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Effect of 1-Methyladenine on Responses of Spermatozoa to Egg Jelly in Starfish — A Convenient Method for Counting the Rate of Acrosome Reaction and for Measuring Sperm Motility —

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ABSTRACT The effect of 1-methyladenine (1-MeAde) on the responses of spermatozoa to egg jelly in *Asterina pectinifera* was investigated. 1-MeAde-treated spermatozoa underwent acrosome reaction of a higher rate than non-treated ones when they were incubated in sea water containing homologous egg jelly. A method for measuring acrosome reaction rates by means of a light microscope was established. For measuring sperm motility, paths of sperm swimming in dark field microscopy were photographed. Spermatozoa taken from testes previously treated with 1-MeAde decreased their beat frequency when they were diluted with sea water or with sea water containing egg jelly compared with non-treated ones. These facts indicate that 1-MeAde exerts an effect on some activities of spermatozoa as well as inducing maturation of oocytes. (*Zool. Mag. 91: 272-280, 1982*)

Starfish egg spawning is induced by 1-methyladenine (1-MeAde) produced by follicle cells under the influence of gonad-stimulating hormone secreted from nervous tissue (Kanatani, 1964; Kanatani and Shirai, 1967; Kanatani *et al.* 1969, Hirai *et al.* 1973; Kanatani, 1979; Shirai *et al.* 1981). 1-MeAde acts on oocytes from the outside (Kanatani and Hiramoto, 1970) and causes them to undergo germinal vesicle breakdown. On the other hand, sperm spawning is also induced by 1-MeAde (Kanatani, 1969) which is produced by interstitial cells (Kubota *et al.* 1977). Therefore spawned spermatozoa have probably been exposed to 1-MeAde. 1-MeAde effect on spermatozoa, however, has not been investigated.

One of the most distinct differences between immature oocytes and maturing oocytes treated with 1-MeAde is that the latter can elevate fertilization membrane (exocytosis of cortical granules) when they are inseminated. Likewise, it is possible to assume that 1-MeAde has some effects on spermatozoa and that these effects are to be manifested in the fertilization phenomena: sperm swimming and the acrosome reaction (exocytosis of acrosomal vesicle).

In this paper we report (1) that spermatozoa treated with 1-MeAde undergo the acrosome reaction in higher rate than those non-treated (control), and (2) that 1-MeAde-treated spermatozoa swim to less extent than those in control when they are diluted with sea water or with sea water containing egg jelly.

Materials and Methods

Starfish, *Asterina pectinifera*, were collected from Kanagawa-ken in May and from Aomori-ken in September and kept in aquaria with circulating cold (14°C) sea water.

Artifitial sea water, Jamarin (SW), was used (Jamarin Laboratory, Osaka, Japan). For pH adjustment, NaOH and HCl were used unless otherwise discribed. Calcium inophore A23187 was a gift from Eli Lilly

and Co. The ionophore was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/m*l* (a stock solution). It was diluted with SW or with SW containing DMSO so that all the test solutions contained 10 % DMSO uniformly. With respect to staining dyes, see the Table 1 shown in Result.

Acrosome reaction assay

A convenient method for measuring acrosome reaction rate was established in advance. Unless otherwise described, 3 drops of 20 % gultarardehyde in deionized water (DW) were added to 0.4 m*l* of sperm suspension. After standing for appropriate durations, the spermatozoa were washed in several m*l* of SW by centrifugation (3,000 rpm, 15 min). Two drops of SW and two drops of the dye solution (stock solution) were added to the sperm pellet. Observation was carried out under an ordinary microscope (bright field, 100 × oil immersion objective). For oxidative fixation, Champy's fluid (Gurr, 1962) was used instead of gultaraldehyde.

Jelly preparation

For induction of the acrosome reaction: Ovaries were immersed in SW containing 1-MeAde (10^{-6}M), then released eggs were collected and washed in SW. Eggs were kept in SW adjusted to pH 5.5 for 1 hr with occasional gentle stirring. Observation of eggs in the presence of India ink showed that the egg jelly dissolved in the acidified SW. The supernatant was filtered with nylon mesh and centrifuged (10,000 × *g*, 15 min) to remove debris. The supernatant was adjusted to pH 8 and concentrated by filtering through an ultrafiltration membrane cone (Centriflo CF25, Amicon Corp.) at 3,000 rpm. Since a diameter of an egg and a thickness of a jelly layer are about 160 μm and 10 μm, respectively, the volume of jelly layer of one egg can be estimated as about 0.9 μl. By counting the eggs used, we estimated the total jelly volume corresponding to the original jelly layer. With the Centriflo technique we reduced the supernatant volume to 1/2 of the original jelly layer volume, thereby concentrating the jelly to twice that of the original jelly. The concentrated jelly solution was diluted with sea water of various pHs. In this case artifitial sea water (van't Hoff's: 2.7 % NaCl, 0.07 % KCl, 0.1 % $CaCl_2$, 0.34 % $MgCl_2$, 0.21 % $MgSO_4$, W/V) was used. pH was adjusted by 10 mM (final concentration) of piperazine-N,N'-(2-ethanesulfonic acid) (PIPES). When 1-MeAde-treated spermatozoa were incubated with these diluted jelly solution (pH 8) for 5 sec, about 90 %, 13 %, 2 %, and 0 % spermatozoa underwent the acrosome reaction at the jelly concentration of 80 %, 26 %, 8 % and 3 %, respectively.

For sperm motility assay: Spawned and washed eggs (about 2 m*l*, sedimented by standing) were kept in acidified SW for 1 hr to dissolve their jelly layer similar to the case described above. The supernatant was diluted to 150 m*l* and adjusted pH to 8. In this case, the jelly concentration corresponded to about 1 % original jelly.

Handling of spermatozoa

Acrosome reaction assay: Two or three alveoli of testes were incubated in 0.5 m*l* of SW or in SW containing 1-MeAde (10^{-6}M) at pH 6.5 for 1 hr. One drop of sperm suspension taken from incubations was added to the diluted jelly solution (26 % original jelly) and mixed. After standing for 5 sec they were fixed as described before.

Motility assay: Ligated testes were incubated in SW or in SW containing 1-MeAde (10^{-6}M) for 1 hr. Then, after the surrounding SW was removed, dry sperm from teased testes was diluted (20,000-fold dilution) with SW or with jelly solution.

Sperm motility assay

Five min after dilution of dry sperm with test solution, 0.2 m*l* sperm suspension was dropped on a glass slide and the spermatozoa were photographed without a

cover glass by means of dark field microscopy (20 x objective). A 200W Hg-lamp was used. Exposure time was 1/2 sec. The glass slides were treated just before use in the following way in order to prevent spermatozoa from adhering to the glass surface. They were (1) immersed for 10 sec in 1 % polyethyleneglycol (MW, 20,000) in DW, (2) rinsed with DW, and (3) dried in the air. By this treatment, the surface of the slides become hydrophilic and the test solutions spread evenly on their surfaces. Eight to sixteen different fields were photographed from each slide. Each swimming path is a wavy line and we can measure the quantities of the following items (Fig. 2): (1) the speed of sperm propulsion by the length of a center axis of a wavy line, (2) the beat frequency by the number of waves in a wavy line, (3) the amplitude of wags by the width of a wave, (4) the whole path which sperm head passed through by the length of a whole wavy line, (5) the beat condition by the radius of curvature of the arc formed by the axis of a wavy line, and (6) the tail bending condition by the continuous images of tail movement. In order to measure the radii of the arcs, the concentric circles drawn on a transparent plate was fitted on the wavy lines. Since spermatozoa tend to roll during their free swimming (Hiramoto and Baba, 1978), the wave number in photographed paths does not always reflect the actual beat frequency. However, those values are comparable in each comparison between certain two conditions.

Results

I. Preliminary observation (agglutination of spermatozoa)

In order to obtain any evidence which indicates 1-MeAde effect on sperm property at the time of fertilization, sperm behavior was observed with respect to capability of agglutinating. Jelly solution was prepared as 1 % original jelly. Ligated testes were preincubated in SW or in SW containing 1-MeAde (final concentration, 10^{-5}M) for 1 hr, then a bit of dry sperm from the preincubated testes was added to drops of SW or of SW containing jelly. Using 4 animals and 4 jelly preparations, direct observations under a microscope showed that spermatozoa treated with 1-MeAde tended to agglutinate in SW containing jelly, this agglutination corresponding sperm swarming as described by Collins (1976). This preliminary result indicated that 1-MeAde had affected sperm behavior which is manifested in the presence of jelly. However, agglutination is a rather complex phenomenon including sperm motility or capability of adhering and is a labile phenomenon affected by the sperm density or by concentration of jelly. Therefore more precise analysis was performed to clarify the 1-MeAde effect on sperm property.

II. Acrosome reaction in 1-MeAde-treated spermatozoa

The most suitable dyes for staining the acrosomal vesicle after gultaraldehyde fixation were phloxine, erythrosin, alizarin red S, orcein, azocarmine G, eosin bluish, eosin yellowish, and eosin (water and alcohol soluble). Comments about other dyes tested are shown in Table 1. In Table 2, suitable dyes after Champy's fixation are listed. In these observations, deeply stained acrosomal vesicles enabled us to judge easily whether the spermatozoa had acrosomal vesicles or not. Gultaraldehyde concentrations from 2 to 8 % made no difference in these observations. Altering the fixation duration (several min to several days) also did not affect the result.

When eggs with intact jelly layer were inseminated, all spermatozoa which became trapped in the jelly underwent the acrosome reaction in starfish. This was observed by washing away all untrapped spermatozoa, dissolving the jelly layer (as described above) in order to liberate the trapped spermatozoa,

Table 1. Dyes suitable for staining the acrosomal vesicle of *Asterina* spermatozoa after gultaraldehyde fixation.

Group	Dyes	Stock solution*	Sources	Observations
I	Phloxine	2 % W	Sako Pure Chem. Ind, Ltd.	These are the most suitable dyes for staining the acrosomal vesicles. Organella are stained in the following order; acrosomal vesicles, mitochondria, and nuclei. Deeply stained acrosomal vesicles enable us to judge easily whether the spermatozoa have acrosomal vesicles or not.
	Erythrosin	2 % W	Merck	
	Alizarin Red S	2 % W	Wako	
	Orcein	2 % W	Merck	
	Azocarmine G	1 % AA10	Merck	
	Eosin Bluish	2 % W	Merck	
	Eosin Yellowich	2 % W	Merck	
	Eosin, water and alcohol soluble	2 % W	Wako	
II	Acid fuchsin	1 % AA1	Merck	The acrosomal vesicles are stained deeply but the appearance is less distinct from the other components than that with the dyes in group I.
	Alizarin	2 % W	Wako	
	Light green yellowish	1 % AA1	Merck	
	Fast green FCF	1 % AA1	Chroma	
	Fluorescein sodium	2 % W	Wako	
III	Azur II	1 % AA1	Merck	The nuclei are stained very deeply and the acrosomal vesicles are easily observed as unstained areas.
	Aniline blue WS	1 % AA2.5	Merck, Wako	
	Alizarin blue S	2 % W	Searle Dianostic Company, Inc.	
	Methyl green	2 % W	Merck	
	Methylene blue	2 % W	Wako	
	Nile blue	2 % W	Wako	
	Crystal violet	2 % W	Merck	
	Janus green	2 % W	Wako	
	Trypan blue	2 % W	Chroma	
	Toluidine blue	2 % W	Tokyo Kasei	
IV	Alcian blue 8GX	2 % W	Wako	Similarly to group III, the nuclei are stained very deeply but the acrosomal vesicles are not very clearly observed.
	Chromotrop 2B	1 % W	Chroma	
	Ponceau S	1 % AA3	Merck	
	Basic fuchsin	2 % W	Wako	
	Neutral red	2 % W	Wako	
	Victoria blue 4R	2 % W	Chroma	
	Victoria blue B	2 % W	Wako	
V	Ponceau 2R	1 % AA3	Wako	Structural components such as acrosomal vesicles, nuclei, mitochondria, and tails are well stained but are not distinguishable from each other.
	Alizarin yellow GG	2 % W	Wako	
	Alizarin yellow R	2 % W	Wako	
	Carmine	2 % W	Merck	
	Hematoxylin	2 % W	Merck	

* 2 % W; 2 % solution in water.
1 % AA10; 1 % solution in 10 % acetic acid.

Table 2. Staining of the acrosomal vesicle after fixation in champy's fixative

Dyes*	Observations
Ponceau 2R Ponceau S Acid fuchsin Chromotrop 2B Light green yellowish Fast green FCF Janus green	The acrosomal vesicles are stained very deeply, but they are not so distinct from the other structural components as in the case in Group I of Table 1.
Azur II Alcian blue 8GX Crystal violet Methyl green Methylene blue Neutral red Toluidine blue	The nuclei are stained very deeply and the acrosomal vesicles are observed as unstained areas similarly to the case of gultaraldehyde fixation (group III and IV).

* Stock soutions are the same as in Table 1.

and staining the spermatozoa. In all cases the spermatozoa had undergone a change of shape and had apparently lost their acrosomal vesicles. These facts are characteristic of the acrosome reaction.

To examine the validity of the staining technique, ionophore-induced acrosome reaction was tested. Figure 1 shows the result. At ionophore concentration of 2×10^{-4}M, 21 % (mean) of spermatozoa reacted during 5 sec. At the same concentration (2×10^{-4}M) when the incubation time was extended to 10 min, the acrosome reaction rate increased to 100 %. At 2×10^{-6}M and 2×10^{-7}M ionophore concentrations, about 50 % and 30 % of spermatozoa reacted, respectively within 10 min.

In order to examine the effect of 1-MeAde on the acrosome reaction, the 26 % jelly solution was used as described in Materials and Methods. In starfish, the acrosome reaction almost never takes place when the spermatozoa are simply diluted in SW alone regardless of 1-MeAde treatment.

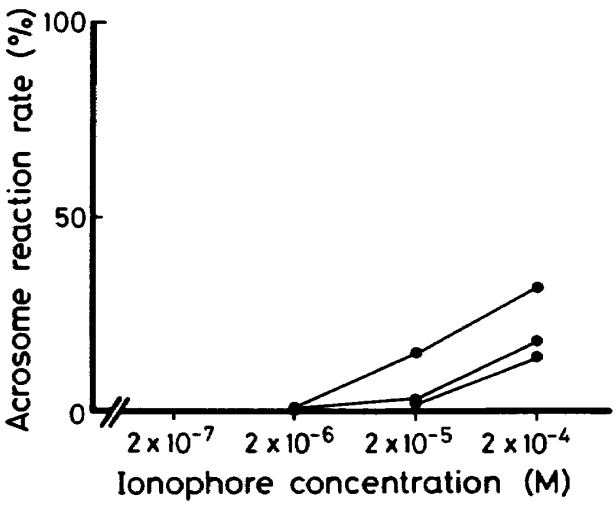

Fig. 1. Acrosome reaction induced by ionophore A23187. Test solutions containing ionophore at various concentrations prepared by dilution of stock solution (2×10^{-3}M in DMSO) with SW or with SW containing 10 % DMSO so that all the test solutions contain 10 % DMSO uniformly. Sperm suspension (0.03 m*l*) was added to 0.3 m*l* of test solution. Fixation was carried out 5 sec after mixing.

Table 3. Increase of acrosome reaction rate induced by jelly in 1-MeAde-treated spermatozoa.

Animal No.	Acrosome reaction rate (%)			
	pH at induction of the reaction			
	6.5		7.5	
	1-MeAde-treated spermatozoa	Non-treated spermatozoa	1-MeAde-treated spermatozoa	Non-treated spermatozoa
1	14 (134)*	6 (117)	26 (145)	8 (116)
2	14 (123)	7 (125)	22 (109)	14 (118)
3	10 (132)	3 (125)	17 (148)	2 (127)
4	13 (250)	4 (120)	15 (126)	10 (124)
Mean ± s.d.	13 ± 2.1	5 ± 2.0	20 ± 5.4	9 ± 5.5

* Number of spermatozoa counted.

1-MeAde effect was significant ($P<0.05$) at both cases (pH 6.5 and pH 7.5).

Table 4. Beat frequency and speed propulsion of spermatozoa in various conditions.

Previous treatment	Diluted with					
	SW alone			Jelly solution**		
	Animal No.	Beat frequency (number/sec)	Speed propulsion (μm/sec)	Animal No.	Beat frequency (number/sec)	Speed propulsion (μm/sec)
SW alone	1	50.0	289	1	46.8	277
	2	50.0	291	2	46.6	270
	3	50.4	291	3	46.8	268
	4	48.6	298	4	46.2	282
	5	42.0	254	5	42.0	240
1-MeAde*	1	48.6	278	1	42.0	246
	2	47.4	286	2	43.8	281
	3	50.2	302	3	45.8	257
	4	49.0	299	4	46.6	268
	5	40.2	227	5	39.4	227

With respect to beat frequency, the effect of pre-incubation with 1-MeAde (*) was statistically significant ($P<0.05$), and the effect of dilution with jelly solution (**) was also significant ($P<0.05$) by the test of three layout of analysis of variance. The two kinds of effects were considered to be cumulative.

With respect to the speed of propulsion, the effect of dilution with jelly solution was significant ($P<0.05$).

The same animal number in groups indicates the same animal.

As shown in Table 3, the acrosome reaction occured to less extent at low pH regardless of 1-MeAde treatment. An increase of the acrosome reaction rate was obtained when the spermatozoa were previously treated with 1-MeAde.

III. Motility in 1-MeAde-treated spermatozoa

Figure 2 shows an example of the photographic paths of swimming spermatozoa. As shown in Fig. 2, we can obtain such values as speed of propulsion, beat frequency, etc. as described in Materials and Methods from each wavy line. In the present study, we mainly dealt with comparisons between the beat frequency and propulsion speed under the different conditions.

The ligated testes were incubated in SW or in SW containing 1-MeAde, and then the dry sperm taken from each incubated testis were diluted with SW or with jelly solution (1 % original jelly). Therefore, measurements were carried out about four kinds of sperm suspensions: SW-treated and SW-diluted one, SW-treated and jelly

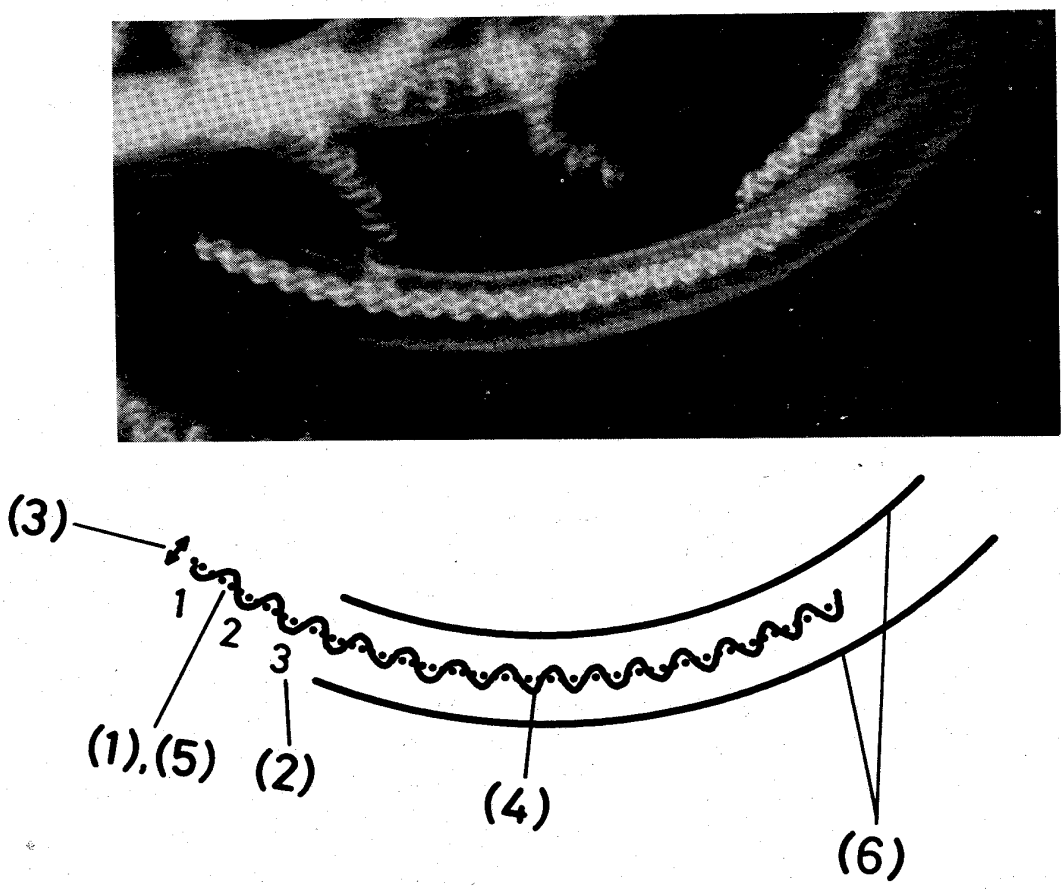

Fig. 2. An example of the path of swimming spermatozoa. Dry sperm (0.5 μl) was diluted with SW (10 ml), and a sperm suspension (0.2 ml) was dropped on a glass slide. Spermatozoa were photographed by means of dark field microscopy (20x objective; 200W Hg lamp, 1/2 sec exposure; Kodak Tri-x film, ASA 400). (1) The length of the axis of a wavy line for the speed of sperm propulsion. (2) The wave number for the beat frequency. (3) The width of the wave for the amplitude of wags. (4) The length of a whole wavy line which a sperm head passed through for the whole path. (5) The arc of the axis of a wavy line for the radius of curvature of sperm propulsion. (6) The continuous image of tail movement.

solution-diluted one, 1-MeAde-treated and SW-diluted one, and 1-MeAde-treated and jelly solution-diluted one. More than 80 % spermatozoa were swimming in these conditions. As shown in Table 4, the beat frequency in spermatozoa treated with 1-MeAde was smaller than that of spermatozoa treated with SW alone, and the beat frequency in spermatozoa diluted with jelly solution was smaller than that of spermatozoa diluted with SW alone. In addition, propulsion speeds in jelly solution-diluted spermatozoa were smaller than those in SW-diluted ones.

Discussion

Acrosome reaction

The electron microscopical technique for counting acrosome reaction rate has many difficulties. For example, setting angles of spermatozoa against supporting membranes are not always appropriate for the observation of the acrosome processes, the reacted spermatozoa bind to surface more easily than non-reacted ones, and morphological changes in spermatozoa without acrosome reaction actually occur in some cases (the changes of the relative position of nuclei, mitochondria, and tails). The measuring method by the light microscope introduced in the present study is based on counting the remaining acrosomal vesicles. With this method all spermatozoa within a certain microscopic field can be confined for the judgment.

As the reason of low rate of acrosome reaction under the condition of low pH, it is considered that existence of H^+ outside (low pH) inhibited the procedure of the acrosome reaction, because the triggering of acrosome reaction requires release of H^+ as previously investigated (Collins and Epel, 1977).

1-MeAde-treated spermatozoa apparently increased their acrosome reaction sensitivity to jelly. Statistical significances at both pHs (6.5 and 7.5) were $P<0.05$. The acrosome reaction consists morphologically of membrane fusion between acrosomal vesicles and sperm cells and of elongation of acrosome processes known as actin polymerization (Tilney *et al.* 1978). On the other hand, as mentioned in Introduction 1-MeAde-treated oocytes acquire capability of forming fertilization membrane upon insemination. Formation of fertilization membrane is based on cortical reaction, that is, exocytosis of cortical granules. This process also consists of membrane fusion between cortical granules and oocyte cells. Furthermore, H^+ release is also induced in the cortical reaction (Aketa, 1963; Ii and Rebhun, 1979). Since facilitation of this exocytosis in oocytes is conditioned by 1-MeAde action, it is worthy to consider the increase of acrosome reaction (exocytosis of acrosomal vesicles) as comparable phenomenon to the cortical reaction.

Sperm motility

In the present study we used the beat frequency and speed propulsion as the indicators of the motility. The statistical test (three way layout analysis of variance) (Campbell, 1974 ; Ishii, 1975) on the result shown in Table 4 revealed that the suppressing effect of 1-MeAde treatment on beat frequency was significant ($P<0.05$) and the suppressing effect of dilution with jelly solution on beat frequency was also significant ($P<0.05$). Furthermore, the test indicates that we can consider that the two kind of effects (1-MeAde treatment and jelly solution dilution) are cumulative each other. In other words, the effect of 1-MeAde treatment was more clealy manifested when spermatozoa were diluted with jelly solution than that in the case of dilution with SW alone. With respect to the speed of propulsion, the effect of dilution with jelly solution was statistically significant ($P<0.05$), whereas 1-MeAde effect was not significant.

Although the mechanism by which 1-MeAde affects acrosome reaction or beat frequency are not yet known, these phenomena are considered to be related in the sperm capacitation as investigated in mammalian spermatozoa.

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