THERIOGENOLOGY (2004) 62(5):897-905.

Ability to activate oocytes and chromosome integrity of mouse spermatozoa preserved in EGTA Tris-HCl buffered solution supplemented with antioxidants

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Acknowledgement:

This study was partially supported by Grant-in-Aid for Scientific Research (C), no. 13680615 (Y.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Abstract

Potential methods for cryopreservation of mouse spermatozoa are freeze-drying, desiccation and suspension in EGTA Tris-HCl buffered solution (ETBS: 50 mM NaCl, 50 mM EGTA and 10 mM Tris-HCl). To examine how long mouse spermatozoa suspended in ETBS-based solutions could retain their normal characteristics without freezing, spermatozoa collected from cauda epididymis were suspended in ETBS or in ETBS supplemented with the antioxidants, dimethyl sulfoxide (DMSO) or *dl*-α-tocopherol acetate (vitamin E acetate: VEA) diluted in DMSO, then held at ambient temperature (22-24^{o}C) for up to 9 days. When oocytes were injected with spermatozoa preserved in ETBS alone, activation rates of oocytes and chromosome integrity at the first cleavage metaphase decreased significantly 1 day ($P< 0.001$) and 2-4 days ($P< 0.01$) following treatment. When oocytes were injected with spermatozoa preserved in ETBS supplemented with DMSO or VEA/DMSO, chromosome integrity had no significant decrease at any preservation time through 9 days. DMSO maintained sperm chromosome integrity more effectively than VEA/DMSO up to 2-4 days (normal karyotypes in DMSO and VEA/DMSO: 91% and 67%, respectively), while VEA/DMSO helped to maintain the ability of spermatozoa to activate oocytes but did not enhance the maintenance of sperm chromosome integrity. These results suggest that deterioration in spermatozoa preserved in ETBS alone was delayed by supplementation with antioxidants.

Introduction

EGTA Tris-HCl buffered solution (ETBS) has been reported to maintain the genetic integrity of freeze-dried mouse spermatozoa [1]. Freeze-dried and desiccated mouse spermatozoa can retain

normal sperm characteristics during storage at ambient temperatures [2]. However, the extent to which spermatozoa suspended in the ETBS retain their ability to activate oocytes and maintain chromosome integrity prior to freeze-drying is not known. Therefore, spermatozoa suspended in ETBS should be freeze-dried as soon after collection as possible.

In addition, it is not known whether antioxidants influence chromosome integrity of freeze-dried and dessicated sperm or their ability to activate oocytes. Vitamin E (tocopherol species) is a hydrophobic antioxidant that can stop the chain reaction of peroxidation by scavenging peroxyl radicals in lipids in the plasma membrane [3]. It can maintain the integrity of human sperm [4] and inhibits chromosome aberrations or DNA damage in mammalian somatic cells [5-9].

In this study, we examine deterioration over time of spermatozoa preserved in ETBS and examine whether this deterioration is ameliorated by two antioxidants, *dl*-α-tocopherol acetate (vitamin E acetate: VEA) and dimethyl sulfoxide (DMSO), known to scavenge hydroxyl radicals generated from hydrogen peroxide [10, 11].

Materials and Methods

Animals

B6D2F1 hybrid male and female mice, 7-12 weeks of age, were used in this study. They were maintained under a 14 h-light/10 h dark photoperiod at a temperature of 22-24^{o}C. Food and water were provided ad libitum. Mice were killed just before use under the animal study protocol approved by the Laboratory Animal Committee, Asahikawa Medical College, Japan.

Media

All chemicals were obtained from Nakalai Tesque (Kyoto, Japan) unless otherwise stated. The medium for preparation of oocytes and sperm injection was Hepes-CZB medium [12], which was modified CZB medium [13] with 20 mM Hepes, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (PVA; cold water soluble; molecular weight: 30,000-50,000, Sigma, St. Louis, MO, USA) instead of BSA.

EGTA Tris-HCl buffered solution (ETBS: 50 mM NaCl, 50 mM ethyleneglycol-bis-[β-aminoethyl ether]-*N,N,N',N'*- tetraacetic acid and 10 mM Tris-HCl) was prepared according to the procedure previously described [1]. EGTA and 1 M Tris-HCl (pH 7.4) were obtained from Sigma (St. Louis, MO, USA) to prepare the ETBS. ETBS was used as a medium for preservation of spermatozoa. In addition, the ETBS was supplemented with 0.5% volume of dimethyl sulfoxide (DMSO, for spectroscopy, Merck, Dermstadt, Germany) to the total volume of the ETBS. Vitamin E acetate (VEA, *dl*-α-tocopherol acetate, Sigma, St. Louis, MO, USA) was diluted in DMSO at 200 mg/ml (close to the limiting concentration in DMSO) and then the ETBS was supplemented with 0.5% volume of the VEA/DMSO to the total volume of the ETBS (final concentration of VEA: 1 mg/ml).

Sperm collection and preservation

A cauda epididymis was removed from a male and punctured with sharply pointed forceps. A dense mass of spermatozoa was squeezed out of the cauda region in a cauda epididymis and put

in a 1.5 ml polypropylene centrifugation tube containing 0.5 ml of preservation media (Hepes-CZB and ETBS-based solutions). The tube was left standing for 10 min at 37°C to allow sperm swim up. After 10 min suspension in ETBS, all spermatozoa lost their motion [1]. Therefore, oocytes invariably were injected with immotile spermatozoa after being suspended in the ETBS-based solutions. After 0.4 ml of the sperm suspension was collected, it was put into another 1.5 ml polypropylene tube. The polypropylene tubes with the sperm suspension were placed in a box to be shaded from light and kept in a room at 22-24°C. To examine the chromosome integrity of motile and immotile spermatozoa preserved for 1 day, Hepes-CZB was also used as a preservation medium.

Intracytoplasmic sperm injection (ICSI)

ICSI was carried out according to procedures previously described [12] with some modifications. All operations were performed at room temperature (18-25°C). Female mice were superovulated with i.p. injection of 10 units of pregnant mare's serum gonadotrophin (Teikokuzouki, Tokyo, Japan) and 10 units of human chorionic gonadotrophin (Mochida, Tokyo, Japan) given 48 h apart. The cumulus-intact oocyte mass was treated with 0.1% hyaluronidase dissolved in Hepes-CZB medium, after which the cumulus-free oocytes were placed in droplets of Hepes-CZB medium. A small volume (1-5 μl) of the sperm suspension was thoroughly mixed with one drop (5-10 μl) of Hepes-CZB medium containing 10% polyvinylpyrrolidone K-90 (Hepes-CZB-PVP). Sperm were picked up with an injection pipette attached to a piezo electric pipette-driving unit [12], after which the sperm head was separated from the tail by electric pulses [12]. Sperm heads were transferred into another droplet of Hepes-CZB-PVP prior to injection in order to dilute the high concentration of ETBS. ICSI was completed within 1 h after

suspending spermatozoa in Hepes-CZB-PVP.

In the present study, motile and immotile spermatozoa preserved in Hepes-CZB were separately picked up and injected into oocytes just after swimming up (fresh sperm) and at the 1-day preservation.

Culture of oocytes

Sperm-injected oocytes were transferred into droplets (50-100 μl) of the modified CZB medium [13] supplemented with 5.56 mM glucose and 5 mg/ml BSA (fraction V, Sigma, St. Louis, MO, USA) under a paraffin oil (Merck, Dermstadt, Germany) overlay in a humidified atmosphere of 5% CO_2 in air. At 5-6 h from the completion of ICSI, oocytes with a second polar body and two pronuclei were considered normally activated (i. e., fertilized). The ability of spermatozoa to activate oocytes was quantified as activation rate, representing the percentage of injected oocytes that become activated and that survived.

Chromosome analysis

Chromosome specimens were prepared at the first cleavage metaphase of activated oocytes according to procedures from previous publications [14, 15]. At 5-6 h following completion of ICSI, oocytes were replaced into CZB medium containing mitotic inhibitor, 0.006 μg/ml vinblastine, and then cultured for an additional 15-18 h. After completion of culture, oocytes were treated for 4-5 min with 0.5% protease (Actinase E, 1000 tyrosine unit/mg; Kaken, Tokyo, Japan) prepared in PBS to remove the zonae pellucidae. Zona-free oocytes were placed into

hypotonic solution (1:1 mixture of 30% fetal bovine serum and 1% sodium citrate) for 4-5 min at room temperature. Fixation of oocytes and spreading of chromosomes onto glass slides were performed according to procedures described previously [16]. Structural chromosome aberrations were scored as outlined previously [17]. Chromatid and chromosome breaks and exchanges were recorded as structural chromosome aberrations. The number of aberrations per oocyte was recorded without discriminating between paternal and maternal pronuclei. Uncountable numbers of structural aberrations such as chromosome fragmentation and multiple exchanges were arbitrarily assigned 10 aberrations per oocyte. Only activated oocytes with 40 chromosomes and no structural chromosome aberrations were judged to be activated oocytes with the normal karyotype.

Analysis of data

Numbers of ICSI oocytes activated normally and the activated oocytes with normal karyotypes were compared by using the chi-square test between oocytes injected with fresh and preserved spermatozoa. When spermatozoa were suspended and preserved in Hepes-CZB, the comparison was also performed between motile and immotile spermatozoa.

The overall rate of sperm integrity (ORSI) was defined as the percentage of oocytes that were injected and survived that had normal karyotypes, as described above. In this computation, the number of normal karyotypes was multiplied by the number of activated oocytes divided by the number of metaphases analyzed, to correct for the loss of activated oocytes that occurred when preparing chromosome spreads.

Results

Spermatozoa preserved in Hepes-CZB with no addition of antioxidants

Results of oocyte activation and chromosomal analysis of ICSI with motile or immotile, fresh or 1-day preserved spermatozoa are shown in Table 1. Nearly all of the oocytes injected with either motile or immotile spermatozoa could be activated after preservation for up to 1 day. However, chromosome integrity in oocytes fertilized by immotile spermatozoa after 1-day preservation was significantly reduced ($P < 0.01$) compared to oocytes from fresh, immotile sperm (40% and 73%, respectively); while oocytes fertilized by motile spermatozoa had higher chromosome integrity (96% and 87% normal karyotypes for fresh and 1-day preserved motile sperm, respectively). Both within the fresh spermatozoa ($P < 0.01$) and spermatozoa preserved for 1 day ($P < 0.0001$), chromosome integrity was higher in oocytes fertilized by motile than by immotile sperm.

Spermatozoa preserved in ETBS-based solutions

The activation rates of oocytes injected with spermatozoa preserved in ETBS-based solutions are shown in Table 2. When oocytes were injected with spermatozoa preserved in ETBS alone for 1 day, the activation rate was 75%, being significantly different ($P<0.001$) from the oocytes injected with fresh spermatozoa (96%). However, when the ETBS was supplemented with DMSO or VEA diluted in DMSO (VEA/DMSO), the spermatozoa preserved for up to 1 day showed no significant decrease of the ability to activate oocytes. After 2-4 days, the activation rates decreased with time (activation rates at 2-4 days to 8-9 days: 63% to 33% in ETBS alone;

66% to 5% in ETBS+DMSO; 85% to 58% in ETBS+VEA/DMSO) and were significantly different (P<0.01) from those of oocytes injected with fresh spermatozoa. In each preservation time from 2-4 days, the activation rates were higher in ETBS supplemented with VEA/DMSO than in ETBS alone and ETBS supplemented with DMSO.

Chromosome integrity of oocytes injected with spermatozoa preserved in ETBS-based solutions is also summarized in Table 2. In ETBS alone, the percentage of activated oocytes with normal karyotypes did not differ significantly between fresh and 1-day preserved spermatozoa (97% and 84%, respectively). However, chromosomal integrity gradually decreased from 2-4 days' to 8-9 days' preservation (74%-58%).

When spermatozoa were preserved in ETBS supplemented with DMSO or VEA/DMSO, no decrease in the percentage of activated oocytes with normal karyotype was seen at any of the preservation times when compared with the oocytes injected with fresh spermatozoa.

Overall rate of sperm integrity (ORSI)

Final evaluation of sperm integrity was done using overall rate of sperm integrity (ORSI) is shown in Figure 1. From 1 to 2-4 days, spermatozoa preserved in ETBS supplemented with DMSO showed the highest ORSI at 1 and at 2-4 days but the lowest at 5-7 and 8-9 days' preservation. From 5 to 9 days' preservation, spermatozoa preserved in ETBS supplemented with VEA/DMSO had higher ORSI than in the other two kinds of ETBS-based solutions. In addition, ORSI was higher in the spermatozoa preserved in ETBS supplemented with VEA/DMSO than in ETBS alone through the preservation times from 1 to 9 days.

Discussion

The integrity of spermatozoa preserved in solution at ambient temperature has been examined in several mammalian species [18-21]. In agreement with those reports, results of this study suggest that quality of spermatozoa preserved at ambient temperatures (e.g. sperm motility, the ability to activate oocytes and chromosome integrity) declines with preservation time. It has been asserted that ETBS inhibits the activation of endogenous sperm nucleases by chelating metal ions in spermatozoa with membrane disruption induced after freezing or freeze-drying, leading to suppression of structural chromosome aberrations in the zygotes [1, 22]. In this study, however, sperm deterioration was not completely prevented by ETBS, suggesting that the mechanism of cumulative damage generated in spermatozoa preserved in solution differs intrinsically from that of DNA damage induced after snap freezing or freeze-drying of spermatozoa suspended in media excluding ETBS.

Chromosome integrity of immotile spermatozoa preserved for 1 day in Hepes-CZB was inferior to that of the spermatozoa preserved for up to 8-9 days in ETBS-based solutions (Tables 1 and 2), in which all spermatozoa became immotile. Thus, ETBS-based solutions were effective in maintaining sperm chromosome integrity after the spermatozoa lost their motion.

In preliminary experiments, Hepes-CZB was a better medium than the ETBS-based solutions to maintain the overall integrity of motile mouse spermatozoa preserved for up to 9 days (data not shown). However, we could not conclude that spermatozoa were positively protected from cumulative damage by the Hepes-CZB, but only that spermatozoa with good quality were

artificially selected and injected into oocytes. This is supported by the fact that cryopreservation using only motile sperm separated from the sperm suspension were more successful in fertilization and development than the population before separation [23].

ETBS alone was less effective than Hepes-CZB in preserving the ability of spermatozoa to activate oocytes. However, when supplemented with VEA/DMSO, the solution became a retardant of sperm deterioration. DMSO is commonly used as a solvent to dilute hydrophobic antioxidants, and it scavenges those radicals causing greatest damage to cellular DNA and hydroxyl radicals [10,11]. On the other hand, vitamin E is known as the most potent lipid peroxyl radical scavenger, reducing chromosome damages induced by free radicals [24]. From data provided in this study, addition of DMSO to sperm preservation media prevents mainly the DNA damage in the spermatozoa, while the VEA may play a partial role in protecting "sperm-borne oocyte-activation factor(s)" (SOAF) [25] from damage such as oxidation. The SOAF exists in the perinuclear region [25] inside the plasma membrane, where VEA is capable of access.

We are unable to explain the high background frequency of chromosome aberrations in fresh spermatozoa suspended in ETBS supplemented with DMSO or VEA/DMSO, as compared to ETBS alone (Table 1). It is possible that spermatozoa were treated with VEA and DMSO at near the cytotoxic doses for these cells. It also is possible that DMSO per se has the potential to damage the sperm membrane, resulting in a steep decrease in the percentage of activated oocytes after injection with spermatozoa preserved for 5-7 days in ETBS supplemented with DMSO (Table 2). Further studies are required to determine the optimal doses of VEA and DMSO to preserve mouse spermatozoa in ETBS at ambient temperature for as long as possible.

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Legend of Figure 1:

Figure 1

Overall rate of sperm integrity (ORSI) defined as the percentage of activated oocytes with normal karyotype to the number of oocytes injected with mouse ($B6D2F_1$) spermatozoa. The spermatozoa were preserved in EGTA Tris-HCl buffered solution (ETBS) and the ETBS supplemented with 0.5% dimethyl sulfoxide (DMSO) or *dl-a*-tocopherol acetate (VEA, final concentration: 1 mg/ml) diluted with DMSO.

Table 1. Oocyte activation and chromosome integrity of ova injected with fresh or 1-day-preserved, motile or immotile mouse spermatozoa

Sperm	Preservation time (22-24°C)	No. oocytes injected and survived (No. ICSI)	% of oocytes activated	Chromosome analysis at 1 st cleavage	
				No. aberrations per oocyte	No. normal karyotypes/ total metaphases analyzed (%)
Motile	Fresh	68 (3)	100	0.041	47/49 (96) [a]
	1 day	63 (3)	100	0.13	46/53 (87) [b]
Immotile	Fresh	66 (3)	100	0.53	33/45 (73) [c]
	1 day	66 (3)	98	2.2	17/47 (40) [d]

Differences between (a) and (c); (c) and (d), $P < 0.01$.

Differences between (a) and (d); (b) and (d), $P < 0.0001$.

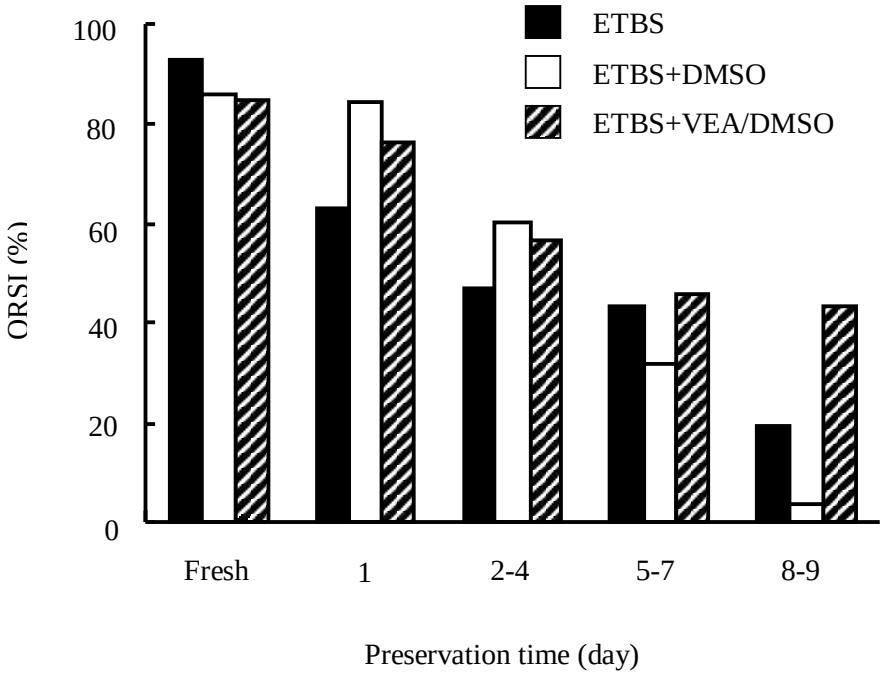
Table 2. Oocyte activation and chromosome integrity of ova injected with fresh or preserved

mouse spermatozoa suspended in EGTA Tris-HCl buffered solution (ETBS) with or without the addition of dimethyl sulfoxide (DMSO) or vitamin E acetate (VEA) diluted in DMSO

ETBS with		Preservation time, day (22-24°C)	No. oocytes injected and survived (No. ICSI)		% of oocytes activated	Chromosome analysis at 1 st cleavage		
DMSO[1)]	VEA[2)]					No. aberrations per oocyte	No. normal karyotypes/ total metaphases analyzed (%)	
-	-	Fresh	73	(3)	96	0.034	57/59	(97)
-	-	1	61	(4)	75 **	0.55	26/31	(84)
-	-	2 to 4	95	(6)	63 ***	0.34	37/50	(74) *
-	-	5 to 7	193	(7)	64 ***	0.95	63/92	(68) ***
-	-	8 to 9	113	(6)	33 ***	1.2	21/36	(58) ***
+	-	Fresh	65	(3)	100	0.17	50/58	(86)
+	-	1	57	(3)	93	0.094	48/53	(91)
+	-	2 to 4	92	(5)	66 ***	0.11	51/56	(91)
+	-	5 to 7	149	(8)	41 ***	1.1	34/44	(77)
+	-	8 to 9	82	(3)	5 ***	2.2	2/3	(67)
+	+	Fresh	73	(3)	100	0.20	51/60	(85)
+	+	1	64	(3)	89	0.16	42/49	(86)
+	+	2 to 4	80	(3)	85 *	0.63	38/57	(67)
+	+	5 to 7	79	(3)	70 **	0.70	30/46	(65)
+	+	8 to 9	67	(3)	58 **	0.44	24/32	(75)

Statistically significant chi-square comparisons, comparing between fresh and preserved spermatozoa (* $P< 0.01$, ** $P< 0.001$, *** $P< 0.0001$).

[1)] Final concentration: 0.5% (v/v); [2)] Final concentration: 1 mg/ml


ETBS
ETBS+DMSO
ETBS+VEA/DMSO
100
80
60
40
20
0
ORSI (%)
Fresh
1
2-4
5-7
8-9
Preservation time (day)