On the Re-insemination upon the Fertilized Eggs of Hemicentrotus pulcherrimus

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Introduction

It has been believed for a long time that the fertilized sea urchin eggs never produce polyspermy, when they are inseminated again after the deprival of their fertilization membranes. According to JUST, this phenomenon originated in the change of egg called "wave of negativity". The negativity means the refusal of the entrance of spermatozoon into the egg ('19). The wave of negativity has been believed to be an irreversible change. Therefore, it has been considered that the re-fertilization of the fertilized eggs has never occurred.

However, SUGIYAMA discovered that the fertilized eggs of sea urchin, *Hemicentrotus pulcherrimus*, produced the polyspermic cleavages, when they were deprived of the fertilization membrane at about 1.5 minute after the insemination in Ca^{++} and Mg^{++} free sea water, then transferred into normal sea water several minutes, and then they were inseminated by more concentrated sperm than in the normal case. When the second insemination was delayed, the insemination at two-cell stage produced the polyspermic cleavages. But in this case the abnormality occurred at second cleavage ('51). SUGIYAMA referred this phenomenon to the re-fertilization of the fertilized eggs. According to him the re-fertilization occurs in the eggs of other species of sea urchin, and in stead of the treatment with Ca^{++} and Mg^{++} free sea water, the treatment with 1 M urea solution also brought the re-fertilization. If the start of the treatment was within 1 minute after insemination, the second insemination brought the polyspermy in spite of the existence of the fertilization membrane.

The discovery of this phenomenon makes a great contribution to clarify the real nature of the mechanism of the refusal of sperm entrance in the fertilized egg.

The experiments presented in this paper were carried out with a purpose of investigating the effects of the mechanical deprival of fertilization membrane, of NaCl-KCl mixture treatment, and of 1 M urea solution treatment on the re-insemination of the fertilized eggs of *Hemicentrotus pulcherrimus* to confirm the

phenomenon which was discovered by SUGIYAMA.

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Material and Methods

The eggs used in the experiments were those of the sea urchin, *Hemicentrotus pulcherrimus*, which were obtained near the station.

The unfertilized eggs used in the experiments were collected as follows. The female animal which was previously deprived of the oral apparatus, Aristotle's lantern, was put upside down onto a frask which was filled with sea water, and then the adequate volume of 5/9 M KCl solution was poured into the body cavity of the animal. Soon after such treatment the animal began to spawn. The unfertilized eggs obtained by such treatment were washed twice with sea water for the preparation.

The sperm used in the experiments was obtained by the operation of the genital organ of the male animal. It was prepared as "dry sperm". The sperm was diluted just before the use with sea water to the definite concentration. In the first insemination when the diluted sperm was mixed to the egg suspension, its concentration was about 5×10^{-5} of the dry sperm. In the second insemination its concentration was about 10^{-3} of initial concentration.

Sea water used was obtained from the open sea with only glass implements.

Every experiment was carried out at room temperature, about 14.5°C.

In each experiment, the ratio of various types of cleaved eggs was calculated at the first cleavage which was given rise at about 2 hours after the insemination. In each case the ratio of various types of cleavages was obtained from the observation of the 200 eggs under the microscope at random.

In each treatment the effect of the re-insemination was obtained from the comparison of two cases, one of which was not re-inseminated and the other was re-inseminated.

To avoid the error given from the use of unsuitable material, the ratio of the fertilization membrane formation, the ratio of the normally cleaved eggs, the ratio of the polyspermic eggs and the ratio of the uncleaved eggs at the first cleavage of the eggs which were not treated but re-inseminated 10 minutes after the first insemination were surveyed.

In the following cases, the results of the re-insemination were investigated.

(1) *The re-insemination upon the eggs which were mechanically deprived of their fertilization membranes.*

The sea water suspension of the unfertilized eggs was inseminated. These eggs were deprived of the fertilization membrane by mechanical process in which

the suspension was flown into and out of a pipett with a fine tip, once a second for 45 seconds 1.5 minute after the insemination. After such treatment, the suspension was divided into two parts. One of them was re-inseminated 10 minutes after the treatment. The other one was kept intact. Both were observed at the first cleavage.

(2) *The re-insemination upon the eggs which were treated with NaCl-KCl mixture 10 minutes after the mechanical deprival of their fertilization membranes.*

The fertilized egg suspension in which the eggs were deprived of their fertilization membranes mechanically by the method descrived above 1.5 minute after the insemination, was treated with NaCl-KCl mixture for 10 minutes after the deprival of the membrane. This suspension was divided into two parts. One of them was re-inseminated in normal sea water and the other was kept intact. At the first cleavage they were observed under the microscope.

NaCl-KCl mixture used in this experiment was composed of 100 volumes of 5/9 M NaCl and 2.2 of 5/9 M KCl. Its pH was prepared 8.2 by adding diluted $NaHCO_3$.

(3) *The re-insemination upon the eggs which were deprived of their ferilization membranes during NaCl-KCl mixture treatment.*

The eggs were transferred into NaCl-KCl mixture about 1.5 minute after the insemination, where the eggs were deprived of their fertilization membranes mechanically as described above. After 10 minutes-treatment of NaCl-KCl mixture, the eggs were transferred again into normal sea water. They were divided into two parts. One was inseminated 5 minutes after the transference and the other was kept intact. They were observed under the microscope at the first cleavage.

(4) *The re-insemination upon the eggs which were treated with 1 M urea solution.*

The eggs were transferred into 1 M urea solution 30 seconds after the insemination. They were transferred again into normal sea water after 5 minutes-treatment. They were divided into two, one of which was re-inseminated, and the other was kept intact. They were observed at the first cleavage.

Results

(1) *The re-insemination upon the eggs which were deprived of their fertilization membranes.*

The deprival of the fertilization membrane by mechanical treatment used in this experiment was complete. No egg remained its fertilization membrane observed after the treatment.

The results of these cases were presented in table 1. In this table, the difference of the ratio of each cleavage between two cases, the one re-inseminated

and the ohter did not, did not appear, except the small increase of uncleaved eggs in re-inseminated one. When the eggs were treated and then re-inseminated, the ratio of the normal cleavage, the ratio of the polyspermic eggs and the ratio of the uncleaved eggs were 86%, 0% and 14% respectively. When the eggs were treated and not re-inseminated, the ratio of the normal cleavage, the ratio of the polyspermic eggs and the ratio of the uncleaved eggs were 92.5%, 1.5% and 6% respectively. When the eggs were not treated and re-inseminated, the ratio of the normal cleavage, the ratio of the polyspermic eggs and the ratio of the uncleaved eggs were 92.5%, 1.5% and 6% respectively. In this case, the ratio of the formation of fertilization membrane was 100%.

Table 1. Abnormality caused by the insemination upon the eggs deprived of the fertilization membrane by mechanical treatment. (*Hemicentrotus pulcherrimus*)

test No.	treatment	re-insemination	formation of f. m.	cleavages		
				normal	polyspermic	uncleaved
I	−	+	100%	92.5%	1.5%	6%
II	+	+	---	86.0	0	14
III	+	−	---	92.5	1.5	6

(2) *The re-insemination upon the eggs which were treated with NaCl-KCl mixture 10 minutes after the mechanical deprival of their fertilization membranes.*

The results were presented in table 2. When the treated eggs were not re-inseminated, the ratio of the normal cleavage, the ratio of the polyspermic ones and the ratio of the uncleaved ones were 84.5%, 2.5% and 13% respectively. When the treated eggs were re-inseminated, that of the normal cleavage, that of the polyspermic ones and that of the uncleaved ones were 85.0%, 0.6% and 14.4% respectively. When the eggs were not treated but re-inseminated, the ratio of the normal ones, that of the polyspermic ones and that of the uncleaved

Table 2. Abnormality caused by the re-insemination upon the eggs which were treated with NaCl-KCl mixture 10 minutes after the mechanical deprival of the fertilization membrane. (*Hemicentrotus pulcherrimus*)

test No.	treatment	re-insemination	formation of f. m.	cleavages		
				normal	polyspermic	uncleaved
I	−	+	100%	92.5%	1.5%	6 %
II	+	+	---	85.0	0.6	14.4
III	+	−	---	84.5	2.5	13.0

ones were 92.5%, 1.5% and 6% respectively. In these eggs the fertilization membrane was formed in all eggs. In these results it is suggested that the re-fertilization does not occur by the re-insemination.

(3) *The re-insemination upon the eggs which were deprived of their fertilization membranes during NaCl-KCl mixture treatment.*

The results were presented in table 3. The following facts were shown in this table. When the treated eggs were kept intact, the ratio of the normal cleavage, that of the polyspermic and that of the uncleaved ones were 92%, 1.5% and 6.5% respectively. When the treated eggs were re-inseminated, the ratio of the normal cleavage, that of the polyspermic and that of the uncleaved ones were 69.5%, 21% and 9.5% respectively. In comparison of two cases, the ratio of the polyspermic eggs of the latter was apparently larger than that of the former. This fact suggests that the re-fertilization occurs in the latter case.

When the eggs were not treated but re-inseminated, the ratio of the normal cleavage, that of the polyspermic and that of the uncleaved ones were 97.5%, 2% and 0.5% respectively. In these eggs the ratio of the fertilization-membrane-formation was 100%.

Table 3. Abnormality caused by the re-insemination upon the eggs which were deprived of the fertilization membrane during NaCl-KCl mixture treatment. (*Hemicentrotus pulcherrimus*)

test No.	treatment	re-insemination	formation of f. m.	cleavages		
				normal	polyspermic	uncleaved
I	−	+	100%	97.5%	2.0%	0.5%
II	+	+	---	69.5	21.0	9.5
III	+	−	---	92.0	1.5	6.5

(4) *The re-insemination upon the eggs which were treated with 1 M urea solution.*

The fertilization membrane of the treated egg was not formed in this experiment. The results of the experiment were presented in table 4. The following facts were shown in this table. When the treated eggs were kept intact, the ratio of the normal cleavage, the ratio of the polyspermic and the ratio of the uncleaved eggs were 87%, 4.5% and 8.5% respectively. When the treated eggs were re-inseminated, the ratio of the normal cleavage, that of the polyspermic and that of the uncleaved ones were 54.5%, 12% and 33.5% respectively. When the eggs were not treated but re-inseminated, the ratio of the normal cleavage, that of the polyspermic and that of the uncleaved ones were 94.5%, 4% and 1.5% respectively. When the eggs were not treated, the ratio of the fertilization-

membrane-formation was 100%.

The comparison among three cases clarifies that the increase of the abnormalities takes place in the treated cases. These facts suggest that the treatment of 1 M urea inhibits the mechanism of negative change to the sperm entrance in the fertilized egg.

Table 4. Abnormality caused by the re-insemination upon the eggs which were treated with 1 M urea solution. (*Hemicentrotus pulcherrimus*)

test No.	treatment	re-insemination	formation of f. m.	cleavages		
				normal	polyspermic	uncleaved
I	−	+	100%	94.5%	4.0%	1.5%
II	+	+	---	54.5	12.0	33.5
III	+	−	---	87.0	4.5	8.5

Discussion

When the unfertilized sea urchin eggs are inseminated, most eggs produce the monospermy. It is considered that this phenomenon originates from the change which refuses the entrance of the spermatozoa in the activation of egg. In general, it is known that soon after the entrance the fertilization membrane starts to grow from the portion where the spermatozoon enters the egg, This elevation of the fertilization membrane is regarded as a sign of the activation of the egg. It is known too that the elevation of the fertilization membrane and the change which inhibits the entrance of the spermatozoon, occur in the artificial activation of the egg. The mechanism of the inhibition of the sperm entrance of the activated egg is not only the mechanical inhibition to the entrance of the spermatozoon of the fertilization membrane but also the change of egg itself, because the re-insemination upon the eggs which were deprived of their fertilization membranes causes no polyspermic eggs. This change of egg itself was called "wave of negativity" by JUST ('19). The real nature of "wave of negativity" is not known completely at present.

ISHIDA analyses this negative change caused in the activation of sea urchin egg into three separated changes. The first is the negative change caused on the surface of the unfertilized egg itself, the second is the negative change caused at the surface of the egg of which fertilization membrane is deprived, and the third is the negative change caused on the surface of the fertilization membrane ('54). The negative change concerned, is the second one, when the egg deprived of its fertilization membrane is re-inseminated.

The relation between the increase of abnormal cleavages in re-insemination

and the treatments of the fertilized sea urchin eggs is presented in table 5. In this table the sign ++ represents the marked increase of the abnormal cleavages, and the sign − represents the case in which the increase of the abnormal cleavages was not caused. The increase of the abnormal cleavages contains the increase of the uncleaved eggs. It may be reasonable to explain that the increase of the abnormal cleavages caused by the re-insemination is due to the increase of the polyspermic eggs resulted from the re-fertilization, because the results obtained from the comparison between these two cases, in one case the eggs were kept intact and in the other the eggs were re-inseminated.

Table 5. Increase of abnormal cleavages caused by re-insemination upon the sea urchin eggs. (*Hemicentrotus pulcherrimus*)

treatment	increase of abnormality
intact	−
mechanical deprival of fertilization mambrane	−
NaCl-KCl treatment after mechanical deprival of fertilization membrane	−
mechanical deprival of membrane during NaCl-KCl treatment	++
1 M urea treatment	++

The sign in this table represents that −: increase of abnormality is not caused, +: increase of abnormality is caused, and ++: marked increase is caused.

The eggs deprived of their membranes with the mechanical treatment, caused no increase of the abnormal cleavages by the re-insemination. The increase of uncleaved eggs was caused by re-insemination, but this may not originate in re-fertilization, because the increase of polyspermic eggs did not occur. This agrees with the fact which has been known since old days. This shows also that the "negative change" of the fertilized sea urchin egg remains after the deprival of the fertilization membrane.

The increase of the abnormal cleavages occurred when the eggs which were deprived of their fertilization membranes mechanically during 10-minutes treatment with NaCl-KCl mixture 1.5 minute after the insemination, were re-inseminated. This fact suggests that this increase of abnomal cleavages originates from the re-fertilization by the re-insemination. According to SUGIYAMA, polyspermy occurs when the sea urchin eggs which are treated with the sea water lacking of both Ca^{++} and Mg^{++} soon after the insemination, are re-inseminated, because the re-fertilization occurs in such a case. The result obtained in the experiment agrees fundamentally with that of SUGIYAMA.

The marked increase of polyspermic eggs did not occur in the case, in which

the eggs were deprived of the fertilization membrane by mechanical treatment 1.5 minute after the insemination, and then were treated for 10 minutes with NaCl-KCl mixture after 10 minutes, and finally were re-inseminated in normal sea water. This suggests that the negative change to the spermatozoon of the egg which is deprived of its fertilization membrane, is completed within 10 minutes after the insemination, and it may become stable to the treatment of NaCl-KCl mixture for 10 minutes.

From the results of the experiments, it is reasonable to assume that the "negative change" of the fertilizied egg of which fertilization membrane is deprived, requires both Ca^{++} and Mg^{++}, and is formed within 10 minutes after the entrance of spermatozoon. According to ISHIDA and NAKANO, polyspermy occurs when the activated sea urchin egg of which fertilization membrane is deprived, is inseminated after the washing with such solution free from Ca^{++} and Mg^{++} ('50).

When the eggs were treated with 1 M urea solution, both the "negative change" of eggs and the formation of the fertilization membrane were inhibited. In these experiments, the increases of abnormal cleavage were larger in the case in which the eggs were re-inseminated, than in the case in which the eggs were kept intact. In these results, it is obvious that the increase of abnormality occurs especially as the increase of uncleaved eggs.

When the treated eggs were kept intact, this increase did not seem to originate from the re-fertilization, because the ratio of the polyspermic eggs was not larger than that of the case in which the eggs were not treated but re-inseminated.

In comparison with the cases noted above, the ircrease of the abnormality caused by the re-insemination upon the treated eggs may originate from the re-fertilization of the fertilized eggs, because in that case, the ratio of polyspermic eggs increases markedly as well as the ratio of uncleaved eggs.

It is concluded from these experiments that the increase of polyspermy occurs in such cases, that the fertilized sea urchin eggs are re-inseminated by somewhat concentrated sperm after the treatment of such solutions as lacking both Ca^{++} and Mg^{++} or 1 M urea solution at a short period after the insemination. This may be referred to the fact that the "negative change" to the sperm-entrance is inhibited by such treatments.

The results obtained in these experiments agree fundamentally with those obtained by SUGIYAMA.

Summary

(1) The effect of the re-insemination upon the eggs which were deprived of the fertilization membranes and treated with various methods, was investigated in *Hemicentrotus pulcherrimus*.

(2) The increase of abnormal cleavages did not occur when the eggs deprived of the fertilization membranes 1.5 minute after the insemination were re-inseminated. This may show that the re-fertilization does not occur in such a case.

(3) The marked increase occurred when the eggs were re-inseminated after the treatment with NaCl-KCl solution and deprival of the fertilization membrane 1.5 minute after the insemination. In this result, the occurrence of re-fertilization of the fertilized eggs may be showed.

(4) The occurrence of re-fertilization was showed in the re-insemination of the eggs treated with 1 M urea solution.

(5) These results agree fundamentally with those of SUGIYAMA.

(6) When the eggs were re-inseminated after the treatment in which the eggs were deprived of their fertilization membranes mechanically in the normal sea water, and then after 10 minutes, those eggs were treated for 10 minutes in the "Ca^{++} and Mg^{++} free" solution such as NaCl-KCl mixture, the increase of polyspermy did not occur.

(7) From these results obtained in these experiments, it may be concluded that the "negative change" of the egg of which fertilization membrane is deprived, requires the existence of both divalent cations, Ca^{++} and Mg^{++}, in surrounding medium, and it is completed within 10 minutes. The change also disappears when the eggs are treated with 1 M urea solution.

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