Burgoyne PS (2001) Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 27,271–276.

Martin RH, Rademaker A and German J (1994) Chromosome breakage in human spermatozoa, a heterozygous effect of the Bloom syndrome mutation. Am J Hum Genet 55,1242–1246.

Matsuda Y and Tobari I (1989) Repair capacity of fertilized mouse eggs for X-ray damage induced in sperm and mature oocytes. Mutat Res 210,35–47.

McPherson SMG and Longo FJ (1992) Localization of DNase I-hypersensitive regions during rat spermiogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. Mol Reprod Dev 31,268–279.

McPherson SMG and Longo FJ (1993) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol 158,122–130.

Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, Derde MC, Devroey P, van Steirteghem AC (1995) The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum Reprod 10,1123–1129.

O'Connell M, McClure N and Lewis SEM (2002) Mitochondrial DNA deletions and nuclear DNA fragmentation in testicular and epididymal human sperm. Hum Reprod 17,1565–1570.

Odorisio I, Rodriguez TA, Evans EP, Clarke AR and Burgoyne PS (1998) The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis. Nat Genet 18,257–261.

Perry AC (2000) Hijacking oocyte DNA repair machinery in transgenesis? Mol Reprod Dev 56,319–324.

Peters AHFM, Plug AW and de Boer P. (1997) Meiosis in carriers of heteromorphic bivalents: sex differences and implications for male fertility. Chromosome Res 5,313–324.

Rybouchkin A, Benijts J, De Sutter P and Dhont M (1997) Disintegration of chromosomes in dead sperm cells as revealed by injection into mouse oocytes. Hum Reprod 12,1693–1698.

Sakkas D, Manicardi G, Bianchi PG, Bizzaro D and Bianchi U. (1995) Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. Biol Reprod 52,1149–1155.

Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y and Campana A. (1998) Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod 13 (suppl. 4),11–19.

Schmiady H, Sperling K, Kentenich H and Stauber M (1996) Prematurely condensed human sperm after in vitro fertilization (IVF). Hum Genet 74,441–443.

Searle AG and Beechey CV (1974) Sperm-count, egg-fertilization and dominant lethality after X-irradiation of mice. Mutat Res 22,63–72.

Sega GA and Sotomayor RE (1982) Unscheduled DNA synthesis in mammalian germ cells – its potential use in mutagenicity testing. In de Serres FJ and Hollaender A (eds) Chemical Mutagens Vol 7, Plenum Press, NY & London, pp. 421–445.

Shimura T, Inoue M, Taga M, Shiraishi K, Uematsu N, Takei N, Yuan Z-M, Shinohara T and Niwa O (2002) p53-Dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. Mol Cell Biol 22,2220–2228.

Speed RM (1989) Heterologous pairing and fertility in humans. In Gillies CB (ed) Fertility and chromosome pairing: recent studies in plants and animals. CRC Press, Boca Raton, Florida, pp. 1–35.

Steele EK, McClure N, Maxwell RJ and Lewis SE (1999) A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. Mol Hum Reprod 5,831–835.

Tarkowski AK (1966) An air drying method for chromosome preparations from mouse eggs. Cytogenetics 5,394–400.

Tarlatzis BC and Bili H (2000) Intracytoplasmic sperm injection. Survey of world results. Ann N Y Acad Sci 900,336–344.

Twigg JP, Irvine DS and Aitken RJ (1998) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 13,1864–1871.

VanSteirteghem AC, Nagy P, Joris H, Liu J, Staessen C, Smitz J, Wisanto A and Devroey P (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. Hum Reprod 8,1061–1066.

Varmuza S, Jursicova A, Okano K, Hudson J, Boekelheide K and Shipp EB (1999) Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1c gamma gene. Dev Biol 205,98–110.

Vernaeve V, Tournaye H, Osmanagaoglu K, Verheyen G, Van Steirteghem A and Devroey MD (2003) Intracytoplasmic sperm injection with testicular spermatozoa is less successful in men with nonobstructive azoospermia than in men with obstructive azoospermia. Fertil Steril 79,529–533.

Wennerholm U-B, Bergh C, Hamberger L, Westlander G, Wikland M and Wood M. (2000) Obstetric outcome of pregnancies following ICSI, classified according to sperm origin and quality. Hum Reprod 15,1189–1194.

WHO. (1999) Cambridge (ed) World Health Organization: WHO Laboratory Manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, UK.

Zenzes MT, de Geyter C, Bordt J, Schneider HPG and Nieschlag E (1990) Abnormalities of sperm chromosome condensation in the cytoplasm of immature human oocytes. Hum Reprod 5,842–846.