JOUR. FAC. SCI., SHINSHU UNIV. Vol. 4, pp. 71—78, Dec. 1969

Effects of the treatments of the spermatozoa with alkaline sea water, low-Ca^{++} solution and uranyl nitrate solution on the insemination of sea urchin eggs, Hemicentrotus pulcherrimus.

by AKIRA KOENUMA

Department of Biology, Faculty of Science,
Shinshu University.
(Received Oct. 8 1969)

Abstract

On the insemination of the sea urchin eggs, *Hemicentrotus pulcherrimus*, the effects of the modified spermatozoa were investigated. In the cases where the eggs were inseminated with the spermatozoa treated with the alkaline sea water, pH 9.2, abnormally cleavaged eggs increased. In these abnormal eggs there seems to be a pretty number of the polyspermic eggs. From the insemination with the low-Ca^{++}-treated spermatozoa a somewhat increase of the abnormal eggs resulted. In the cases of the insemination with the spermatozoa treated with uranyl nitrate solution an increase of the abnormal eggs did not result from the first insemination with the modified sperm, but from the second insemination. These results show a possibility that those treatments of the spermatozoa affect in an early process of fertilization. As to the results of these experiments, effects of treatments of the spermatozoa were discussed with a relation to the acrosome reaction of the spermatozoa.

Introduction

It has been well known that the polyspermy resulted frequently from the various treatments of the sea urchin eggs. We have fairly known the many factors of polyspermy-formation in the fertilization of sea urchin eggs[2,6,10,11]. Of these factors, some ones possiblly induce the polyspermy with the treatment of the sperm alone not involving the eggs.

The polyspermy occurring in the insemination of the modified spermatozoa was first reported by AMOROSO & PARKES in rabbit's egg[1]. Polyspermy was also found in the insemination of the eggs of a echuroid worm, *Urechis unicinctus*, with the modified spermatozoa[8]. According to KOENUMA, an increase of poly-

spermic cleavaged eggs of a sea urchin resulted from the insemination of the spermatozoa treated with acidified sea water, pH 7.1[9]. It is quite interesting to investigate whether the polyspermic cleavage of sea urchin eggs increases with different treatments of the sperm. The present work is an attempt to find the more effective treatment of the spermatozoa for the polyspermy-induction in a sea urchin other than that with an acidified sea water.

Material and Methods

The experiments were made by using the eggs of sea urchin, *Hemicentrotus pulcherrimus*, which were obtained near the marine biological station of Misaki, Kanagawa. Eggs were collected by the spawing caused from the treatment with KCI solution. Spermatozoa were obtained from the gonads removed from male animals.

Insemination was carried out by the method previously mentioned[9]. The sperm concentration used in the insemination was 10^{-4} of the "dry" sperm. To deprive of the fertilization membrane, the eggs were filtered through the double cheese cloth sheets 2 minutes after the insemination. About 75 % of the eggs was deprived of the fertilization membrane by means of this method. Statistical test was made by using more than two hundreds eggs in each experiment. In the cases of the eggs deprived of fertilization membrane, only the eggs succesfully deprived of membrane were observed.

Eggs were observed at the first cleaval stage. A room temperature during the course of this experiment showed the range between 9°C and 12°C.

Experimental Results

1. *Insemination with the spermatozoa treated with alkaline sea water.*

The cleavages of the sea urchin eggs inseminated with the spermatozoa treated with alkaline sea water were observed. Alkaline sea water was prepared from the addition of a proper volume of the diluted sodium hydroxide solution, and its pH was 9.2. Before the insemination the treatment was carried out by means of the dilution of the "dry" sperm with 100 volumes of alkaline sea water. The duration of the treatment was about 25 seconds. After the treatment of spermatozoa, the one volume of sperm suspension was mixed with 100 volumes of egg suspension of the normal sea water. Thus the ultimate density of the spermatozoa at the insemination became 10^{-4} of the initial density of the "dry" sperm. When the intact eggs were inseminated with such density of the intact spermatozoa, the fertilization membrane occurred in 99—100 % of the eggs. 94.5 % of those eggs cleavaged normally.

The result was present in table 1, where the following facts were shown.

Table 1. Results of the insemination with the modified spermatozoa treated with the alkaline sea water, pH 9.2, in the eggs of the sea urchin, *Hemicentrotus pulcherrimus*.

Treatments of eggs				Cleavage of eggs		
1 st. insemination	deprival of F. M.	treatment with Na-K mixture	2 nd. insemination	uncleavaged eggs (%)	normal eggs (%)	abnormal eggs (%)
sw	−	−	−	3.5	94.5	2.0
sw	+	−	−	0.4	98.8	0.8
sw	+	−	sw	1.3	96.8	2.0
sw	+	−	alk	2.0	93.0	5.0
sw	+	+	−	0.5	97.0	2.5
sw	+	+	sw	3.3	94.5	2.3
sw	+	+	alk	2.5	92.5	5.0
alk	−	−	−	24.8	54.8	20.4
alk	+	−	−	3.2	93.6	3.2
alk	+	−	sw	1.8	95.6	2.6
alk	+	−	alk	2.0	93.0	5.0
alk	+	+	−	3.5	95.0	1.5
alk	+	+	sw	4.9	92.0	3.1
alk	+	+	alk	2.5	91.7	5.8

sw : sea water diluted sperm,
alk : alkaline sea water treated sperm,
− : a treatment was not carried out.
+ : a treatment was carried out.

The percentages of the uncleavaged eggs and the abnormally cleavaged eggs increased remarkablly when the unfertilized eggs were inseminated with the treated spermatozoa. Such remarkable increase of the abnormal cleavage, however, did not occur in the eggs deprived of the fertilization membrane after insemination. In a comparison between the cleavages of the eggs inseminated with the normal spermatozoa and those with the modified spermatozoa, somewhat decrease of the normal cleavage was appreciable in the latter cases. When the eggs were inseminated twice, a slight decrease of the normal cleavage could be recognizable only in the eggs inseminated with the modified spermatozoa as second insemination.

2. *Insemination with the spermatozoa treated with uranyl nitrate solution.*

The cleavages of the sea urchin eggs inseminated with the spermatozoa treated with uranyl nitrate solution were investigated. The spermatozoa were treated with 10^{-3}M sulution, and its pH was 7.2. For the treatment of the spermatozoa, the dilution of "dry" sperm with a proper volume of the reagent was employed. The duration of the treatment was about 5 minutes. After the treatment of

Table 2. Results of the insemination with the modified spermatozoa treated with 10^{-3}M. uranyl nitrate solution in the eggs of the sea urchin, *Hemicentrotus pulcherrimus*.

Treatments of eggs				Cleavage of eggs		
1 st. insemination	deprival of F. M.	treatment with Na-K solution	2 nd. insemination	uncleavaged eggs (%)	normal eggs (%)	abnormal eggs (%)
sw	−	−	−	0.0	100.0	0.0
sw	+	−	−	4.5	94.5	1.0
sw	+	−	sw	2.5	96.0	1.5
sw	+	−	un	8.0	90.5	1.5
sw	+	+	−	2.7	96.0	1.2
sw	+	+	sw	1.7	93.8	4.5
sw	+	+	un	2.8	94.8	2.5
un	−	−	−	0.0	99.5	0.5
un	+	−	−	1.0	96.0	3.0
un	+	−	sw	2.0	96.5	0.5
un	+	−	un	2.5	91.5	6.0
un	+	+	−	1.0	97.5	1.4
un	+	+	sw	2.7	91.7	5.7
un	+	+	un	2.4	94.6	3.0

sw : sea water diluted sperm,
un : uranyl nitrate treated sperm,
− : a treatment was not carried out.
+ : a treatment was carried out.

spermatozoa the one volume of the sperm suspension was mixed with the 100 volumes of the egg suspension of normal sea water, thus the final density of the spermatozoa at the fertilization became 10^{-4} of the initial density of the "dry" sperm.

The fertilization membrane was formed in 99—100 % of the eggs, when the unfertilized eggs were inseminated with such density of the intact spermatozoa. Of these eggs 100 % cleavaged normally.

Table 2 showed the result obtained. In the first insemination, little effect resulted from the treatment of spermatozoa with uranyl nitrate solution. Because any significant difference was not found between the intact spermatozoa and the modified ones. In the second insemination, however, those modified spermatozoa seems to be effective to increase the abnormal eggs. Because a somewhat decrease of the normal eggs resulted from the second insemination of the modified spermatozoa to the eggs fertilized and kept without the treatment of the Na-K mixture.

3. *Insemination with the spermatozoa treated with low-Ca^{++} solution*

The cleavages of the sea urchin eggs inseminated with the spermatozoa treated with the low-Ca^{++} solution were observed. As a low-Ca^{++} solution, 5/9 M Nacl-KCl mixture, 100 : 2, pH 8.2 with $NaHCO_3$, was used. The duration of the treatment of spermatozoa was about 5 minutes. The method of the treatment was a dilution method previously mentioned. The density of the spermatozoa at fertilization was kept to about 10^{-4} of the initial "dry" sperm.

The fertilization membrane was formed in 98—100 % of the eggs when the eggs were inseminated with such density of the normal spermatozoa. 99% of the eggs in the normal sea water cleavaged normally.

Table 3. Results of the insemination with the modified spermatozoa treated with the low-Ca^{++} solution, pH 8.2, in the eggs of the sea urchin, *Hemicentrotus pulcherrimus.*

Treatments of eggs				Cleavage of eggs		
1 st. insemination	deprival of F. M.	treatment with Na-K solution	2 nd. insemination	uncleavaged eggs (%)	normal eggs (%)	abnormal eggs (%)
sw	−	−	−	0.0	99.0	1.0
sw	+	−	−	2.6	94.0	3.4
sw	+	−	sw	2.5	95.2	2.3
sw	+	−	l-Ca	6.0	91.0	3.0
sw	+	+	−	5.0	93.0	2.0
sw	+	+	sw	4.0	92.5	3.5
sw	+	+	l-Ca	2.4	94.3	3.3
l-Ca	−	−	−	3.8	90.8	5.4
l-Ca	+	−	−	2.3	94.0	3.7
l-Ca	+	−	sw	4.0	91.0	5.0
l-Ca	+	−	l-Ca	1.5	95.0	3.5
l-Ca	+	+	−	5.5	91.2	3.3
l-Ca	+	+	sw	2.3	93.7	3.9
l-Ca	+	+	l-Ca	4.4	89.6	6.0

sw : sea water diluted sperm,
l-Ca : low Ca^{++} treated sperm,
− : a treatment was not carried out.
+ : a treatment was carried out.

The result was present in table 3. From this table the following facts were obtained. When the eggs were inseminated with the spermatozoa treated with the low-Ca^{++} slution in the first insemination, some decrease of the normally cleavaged eggs was observed. In the eggs deprived of the fertilization membrane after the insemination with the modified spermatozoa and then kept in the normal sea water, somewhat recovery of the decrease of the normally cleavaged eggs

seems to occur. This fact may suggest that the cause of the decrease of the normal eggs is concerned with the early process of the fertilization. In the second insemination, the effect of the low-Ca^{++} solution was insignificant, because the definite difference did not occur.

Discussion

Concerning the polyspermic fertilization, many factors have been known for a long time. There is a detailed study by CLARK, J. M. on those factors.[2] According to her, the following treatments of the sea urchin eggs produced the polyspermy. Those treatments were the exposure of the eggs to higher temperatures, lower ones, acidified sea water, the egg and sperm extracts, single cation solutions of Na^{+}, K^{+}, Ca^{++} and Mg^{++}, the exposure to alkaloides, nicotine, morphine, cocaine, strychinine, etc., the exposure to the fat solvents, chloroform, alcohols and the exposure to a narcotics. Polyspermy also resulted from the fertilization of the injurious eggs, the aged eggs, the immatured eggs and the eggs obtained from the animal kept in an inadequate condition. According to SMITH, H. and CLOWES, H. A.[12], the increase of the percentages of the polyspermy occurred when the eggs of a sea urchin, *Arbacia punctulata*, were inseminated in the acidified sea water ranging between pH 7.0 and 7.2. In the experiments of the refertilization of the parthenogenetically activated sea urchin eggs, ISHIDA, J. and NAKANO, E.[6] indicated that the insemination with a dense sperm was effective to produce polyspermy. According to RUNSTROM, J. and KRISZAT, G.[11], the treatment of the sea urchin eggs with PCMB was effective to produce the polyspermy.

It is quite important that the increase of the polyspermic cleavage of the sea urchin eggs occurs when the intact eggs are inseminated with the modified spermatozoa. According to KOENUMA, A.[9] polyspermic cleavage increased when the sea urchin eggs were inseminated with the spermatozoa treated with an acidified sea water, pH 7.1. In the present experiments, the percentages of the abnormally cleavaged eggs increased when the unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were inseminated with the alkaline treated or low-Ca^{++} treated spermatozoa. In these abnormal eggs, there seems to be a pretty number of polyspermic eggs. Therefore, it may be suggested that spermatozoa take a part in the establishment of the sperm-block mechanism in the fertilized egg of the sea urchin. It may be important in the relation between these results and the fact reported by AMOROSO and PARKES[1] that the polyspermy produced when the rabbit's eggs was fertilized by the spermatozoa irradiated by X-ray. The effect of the uranyl nitrate treatment of the spermatozoa on the increase of the polyspermic eggs was observed. When the unfertilized eggs were inseminated

with the uranyl nitrate treated spermatozoa, the increase of the polyspermic eggs was hardly evident. This result is essentially consistent with the KOENUMA's previous report and ISHIDA's observation[5]. When the eggs previously fertilized were further inseminated, the increase of the polyspermic eggs occurred in some cases. The insemination with the spermatozoa treated with uranyl nitrate was the one of those cases. This result was similar to the result of the previous experiment in which the sea urchin eggs were inseminated by the modified spermatozoa treated with acidified sea water.

In the experiments of the alkaline treated sperm and the low-Ca^{++} treated one, there was a tendency that the abnormal eggs rather decreased when the inseminated eggs were deprived of their fertilization membrane and they were treated with Na-K mixture shortly after the insemination. It is probable that the very early fertilization process was affected by the treatment of the spermatozoa. It is well known that in the sea urchin spermatozoa the acrosome reaction takes place when they meet with the unfertilized eggs. This acrosome reaction is believed to be the one of the important reactions for the early fertilization process of the sea urchin eggs. According to DAN, J.[3], the acrosome reaction of the sea urchin spermatozoa is induced by the contact of the spermatozoa with egg sea water, alkaline sea water and solid matters. In the present result, the increase of polyspermic cleavage by the insemination with the alkaline sea water treated spermatozoa is interesting in the relation to the effect of the alkaline sea water on the acrosome reaction of sea urchin spermatozoa.

Although no acrosome reaction was induced by the contact with low-Ca^{++} solution, the effect of the sperm treatment with such solution on the fertilization was evident. According to DAN, J.[4], the acrosome reaction was inhibited in the Ca^{++}-deficient solutions in many cases. This phenomenon may be reversible, since eggs were fertilized by the spermatozoa treated with low-Ca^{++} solution in the normal sea water. Somewhat increase of the polyspermic cleavage in the insemination with the low-Ca^{++} treated spermatozoa suggests a possibility that the acrosome reaction of the spermatozoa was affected by the treatment.

If the acrosome reaction of the uranyl nitrate treated spermatozoa is retarded fairly after the transferrence of the sperm to normal sea water, the result of the present experiment may be explainable as the same effect of the insemination with dense sperm in the experiments of ISHIDA, J and NAKANO, E.[6]. According to KOENUMA, A.[9], the similar tendency of the increase of the polyspermic cleavage of sea urchin eggs was caused when the eggs were inseminated with the spermatozoa treated with an acidified sea water. That result of the insemination of the acidified sea water may be explainable likewise with the uranyl nitrate treated cases, if the treatment of the spermatozoa gives an similar after-effect on the acrosome reaction. The effect of the uranyl nitrate and the acidified

sea water on the acrosome reaction, however, is still obscure.

These results may suggest a possibility that the acrosome reaction of sea urchin spermatozoa is partially responsible to the triggerring process for the establishment of the sperm-block mechanism. There is no doubt that the participation of the spermatozoa for the establishment of the sperm-block mechanism of the egg is not indispensable, because the sperm-block mechanism of the egg is caused from the artificial activation of the egg[6,10]. To clarify the possibility the direct observation of the acrosome reaction of the modified spermatozoa seems to be valuable.

The author expresses his thanks to Dr. TOMIYAMA, ICHIRO and the stuffs of Misaki Marine Biolegical Station for their kind help to this study, the author also expresses his thanks to Prof. KURASAWA, HIDEO of Shinshu University for his correction of this manuscript.

Literature Cited

1) AMOROSO, E. C. & A. S. PARKES (1947) Effects on embryonic development of X-irradiation of rabbit spermatozoa *in vitro*. *Proc. Roy. Soc. L. B.* **134**, 57–78.

2) CLARK J. M. (1936) An experimental study of polyspermy. *Biol. Bull.* **70**, 361–384.

3) DAN, J. C. (1952) Studies on the acrosome. I. Reaction to egg water and other stimuli. *Biol. Bull.* **103**, 54–66.

4) ——— (1954) Studies on the acrosome. III. Effect of calcium defficiency. *Biol. Bull.* **107**, 335–349.

5) ISHIDA, J. (1954) Physiological chemistry of the cell-surface of the sea urchin egg. *Biochemistry* **25**, 389–396.

6) ISHIDA, J. & E. NAKANO (1950) Fertilization of activated sea urchin eggs deprived of fertilization membrane with Ca-Mg-free media. *Annot. zool. Jap.* **23**, 43–48.

7) KOENUMA, A. (1956) The effect of uranyl nitrate on fertilization in the echiuroid and sea urchin eggs. *Zool. Mag.* **65**, 281–286.

8) ——— (1959) On the insemination with modified spermatozoa in *Urechis unicinctus*. *Jour. Fac. Liberal Arts and Science, Shinshu Univ.*, **9**, *Part* II, *Nat. Sci.*, 15–19.

9) ——— (1963) The effects of the treatment of spermatozoa with acid sea water on re-insemination of sea urchin eggs, *Hemicentrotus pulcherrimus*. *Jour. Fac. Liberal Arts and Science, Shinshu Univ.* **13**, 9–13.

10) NAKANO, E. (1954) Further studies on the fertilization of activated sea urchin eggs. *Jap. Jour. Zool.* **11**, 245–251.

11) RÜNSTRÖM, J. & G. KRISZAT (1953) The action of SH-reagents on the activation process in the sea urchin egg. *Ark. Zool. Andra Serien* Bd. **4**, Häfte 2, 165–185.

12) SMITH, H. & H. A. CLOWES (1924) The influence of hydrogen ion concentration on the fertilization process in *Arbacia*, *Asterias* and *Chaetopterus* eggs. *Biol. Bull.* **47**, 333–344.