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Chromosome Analysis of BALB/c Mouse Spermatozoa with Normal and Abnormal Head Morphology[1]

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ABSTRACT

To assess the relationship between mouse sperm head morphology and karyotype, sperm heads with either a normal or an abnormal morphology were injected individually into enucleated mouse oocytes that were karyotyped at the metaphase of the first cleavage. BALB/c male mice that produce an unusually high proportion of morphologically abnormal spermatozoa were used as sperm donors. Abnormal karyotypes were found in a significantly higher proportion of eggs injected with severely misshapen sperm heads (36–38%) as compared to those injected with normal and quasi-normal heads (15–21%) ($p < 0.01$). Most karyotype abnormalities were structural rather than numerical, the most common being breaks and exchanges of chromosome type in both normal and abnormal spermatozoa.

INTRODUCTION

In many mammalian species, including humans and the great apes, males produce a considerably large number of morphologically abnormal spermatozoa. Reportedly, as compared to normal spermatozoa, morphologically abnormal spermatozoa are less able to pass through the cervix [1, 2], the uterotubal junction [3–5], and the oocyte vestments [6, 7]. Some strains of mice (e.g., C57BL/Kw, PL/J2-azh/azh, KE, and BALB/c) are known to produce very high proportions of structurally abnormal spermatozoa. However, it is not certain whether all morphologically abnormal spermatozoa are necessarily genetically defective. Burruel et al. [8] microsurgically injected BALB/c mouse spermatozoa into mouse oocytes and found that spermatozoa with grossly misshapen heads were able to produce normal offspring. In the present study, we karyo-analyzed BALB/c mouse spermatozoa with normal and abnormal head morphologies.

MATERIALS AND METHODS

Animals

Spermatozoa were collected from caudae epididymides of BALB/cAnNCr mice and hybrid mice B6D2F1 (C57BL/6 × DBA/2J). B6D2F1 females were used as oocyte donors. All mice were purchased through the National Cancer Institute and maintained in a temperature- and light-controlled room (14L:10D) for at least 2 wk before use. When used in experiments B6D2F1 mice were 6–12 wk old, and BALB/c mice were 6–9 mo old. All animals were maintained in accordance with the guidelines of the Laboratory Animal Service at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEW publication no. [NIH] 80–23, revised in 1985). The protocol of our animal handling and treatment was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Reagents

Polyvinyl alcohol (PVA, cold water soluble, molecular weight about 10 000) and polyvinyl pyrrolidone (PVP, molecular weight about 360 000) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine testicular hyaluronidase (200 USP U/mg) was obtained from ICN Biochemicals (Costa Mesa, CA). BSA (fraction V) was purchased from Calbiochem (La Jolla, CA) and mineral oil from Squibb and Sons (Princeton, NJ). All other reagents were obtained from Sigma unless otherwise stated.

Media

CZB medium [9] supplemented with 5.56 mM D-glucose was used for the culture of mouse oocytes after microsurgery. The medium for collection of oocytes from oviducts and subsequent oocyte treatments, including micromanipulation, was a modified CZB (Hepes-CZB [10]) containing 20 mM Hepes-HCl, a reduced amount of $NaHCO_3$ (5 mM), and 0.1 mg/ml PVA instead of BSA. CZB was used under 5% CO_2 in air, and Hepes-CZB was used under air.

Preparation of Oocytes

B6D2F1 females were each injected with 5 IU eCG followed by 5 IU hCG 48 h later. Mature oocytes were collected from oviducts about 15 h after the hCG injection. They were freed from the cumulus cells by 5-min treatment with 0.1% bovine testicular hyaluronidase in Hepes-CZB. The oocytes were rinsed, and before micromanipulation they were kept in CZB medium for up to 4 h at 37°C in 5% CO_2 in air.

Classification of Head Morphology in BALB/c Spermatozoa

Sperm head morphology was classified according to Burruel et al. [8] as normal, quasi-normal, and grossly abnormal. Quasi-normal heads included those missing the rostral part of the acrosome and/or the posterolateral region of the postacrosomal region. Grossly abnormal heads included collapsed and triangular heads with highly deformed acrosomal caps and nuclei (Fig. 1).

Enucleation of Oocytes

The oocytes were transferred into a droplet of Hepes-CZB containing 5 μg/ml cytochalasin B, which had previously been placed in the operation chamber on the mi-

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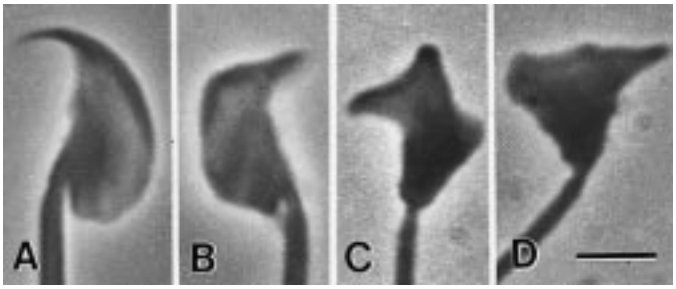

FIG. 1. Head morphology of BALB/c spermatozoa. **A**) Normal, **B**) quasi-normal, **C**) collapsed, **D**) triangular. Bar = 3 μm.

croscope stage and kept there for 5–10 min. Enucleation of the oocytes was performed by aspirating the metaphase II chromosome-spindle complex up into a pipette (8–10 μm in the inner diameter) with a minimal volume of oocyte cytoplasm [11]. Enucleated oocytes were then transferred into cytochalasin B-free CZB and kept there for up to 3 h at 37°C before sperm injection.

Intracytoplasmic Sperm Injection (ICSI)

ICSI was carried out according to Kimura and Yanagimachi [10], but with some modifications. A drop (50 μl) of sperm suspension was mixed thoroughly with 100 μl of Hepes-CZB medium containing 12% (w:v) PVP (PVP-Hepes). Part of this mixture was transferred to the micromanipulation chamber on the microscope stage. To examine whether the prior separation of the sperm head from the tail and the enucleation of mature oocytes affected the sperm chromosome complement, spermatozoa were injected in three different ways. In the first (I), a single motile spermatozoon with normal head was selected and drawn into an injection pipette of inner diameter of 7–8 μm at the tip. Immediately before injection, a few piezo-pulses were applied to the midpiece region of the spermatozoon to immobilize it; then its whole body was injected into an intact oocyte. In the second procedure (II), a motile spermatozoon with normal head was selected, its head was separated from tail by applying one or a few piezo-pulses to the neck region [12], and the sperm head alone was injected into an intact oocyte. In the third (III), an isolated sperm head obtained as above was injected into an enucleated oocyte. All microsurgical operations were performed at 25°C in Hepes-CZB. Sperm-injected oocytes were cultured in 50-μl droplets of CZB medium under mineral oil at 37°C.

TABLE 1. Analyses of sperm chromosomes after injection into oocytes using three different methods.

Strain of sperm donor	Method of sperm injection*	No. of experiments	No. of eggs analyzed	No. (%) of eggs with normal sperm chromosomes‡	Sperm's X/Y ratio
B6D2F1	I	3	40	35 (87.5%)	0.87
	II	3	98	90 (91.8%)	0.92
	III	3	42	36 (85.7%)	1.06
Balb/c†	I	4	60	50 (83.3%)	1.88
	II	3	89	76 (85.4%)	0.90
	III	11	184	157 (85.3%)	1.31

* I: Whole sperm injection into intact MET II oocyte; II: Isolated sperm head injection into intact Met II oocyte; III: Isolated sperm head injection into enucleated oocyte.
† Only the spermatozoa with normal head morphology were injected into oocytes.
‡ There were no significant differences among all different groups.

Chromosome Examination

Seven to eight hours after ICSI, the oocytes were transferred into an another droplet (0.2 ml) of CZB containing 0.006 μg/ml vinblastine. Vinblastine prevented mingling of sperm and egg chromosomes by disrupting ooplasmic microtubules. Between 19 and 21 h after ICSI, eggs arrested at metaphase of the first cleavage were treated with 1% (w:v) Pronase (Kaken Pharmaceuticals, Tokyo, Japan) for 5 min to remove the zona pellucida, then with a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% fetal bovine serum) for 10 min at room temperature. Fixation of eggs and spreading of chromosomes were performed according to Mikamo and Kamiguchi [13]. The chromosomes on slides were stained with 2% Giemsa solution for 8 min. After conventional chromosome analysis, they were C-banded [14] to detect acentric and dicentric chromosomes [13]. With use of ICSI methods I and II, an oocyte injected with a spermatozoon was considered chromosomally normal when it contained 40 structurally normal chromosomes at the metaphase of the first mitotic division, as we could not always distinguish between chromosomes derived from the male pronucleus and those from the female pronucleus. When method III was used, all the chromosomes were of sperm origin. Thus, with use of method III the normality and abnormality of sperm chromosomes could be assessed without intervention of oocyte chromosomes.

Statistical analysis was done using the chi-square test.

RESULTS

As shown in Table 1, there was no significant difference in the incidence of sperm chromosome abnormalities among the three experimental groups. Previous investigators reported that the rate of chromosome abnormality including structural and numerical aberrations derived from the female pronucleus was less than 5% using in vitro fertilization technique [15, 16]. In this study, it was also low (3 of 72, 4.17%) in the oocytes in which male and female pronuclei were clearly separated and chromosomes from male pronuclei contained the Y chromosome. Therefore, we ignored the influence of chromosome abnormalities derived from the female pronucleus when we compared the three methods of injection. Thus, the chromosome complements were not changed either by isolation of sperm heads from tails or by enucleation of oocytes prior to their injection with a spermatozoon.

Table 2 summarizes chromosome analyses of BALB/c spermatozoa with normal and abnormal head morphologies using method III. The incidence of a normal karyotype was significantly higher in the spermatozoa with normal and quasi-normal heads than in those with severely misshapen heads ($p < 0.01$). The difference between normal-headed and quasi-normal-headed spermatozoa was not significant with respect to the incidence of normal/abnormal karyotypes. Regardless of sperm head morphology, the predominant types of structural chromosome aberration were breaks and exchanges of chromosome type. There was no great difference in the distribution of aberration types among the four groups of spermatozoa. X/Y ratios were not significantly different from the expected X/Y ratio (= 1) in all the groups of spermatozoa. Finally, no significant increase in aneuploidy was evident in any of the groups. The

TABLE 2. Chromosome analysis of Balb/c mouse spermatozoa with normal and abnormal head morphologies: Method III was used.

Sperm head morphology	Exp. no.	No. of eggs analyzed	Sperm with normal karyotype		Sperm with structural chromosome aberrations		Total no. of structural aberrations				
							Chromosome-type		Chromatid-type		
			No. (%)	X/Y ratio	No. (%)	X/Y ratio	Break	Exchange	Break	Exchange	Aneuploidy
Normal	11	184	157 (85.3%)[a]	1.31	26 (14.1%)	0.44	25	22	10	0	1 (0.5%)
Quasi-normal	10	130	103 (79.2%)[b]	0.94	26 (20.0%)	1.89	18	11	4	0	1 (0.8%)
Collapsed	12	161	104 (64.6%)[c]	0.79	54 (33.5%)	0.86	68	60	35	2	2 (1.9%)
Triangular	8	98	61 (62.2%)[c]	0.58	37 (37.8%)	0.68	43	38	8	1	0

[a–c] a vs c: $p < 0.0001$; b vs c: $p < 0.01$ (chi-square test).

aneuploidy that did occur in this study involved chromosomes 19 (n−1) or 21 (n+1).

DISCUSSION

The present study shows that about 15% of morphologically normal BALB/c spermatozoa carry chromosomal abnormalities. This percentage was considerably higher than we had anticipated. Several previous groups [15–18] have investigated the incidence of chromosomally abnormal spermatozoa by examining sperm chromosomes at the metaphase of the first cleavage of oocytes inseminated in vitro. The values obtained were 1.43% in outbred TO mouse [15], 0.9% in the RFM mouse [16], 4.29% in the C57B1/6J × CBA/Ca hybrid mouse [17], and 8.17% in the ICR mouse [18]. As structurally abnormal mouse spermatozoa seldom fertilize oocytes under ordinary in vivo and in vitro conditions [6], the figures cited above must represent the incidence of chromosomal abnormality in structurally normal spermatozoa.

We found that about 35% of BALB/c spermatozoa with grossly misshapen heads (collapsed and triangular) had chromosome abnormalities, significantly higher than for those with normal or quasi-normal heads (15–20%) ($p <$ 0.01–0.0001) (Table 2). There were no significant differences, however, between normal and quasi-normal spermatozoa in that regard (Table 2). All aneuploidy that we found in this study involved either 19 (n−1), or 21 (n+1), and its incidence was 0–1.9%. This is in agreement with a previous report indicating that hyperploidy occurs in less than 0.4% of secondary spermatocytes in BALB/c [19]. Lee et al. [20] found that the incidence of structural chromosome aberrations in human spermatozoa with deformed heads (26.1%) was almost four times higher than in those with normal heads (6.9%). Although it has been believed that there are no correlations between sperm (head) morphology, concentrations of DNA/non-DNA materials, and the sperm karyotype [4, 18, 21–23], our results suggest that structurally abnormal spermatozoa are apt to have chromosome abnormalities.

According to the study by Hugenholtz and Bruce [24], various mutagenic agents can induce misshapen mouse spermatozoa. These investigators maintain that sperm shape is highly controlled by various autosomal and sex chromosomal factors and is highly heritable. The high incidence of chromosome aberrations reported here in structurally abnormal spermatozoa could be due to the action of defective genes that cause abnormalities in sperm chromosomes as well as in sperm head morphology.

The nuclei of structurally normal and abnormal spermatozoa may differ in the rate of decondensation after entry in the oocytes. According to Tateno and Kamiguchi [25], sperm chromosomes tend to break when decondensation of sperm nuclei within activated oocytes is delayed. Even though there may be no strict relationship between sperm head morphology and S-S concentration of sperm protamine [21, 23], differences in some other structural or chemical components of normal and abnormal spermatozoa may result in marked differences in the behavior of their nuclei within oocytes.

Finally, the unexpectedly high incidence of chromosomal abnormality reported here may be due, in part, to the techniques employed. Whole sperm heads or even entire spermatozoa were injected directly into oocytes. The intact acrosome and the overlying sperm plasma membrane, which never enter the ooplasm during normal fertilization [26], were injected into the ooplasm. Kimura et al. [27] showed that mouse oocytes injected with mouse spermatozoa survived and developed into normal offspring, whereas those injected with hamster or rabbit spermatozoa did not. Even though mouse oocytes were activated by hamster and rabbit spermatozoa, all of them became deformed and then degenerated (see Figs. 1 and 2 of Kimura et al. [27]). Interestingly, the oocytes survived and did not degenerate when hamster and rabbit spermatozoa were sonicated before injection. As sonication removes almost all of the acrosomal contents from sperm heads, it could be that the acrosomal contents (enzymes) in hamster and rabbit spermatozoa damaged the mouse oocytes. It is known that the acrosomal enzymes are present in the principal segment of the acrosome. As this region of the acrosome is much smaller in mouse spermatozoa than in hamster and rabbit spermatozoa [26], the survival of mouse oocytes after injection of intact mouse spermatozoa may be related to the small size of this region of the acrosome. Even though the amount of acrosomal enzymes introduced into each oocyte is very small, such exogenous enzymes may nevertheless damage the sperm nucleus. Thousands of apparently healthy human offspring have been born through the ICSI procedure [28, 29], which involves direct injection of acrosome-intact spermatozoa into oocytes. Many normal mouse offspring were also born after ICSI using acrosome-intact spermatozoa [10, 30, 31]. However, this does not imply that all the oocytes injected with acrosome-intact spermatozoa had developed normally. It is highly probable that some sperm-injected oocytes did not develop normally and were aborted before or after implantation. It is possible that the acrosome (acrosomal enzymes) introduced into an oocyte by ICSI damages the oocyte's cytoplasm and/or nucleus. Under normal conditions, the fertilizing spermatozoon discards virtually all acrosome material before it unites with an oocyte [26]. Two reports indicated that mouse and human oocytes developed better when acrosome-reacted spermatozoa were injected rather than acrosome-intact ones [31, 32]. The reason for this is unknown, but it could be that the acrosome contents (enzymes) introduced into the oocyte's cytoplasm interfere with normal embryo development. Some mouse and human oocytes may be able to tolerate exotic acrosomal enzymes, but oth-

ers may not. We are now investigating whether this assumption is valid.

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