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ASSISTED REPRODUCTION

Chromosome analysis of mouse zygotes produced by intracytoplasmic injection of spermatozoa exposed to acrosome reaction inducing agents methyl-β-cyclodextrin and calcium ionophore A23187

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Abstract
Purpose This study was performed to investigate whether removal of cholesterol from the plasma membrane and collapse of the acrosome can prevent structural chromosome aberrations of paternal origin in mouse zygotes produced by intracytoplasmic sperm injection (ICSI).
Methods Mouse spermatozoa were treated with methyl-β-cyclodextrin (MβCD) to remove cholesterol from the plasma membrane and with calcium ionophore A23187 to collapse the acrosome. Chromosomes of zygotes derived from MβCD- and ionophore-treated spermatozoa were analyzed at the first mitotic metaphase.
Results Both chemical agents effectively induced the acrosome reaction. Incidence of structural chromosome aberrations in ICSI zygotes derived from MβCD-treated spermatozoa was similar to that in zygotes produced by in vitro fertilization (IVF) with the same spermatozoa, but significantly lower compared to ICSI zygotes derived from acrosome-intact spermatozoa. Chromosome aberration rates in ICSI zygotes derived from ionophore-treated spermatozoa were evidently high compared to IVF zygotes.
Conclusions Induction of the acrosome reaction through cholesterol efflux by MβCD can prevent chromosome aberrations of paternal origin, while use of ionophore to induce the acrosome reaction exerts detrimental effect on paternal chromosomes in ICSI zygotes.

Capsule Structural chromosome aberrations in ICSI zygotes were prevented when acrosome reaction was induced by methyl-β-cyclodextrin, but generated when ionophore was used to induce the reaction.

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Introduction

With fertilization in mammals, spermatozoa undergo the acrosome reaction, in which hydrolytic enzymes help the passage of sperm through the zona pellucida enclosing the oocyte. Prior to the acrosome reaction, spermatozoa undergo capacitation, in which cholesterol dissociates from the plasma membrane overlying the acrosomal region [1]. With the intracytoplasmic sperm injection (ICSI) technique, uncapacitated spermatozoa with intact acrosomes are usually used to produce embryos, so the cholesterol-rich plasma membrane and acrosome enzymes are injected into the ooplasm. These spermatozoal structures have been suggested to affect sperm chromatin remodeling in mice [2], pigs [3] and rhesus monkeys [4–6]. While injection of uncapacitated spermatozoa with intact acrosomes affects development of mouse ICSI embryos [7], simultaneous removal of the plasma membrane and acrosome before ICSI improves embryonic development [8]. Supportive evidence has been presented for the rat [9].

Previous study found that when mouse spermatozoa were used for ICSI shortly after collection from the cauda epididymis, incidences of structural chromosome aberrations in resultant zygotes were high compared to zygotes conventionally produced by in vitro fertilization (IVF) procedure [10]. However, the aberration rate was reduced when spermatozoa were incubated for 2 h or more in bicarbonate-buffered TYH medium, which can effectively induce the capacitation and acrosome reaction in mouse spermatozoa [11]. Furthermore, when sperm incubation was carried out in hepes and phosphate-buffered media that

never induce the capacitation and acrosome reaction, the chromosome aberration rate in resultant ICSI zygotes was raised in a time-dependent manner, but the time-dependent increase in chromosome aberrations disappeared when testicular spermatozoa with a low content of cholesterol in the plasma membrane were used [12]. These findings suggest that the membrane cholesterol and acrosome enzymes are involved in generation of structural chromosome aberrations of paternal origin in ICSI zygotes, and removal of cholesterol from the plasma membrane and induction of the acrosome reaction before ICSI can reduce risk of generating chromosome aberrations.

It has been demonstrated that methyl-β-cyclodextrin (MβCD) allows mouse spermatozoa to capacitate by promotion of cholesterol efflux from the plasma membrane [13–15]. Calcium ionophore A23187 is commonly used to induce the acrosome reaction in mammalian spermatozoa. In the present study, attempts were made to examine whether treatment of mouse spermatozoa with MβCD and ionophore before ICSI can prevent structural chromosome aberrations of paternal origin in ICSI zygotes.

Materials and methods

Chemicals

All inorganic reagents and polyvinylpyrrolidone (PVP) were purchased from Nacalai Tesque (Kyoto, Japan). Lipid-rich bovine serum albumin (BSA) (AlbuMax; Gibco BRL, Auckland, New Zealand) was used instead of conventional fraction V albumin. Polyvinyl alcohol (PVA) (cold-water soluble), hyaluronidase, antibiotics, vinblastine sulfate, methyl-β-cyclodextrin (MβCD), FITC-conjugate peanut agglutinin (PNA), and fetal bovine serum (FBS) were products of Sigma–Aldrich (St. Louis, MO, USA). Actinase E (Kaken Pharmaceuticals, Tokyo, Japan) was used as a protease. Calcium ionophore A23187 and Hoechst 33258 were products of Calbiochem (Merck KGaA, Darmstadt, Germany). Paraffin oil (Art. 1.07162.1000) was purchased from Merck Japan (Tokyo) and Vectashield from Vector Laboratories (Burlingame, CA, USA). Gonadotropic hormones eCG and hCG were products of Teikoku–Zoki Pharmaceuticals (Tokyo) and Aska Pharmaceuticals (Tokyo), respectively.

Media

Bicarbonate-buffered TYH [16] and hepes-buffered H-TYH [10] were used as media for preparing spermatozoa. When spermatozoa were treated with ionophore, the concentration of calcium in TYH was doubled to effectively induce the acrosome reaction [17]. This modified TYH with two-fold of Ca^{2+} was designated as mTYH. Hepes-buffered H-mCZB [18] was used for preparing oocytes where appropriate. Temporary storage of oocytes and cultivation of fertilized ova were performed using bicarbonate-buffered CZB [19] with modification by addition of 5.56 mM glucose. This was designated as mCZB. H-TYH and H-mCZB were used under 100% air, and TYH, mTYH and mCZB were used under 5% CO_2 in air.

Animals

Oocytes and spermatozoa were collected from hybrid B6D2F1 mice. Animals were maintained under conditions of 14 h-lighting and a temperature of 23±2°C, and provided with ad libitum access to food and water. All experiments were performed according to the Guidelines for Animal Experiments of Asahikawa Medical College.

Preparation of oocytes and spermatozoa

Female mice at 7–12 weeks old were intraperitoneally injected with 8–10 IU eCG, followed 48 h later with an injection of 8–10 IU hCG to induce superovulation. Approximately 16 h after hCG injection, MII oocytes were collected from the oviducts and placed in H-mCZB with 0.1% hyaluronidase for 3–5 min at 37°C to remove cumulus cells. After thorough washing in mCZB, cumulus-free oocytes were kept in the same medium under paraffin oil at 37°C. Mature spermatozoa were collected from the cauda epididymis of male mice at 7–12 weeks old.

Treatment of spermatozoa with MβCD and ionophore

For treatment with MβCD, spermatozoa were directly collected from the epididymis into a droplet (150 μl) of H-TYH with 1 mM of MβCD under paraffin oil at a concentration of $2–4\times10^7$ cells/ml. Spermatazoa were incubated for 2 h at 37°C in the experiment for IVF and for 3–3.5 h in the experiment for ICSI.

Treatment of spermatozoa with ionophore was performed under three different sperm incubation conditions: no-incubation, post-incubation and pre-incubation. With no-incubation, spermatozoa were directly released from the epididymis into a droplet (150 μl) of TYH at a concentration of $2–4\times10^7$ cells/ml under paraffin oil. After dispersing for 5 min, an aliquot (100 μl) of sperm suspension was transferred to 2.5 ml of mTYH with 20 μM of ionophore, and treated for 10 min at 37°C. Spermatozoa were washed twice with normal TYH by centrifugation of 350*g* and immediately used for ICSI and IVF. With post-incubation, spermatozoa were additionally incubated in TYH for 2 h at 37°C after treatment with ionophore, then used for ICSI and IVF. With pre-incubation, spermatozoa were incubated in

TYH for 2 h at 37°C before treatment with ionophore and used for ICSI and IVF without additional incubation.

Immunocytological staining of acrosome

Responsiveness of the acrosome to MβCD was examined after treatment for 3 h. Acrosome status of ionophore-treated spermatozoa was examined immediately before use for IVF or ICSI under the three different sperm incubation conditions. The procedure for staining the acrosome was basically performed as described by Mendosa et al. [20]. In brief, an aliquot (50 μl) of sperm suspension was put in a 15-ml plastic tube, and the same volume of Hoechst 33258 (4 μg/ml) was added to the tube. The staining reaction was continued for 5 min at 37°C to distinguish live spermatozoa from dead spermatozoa. Spermatozoa were washed once with H-TYH, then fixed with 1% paraformaldehyde for 1 h at room temperature (RT). After washing twice with H-TYH, spermatozoa were smeared on a slide glass and treated with 100% methanol for 15 min at RT in the dark. The smear was dried and treated with FITC-PNA (50 μg/ml) for 30 min at RT in the dark. After washing once with distilled water, the smear was dried, covered with Vectashield and immediately examined using a fluorescent microscope. The ratio of live spermatozoa with different acrosome statuses was determined by counting more than 900 cells in five trials.

ICSI using spermatozoa treated with MβCD and ionophore

When spermatozoa were incubated in H-TYH with MβCD for 3–3.5 h, 10 μl of the sperm suspension was first transferred to a droplet (100 μl) of normal H-TYH to dilute MβCD. A small amount (approximately 5 μl) of diluted sperm suspension was then transferred into a droplet (20–30 μl) of H-TYH containing 10% PVP under paraffin oil, which was prepared in a plastic chamber on an inverted microscope with a piezo micromanipulator. A motile spermatozoon with morphologically normal feature was selected under ×200 magnification, and the head was separated from the tail by applying piezo pulses, then immediately injected into an oocyte using a piezo micromanipulator as described by Kimura and Yanagimachi [18]. Injected oocytes were transferred into mCZB for cultivation within 30 min of the manipulation.

The ICSI procedure using ionophore-treated spermatozoa was the same with MβCD-treated spermatozoa.

IVF using spermatozoa treated with MβCD and ionophore

IVF zygotes were conventionally produced to compare the incidence of chromosome aberrations with that of ICSI zygotes. When spermatozoa were incubated in H-TYH with MβCD for 2 h, a small amount (10 μl) of sperm suspension ($2–4\times10^7$ cells/ml) was added to a droplet (100 μl) of H-TYH containing cumulus-free oocytes under paraffin oil. Co-culture of spermatozoa and oocytes was maintained for 4 h at 37°C. Fertilized ova were thoroughly washed with mCZB and transferred into the same medium at 37°C for further cultivation.

In the IVF experiment using ionophore-treated spermatozoa, cumulus-free oocytes were inseminated with spermatozoa at a concentration of approximately 10^6 cells/ml in TYH and kept for 2 h at 37°C. After being washed with mCZB, fertilized ova were cultured in the same medium at 37°C.

IVF zygotes derived from spermatozoa that underwent capacitation in normal TYH served as control.

Preparation of chromosome slides and analysis

At 6–8 h after ICSI or IVF, zygotes were exposed to vinblastine sulfate (0.02 μg/ml) to prevent syngamy and spindle formation. At the first cleavage metaphase, zygotes were processed for chromosome preparations. In brief, zygotes were treated with 0.5% actinase E to digest the zona pellucida, then placed in hypotonic solution (1:1 mixture of 1% sodium citrate solution and 30% FBS) for 8 min at RT. Chromosome slides were made by the gradual fixation-air drying method [21]. Chromosome slides were stained with 2% Giemsa solution for 8 min. After conventional analysis to detect chromosome break, gap, ring and chromatid exchange, slides were stained using the C-banding technique to detect dicentric aberration and to distinguish between structural chromosome aberration and aneuploidy [22]. In the present study, incidence of chromosome aberrations was represented as that in zygotes, because paternal chromosome complements were not always separated from maternal ones in some chromosome preparations. As described elsewhere [23], the incidence of structural chromosome aberrations of maternal origin in mouse ICSI zygotes remains fairly constant (0–2.8%) despite the occurrence of aberrations in paternal chromosomes. In the present study, therefore, increased incidence of structural chromosome aberrations in zygotes derived from treated spermatozoa was considered to have been caused by the increase of structural chromosome aberrations of paternal origin. Polyploid zygotes due to polyspermy in the IVF experiment and triploid zygotes due to suppression of the second polar body in the ICSI experiment were eliminated from chromosome analysis.

Statistical analysis

The χ^2 test or Fisher's exact probability test was used to compare the percentage of zygotes with chromosome aberrations. Differences in frequency of acrosome-reacted spermatozoa were examined using the Mann–Whitney *U*-

test. When more than one pair of frequencies was to be compared, a non-parametric Kruskal–Wallis one-way analysis of variance and Scheffe's method were used. Differences were considered significant at the level of $P<0.05$.

Results

Induction of the acrosome reaction by MβCD and ionophore

In the present results, acrosome status was divided into four categories: intact (type A); condensation (type B); collapse (type C); and elimination (type D) (Fig. 1). Spermatozoa of types C and D were regarded as acrosome-reacted spermatozoa. Over 95% of spermatozoa incubated in H-TYH without MβCD for 3 h had an intact acrosome (type A) (Fig. 2). When sperm incubation was carried out in H-TYH with MβCD for the same period, the percentage of acrosome-intact (type A) spermatozoa was extremely reduced, and in contrast that of acrosome-reacted (types C and D) spermatozoa was markedly raised. Thus the cholesterol efflux by MβCD was closely coupled with the acrosome reaction in mouse spermatozoa.

Morphological changes of the acrosome following treatment with ionophore appeared similar to those resulting from treatment with MβCD. As expected, ionophore well induced the acrosome reaction (Fig. 3). Spermatozoa that underwent pre-incubation were most sensitive to ionophore, showing the highest percentage (96%) of completely acrosome-reacted (type D) spermatozoa.

Chromosome analysis of zygotes derived from MβCD- and ionophore-treated spermatozoa

When IVF were performed with spermatozoa incubated in H-TYH with MβCD for 2 h, 45–65% of oocytes were fertilized. Incidence (2.8%) of structural chromosome aberrations in IVF zygotes derived from MβCD-treated spermatozoa was not significantly different from that (1.6%) in control IVF zygotes (Table 1). When spermatozoa were used for ICSI after incubation in H-TYH with MβCD for 3–3.5 h, incidence (4.5%) of structural chromosome aberrations in resultant zygotes was close to that in IVF zygotes. However, when spermatozoa were incubated in H-TYH without MβCD for the same time, incidence (10.8%) of structural chromosome aberrations in resultant ICSI zygotes was significantly raised. Chromosome breaks, dicentrics and chromatid breaks discernibly occurred. In the present results, no significant increase of aneuploidy was identified in any group.

Ionophore-treated spermatozoa showed high fertilizing capacity, as more than 80% of inseminated oocytes were successfully fertilized in any sperm incubation condition. Incidences of structural chromosome aberrations in IVF zygotes derived from ionophore-treated spermatozoa were low (1.1–2.8%) regardless of sperm incubation conditions (Table 2). The incidences were similar to that in control IVF zygotes (see Table 1). In ICSI zygotes of ionophore-treated sperm origin, however, structural chromosome aberration rates were markedly higher than in IVF zygotes of matched controls. The chromosome aberration rate of ICSI zygotes in the no-incubation condition was significantly higher than that of ICSI zygotes in the other two incubation conditions. Breakage-type aberrations were predominantly observed in any incubation condition. No increase in aneuploidy was seen for any zygotes produced by IVF and ICSI with ionophore-treated spermatozoa.

Discussion

There was no significant increase in incidence of structural chromosome aberrations in IVF zygotes derived from

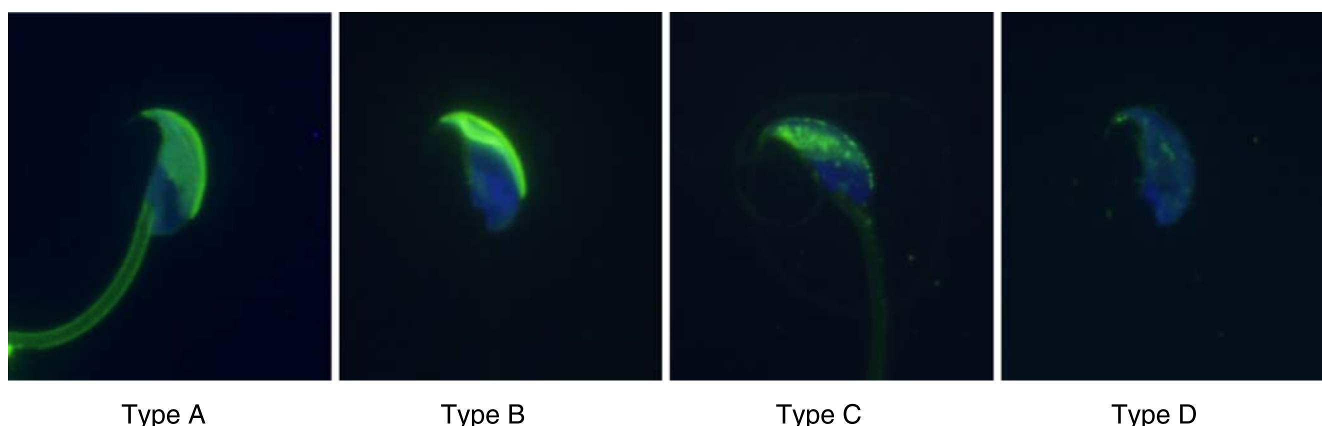

Fig. 1 Classification of acrosome status of mouse spermatozoa after treatment with 1 mM MβCD for 3 h in H-TYH. Type A, intact acrosome; type B, condensed acrosome; type C, collapsed acrosome; and type D, elimination of the acrosome

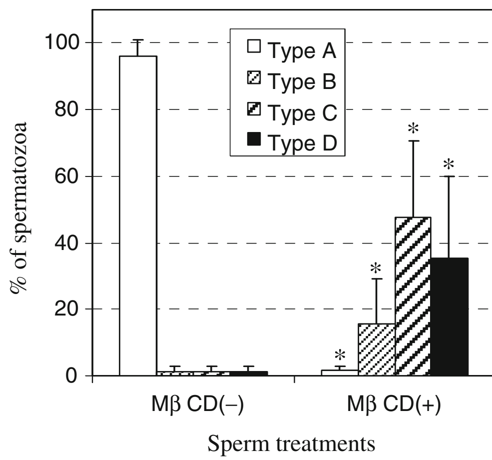

Fig. 2 Comparison between incubations in H-TYH with and without MβCD regarding the ratio of spermatozoa with different acrosome statuses. *$P<0.01$: significant difference compared to MβCD(−) in each type

MβCD-treated spermatozoa and ICSI zygotes derived from MβCD-treated spermatozoa. These findings demonstrate that MβCD has no detrimental effect on sperm chromatin, and cytogenetically supports the previous finding that IVF embryos derived from MβCD-treated spermatozoa show normal developmental ability [15]. In contrast, the increased incidence of structural chromosome aberrations was found in ICSI zygotes derived from acrosome-intact spermatozoa after incubation in H-TYH without MβCD. The incidence was significantly higher than that in ICSI zygotes derived from MβCD-treated spermatozoa. This indicates that induction of the acrosome reaction through the cholesterol efflux by MβCD before ICSI can effectively prevent structural chromosome aberrations of paternal origin in mouse ICSI zygotes. It is probable that removal of the membrane cholesterol and acrosome before ICSI would enable normal sperm chromatin remodeling within

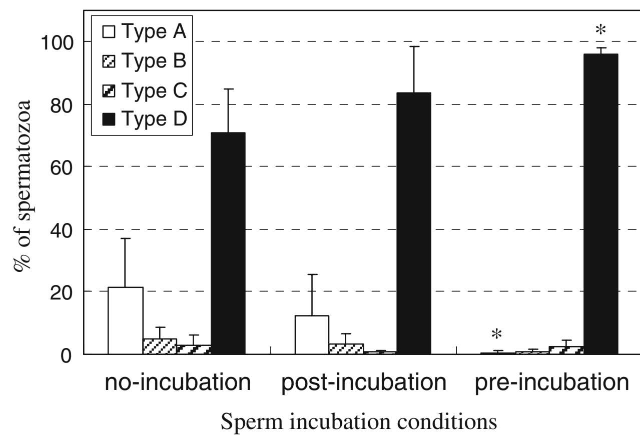

Fig. 3 Comparison among sperm incubation conditions regarding the ratio of spermatozoa with different acrosome statuses after treatment with ionophore A23187. *$P<0.05$, significant difference vs. no-incubation group in each type of acrosome statuses

Table 1 Chromosome analysis of IVF zygotes and ICSI zygotes derived from MβCD-treated spermatozoa

Fertilization method	Sperm treatment with MβCD	No. of zygotes analyzed (no. exp.)	No. of aneuploid zygotes (%)	No. of zygotes with structural chromosome aberrations (%)	No. of aberrant chromosomes								
					Chromosome type					Chromatid type			
					Break	Gap	Dicentric	Translocation	Ring	Break	Gap	Exchange	Total
IVF	Control	253 (4)	2 (0.8)	4 (1.6)	1	2	0	0	0	1	0	0	4
IVF	1 mM, 2 h	211 (5)	3 (1.4)	6 (2.8)	4	0	0	0	0	1	1	0	6
ICSI	0 mM, 3–3.5 h	212 (6)	6 (2.8)	23 (10.8)[a]	15	4	16	0	1	10	0	0	46
ICSI	1 mM, 3–3.5 h	200 (7)	5 (2.5)	9 (4.5)[b]	2	2	4	0	0	0	1	0	9

[a] Significantly high ($P<0.001$) compared to control IVF zygotes derived from spermatozoa incubated without MβCD

[b] Significantly low ($P<0.05$) compared to ICSI zygotes derived from spermatozoa incubated without MβCD

Table 2 Chromosome analysis of IVF zygotes and ICSI zygotes derived from ionophore-treated spermatozoa under different sperm incubation conditions

Fertilization method	Sperm incubation conditions	No. of zygotes analyzed (no. exp.)	No. of aneuploid zygotes (%)	No. of zygotes with structural chromosome aberrations (%)	No. of aberrant chromosomes								
					Chromosome type					Chromatid type			
					Break	Gap	Dicentric	Translocation	Ring	Break	Gap	Exchange	Total
IVF	No-inc.	218 (6)	4 (1.8)	6 (2.8)	3	3	1	0	0	1	0	0	8
	Post-inc.	324 (6)	9 (2.8)	9 (2.8)	6	0	2	2	0	0	0	0	10
	Pre-inc.	270 (7)	3 (1.1)	3 (1.1)	1	1	0	0	0	0	0	1	3
ICSI	No-inc.	209 (8)	4 (1.9)	49 (23.4)[a,b,c]	32	16	13	6	0	6	3	3	79
	Post-inc.	215 (8)	6 (2.8)	34 (15.8)[a]	15	16	6	2	1	4	2	1	47
	Pre-inc.	201 (8)	4 (2.0)	26 (12.9)[a]	11	14	2	0	0	0	3	0	30

[a] $P<0.001$ compared to IVF zygotes of the matched control

[b] $P<0.05$ and [c] $P<0.01$ compared to ICSI zygotes in post-incubation and pre-incubation, respectively

the ooplasm after ICSI. MβCD is known to induce the acrosome reaction in human spermatozoa [24]. If structural chromosome aberrations of paternal origin in human ICSI zygotes are caused by the same mechanism as seen in mouse ICSI zygotes, treatment of human spermatozoa with MβCD may reduce chromosomal risk.

Ionophore effectively induced the acrosome reaction in mouse spermatozoa, and incidences of structural chromosome aberrations in IVF zygotes derived from ionophore-treated spermatozoa were similar to that in control IVF zygotes. However, when ionophore-treated spermatozoa were used for ICSI, incidences of structural chromosome aberrations in resultant zygotes were evidently raised in any sperm incubation condition. The previous studies found that when mouse uncapacitated spermatozoa with intact acrosome were used for ICSI following short incubation (≤0.5 h) in TYH, incidence of structural chromosome aberrations in resultant zygotes was approximately 7%. When spermatozoa underwent capacitation and acrosome reaction following incubation in TYH for 2–2.5 h, the incidence was reduced to 3.8% [10, 23]. In the present study, however, the incidence (23.4%) was unaccountably high when spermatozoa were treated with ionophore under the no-incubation. The chromosomal disorder was not sufficiently conquered even when spermatozoa were incubated in TYH for 2 h before or after the ionophore treatment. Therefore, it seems likely that causal factors other than the membrane cholesterol and the acrosome may be involved in generation of structural chromosome aberrations of paternal origin in mouse ICSI zygotes derived from ionophore-treated spermatozoa.

Endonucleases in mature epididymal spermatozoa of mice can be activated by ionophore, resulting in degradation of sperm DNA [25]. Phosphatidylserine externalization of the plasma membrane, one of the earliest signs of apoptosis, has been reported in human spermatozoa after treatment with ionophore [26]. Assuming that these alterations caused by ionophore are closely linked to generation of paternal chromosome aberrations, a possible explanation for the discrepancy in chromosome damage between IVF zygotes and ICSI zygotes is that the altered spermatozoa fail to pass through the zona pullucida. Chromosome analysis of IVF zygotes from zona-free oocytes fertilized with ionophore-treated spermatozoa and ICSI zygotes derived from ionophore-treated spermatozoa that have passed through the zona pellucida may provide clues to resolving this issue.

In conclusion, MβCD effectively induces the acrosome reaction in mouse spermatozoa through cholesterol efflux from the plasma membrane. This treatment can reduce risk of generating structural chromosome aberrations of paternal origin in resultant ICSI zygotes. Ionophore is also effective in inducing the acrosome reaction, but the treatment exerts detrimental effect on paternal chromosomes in ICSI zygotes.

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Conflict of interest statement The author declares that there are no conflicts of interest.

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