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Cyclodextrin Removes Cholesterol from Mouse Sperm and Induces Capacitation in a Protein-Free Medium[1]

Young-Ho Choi[2] and Yutaka Toyoda

Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan

ABSTRACT

Cyclodextrin, which stimulates cholesterol efflux from cells, was examined for its ability to induce capacitation of mouse spermatozoa. A chemically defined, protein-free medium was used for in vitro fertilization of cumulus-free mouse eggs. Fertilization did not occur in modified Krebs-Ringer bicarbonate medium (TYH) supplemented with 1 mg/ml polyvinylalcohol instead of BSA. However, fertilization was observed when spermatozoa were preincubated with methyl-β-cyclodextrin (MBCD); fertilization rates increased dose-dependently from 0.25 to 0.75 mM MBCD. The fertilization rate decreased when 0.75 mM MBCD was added to both preincubation and fertilization media versus only the preincubation medium (21% vs. 53%); in sharp contrast, fertilization increased when 4 mg/ml BSA was present in both of the media versus the preincubation medium only (66% vs. 25%). At 0.75 mM, 2-hydroxy-β-cyclodextrin had a lower ability to capacitate spermatozoa in vitro than MBCD (14% vs. 41%). Eggs fertilized by spermatozoa treated with MBCD (0.75 mM) developed to blastocysts (45%, 36 of 80) when cultured in KSOM. When 160 fertilized eggs were transferred to ICR recipients, 62 live offspring were born. After incubation of mouse spermatozoa for 90 min in 0.75 mM MBCD in TYH medium, the cholesterol content of the spermatozoa was significantly ($p < 0.01$) lower than that of the control (2.27 ± 0.09 vs. 4.13 ± 0.09 nmol unesterified cholesterol/10^7 sperm; mean ± SEM, n = 5). The proportion of capacitated (B pattern) spermatozoa determined by chlortetracycline fluorescence was higher with MBCD treatment for 90 min than for the control (45% vs. 15%; $p < 0.01$). The proportion of acrosome-reacted (AR pattern) spermatozoa was not different between MBCD treatment and the control. Therefore, MBCD increased capacitation rather than the acrosome reaction of spermatozoa.

INTRODUCTION

Mammalian sperm undergo biochemical and physiological changes in the female reproductive tract, termed capacitation, that enable fertilization [1, 2]. Capacitated spermatozoa then undergo the acrosome reaction, a morphological alteration consisting of a series of point fusions between the outer acrosomal membrane and the overlying plasma membrane [3, 4]. Cholesterol loss from the surface membrane of spermatozoa is one of the molecular events of capacitation in vitro [5–9]. Many studies have shown that removal or loss of cholesterol from the plasma membranes of sperm can be mediated in vitro by cholesterol acceptors present in fluids of the female reproductive tract, such as albumin [5, 7, 10, 11] and sterol-binding protein (high-density lipoprotein [12, 13]).

Serum albumin has been widely used in media for capacitation of spermatozoa and the acrosome reaction [14–17]. The role of BSA may be as a carrier molecule (e.g., for cholesterol, hormones, ions, fatty acids, amino acids, etc.), a buffer for pH, an osmotic pressure regulator, and/or a chelator of heavy metal ions [18, 19]. However, the molecular mechanisms of BSA-induced changes have not been clearly determined, partly because BSA is contaminated by other serum components including enzymes, hormones, lipids, and organic and inorganic ions [19, 20].

Cyclodextrins, cyclic oligosaccharides consisting of 6, 7, or 8 glucopyranose units (referred to as α-, β-, and γ-cyclodextrins), originated from enzymatic degradation of starch through the action of cyclodextrin-glucano-transferase [21]. Cyclodextrins function as carrier molecules and dissolve lipophiles in their hydrophobic core [22]. Among the three kinds of cyclodextrins, β-cyclodextrin has a suitable structure to encase natural and synthetic molecules such as hormones, vitamins, and lipid components [23]. Further derivatives of β-cyclodextrin were made by chemical modifications of the hydroxyl groups, which greatly improved their solubility and ability to dissolve sterols in aqueous solution and also reduced their toxicity [24, 25]. Recently, cyclodextrin was shown to stimulate efflux of cholesterol from cultured cells with high efficiency [26–28].

We report successful in vitro fertilization in mice using methyl-β-cyclodextrin (MBCD) in a chemically defined, protein-free medium. Furthermore, we show that MBCD treatment removes unesterified cholesterol from mouse sperm.

MATERIALS AND METHODS

Media

The medium used for sperm preincubation and fertilization was the same as modified Krebs-Ringer bicarbonate medium (TYH) [14] except that it did not contain BSA. This medium, designated c-TYH (Table 1), contained 0.75 mM MBCD and 1.0 mg/ml polyvinylalcohol (cold water soluble; Sigma Chemical Co., St. Louis, MO) as a substitute for BSA. KSOM was used for in vitro culture of fertilized eggs [29].

Preparation of Spermatozoa

Outbred Jcl:ICR mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Males (more than 2 mo old) were killed by cervical dislocation. Caudae epididymides were excised from two mice, punctured with a 23-gauge needle, and submerged into 400-μl drops of c-TYH including 5 different concentrations of MBCD (experiment 1), hydroxy-β-cyclodextrin (HBCD; 0.75 mM; experiment 2), or 4 mg/ml BSA (fatty acid-free; Yagai Co., Tokyo, Japan; experiment 3). The spermatozoa were allowed to disperse

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[2]Correspondence and current address: Young-Ho Choi, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO 80523. FAX: 970 491 3557; e-mail: ychoi@cvmbs.colostate.edu

Table 1. Composition of c-TYH.*

Component	Concentration	
	mg/100 ml	mM
NaCl	697.6	119.37
KCl	35.6	4.78
$CaCl_2.2H_2O$	25.1	1.71
$MgSO_4.7H_2O$	29.3	1.19
KH_2PO_4	16.2	1.19
$NaHCO_3$	210.6	25.07
Glucose	100.0	5.56
Sodium pyruvate	5.5	0.5
Potassium penicillin G	7.5	
Streptomycin sulfate	5.0	
Phenol red	0.2	
Polyvinylalcohol	1 mg/ml	
Methyl-β-cyclodextrin	1 mg/ml	0.75

* Modified from Toyoda et al. [14].

for 5–10 min, resulting in 0.6–2 $\times$ 10^7 sperm/ml. Spermatozoa were then incubated for 90 min under 5% CO_2 in air at 37°C.

Preparation of Eggs

ICR female mice (more than 2 mo old) were superovulated with 5 IU eCG i.p., followed 48 h later by 5 IU hCG i.p. Fifteen to seventeen hours after hCG injection, females were killed by cervical dislocation. Cumulus-intact eggs released from the swollen ampulla of the oviducts were transferred to 400-μl drops of c-TYH without MBCD. Cumulus cells were removed with 0.05% hyaluronidase (Sigma) and 3 protease inhibitors (50 μM benzamidine, 50 μM PMSF, and 0.25 mg/ml aprotinin; Sigma) in c-TYH without MBCD. Eggs were washed three times and transferred to 400-μl drops of c-TYH without MBCD.

In Vitro Fertilization and Culture

Cumulus-free mouse eggs were transferred into 400-μl drops of c-TYH without MBCD. Sperm suspension (7–10 μl) preincubated for 90 min was added to the 400 μl of c-TYH without MBCD containing cumulus-free mouse eggs. The final concentration of spermatozoa was 2–5 $\times$ 10^5 cells/ml. After 6 h of coincubation of gametes, cumulus-free eggs were washed and mounted whole on a glass slide, fixed with 2.5% glutaraldehyde for 2 h at room temperature, and stained with 0.25% acetolacmoid [30]. The eggs possessing enlarged sperm heads and/or a male pronucleus with a sperm tail were considered to be fertilized.

To obtain embryos, in vitro fertilization was conducted in c-TYH without MBCD containing 0.75 mM MBCD-treated spermatozoa. At 6 h postinsemination, ova (presumptive zygotes) having a second polar body were washed three times with KSOM; they were then transferred into 100 μl of the same medium and cultured for 90 h under 5% CO_2 in air at 37°C. Embryo development was observed at 24-h intervals.

Embryo Transfer

ICR females, more than 2 mo old, were mated with vasectomized ICR males to serve as embryo recipients. To each infundibulum on Day 1 of pseudopregnancy (the day when a vaginal plug was noted) were transferred 10 embryos at the 2-cell stage that had been fertilized by spermatozoa treated with 0.75 mM MBCD and cultured in KSOM. Anesthesia was with 0.015–0.017 ml/g BW of tribromoethanol [31].

Table 2. In vitro fertilization of cumulus-free mouse eggs by epididymal spermatozoa treated with MBCD.

MBCD concentration (mM)	No. of eggs examined	No. of replicates	No. (%) of eggs fertilized		
			Monospermic	Polyspermic	Total
0	107	8	0 (0)	0 (0)	0 (0)[a]
0.25	54	4	6 (11)	0 (0)	6 (11)[b]
0.50	79	5	23 (29)	1 (1)	24 (30)[c]
0.75	100	6	48 (48)	2 (2)	50 (50)[d]
1.0	107	7	48 (45)	1 (1)	49 (46)[d]
2.0	62	4	2 (3)	0 (0)	2 (3)[a,b]

[a–d] Values without common superscripts differ ($p < 0.05$).

Measuring Unesterified Cholesterol Content of Sperm

Unesterified cholesterol content of sperm was assayed after incubation for 90 min in 400 μl of c-TYH containing 0.0 or 0.75 mM MBCD. Aliquots of sperm suspension were washed (300 $\times$ *g*, 10 min) twice with Dulbecco's PBS (138 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$) and then stored frozen (−20°C) in 1 ml of PBS at 1 $\times$ 10^7 sperm/ml. For assay, aliquots were thawed and mixed well. Cholesterol was extracted by modification of a previously described procedure [32]. To 1.0 ml of the suspension, chloroform: methanol (1:2 [v:v]) was added (3.75 ml), and the solution was vortexed for 2 min. The insoluble material was centrifuged (1000 $\times$ *g*, 10 min) and extracted again with 4.75 ml of methanol:chloroform:water (2:1:0.8 [v:v:v]) for 2 min. After centrifugation (1000 $\times$ *g*, 10 min), the first and second supernatants were combined and partitioned by addition of 2.5 ml chloroform and 2.5 ml of 0.88% KCl. The phases were separated by centrifugation (1000 $\times$ *g*, 10 min), and the chloroform-rich phase was removed; 15 μg of 5α cholestan were added as an internal standard. The suspension was dried under N_2, and the residue was dissolved in 1 ml of chloroform:methanol (2:1 [v:v]). Unesterified cholesterol content was analyzed through use of a GC-9A gas chromatograph fitted with an NB-1 column (i.d. 0.53 μm, length 15 m; Shimadzu, Kyoto, Japan) using helium as carrier. The inlet and flame ionization detector were maintained at 295°C, and the column was at 240°C. Peak areas of cholesterol and 5α cholestan were measured with a C-R3A Chromato pack integrator (Shimadzu). A standard curve was constructed with pure standard.

Chlortetracycline Fluorescence Assay

The fluorescence assay was performed with a slight modification of the method described previously [33]. The buffer contained 750 μM chlortetracycline (CTC, C-4881; Sigma), 20 mM Tris, 130 mM NaCl, and 5 mM cysteine and was kept at 4°C and shielded from light. The sperm suspension (20 μl) was mixed with an equal volume of the CTC solution. After 10 sec, 5 μl of 1% glutaraldehyde in 1 M Tris buffer was added. One drop (5 μl) of the suspension was put on a glass slide and covered with a coverslip. Slides were examined under a microscope equipped with epifluorescence optics (Nikon, Tokyo, Japan) using a 380- to 420-nm filter and a DM 430 dichroic mirror. Aliquots of sperm suspension were removed after 5 and 90 min of incubation. At least 200 spermatozoa were scored on each

Table 3. Comparison of MBCD and HBCD on in vitro fertilization of cumulus-free mouse eggs.

Chemicals*	No. of eggs	No. (%) of eggs fertilized		
		Monospermic	Polyspermic	Total
MBCD	74	27 (37)	3 (4)	30 (41)[a]
HBCD	63	9 (14)	0 (0)	9 (14)[b]

* The dose used was 0.75 mM for both chemicals. a vs. b: $p < 0.01$ (n = 4 replicates).

Table 5. In vivo development of cumulus-free mouse eggs fertilized by epididymal spermatozoa treated with MBCD.

No. of experiment	No. of eggs transferred	Recipients pregnant	No. of offspring
1	60	2/3	19
2	60	3/3	31
3	20	1/1	7
4	20	1/1	5
Total	160	7 (88%)	62 (39%)

slide. Spermatozoa were classified as uncapacitated (F pattern), having uniform fluorescence over the entire head; capacitated (B pattern), having a fluorescence-free dark band in the postacrosomal region; or acrosome-reacted (AR), having barely detectable fluorescence over the whole surface of the head.

Statistical Analysis

Each experiment was carried out with 3–8 replicates. Fertilization rates were compared by chi-square analyses. Concentrations of cholesterol were analyzed by Student's *t*-test. The proportion of B pattern spermatozoa among each treatment was subjected to an arcsin transformation followed by one-way ANOVA.

RESULTS

Experiment 1. Effect of MBCD on In Vitro Fertilization

The effect of MBCD on fertilization of eggs in vitro was determined. Spermatozoa were preincubated for 90 min in c-TYH containing 0, 0.25, 0.5, 0.75, 1, or 2 mM of MBCD. In this system, in vitro fertilization did not occur without MBCD (Table 2). The fertilization rate was highest at 0.75 mM MBCD (50%). The fertilization rate at 2 mM MBCD was lower ($p < 0.05$) than at 0.75 mM. Further experiments were therefore done at 0.75 mM MBCD.

Experiment 2. Comparison of MBCD and HBCD

The fertilizing abilities of 0.75 mM MBCD and 0.75 mM HBCD were compared. We also tried to examine effects of β-cyclodextrin, but it did not fully dissolve in the medium. When HBCD was present during capacitation of spermatozoa instead of MBCD, the fertilization rate was significantly ($p < 0.01$) lower (Table 3).

Experiment 3. Comparison of MBCD and BSA

The effects of addition of MBCD (0.75 mM) and of BSA (4 mg/ml) to preincubation and/or fertilization media were compared (Table 4). The fertilization rate obtained after addition of MBCD to only the preincubation medium was higher ($p < 0.01$) than the rate obtained by addition to both media (53% vs. 21%). The former rate was similar to that resulting from use of BSA in both media (53% vs. 66%).

Experiment 4. In Vitro and In Vivo Development of Fertilized Eggs

Viability of fertilized eggs was examined by in vitro culture and embryo transfer. Eggs fertilized by spermatozoa treated with 0.75 mM MBCD were cultured in KSOM for 4 days or transferred to pseudopregnant ICR recipients at the 2-cell stage. In vitro development of cumulus-free mouse eggs fertilized in c-TYH plus 0.75 mM MBCD was examined after 4 days of culture in KSOM. The experiment was repeated three times. Among 80 eggs cultured, 53 (66%) developed to the 2-cell stage, and 36 (45%) developed to the blastocyst stage. When mouse eggs (2 cell) were transferred to ICR recipient mice, 7 of 8 recipients were pregnant, and 62 live offspring were born from 160 eggs (Table 5).

Experiment 5. Sperm Cholesterol Content

Unesterified cholesterol loss from spermatozoa treated with MBCD was determined. Immediately after the sperm suspension was obtained, spermatozoa contained 4.19 ± 0.20 nmol unesterified cholesterol/10^7 sperm (Table 6; mean ± SEM, n = 5). The loss of unesterified cholesterol from spermatozoa incubated for 90 min increased ($p < 0.01$) in medium with 0.75 mM MBCD compared to the control (2.27 ± 0.09 vs. 4.13 ± 0.09 nmol unesterified cholesterol/10^7 sperm; mean ± SEM, n = 5). Therefore, 45 ± 3.7% (mean ± SEM, n = 5) of unesterified cholesterol was depleted after incubation of sperm for 90 min in 0.75 mM MBCD.

Experiment 6. Percentage of Capacitated spermatozoa

The role of MBCD during sperm preincubation was studied using the CTC test. Capacitation rates for each treatment, estimated by CTC fluorescence and calculated by proportions of the B pattern, are shown in Table 7. Immediately after collection, the capacitation pattern was found in 13–16% of spermatozoa. The capacitation rate of MBCD-treated spermatozoa was higher ($p < 0.01$) than that of the control (45% vs. 15%) after 90 min of incuba-

Table 4. Comparison of MBCD and BSA addition to preincubation or fertilization media in relation to in vitro fertilization.

Preincubation	Fertilization*	No. of eggs examined	No. (%) of eggs fertilized		
			Monospermic	Polyspermic	Total
MBCD	PVA	59	30 (51)	1 (2)	31 (53)[a]
MBCD	MBCD	57	12 (21)	0 (0)	12 (21)[b]
BSA	PVA	60	15 (25)	0 (0)	15 (25)[b]
BSA	BSA	59	37 (63)	2 (3)	39 (66)[a]

* PVA, polyvinylalcohol. a vs. b: $p < 0.01$ (n = 4 replicates).

Table 6. Loss of cholesterol from spermatozoa after 90 min incubation in c-TYH.*

Preincubation (min)	Concentration of MBCD (mM)	Unesterified cholesterol content per 10^7 cells	
		μg	nmol
0	0	1.56 ± 0.08	4.19 ± 0.20
90	0	1.54 ± 0.03	4.13 ± 0.09
90	0.75	0.85 ± 0.03	2.27 ± 0.09

* Data are mean ± SEM of 5 replicates.

Table 7. Changes of CTC fluorescence patterns of spermatozoa after 90 min incubation in c-TYH containing MBCD.*

Incubation time (min)	Concentration of MBCD (mM)	Percentage of CTC patterns (mean ± SEM)	
		B pattern	AR pattern
5	0	12.9 ± 1.8[a]	1.2 ± 0.5[a]
5	0.75	16.0 ± 2.4[a]	1.3 ± 0.5[a]
90	0	15.1 ± 1.9[a]	6.8 ± 1.8[a,b]
90	0.75	45.2 ± 5.5[b]	12.2 ± 3.1[b]

* Experiments were repeated 4 times.
[a–b] Values within columns without common superscripts differ ($p < 0.01$).

tion. Proportions of AR pattern increased during incubation in both treatments. The proportion with AR pattern after MBCD treatment at 90 min was higher ($p < 0.01$) than for control and MBCD treatment at 5 min. The motility of spermatozoa before and after incubation was 70 ± 10% in both treatments.

DISCUSSION

This is the first report of in vitro capacitation of mammalian spermatozoa by MBCD. Our results support the concept that removal of cholesterol from the sperm membrane is essential for capacitation. Therefore, MBCD could be utilized as a BSA substitute to provide a chemically defined, protein-free medium for in vitro fertilization.

Earlier research on capacitation in vitro was performed using various biological fluids (e.g., oviductal fluid, follicular fluid, and blood serum). The composition of these fluids is so complex that it was difficult to determine which component was involved in inducing capacitation [4]. Toyoda et al. [14] reported the first successful in vitro fertilization of mouse eggs using a chemically defined medium supplemented with BSA. Moreover, serum albumin is used in most media for capacitating sperm [34], serving to maintain sperm motility, to induce the acrosome reaction [16], and to promote membrane cholesterol efflux [7, 12, 35]. However, serum albumin can contaminate the medium with various molecules [19, 20].

Several efforts have been made to use a chemically defined, protein-free medium for in vitro fertilization of cumulus-enclosed oocytes in several species, including hamsters [36], mice [16], and cattle [37]. However, cumulus cells have complex cellular and extracellular components that play positive roles in fertilization [38]. Recently, cumulus cells have been removed from oocytes in order to evaluate which factor(s) can induce capacitation and affect the penetration of oocytes by spermatozoa in pigs [39] and cattle [40].

In the present study, we used a chemically defined, protein-free medium for in vitro fertilization of cumulus-free mouse eggs and demonstrated the in vivo development to neonates of in vitro-fertilized mouse eggs. Furthermore, MBCD is a substitute for BSA that removes cholesterol from spermatozoa and induces capacitation in vitro. Removal of cholesterol from spermatozoa coincided with the increased proportion of B pattern (capacitated) sperm in medium containing MBCD after 90-min incubation as evidenced by the CTC fluorescence test. Therefore, MBCD can be used to provide a chemically defined, protein-free medium to analyze more detailed molecular events of capacitation and fertilization in vitro.

The fertilization rate with 0.75 mM MBCD was superior to that with 0.75 mM HBCD. This is consistent with the more efficient cholesterol efflux with use of MBCD as compared to HBCD in cultured cells [26] and myometrial plasma membranes [41]. However, dose comparisons were not done with HBCD; therefore, optimal concentration of HBCD may not have been used in the present experiment. The fertilization rate declined when MBCD was present in both the capacitation and fertilization media or when the MBCD concentration was 2 mM in capacitation medium. After 6-h coincubation of gametes, all spermatozoa were dead when MBCD was present in both media. The effect of MBCD on viability of sperm is likely dependent on the concentration and the incubation time.

A role for cholesterol in sperm fertilizing ability was suggested by Davis [42], who first showed that cholesterol-containing vesicles from rabbit seminal plasma render capacitated sperm incapable of fertilizing eggs in vivo. In his model, when sperm are removed from seminal plasma, the cholesterol:phospholipid ratio of the sperm plasma membrane decreases, and the altered lipid concentration induces an acrosome reaction, allowing the sperm to fertilize [42]. This hypothesis has been supported by several researchers using several animal models [7, 10, 12, 43-45]. Moreover, the inhibitory role of cholesterol in fertilization has been examined by several investigators [44, 46, 47]. In addition, the distribution of cholesterol during capacitation in vitro has been shown by Suzuki and Yanagimachi [48] and Lin and Kan [49]. Our present study also supports the idea that cholesterol exerts an inhibitory influence on capacitation. There was no change in cholesterol content after incubation for 90 min in c-TYH without MBCD, which correlated with no fertilization in vitro. Furthermore, the proportion of the B pattern in sperm as estimated by CTC test did not increase during incubation when cholesterol was not lost.

When bovine sperm were incubated with 50% estrous oviductal fluid for 2 h, the net transfer of sperm unesterified cholesterol to high-density lipoproteins in the oviductal fluid was about 25% of total sperm cholesterol [13]. This result was similar to that of Zarintash and Cross [32], who calculated a 29% cholesterol loss in human sperm incubated with 26 mg/ml BSA for 24 h in vitro. In our study, spermatozoa lost 45% of their unesterified cholesterol after incubation for only 90 min in 0.75 mM MBCD in a protein-free medium. This clearly indicates that the cholesterol-binding capacity of cyclodextrin is higher than that of albumin or lipoprotein at concentrations in the female reproductive tract fluid. The high efficiency of cyclodextrins in cholesterol efflux from cultured cells as compared to that of high-density lipoprotein was also reported by Kilsdonk et al. [26]. They obtained 50–80% loss of the labeled cholesterol from L-cell mouse fibroblasts with cyclodextrins. It has been previously suggested that α-cyclodextrin and β-cyclodextrin could substitute for albumin in serum-free culture of mammalian cells [50, 51].

The role of cholesterol in capacitation in vivo is not clear. Some reports show that cholesterol removal is strong-

ly related to in vivo capacitation. Radiolabeled cholesterol transfers from sperm to lipoproteins and albumin in uterine fluid and follicular fluid [12, 52]. Due to the ability of cyclodextrins to encapsulate and solubilize hydrophobic molecules, they have been extensively used as drug delivery systems [23, 53, 54]. It has been also suggested that they might be used as food additives [55]. The relatively high specificity of β-cyclodextrin for cholesterol indicates that these compounds might be effective in modifying cholesterol metabolism in vivo [24, 56]. Investigations of the effects of these chemicals on fertilization in vivo when the diet contains water-soluble cyclodextrins might prove interesting.

In conclusion, MBCD likely induced capacitation in vitro by removal of cholesterol from sperm, enabling the use of a chemically defined, protein-free medium for fertilization in vitro.

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