Journal of the American Association for Laboratory Animal Science
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Vol 49, No 2
March 2010
Pages 167–172

Extracellular ATP and Dibutyryl cAMP Enhance the Freezability of Rat Epididymal Sperm

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We studied the effects of ATP, ionomycin, and dibutyryl cAMP (dbcAMP) on the motility, freezability, and oxygen consumption of rat epididymal sperm. In vitro fertilization and intrauterine insemination were performed by using frozen–thawed rat sperm. Frozen–thawed sperm diluted in raffinose–modified Krebs-Ringer bicarbonate solution–egg yolk extender containing 1.85 mM ATP and 100 μM dbcAMP exhibited considerably higher motility and viability than sperm diluted in dbcAMP-free extender. Addition of ionomycin and dbcAMP to ATP-containing extenders did not alter the oxygen consumption rate of sperm, suggesting that extracellular ionomycin and dbcAMP are not involved in the mobilization of mitochondrial energy substrates in sperm. Further, high rates of pronucleus formation and progression to the blastocyst stage were observed in embryos produced by the fertilization of oocytes with fresh sperm in an in vitro fertilization medium supplemented with ATP and dbcAMP. Oocytes were not penetrated by frozen–thawed sperm when cocultured with cumulus-oocyte complexes in a medium without ATP and dbcAMP. In contrast, cryopreserved sperm penetrated oocytes when the gametes were cultured in an ATP- and dbcAMP-containing medium, and the resultant embryos formed blastocysts. Our results show that the dilution of rat sperm in raffinose–modified Krebs-Ringer bicarbonate solution–egg yolk extender supplemented with ATP and dbcAMP prior to sperm cryopreservation enhances the freezability of the cryopreserved sperm. Furthermore, the in vitro fertilization medium we developed effectively supports the production of embryos from both fresh and cryopreserved rat sperm.

Abbreviations: BSA, bovine serum albumin; dbcAMP, dibutyryl cAMP; IVF, in vitro fertilization; mKRB, modified Krebs–Ringer bicarbonate.

The basic mechanochemical event underlying sperm motility is ATP-induced microtubule sliding.[4] Adenosine triphosphatase associated with the dynein arms on outer-doublet microtubules provides the energy required for this process.[30]

ATP, calcium, and cAMP have received considerable attention as potential primary regulators of sperm motility in several species of animals.[2,15,16] Extracellular ATP acts on sperm by triggering a purinergic receptor-mediated increase in the intracellular calcium level; this increase may produce several downstream effects that enhance sperm motility.[18,26] Increased calcium levels presumably activate soluble adenylyl cyclase, thereby increasing the cAMP concentration in sperm.[1,3,17] cAMP induces protein phosphorylation by activating protein kinase A[9,13] and mediates calcium influx into sperm via the CatSper calcium ion channels.[6,10,23] In addition, cAMP may elevate mitochondrial calcium levels,[8] thereby activating the calcium-dependent dehydrogenases involved in the Krebs cycle and providing ATP required for sperm motility.

Previously, we showed that rat sperm become freezable when diluted in ATP-containing raffinose–modified Krebs-Ringer bicarbonate solution (mKRB)–egg yolk extender.[32] This finding indicates the existence of a unique pathway that utilizes extracellular ATP in rat sperm and suggests that extracellular ATP produces several downstream effects that improve sperm motility by increasing calcium levels or by activating cAMP signal transduction pathways. Further elucidation of the role of extracellular ATP in the energy-synthetic processes and motility-regulation system of rat sperm could lead to improved motility, freezability, and fertilizing ability of the sperm.

We, therefore, evaluated the freezability of rat epididymal sperm preserved in raffinose–mKRB–egg yolk extender with ATP, ionomycin (a calcium ionophore), and dibutyryl cAMP (dbcAMP; a membrane-permeable cAMP analog) under various conditions. We also determined the effects of these agents on oxygen consumption by sperm. Sperm cryopreservation was considered successful if frozen–thawed sperm fertilized oocytes. To improve the effectiveness of in vitro fertilization (IVF), we determined whether ATP- and dbcAMP-supplemented IVF media improve the fertilizing ability of sperm. We also attempted artificial insemination with frozen–thawed rat sperm.

Materials and Methods

Principles of laboratory animal care were followed during this study, and all procedures were conducted in accordance with guidelines of the Ethics Committee for Care and Use of Laboratory Animals for Research of the Graduate School of Agricultural Science (Tohoku University, Japan). Wistar rats were used throughout the experiments. Animals were kept in polycarbonate cages (25 × 40 × 20 cm) under controlled conditions with lights on at 0800 and off at 2000 h. They were given food and tap water ad libitum.

Raffinose–mKRB–egg yolk extender. We used raffinose–mKRB–egg yolk freezing solution containing 0.1 M raffinose, 32.37 mM sodium DL-lactate, 50 μg/mL streptomycin, and 75 μg/mL penicillin (all from Sigma, St Louis, MO) and 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM

Received: 02 Jul 2009. Revision requested: 06 Aug 2009. Accepted: 08 Sep 2009.
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$MgSO_4 \cdot 7H_2O$, 1.19 mM KH_2PO_4, 25.07 mM $NaHCO_3$, 0.5 mM sodium pyruvate, and 5.56 mM glucose (all from Wako Pure Chemical Industries, Osaka, Japan). Egg yolk (20% v/v) was added to the solution. Egg-yolk lipids were solubilized by adding 0.04% (w/v) SDS (Wako) as described previously.[31,32]

Sperm motility. Sperm motility was assessed by using a sperm motility analysis system (version 1.0, Kashimura, Tokyo, Japan) and a previously described protocol.[5,32]Acrosome integrity.

Acrosomes of fresh and frozen–thawed sperm were assessed by staining with FITC-conjugated peanut agglutinin (Wako).[31]

Rat epididymal sperm collection. Sexually mature Wistar male rats older than 15 wk were euthanized by cervical dislocation. Both caudae epididymides were excised, rinsed, and carefully blotted free of blood and adipose tissues. A small part of the cauda epididymis was excised with fine scissors, and the sperm droplet that subsequently welled up was transferred to a 1.5-mL microfuge tube containing 1 mL freezing medium at 37 °C. After 5 min, the tube was assessed macroscopically to determine whether the sperm were dispersed adequately.

Cryopreservation and thawing. We investigated the effect of various concentrations of ionomycin (Sigma) in ATP-containing raffinose–mKRB–egg yolk extenders on the motility of fresh and frozen–thawed sperm. Sperm samples from both caudae epididymides of 3 rats were collected and immediately suspended in 1 mL raffinose–mKRB–egg yolk extender containing 1.85 mM ATP and 0, 5, 10, 15, or 20 µM ionomycin. Each suspension was incubated at 37 °C for 5 min to ensure sperm dispersal, and the sperm concentration and motility then were evaluated with the sperm motility analysis system. Sperm were processed and frozen by using a previously described method with modifications.[31] Diluted sperm samples were cooled to 5 °C for 90 min and further diluted to a ratio of 1:1 with the previously mentioned extenders to which a commercial cryoprotectant (Equex STM, Nova Chemical Sales, Scituate, MA) was added to 1.5%. The sperm concentration after dilution was 5×10^6 sperm/mL. The samples were equilibrated at 5 °C for 30 min before freezing and loaded into standard 0.5-mL straws. Next, a tube rack made from stainless steel was put into the polystyrene box (17 × 27 × 17 cm), and the box was filled with liquid nitrogen until 2 cm below the level of the tube rack. The heat-sealed straws were placed in the tube rack, exposed to liquid nitrogen vapor for 10 min, plunged into liquid nitrogen (–196 °C), and stored for 3 d at this temperature. The straws were thawed in water (37 °C) for 10 s, transferred to 1.5-mL microfuge tubes, and incubated at 37 °C for 5 min. Thereafter, sperm motility parameters were assessed.

We next analyzed the characteristics of fresh and frozen–thawed sperm stored in ATP-containing raffinose–mKRB–egg yolk extenders supplemented with dbcAMP (Sigma) at various concentrations. In brief, sperm samples were collected from the caudae epididymides of 3 male rats, divided into 5 aliquots, and suspended in extender solution containing 1.85 mM ATP and 0, 100, 200, 300, or 400 µM dbcAMP. The suspensions were cooled to 5 °C for 90 min, and equal volumes of the previously mentioned extender solutions containing 1.5% cryoprotectant were added. The resultant solutions were equilibrated at 5 °C for 30 min, frozen, and stored in liquid nitrogen. The straws were thawed rapidly by holding them in water at 37 °C for 10 s. Sperm were transferred to a 1.5-mL microfuge tube and incubated at 37 °C for 5 min. Sperm motility (%), straight-line velocity (µm/s), curvilinear velocity (µm/s), and amplitude of lateral head displacement (µm) were evaluated after incubation for 3 h. Acrosome integrity was evaluated by staining frozen–thawed sperm with FITC-conjugated peanut agglutinin.

Oxygen consumption by sperm. We assessed the effects of ionomycin and dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on sperm mitochondrial activity. Sperm samples were collected from 5 mature male rats and diluted in ATP-free raffinose–mKRB–egg yolk medium at 37 °C for 5 min to permit sperm dispersal. Equal aliquots of the samples were resuspended in raffinose–mKRB–egg yolk medium containing 3.7 mM ATP (control), 20 µM ionomycin, or 200 µM dbcAMP; thus the final ATP, ionomycin, and dbcAMP concentrations were halved. Oxygen-consumption rates were measured using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK); the electrodes were maintained at 37 °C for 10 min and calibrated with air-saturated water (presumed oxygen concentration, 406 nmol/mL) at 37 °C. Sperm samples (1 mL each) were suspended in a reaction chamber (final concentration, approximately 1×10^7 sperm/mL) and carefully stirred to avoid introduction of external air. Data were acquired by using a commercial software program (LabChart version 5.2, AD Instruments, Castle Hill, Australia). The oxygen consumed by the sperm were calculated as described previously:[25]

> Oxygen concentration (nmol oxygen/mL) = oxygen (U) × oxygen concentration of air-saturated water (that is, 406 nmol oxygen/mL) ÷ oxygen full-chart span (U)

The rates of oxygen consumption by sperm were expressed as nmol/min/1×10^7 sperm.

IVF and embryo culture. After IVF and culture, embryo development was examined as described previously.[12] mKRB medium containing 4 mg/mL bovine serum albumin (BSA, Sigma) was used for fertilization. For subsequent culture, we used modified rat 1-cell embryo culture medium (ARK Resource, Kumamoto, Japan). The fertilization and culture media were prepared in polystyrene culture dishes covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) and equilibrated overnight in a CO_2 incubator (Hirasawa, Tokyo, Japan; 5% CO_2 in air at 37 °C).

Superovulation was induced in immature rats as described previously.[19] In brief, female rats (age, 28 to 33 d) were injected intraperitoneally with 10 IU pregnant mare serum gonadotropin (Serotropin, Teikoku Zoki, Tokyo, Japan) and, after 48 h, with 10 IU human chorionic gonadotropin (Puberogen, Sankyo, Tokyo, Japan). After 13 to 15 h, the rats were euthanized by cervical dislocation. Their oviducts were isolated, blotted with sterilized filter paper to remove the liquid and blood on the external surfaces, and added to dishes containing 100 µL mKRB–BSA medium. The cumulus–oocyte complexes in the oviducts were collected by flushing excised oviducts with mKRB–BSA.

Fresh sperm were obtained from male rats as described previously. Frozen–thawed sperm in dbcAMP- and ATP-containing raffinose–mKRB–egg yolk extenders were used. In brief, fresh epididymal sperm were suspended in 1 mL mKRB–BSA medium with or without 1.85 mM ATP and 100 µM dbcAMP (concentration, 1×10^7 sperm/mL). Both sperm suspensions were warmed in an incubator for 5 min, and 10 µL of each was transferred to fresh dishes containing drops of mKRB with 4 mg/mL BSA (90 µL; final concentration, 1×10^6 sperm/mL). Frozen sperm were thawed in a water bath (37 °C) for 5 min and suspended in the aforementioned media. Next, 10 µL of each suspension was transferred to a fresh dish containing drops of mKRB-BSA medium (final frozen–thawed sperm concentration, 2 to 3×10^6 sperm/mL). Immediately after sperm were added to the insemination medium, cumulus–oocyte complexes col-

lected from the oviducts of superovulated immature rats were carefully released into the sperm suspensions and incubated in the insemination medium at 37 °C in 5% CO_2 for 12 h. The eggs were freed from the cumulus cells, washed thrice with rat 1-cell embryo culture medium, and examined under phase-contrast microscopy to detect penetration and fertilization. Eggs containing sperm within the perivitelline space were defined as penetrated eggs. Penetrated eggs that exhibited pronucleus formation were considered to be undergoing fertilization. Eggs (20 to 30) with both female and male pronuclei with corresponding tails were cultured in 100 µL culture medium for 5 d in a CO_2 incubator. Embryos that progressed to the 2-cell, 4-cell, and blastocyst stages were counted at 24, 72, and 120 h of culture, respectively.

Intrauterine insemination of frozen–thawed sperm. Sperm were frozen in dbcAMP- and ATP-containing raffinose–mKRB–egg yolk extenders, as described earlier. By using a previously described method,[20] fresh sperm collected from 6 mature male rats were injected into the uterine horns of 6 female Wistar rats, and frozen–thawed sperm were injected into the uterine horns of 27 female rats. In brief, sexually mature female rats were maintained on a 12:12-h light:dark cycle (lights on, 0800 to 2000 h) and introduced into cages containing sterile vasectomized male rats. In the evening (2200 to 2300 h) of day 0 (day of pseudopregnancy induction), fresh and thawed sperm (50 µL each; 20 to 30×10^6 sperm/mL) were injected into the oviductal ends of the uterine horns of pseudopregnant rats.

Statistical analysis. The data were subjected to ANOVA and Fisher protected least-significant difference posthoc test (StatView, Abacus Concepts, Berkeley, CA). All data are expressed as the mean ± SEM. A P level of less than 0.05 was considered statistically significant.

Results

Effect of ionomycin in freezing extenders on cryopreserved rat sperm. We first examined the effect of ionomycin (0, 5, 10, 15, and 20 µM) in and ATP-containing raffinose–mKRB–egg yolk freezing extenders on the motility of fresh and frozen–thawed sperm. Sperm motility did not significantly differ between fresh and frozen–thawed sperm that were extended in ATP with raffinose–mKRB–egg yolk extenders containing 0, 5, 10, 15 and 20 µM ionomycin (Table 1).

Effect of dbcAMP in freezing extenders on cryopreserved rat sperm. This experiment aimed to determine whether the addition of the sperm-motility regulator dbcAMP (0, 100, 200, 300, and 400 µM) to ATP-containing raffinose–mKRB–egg yolk extenders improves postthaw sperm motility. Frozen sperm in extender containing ATP and 100 µM dbcAMP were more motile than those in dbcAMP-free extender (Table 2). Frozen–thawed sperm incubated for 3 h at 37 °C in the ATP-containing extender supplemented with 100 µM dbcAMP exhibited significantly ($P < 0.05$) greater motility, straight-line velocity, and amplitude of lateral head displacement than did those in dbcAMP-free ATP-containing extender (Figure 1). Both before and after thawing, the proportion of sperm with intact acrosomes did not significantly differ among the sperm diluted in the various dbcAMP-containing extenders (Table 2).

Effect of ionomycin and dbcAMP in ATP-containing media on the oxygen consumption of sperm. Oxygen consumption by sperm was evaluated by incubation of sperm at 37 °C for 10 min in ATP-raffinose–mKRB–egg yolk medium with or without 10 µM ionomycin and 100 µM dbcAMP. No significant differences were noted in the oxygen-consumption rates among sperm cultured in any of the tested media (Figure 2).

Effects of ATP and dbcAMP in fertilization medium on the fertilizing ability of fresh and frozen–thawed sperm and on oocytes in vitro. To determine the optimal conditions for successful IVF, oocytes were fertilized with fresh and frozen–thawed sperm in mKRB-BSA medium with or without ATP and dbcAMP (Table 3). In medium without dbcAMP, 55% of oocytes were penetrated by fresh sperm, 48% exhibited pronuclei, and 22 % developed into blastocysts. However when ATP and dbcAMP were added, these proportions were increased significantly ($P < 0.05$): 75% of oocytes were penetrated by fresh sperm, 67% had pronuclei, and 33% developed into blastocysts. Oocytes were not penetrated after coculture of cumulus–oocyte complexes with frozen–thawed sperm in mKRB-BSA medium. However, oocytes were penetrated (3%) when cocultured with frozen–thawed sperm in mKRB-BSA medium containing ATP and dbcAMP. Of the penetrated oocytes, 3%, 3%, 0.9%, and 0.9% progressed to the pronuclear, 2-cell, 4-cell, and blastocyst stages, respectively.

Fertilization after intrauterine insemination with frozen–thawed sperm. The fertilizing ability of frozen–thawed sperm after intrauterine insemination was assessed. After the injection of fresh sperm into 6 rats, 3 became pregnant and delivered 17 live rat pups. In contrast, when cryopreserved sperm were inseminated into the uteri of 27 rats, no live offspring were obtained.

Discussion

Both intracellular and extracellular ATP molecules play key roles in sperm function. Intracellular ATP is the main energy source driving sperm motility.[26] Extracellular ATP increases the calcium and cAMP levels in sperm.[18] Intra- and extracellular calcium are crucial for functional sperm motility in several animal species.[7,21,22] cAMP is generated from ATP during an adenylate-cyclase-mediated reaction in the protein kinase A pathway.[1,27] Although cAMP also is associated with sperm motility,[15] its role in the freezability of rat sperm has not been reported thus far.

We recently showed that rat sperm can be frozen successfully after incubating them in ATP-containing raffinose–mKRB–egg yolk extender.[32] This finding suggests that extracellular ATP has several downstream effects that increase the calcium and cAMP levels. These molecules may enhance sperm motility directly before cryopreservation or may be used as oxidative substrates in the mitochondria for ATP production and contribute to the remobilization of frozen–thawed sperm. We hypothesized that increasing the intracellular calcium and cAMP levels in rat sperm before cryopreservation would increase the oxygen-consumption rates of the sperm, enhance their motility, and ensure the survival of the cryopreserved sperm. To test this hypothesis, we first determined the effect of the calcium ionophore ionomycin in ATP-containing raffinose–mKRB–egg yolk freezing extenders on the motility of fresh and frozen–thawed sperm. The motility of fresh and frozen–thawed sperm diluted in ATP-containing extender supplemented with ionomycin did not significantly differ from that of sperm diluted in ionomycin-free extender. This result suggests even in the presence of ATP, ionomycin did not enhance the freezability of rat sperm.

In the next experiment, we attempted to determine whether addition of the sperm-motility regulator dbcAMP to ATP-containing extenders improves sperm motility after thawing. Frozen–thawed sperm in ATP-containing extender supplemented with 100 µM dbcAMP were significantly more motile than those in dbcAMP-free extender. Moreover, the frozen–thawed sperm incubated for 3 h at 37 °C exhibited greater motility, straight-line velocity, and amplitude of lateral head displace-

Table 1. Effect of ionomycin in ATP-containing raffinose–mKRB–egg yolk medium on various sperm characteristics after collection and thawing

		Ionomycin (μM)				
	Sperm characteristics	0	5	10	15	20
After collection	Motility (%)	73.9 ± 8.0	78.9 ± 2.4	81.4 ± 0.4	71.2 ± 9.9	76.6 ± 2.9
	VSL (μm/s)	14.5 ± 1.2	14.2 ± 5.0	8.0 ± 2.9	10.5 ± 1.3	11.1 ± 2.5
	VCL (μm/s)	138.4 ± 3.3	129.0 ± 13.3	103.4 ± 14.4	102.6 ± 6.6[a]	104.8 ± 16.0
	ALD (μm)	6.7 ± 0.5	6.8 ± 0.3	6.1 ± 0.5	5.8 ± 0.4	5.5 ± 0.7
	BCF (Hz)	32.4 ± 1.3	31.6 ± 2.8	32.1 ± 2.2	30.1 ± 1.7	30.9 ± 2.3
After thawing	Motility (%)	31.4 ± 3.6	31.9 ± 4.7	35.9 ± 1.7	25.4 ± 2.4	27.6 ± 7.3
	VSL (μm/s)	3.5 ± 0.5	5.9 ± 1.0	4.5 ± 0.2	5.9 ± 1.1	3.9 ± 0.4
	VCL (μm/s)	66.5 ± 5.2	83.6 ± 2.9	91.7 ± 1.2	77.3 ± 7.3	101.7 ± 22.2
	ALD (μm)	2.9 ± 0.2	4.0 ± 0.2	4.1 ± 0.1	3.7 ± 0.5	5.8 ± 2.0
	BCF (Hz)	35.1 ± 2.7	33.7 ± 2.8	37.8 ± 4.2	31.9 ± 0.7	29.7 ± 6.5

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity
Values are expressed as the mean ± SEM (n = 3).
[a]Value significantly ($P < 0.05$) different from control value.

Table 2. Effect of dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on sperm characteristics after collection and thawing

		dbcAMP concentration (μM)				
	Sperm characteristics	0	100	200	300	400
After collection	Motility (%)	63.3 ± 9.1	80.0 ± 5.8	71.4 ± 10.7	52.5 ± 15.1	29.6 ± 9.9[a]
	VSL (μm/s)	12.5 ± 0.8	13.5 ± 2.4	13.9 ± 3.9	12.8 ± 3.9	8.5 ± 2.2
	VCL (μm/s)	117.4 ± 0.9	100.9 ± 7.7	116.8 ± 14.7	93.4 ± 23.4	95.8 ± 9.8
	ALD (μm)	6.7 ± 0.3	5.1 ± 0.4[a]	5.6 ± 0.3	4.5 ± 1.3[a]	4.4 ± 0.6[a]
	BCF (Hz)	27.7 ± 0.9	24.6 ± 2.5	23.4 ± 1.1	26.2 ± 1.3	28.4 ± 3.3
	Acrosome integrity (%)	87.9 ± 5.8	90.5 ± 4.5	86.4 ± 5.1	88.0 ± 4.9	89.7 ± 4.2
After thawing	Motility (%)	28.5 ± 8.3	43.3 ± 2.8	34.9 ± 3.3	29.0 ± 3.8	14.1 ± 0.9[a]
	VSL (μm/s)	4.4 ± 0.5	5.1 ± 0.8	4.3 ± 0.2	4.3 ± 0.2	4.9 ± 1.9
	VCL (μm/s)	88.9 ± 12.9	89.3 ± 10.2	72.5 ± 4.4	88.4 ± 5.8	74.6 ± 9.5
	ALD (μm)	4.1 ± 0.4	3.9 ± 0.5	3.9 ± 0.6	4.4 ± 0.3	4.1 ± 0.5
	BCF (Hz)	36.8 ± 2.9	32.6 ± 2.2	35.6 ± 2.9	36.0 ± 1.0	36.2 ± 2.3
	Acrosome integrity (%)	76.0 ± 7.8	77.7 ± 8.2	76.9 ± 4.6	71.4 ± 8.0	69.3 ± 7.1

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity
Values are expressed as the mean ± SEM (n = 3).
[a]Value significantly ($P < 0.05$) different from control value.

ment than those incubated in dbcAMP-free ATP-containing extender. Both before and after thawing, the proportion of sperm with intact acrosomes did not differ among sperm diluted in the various dbcAMP-containing extenders. Therefore, the addition of dbcAMP to ATP-containing extenders provided the best results with regard to the postthaw sperm motility, thermal resistance of sperm, and acrosome integrity. This finding may be attributed to the activation of the cAMP-dependent molecular signaling or signal-transduction systems in sperm by extracellular ATP before sperm cryopreservation.[18,26] This activation may contribute to the remobilization of rat sperm after freezing and thawing. Oxygen consumption by sperm was evaluated after their incubation 37 °C for 10 min in ATP-containing medium with or without 10 μM ionomycin and 100 μM dbcAMP. Oxygen-consumption rates did not differ among sperm cultured in the media tested, suggesting that extracellular ionomycin and dbcAMP are not involved in the mobilization of mitochondrial energy substrates in sperm.

The role of cAMP in the enhancement of sperm motility is well established; further, cAMP is an important regulator of capacitation and the acrosome reaction.[3,14] We sought to determine the optimal conditions for successful IVF with fresh and frozen–thawed rat sperm. Oocytes were fertilized with fresh and frozen–thawed sperm in mKRB-BSA medium with or without ATP and dbcAMP. When IVF was performed with fresh sperm, the resultant fertilization rates and the percentage of embryos that progressed to blastocysts were higher when mKRB-BSA medium containing ATP and dbcAMP was used than when the medium lacked ATP and dbcAMP. Frozen–thawed sperm failed to penetrated oocytes after coculture with cumulus–oocyte complexes in mKRB-BSA medium. In contrast, cryopreserved sperm cultured in ATP- and dbcAMP-containing mKRB-BSA medium successfully penetrated oocytes, and the resultant embryos progressed to blastocysts. These results indicate ATP and dbcAMP in the IVF medium may regulate intracellular cAMP levels. An increase in intracellular cAMP levels may mediate calcium influx into sperm through CatSper calcium

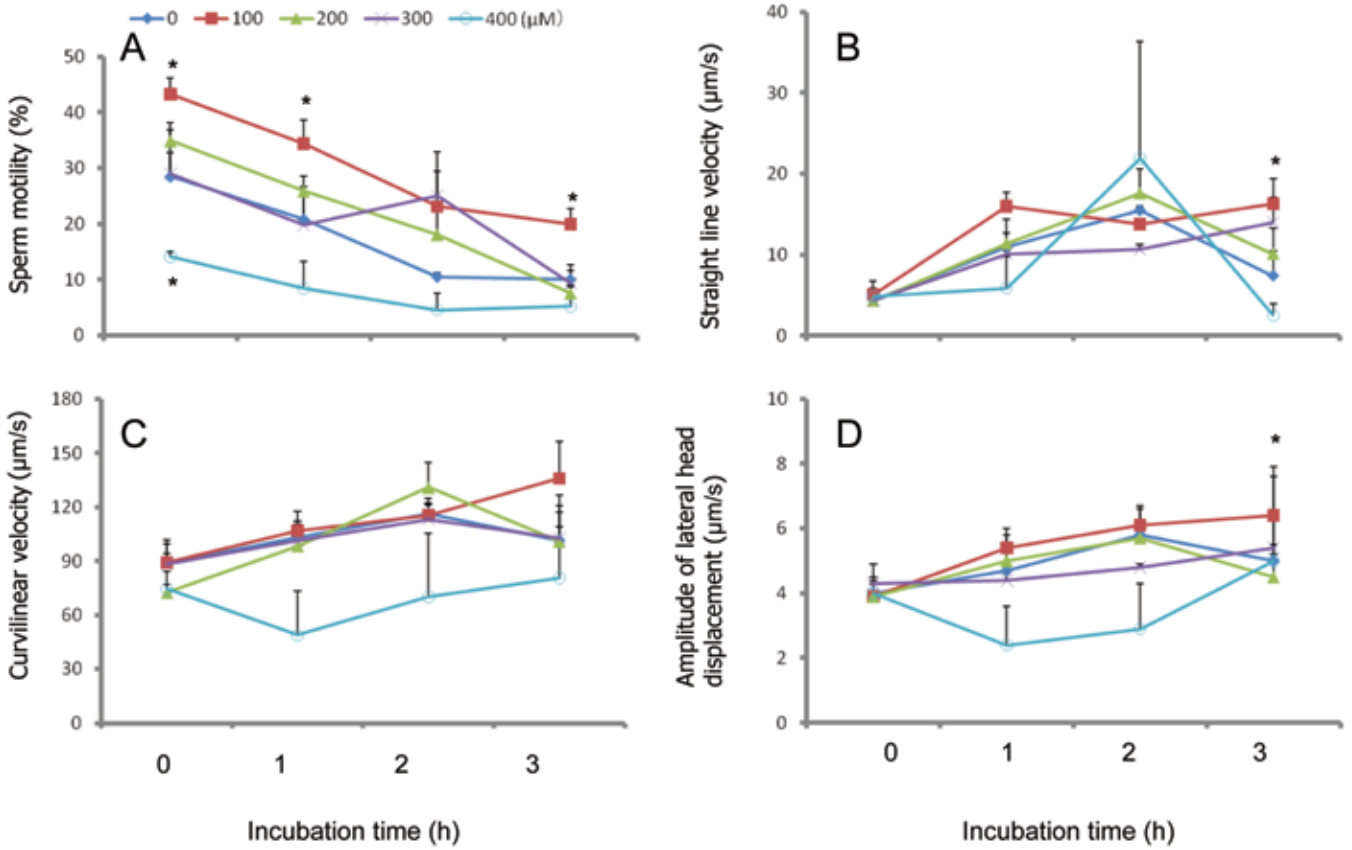

Figure 1. Effect of dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on the (A) motility, (B) straight-line velocity, (C) curvilinear velocity, and (D) amplitude of lateral head displacement of frozen–thawed sperm after incubation at 37 °C for 3 h. Values are presented as mean ± SEM (n = 3). *, value significantly ($P < 0.05$) different from control value.

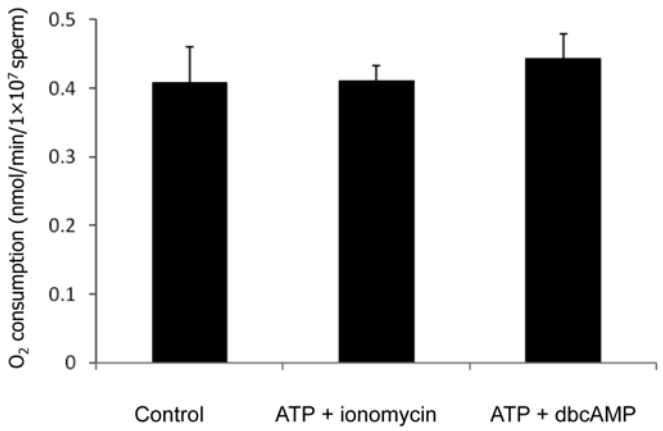

Figure 2. Effect of ionomycin and dbcAMP in raffinose–mKRB–egg yolk medium containing ATP on the oxygen consumption of fresh sperm after incubation at 37 °C for 10 min. Values are presented as mean ± SEM (n = 5). Supplementing ATP-containing medium with ionomycin and dbcAMP did not alter the oxygen consumption of fresh sperm.

ion channels[6,10,23] or activate adenosine triphosphatase; sperm then become hyperactive and undergo the acrosome reaction.[2,27] These changes augment the fertilizing ability of fresh and cryopreserved rat sperm. A recent article[29] also addressed the importance of cAMP and reported increased fertilization rates with frozen–thawed sperm that had been treated with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthin.

We also assessed the fertilizing ability of frozen–thawed sperm by using intrauterine insemination. Three of the six Wistar rats injected with fresh sperm became pregnant and delivered 17 live rat pups. In contrast, when cryopreserved sperm were inseminated into the uteri of 27 rats, no live offspring were obtained. Successful artificial insemination by using frozen–thawed rat sperm has been reported previously,[20] and a more recent publication by same group[28] confirmed that cryopreserved rat sperm can be revitalized and result in the birth of live offspring through embryo transfer after IVF. Although the authors[28] mentioned that intracytoplasmic sperm injection is the only way to routinely obtain offspring routinely derived from oocytes fertilized in vitro, the use of fresh and cryopreserved sperm in an in vitro fertilization protocol is not yet widespread.[11] Taken together, these findings indicate that protocols for sperm cryopreservation and oocytes fertilized in vitro by using cryopreserved sperm are still under development for safe preservation of most rat strains.

In conclusion, the cryopreservation of rat epididymal sperm in raffinose–mKRB–egg yolk extender supplemented with ATP and dbcAMP rendered sperm from Wistar rats freezable. Oocyte fertilization by fresh sperm and blastocyst formation from the fertilized oocytes were enhanced when the fertilization medium was supplemented with ATP and dbcAMP. Frozen–thawed sperm incubated in mKRB-BSA medium containing ATP and dbcAMP penetrated oocytes, and resultant embryos progressed to the blastocyst stage. This finding indicates that ATP- and dbcAMP-containing extenders improved the postthaw motility and fertilizing ability of cryopreserved rat sperm. Moreover, the IVF medium developed in the current study may be effective for the in vitro production of embryos from cryopreserved rat sperm. Further experiments are required to develop more

Table 3. Development of oocytes after IVF with fresh and cryopreserved rat sperm

			No. (%) of total oocytes examined				
	ATP+ dbcAMP	Total	Penetration	Pronuclear formation[a]	2-cell embryos	4-cell embryos	Blastocysts
Fresh sperm	–	146	81 (55.5)[b]	71 (48.6)[b]	70 (48.0)[b]	57 (39.0)[b]	33 (22.6)[b]
	+	169	128 (75.7)[c]	113 (66.7)[c]	114 (67.5)[c]	104 (61.5)[c]	57 (33.7)[c]
Frozen sperm	–	132	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	+	102	4 (3.9)	4 (3.9)	4 (3.9)	1 (0.9)	1 (0.9)

[a]Two pronuclei with a sperm tail.
[b,c]Values with different superscripts within the same column are significantly ($P < 0.05$) different.

effective methods for artificial insemination and IVF using cryopreserved rat sperm.

Acknowledgment

This study was partly supported by grants 19658102 and 16108003 (to ES) from the Japan Society for the Promotion of Science.

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