Human sperm chromosome complements after microinjection of hamster eggs

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Summary. A technique was developed for microinjection of human spermatozoa into golden hamster (*Mesocricetus auratus*) eggs to obtain human pronuclear chromosome complements. Before microinjection the spermatozoa were treated by brief sonication or incubation in TEST–yolk buffer to reduce motility. Very few sperm chromosome complements developed after sperm treatment with sonication and the frequency of spermatozoa with structural chromosomal abnormalities was exceedingly high (91%). The majority of sperm chromosome complements analysed had multiple breaks and rearrangements. Sperm incubation in TEST–yolk buffer before microinjection provided more analysable sperm karyotypes with a significantly lower frequency of structural chromosomal abnormalities (39%, $P < 0.001$). Our results therefore suggest that sonication induces structural chromosomal abnormalities in spermatozoa. Since the frequency of chromosomal abnormalities after microinjection was higher than after sperm fertilization of hamster eggs, it appears that microinjection *per se* may also increase the frequency of chromosomal abnormalities in spermatozoa. These results are based on small numbers and must be confirmed on larger sample sizes, but our study suggests that microinjection of spermatozoa into eggs should not be recommended for clinical use until fully evaluated.

Keywords: human sperm chromosomes; microinjected hamster eggs; pronuclear chromosomes

Introduction

The technique of microinjection of mammalian eggs to introduce homologous and heterologous spermatozoa has been used to study various aspects of developmental biology (Uehara & Yanagimachi, 1976, 1977; Thadani, 1980; Perreault & Zirkin, 1982; Barg *et al.*, 1986; Naish *et al.*, 1987a, b; Perreault *et al.*, 1987). This technique has also been used experimentally for human in-vitro fertilization programmes. Metka *et al.* (1985), Laws-King *et al.* (1987) and Lassalle *et al.* (1987) microinjected spermatozoa into the peri-vitelline space of eggs; Lazendorf *et al.* (1987) microinjected human spermatozoa into the ooplasm.

To our knowledge, there have been no reports of karyotyped human chromosome complements after microinjection of human spermatozoa into mammalian eggs. Karyotyping of microinjected eggs would ensure that fertilization had actually occurred (as opposed to parthenogenetic activation of the eggs) and it could also determine whether microinjected spermatozoa had an increased frequency of chromosomal abnormalities.

In this paper, we report our experience in developing a technique which allows chromosomal analysis of human spermatozoa after microinjection into hamster eggs.

Materials and Methods

A detailed technique for obtaining pronuclear human sperm chromosome complements after sperm penetration of golden hamster eggs which have had the zona pellucida removed has been published (Martin, 1983). The methods reported here are similar but with alterations in the preparation of spermatozoa, manipulation of eggs, culture of injected eggs in colcemid and the actual techniques for microinjection of the human spermatozoa into the hamster eggs.

Media

Biggers–Whitten–Whittingham medium (BWV) (Biggers *et al.*, 1971) with some modifications (Martin, 1983) was used for sperm preparation and egg preparation. This medium was sterilized by filtration through a cellulose acetate membrane (pore size 0.22 µm). The pH was adjusted to 7.4–7.5 with a small amount (<0.1 ml) of acid or base Hepes solution. The medium used for egg culture was Ham's F10 (Flow Laboratories, McLean, VA 22102, U.S.A.) supplemented with 15% fetal bovine serum (Flow Laboratories; heat inactivated at 60°C for 1 h, 100 i.u. penicillin G/ml and 50 µg streptomycin sulphate/ml). Just before use, the pH was adjusted to 7.2 with 1 N-HCl.

Human sperm preparation

Informed written consent was obtained from all sperm donors. The semen sample was collected in a sterile container and processed as soon as liquefaction had occurred (30 min at 37°C). Approximately 10 ml Medium BWV at 37°C were added to the semen, the components were mixed, the suspension was centrifuged at 600 g for 6 min, the supernatant was decanted, and the pellet resuspended in 10 ml Medium BWV. The spermatozoa were washed twice more to remove seminal fluids and debris. Two different methods of sperm preparation were used to effect capacitation and to decrease motility of the spermatozoa in order to facilitate microinjection.

Sonication. The final sperm pellet was resuspended in 5 ml Medium BWV in a 100-ml specimen cup. The cup was placed in an ice-water bath and the probe of a sonicator (Sonifier Cell Disruptor, model W185; Branson Sonic Power Co., Danbury, CT 06810, U.S.A.), was inserted in the sperm suspension. The spermatozoa were sonicated at maximum power for 2–10 sec (median 4 sec) until the sperm tails were bent but not sheared from the sperm head. The sperm suspension was centrifuged at 600 g for 6 min and ~1 ml Medium BWV was added to the pellet to provide a final sperm concentration of about 200×10^6 /ml.

TEST–yolk buffer. After washing by centrifugation, the final sperm pellet was resuspended in Medium BWV to the original semen volume and an equal amount of TES–Tris (TEST)–yolk buffer (Bolanos *et al.*, 1983; Brandriff *et al.*, 1985a) at 37°C was added; the sample was well mixed with the buffer and sealed in a tube which was immersed in a jar of water at room temperature. This jar, in turn, was sealed and plunged into a styrofoam box of crushed ice which was closed and refrigerated at 4°C for 1–3 days (until the day of the experiment). On the experiment day, the spermatozoa in TEST–yolk buffer were removed from refrigeration and warmed at 37°C for 1 h. The spermatozoa were washed three times by centrifugation at 600 g as described above. The final sperm pellet was resuspended in ~0.5 ml Medium BWV for a final concentration of about 200×10^6 /ml.

Hamster egg preparation

The hamsters (*Mesocricetus auratus*) were superovulated with an intraperitoneal injection of 25–30 i.u. PMSG (Sigma (G-4877); St Louis, MO 63178, U.S.A.) 69–73 h before egg retrieval followed by an intraperitoneal injection of 25 i.u. hCG (A.P.L., Ayerst Laboratories, New York, NY, U.S.A.) 16–17 h before egg retrieval. Each hamster yielded 40–60 eggs. The hamsters were stunned with ether and killed by cervical dislocation about 16–17 h after hCG injection. The oviducts were punctured, the cumulus cell mass was removed and 0.1% hyaluronidase (Type 1-S; Sigma) in Medium BWV was added. The hyaluronidase dispersed the cumulus cells in 2–3 min, freeing the eggs, which were then washed 3 times in Medium BWV. After washing, the eggs were transferred to a watchglass of F10 medium under paraffin oil (warmed to 37°C) to await transfer to the microinjection slide. Hamsters were killed one at a time just before microinjection of the eggs. Two hamsters were used routinely for each experiment.

Equipment for microinjection

The gametes were handled with micromanipulators (Wild Leitz Canada Ltd, Calgary, Alberta, Canada T2H 2K1) under $\times 200$ magnification using a Wild Leitz Diavert microscope with a fixed stage modification.

The micropipettes were made of glass tubing (Kimble) with an outer diameter of 1.0 mm and an inner diameter of 0.5 mm. The glass tubing was pulled on a horizontal micropipette puller (Industrial Science Associates, Flushing, NY 11358, U.S.A.) set to a pull time of ~2 min, which resulted in sharp-shouldered, short-shanked pipettes.

The tips of the micropipettes were opened and cleaned as follows. The pipette was attached via a short length of intramedic P-90 tubing (Fisher Scientific, Edmonton, Alberta, Canada T5S 1J3) to a 5-ml syringe equipped with a blunt 20-gauge needle. The 5 ml of air in the syringe was subsequently compressed to ~2.5 ml while the pipette tip was held in 20–25% hydrofluoric acid in water until very tiny bubbles appeared. Then the hydrofluoric acid was flushed in and out of the pipette 5 times, water was rinsed in and out 5 times and methanol was rinsed in and out 5 times. These procedures produced microinjection pipettes with glass that was extremely thin and sharp on the tip. The final inner diameter of the microinjection pipette was 6–7 μm . The injection pipette was filled with Fluorinert (FC-77: Sigma) and mercury (Fisher) was used to control the suction. Rejected injection micropipettes were used to fashion egg-holding pipettes. The pipettes were broken at approximately 100 μm diameter using a de Fonbrune (Beaudouin, Paris, France) microforge. The holding pipettes were fire-polished in a Bunsen burner flame until the inner diameter of the tip was 5–10 μm .

Just before injection, 20 μl 10% polyvinylpyrrolidone (PVP) (average molecular weight 360 000) (Sigma) in 0.9% (w/v) NaCl was mixed with 20 μl of the sperm suspension on a glass slide to facilitate microinjection (Thadini, 1980). The PVP is very viscous and further slows sperm motility. Drops of PVP–sperm suspension were placed in a row on a 50 $\times$ 76 mm siliconized glass slide alternating with drops of Medium BWB medium (for hamster eggs). A rectangular wax wall composed of a mixture of 10 parts Vaseline to 1 part paraffin wax (approximately 1 mm in height) surrounding the sperm and egg drops had previously been applied to the slide. This was flooded with paraffin oil.

Microinjection

The eggs were transferred, about 25% of the total number at a time, from the F10 medium to the drop of Medium BWB on the microinjection slide. The spermatozoa were picked up with the injection pipette and the stage was moved so that the pipette travelled to the top of the drop of Medium BWB where an egg was picked up with the holding pipette. The microscope stage was then altered so that the egg and both pipettes were moved to the bottom of the drop of Medium BWB. The egg and the injection pipette were aligned such that the injection pipette was midway between the top and bottom of the egg vertically and horizontally. A spermatozoon was placed near the tip of the injection pipette. The injection pipette was quickly poked through the zona pellucida and the egg membrane until the tip of the pipette was almost to the opposite side of the egg. A small amount of egg cytoplasm was pulled into the injection pipette, the cytoplasm and one or occasionally two spermatozoa were ejected into the egg, and the pipette was withdrawn quickly from the egg. The egg was released from the holding pipette at the bottom of the drop of Medium BWB and the pipette was withdrawn from the drop. With the holding pipette in the oil outside the drop of Medium BWB, the stage was moved to the top of the drop of Medium BWB. The process was repeated until all of the eggs in the drop had been injected and were at the bottom of the drop of Medium BWB. The injected eggs were then removed from the injection drop and were transferred to Medium F10 under oil at 37°C. The remaining eggs were transferred to the injection slide and injected as described above. The average time taken to inject the eggs from one hamster was 50 min (~1 egg/min).

Egg culture

Eggs with a healthy appearance (i.e. those without a flat, two-dimensional appearance) from one hamster were divided equally among four 50- μl drops of Medium F10 under oil in a 5-cm Petri dish, prewarmed to 37°C. The injected eggs were incubated at 37°C in 5% CO_2 in air, 95% humidity for 5 (eggs from second hamster) to 7 h (eggs from first hamster). Colcemid (Gibco/BRL, Burlington, Ontario, Canada L7P 1A1) was added to the drops of F10 medium to a concentration of 0.2 $\mu\text{g/ml}$ and incubation was continued for a further 14–15 h.

Chromosome preparation

The eggs were inspected using the microinjection microscope (magnification $\times 200$) for pronuclei. Those lacking pronuclei were treated with 0.2% trypsin (Type XII, Sigma) in Medium BWB with colcemid to remove the zona pellucida and then washed once in Medium BWB with colcemid. The eggs were transferred to a hypotonic solution (1% sodium citrate) for 3–6 min. A maximum of 5 eggs was transferred in a small drop of hypotonic solution (2 mm diameter) to the centre of a glass slide pre-cleaned with alcohol. Four drops of fixative (95% ethanol:glacial acetic; 3:1, v/v), each 20 μl , were dropped over the eggs (Tarkowski, 1966). After 2 weeks the chromosomes were stained with 0.5% quinacrine dihydrochloride (pH 4.5) for 25 min followed by three rinses in distilled H_2O (pH 4.5) for a total of 10 min. The slides were mounted in distilled H_2O at pH 4.5 and examined and photographed with a Zeiss fluorescent microscope with a D.C.-powered HBO W2 mercury lamp. The barrier filter was set at 47 and the excitation filter at BG3. The slides were subsequently stained with 3 ml Giemsa (Harleco, Sibbstown, NJ, U.S.A.) in 47 ml Gurr buffer (pH 6.8) for 5 min.

Sperm chromosome complements by human sperm fertilization of hamster eggs

Sperm chromosome complements were also obtained by our usual technique of sperm fertilization of zona pellucida-free hamster eggs (Martin, 1983) except that TEST–yolk buffer (Brandriff *et al.*, 1985a) was used for sperm

capacitation for Donors C and D. These sperm karyotypes were prepared for the donors who participated in the microinjection experiments to ensure that none of the donors had an unusually high frequency of chromosomal abnormalities which might confound results from the microinjection experiments.

Results

After microinjection, about 90% of the eggs looked healthy; the site of the injection appeared to seal immediately and the spermatozoa remained in one position. At the present time the efficiency of the technique is poor; approximately 100 hamster eggs were microinjected for a mean yield of 3·4 human sperm karyotypes per experiment. However, we expect an improvement in efficiency as we gain experience with the technique.

Normal men with no known exposure to a mutagen were sperm donors for the experiments. Two donors (A, 37 years; B, 28 years) were used for the experiments with sonicated spermatozoa and 3 donors (A, 37 years; C, 28 years; D, 25 years) were used for the experiments in which spermatozoa were stored in TEST–yolk buffer. Donor A participated in both types of experiments.

The detailed results from sonicated spermatozoa and spermatozoa treated with TEST–yolk buffer are presented in Table 1. The proportion of spermatozoa with a normal chromosome complement was only 9% (1/11) after sonication. After treatment with TEST–yolk buffer, 58% (19/33) of the spermatozoa had a normal karyotype. The proportion of spermatozoa with structural chromosomal abnormalities was significantly higher in spermatozoa treated with sonication (91%) compared to spermatozoa treated with TEST–yolk buffer (39%) ($\chi^2 = 40\cdot03$, d.f. = 1, $P < 0\cdot0001$). Two types of structural abnormalities were present: simple abnormalities such as a chromosome break and spermatozoa with multiple breaks and rearrangements. Both of these types of structural abnormalities were observed in spermatozoa from both treatment methods but the frequency of each was elevated in sonicated spermatozoa. Simple structural abnormalities were observed in 15% of spermatozoa treated with TEST–yolk buffer compared to 27% of sonicated spermatozoa and multiple breaks and rearrangements were seen in 24% of TEST–yolk-buffer spermatozoa compared to 64% of sonicated spermatozoa.

Table 1. Sperm chromosome complements obtained after microinjection of hamster eggs with human spermatozoa

	Sperm treatment	
	Sonication	TEST–yolk buffer
Total spreads	11	33
Normal spreads		
23, X	1	11
23, Y		8
Abnormal spreads		
Numerical	0	1 (24,X,+9)
Structural	23,X,2csb(3)(q)+ace 23,Y,csb(5)(p15) 23,Y,+ace Multiple breaks and rearrangements (7)	23,X,csb(2)(q3) 23,Y,csg(3)(q26)csb(5)(q14) 23,X,csb(4)(q31) 22,X,+8,-4,+ace(4q) 23,X,-4,-18,+dic(4;18)(pter--q13::q12--pter)+ace(18q ter--18q12::4q13--4q ter) Y, Multiple breaks and rearrangements (3) Multiple breaks and rearrangements (5)
Total	10	13

The hamster egg chromosomes did not demonstrate any of the structural chromosomal abnormalities observed in the human sperm complements. An example of a normal sperm chromosome complement (obtained after TEST–yolk treatment of the spermatozoa) is presented in Fig. 1.

Sperm chromosome complements were also obtained by allowing human spermatozoa to fertilize hamster eggs (Martin, 1983). The results for the 4 donors are presented in Table 2. The frequency of spermatozoa with chromosomal abnormalities varied from 11 to 12% including all types of abnormalities. For numerical abnormalities the range was 0 to 11% and for structural abnormalities 0 to 12%. These donors were within the normal range observed for control donors in our laboratory (Martin *et al.*, 1987).

Fig. 1. A normal 23,Y, Q-banded human sperm chromosome complement from a microinjected hamster egg (pretreatment of spermatozoa with TEST-yolk buffer). The unmarked chromosomes are hamster chromosomes. The centromeres of pronuclear chromosomes are often decondensed. $\times 1400$.

Table 2. Sperm chromosome complements obtained after fertilization of hamster eggs with human spermatozoa

Donor	Total complements	Normal	Abnormal		
			Numerical (%)	Structural (%)	Total (%)
A	83	74	2 (2)	7 (8)	9 (11)
B	27	24	3 (11)	0	3 (11)
C	91	80	4 (4)	7 (8)	11 (12)
D	25	22	0	3 (12)	3 (12)

Discussion

It is necessary to treat spermatozoa before microinjection to remove the tails or decrease motility because untreated spermatozoa continue movement in the egg cytoplasm (unpublished observations). Since most studies (Thadani, 1980; Perreault & Zirkin, 1982; Lanzendorf *et al.*, 1987) have used sonication to remove the sperm tails before microinjection, we attempted this technique first with human spermatozoa. However, very few sperm chromosome complements developed after microinjection of sonicated spermatozoa and those that did had an exceedingly high frequency of structural chromosomal aberrations (91%). The majority of spermatozoa had multiple breaks and rearrangements rendering detailed accurate analysis impossible.

Since treatment of spermatozoa in TEST-yolk buffer (Bolanos *et al.*, 1983; Brandriff *et al.*, 1985a) slows sperm motility, we tried this method of sperm treatment before microinjection into hamster eggs. The frequency of spermatozoa with structural chromosomal abnormalities (39%) was significantly lower using TEST-yolk buffer and more of the spermatozoa could be analysed with precision.

This is the first report of human sperm chromosome complements obtained after microinjection of human spermatozoa into hamster eggs. To our knowledge there is only one other report of chromosome studies performed after microinjection of mammalian eggs. Libbus *et al.* (1987) studied the incidence of chromosomal aberrations in hamster (*Mesocricetus auratus*) and vole (*Microtus oregoni*) spermatozoa after microinjection into hamster eggs (*Mesocricetus auratus*). In this study the spermatozoa were sonicated and flow-sorted using u.v.-laser irradiation to attempt to separate X- and Y-chromosome bearing spermatozoa. As observed in our study, the hamster egg chromosomes exhibited a low frequency of chromosomal aberrations (4–7%) but the microinjected spermatozoa had a very high frequency of structural chromosomal abnormalities. The flow-sorted spermatozoa had an average of 50% abnormalities; but even the control spermatozoa, treated only by sonication, had 20% (hamster) and 21% (vole) abnormalities. Libbus *et al.* (1987) suggested that either the sperm sonication or the microinjection procedure could have caused these chromosomal abnormalities. Our results suggest that sonication induces chromosomal abnormalities in human spermatozoa since we observed a significant increase in the frequency of abnormalities in spermatozoa treated with sonication (91%) compared to TEST-yolk buffer (39%). Since exactly the same donors were not used in both types of experiments (only Donor A participated in both experiments), it is possible that donor age or individual variation in the frequency of sperm chromosomal abnormalities could have had some effect on the observed different frequencies of abnormalities after sperm treatment by sonication or TEST-yolk buffer, although we have never seen such high frequencies of sperm chromosomal abnormalities in previous studies using the technique of fertilization of hamster eggs (Martin *et al.*, 1982, 1983, 1987; Martin, 1984; Martin & Rademaker, 1987). We have previously demonstrated that the frequency of structural chromosomal abnormalities in spermatozoa increases with the age of the donor (Martin & Rademaker, 1987) but there was no significant difference in the ages of the donors who participated in the sonication experiments (37 and 28 years) and those who participated in the TEST-yolk buffer experiments (37, 28 and 25 years). In 35 normal men, the variation in the frequency of structural chromosomal abnormalities is 0–23% (Martin *et al.*, 1987). To ensure that none of the donors who participated in the microinjection experiments had abnormally high frequencies of sperm chromosomal abnormalities with the technique of sperm fertilization, all the donors were tested by this method (Martin, 1983) and found to have normal frequencies of structural chromosomal abnormalities in spermatozoa (0–12%). Therefore, although our numbers are small, our results suggest that sonication induces chromosomal abnormalities in spermatozoa. Our study also suggests that human spermatozoa are more susceptible to sonication damage (or damage caused by a combination of sonication and microinjection) since we observed a much higher frequency of chromosomal abnormalities in human spermatozoa (91%) than has been reported in hamster (20%) or vole (21%) spermatozoa (Libbus *et al.*, 1987).

However, even spermatozoa treated with TEST-yolk buffer before microinjection appear to have an elevated frequency of structural chromosomal abnormalities (39%) compared to sperm chromosome karyotypes obtained after sperm fertilization of hamster eggs (3–13% in four large studies: Martin *et al.*, 1983, 1987; Brandriff *et al.*, 1985b; Kamiguchi & Mikamo, 1986). Various techniques for sperm capacitation have been used to obtain sperm karyotypes after sperm penetration of hamster eggs, including TEST-yolk buffer (Brandriff *et al.*, 1985b) and none of them have resulted in a frequency of structural abnormalities as high as observed after microinjection. Our results therefore suggest that microinjection of spermatozoa may induce structural chromosomal abnormalities. The donors who participated in the microinjection experiments also had sperm chromosome complements studied by the technique of sperm fertilization of hamster eggs. Unfortunately not all the donors could be studied using TEST-yolk buffer as the capacitation medium, but 2 of the donors who participated in the microinjection TEST-yolk buffer experiments were also used for fertilization experiments with TEST-yolk as the capacitation medium. The frequency of structural abnormalities in these experiments was 0–12%. Therefore neither the TEST-yolk buffer treatment nor individual donor variation in the frequency of abnormalities appears to be the explanation for the high frequency of structural chromosomal abnormalities after microinjection. However, we have studied only a small number of sperm chromosome complements after microinjection and it is possible that statistical fluctuation or some factor other than the microinjection was responsible for the high frequency of structural abnormalities observed.

Microinjection of spermatozoa into homologous and heterologous eggs is a valuable technique to study early interactions of gametes and embryonic development. Microinjection of human spermatozoa into the ooplasm has been used experimentally for diagnosing male infertility in human in-vitro fertilization programmes (Lazendorf *et al.*, 1987) and microinjection of spermatozoa into the perivitelline space has been suggested as a potential treatment of human infertility (Metka *et al.*, 1985; Laws-King *et al.*, 1987). Our study demonstrates that it is possible to analyse human sperm chromosome complements after microinjection into the ooplasm of hamster eggs. Although our results are preliminary, our study also suggests that sonication induces a high frequency of structural chromosomal abnormalities in human spermatozoa and that microinjection into the ooplasm *per se* may cause an increased frequency of chromosomal abnormalities. Before the technique of microinjection of human spermatozoa into eggs is used clinically in in-vitro fertilization programmes, it will be necessary to assess the chromosomal normality of an adequate sample size of microinjected eggs.

We thank Leona Barclay, Teresa Beckett, Judy Chernos, Kathy Hildebrand and Leslie Long-Simpson for helpful discussions and suggestions about this project; Dr Brigitte Brandriff for help with the TEST-yolk buffer procedure; and Dr Sally Perreault for help with the microinjection technique.

This study was supported by the Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research and the Alberta Children's Hospital Research Foundation.

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Received 6 January 1988