RE-FERTILIZATION OF THE FERTILIZED EGGS OF THE SEA URCHIN [1]

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It has up to now been believed that spermatozoa cannot penetrate into the fertilized eggs of the sea urchin even if the fertilization membrane has been removed. Loeb says (1916, p. 85) on this point: "It is well known that if an egg is once fertilized it becomes impermeable for other spermatozoa." In regard to the same point, Lillie (1919, p. 161) states: "The fertilized egg does not react to fresh spermatozoa, and these do not enter it . . . and it has been repeatedly shown that removal of the fertilization membrane does not render the egg more susceptible to superimposed insemination." Just (1939, p. 190) also expresses a similar belief in his publication as follows: "With fertilization, they pass beyond the fertilizable condition. I know of only one report (Morgan, 1895), based on insufficient evidence, which claims that fertilized eggs can be re-fertilized. In my experience, eggs having been fertilized lose capacity for fertilization for neither they nor their fragments can be fertilized again."

The present author has found that when the fertilized eggs have been washed with Ca-Mg-free media after removal of the fertilization membrane, they again become permeable to other spermatozoa. In 1947, some results of the experiments were preliminarily reported by him in Japanese. More recently he has found that spermatozoa can enter the fertilized eggs without removal of the fertilization membrane if the eggs are treated with Ca-Mg-free media before the hardening of the membrane. Since these results seem to pose certain interesting problems, it should be worthwhile to report them somewhat in detail.

Material and Methods

The following sea urchins were employed: *Strongylocentrotus pulcherrimus, Heliocidaris crassispina, Temnopleurus toreumaticus* and *Pseudocentrotus depressus*. The egg of *Strongylocentrotus pulcherrimus* proved the best one for the investigation since the fertilization membrane can be easily removed and the clear protoplasm renders mitotic asters easily visible in the living egg.

The first insemination was performed in the usual manner. In the first series of experiments, the fertilization membrane was removed by repeatedly sucking and blowing the eggs with a pipette which had a diameter slightly exceeding that of the egg. The denuded eggs were then exposed to a Ca-Mg-free solution. After immersion for several minutes, the eggs were put back into the normal sea water and then the second insemination was carried out.

In another series of experiments, fertilized eggs were put into a Ca-Mg-free

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solution before hardening of the fertilization membrane. On their return to sea water, superimposed insemination was performed without removal of the membrane. Whether or not spermatozoa penetrated into the eggs at the time of the second insemination and afterwards took part in the cleavage was examined before and after the first or second cleavage. The re-fertilized eggs showed abnormal mitotic figures and cleavages characteristic of the polyspermic eggs.

Experimental Results

Re-fertilization of the eggs deprived of the fertilization membrane

A. Experiments in Strongylocentrotus pulcherrimus

The eggs of *Strongylocentrotus* which had been normally fertilized were deprived of the fertilization membrane mechanically. This procedure was carried out two minutes after insemination, just after the membrane had been raised and before

Table I

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed with Ca-Mg-free sea water (6° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	1%	99%	1%
10^{-3}	0	8	92	
10^{-4}	0	82	18	
10^{-5}	1	98	1	

it had hardened. A fresh sperm suspension was added to the denuded eggs thus obtained and the development of these eggs was observed. It was found that in these eggs the cleavage was quite normal and polyspermic division was as infrequent as in the control. The result of this experiment indicates that the denuded eggs in that condition cannot be fertilized again, and coincides perfectly with those of the earlier workers.

In the next experiments, the denuded eggs obtained by the above-mentioned procedure were washed with Ca-Mg-free sea water for 5 minutes, and were put back into the normal sea water. The second insemination was carried out in watch glasses. The undiluted sperm, taken directly from the testes of freshly opened males, was taken as a standard and various concentrations of sperm were made by ten-fold serial dilutions. To 4.5 cc. of the egg suspension was added 0.5 cc. of a sperm suspension. In all the experiments, parallel controls were run with eggs which were not inseminated again after washing with Ca-Mg-free sea water. The eggs were examined after two or three hours. One of the results of the experiments is shown in Table I.

It can be seen that more than 90 per cent of the eggs showed polyspermic features at the first cleavage if the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}. Some of such eggs showed the irregular division

characteristic of polyspermic eggs while the other heavily polyspermic eggs failed to divide, showing numerous asters in the protoplasm. In the control, polyspermic eggs were very few. The result indicates that spermatozoa penetrated into the previously fertilized eggs at the time of superimposed insemination and afterwards took part in the formation of the mitotic figure. The present author wishes to call this phenomenon "re-fertilization," since spermatozoa not only enter fertilized eggs but also participate in the mitotic process of the eggs.

A number of similar experiments were also conducted using a molar urea solution (pH 7.0) instead of Ca-Mg-free sea water. It was found that a molar urea solution was more effective than Ca-Mg-free sea water. One of the results is shown in Table II.

When the concentration of sperm used in the superimposed insemination was 10^{-3} or 10^{-2}, 100 per cent of the eggs were re-fertilized and heavy polyspermy occurred in high percentages. Such heavily polyspermic eggs formed many asters in the protoplasm, but they did not cleave. It appears that the formation of too

TABLE II

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in normal sea water and washed in a molar urea solution (8° C.)

Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
10^{-2}	0%	0%	100%	1%
10^{-3}	0	0	100	
10^{-4}	0	32	68	
10^{-5}	1	96	3	

many asters caused the failure of cytoplasmic division. It is also seen in Tables I and II that re-fertilization was hard to induce when the concentration of sperm in the second insemination was 10^{-5}. This is a noteworthy fact since in the usual experiments fertilization can occur with a much lower concentration of sperm.

In Figure 1, microphotographs of re-fertilized eggs and controls are shown. In normal eggs polyspermy is not found (Fig. 1a). The eggs which were inseminated again after the removal of the fertilization membrane in normal sea water cleave normally (Fig. 1b). This means that re-fertilization has not taken place. In the eggs deprived of the membrane after fertilization and washed with urea solution, no irregularity of cleavage is found, though the blastomeres are spherical and well separated from each other owing to the lack of the hyaline layer (Fig. 1c). The eggs deprived of the fertilization membrane will, when washed with urea solution and inseminated again, become polyspermic (Fig. 1d). It is clear that re-fertilization has occurred in these eggs.

In a group of experiments, the removal of the fertilization membrane was done in a Ca-Mg-free medium. This method was found to be simpler and more convenient than that of the above-mentioned experiments, because the fertilization membrane was easily removable and both the removal of the membrane and washing with Ca-Mg-free medium were done at the same time. It is a well-known fact that Ca-ion is

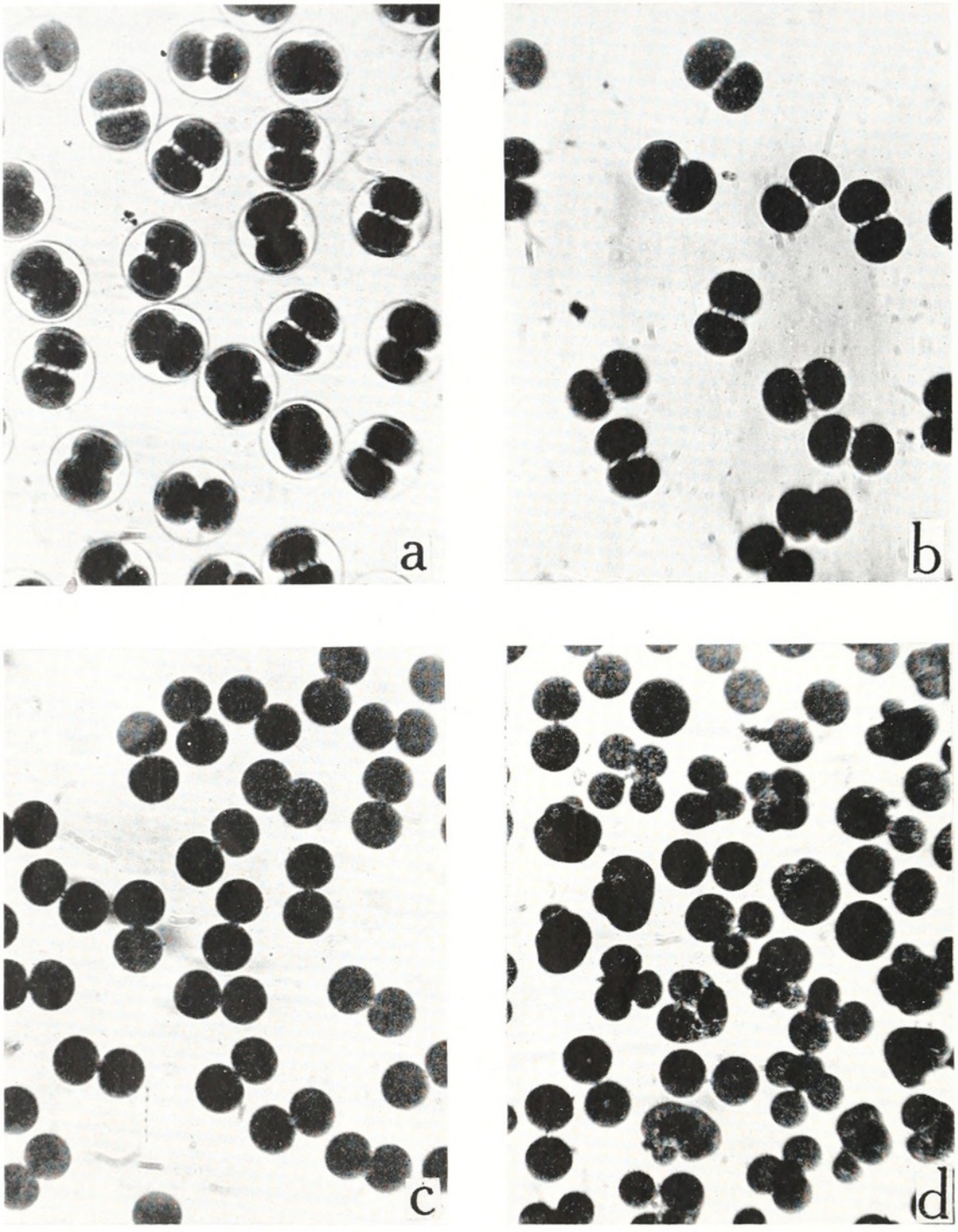

FIGURE 1. Re-fertilization of Strongylocentrotus eggs. The photographs were all taken of living eggs after the first cleavage. a. Normal eggs (control). b. Eggs which were inseminated again after removal of the fertilization membranes in normal sea water. c. Eggs which were deprived of the membrane and washed with urea solution, but not re-inseminated. d. Eggs which were deprived of the membrane, washed with urea solution and re-inseminated.

indispensable for hardening of the fertilization membrane. If the fertilized eggs are transferred into a Ca-Mg- free medium before the membrane has hardened, the membrane will not become tough and is elevated very high so that its removal is easy. One of the results is shown in Table III. It is evident from this that re-fertilization can be induced by this method.

TABLE III

Re-fertilization of once-fertilized eggs which have been deprived of the fertilization membranes in a Ca-Mg-free medium (9° C.)

Medium in which membrane was removed	Concentration of sperm in 2nd insemination	Eggs 3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10^{-2}	1%	1%	98%	1%
	10^{-3}	0	5	95	
	10^{-4}	0	80	20	
	10^{-5}	1	98	1	
Urea solution (1 *M*)	10^{-2}	0	0	100	0
	10^{-3}	0	1	99	
	10^{-4}	0	49	51	
	10^{-5}	0	94	6	

In the above-mentioned experiments, superimposed insemination was carried out in sea water immediately after washing with a Ca-Mg-free medium. It was of some importance to ascertain whether or not re-fertilization could take place when superimposed insemination was induced at a later stage of cell division. Some experiments were carried out accordingly. The fertilized eggs were put into a Ca-Mg-free medium before the fertilization membrane had hardened, and the membrane was mechanically removed. After that they were put back into sea water in a few minutes and fresh sperm suspensions were added again at various stages of the early development of the eggs. The results are summarized in Table IV.

There is clearly indicated, by the inspection of these data, the possibility of re-fertilization taking place at any stage of the first cleavage. If the eggs are re-

TABLE IV

Re-fertilization of once-fertilized eggs at various stages of development. The membranes were removed in urea solution immediately after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3}, temp.: 13° C.)

Time from 1st insemination to 2nd insemination	Stage when observed	Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs
6 m.	After 1st cleavage	1%	7%	92%
25 m.	After 1st cleavage	3	2	95
50 m.	After 1st cleavage	2	3	95
1 hr. 5 m.	After 1st cleavage	3	93	4
	After 2nd cleavage	2	8	90
2 hrs. (2-cell stage)	After 2nd cleavage	2	5	93
Control	After 2nd cleavage	2	98	0

fertilized before the anaphase, the polyspermic division takes place at the time of the first cleavage. However, if the eggs are re-fertilized after the full growth of the amphiaster, the process of the first cleavage goes on normally and polyspermic irregularity becomes visible at the second cleavage. It is of especial interest to note that the eggs are re-fertilizable even at the 2-cell stage if they have been allowed to develop in sea water after the treatment mentioned above. As shown in Figure 2, the eggs re-fertilized at the 2-cell stage become abnormal later on.

Isotonic solutions also of glycerine or thiourea are found to be effective for making the fertilized eggs re-fertilizable, while sea water containing ether, Cu-ion, trypsin, lipase, NaOH or HCl is not. Ca-Mg-free sea water becomes ineffective when any of Ca^{++}, Mg^{++}, Sr^{++} or Ba^{++} is added. It may be concluded that those media which contain any divalent cation are ineffective.

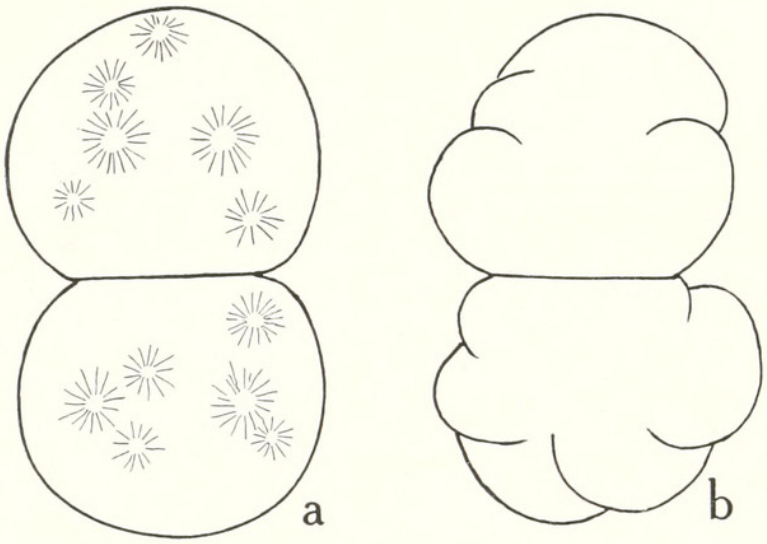

FIGURE 2. Eggs re-fertilized 5 minutes after the first cleavage. a. Thirty minutes after re-fertilization. Note many asters in each blastomere. b. One hour after re-fertilization. The second cleavage is polyspermic in each blastomere.

B. Experiments on Pseudocentrotus depressus, Heliocidaris crassispina and Temnopleurus toreumaticus

A further investigation of re-fertilization was made with three other species of sea urchins, its possibility being demonstrated to be fairly general. The three species used were *Pseudocentrotus depressus, Heliocidaris crassispina* and *Temnopleurus toreumaticus*. The procedure of the experiment was the same as was carried out with *S. pulcherrimus*. The fertilized eggs were put into Ca-Mg-free sea water and the removal of the fertilization membranes was performed in that solution. The results of the experiments, as summarized in Table V, show that all of the three species are capable of re-fertilization.

In several other experiments, Ca-Mg-free sea water was replaced with a molar urea solution with successful results.

Re-fertilization of the eggs without removal of the fertilization membrane

In recent years it has come to be recognized as a result of the works of Motomura (1941) and Runnström (1947) that the fertilization membrane is formed

TABLE V

Re-fertilization in several species of sea urchins. The fertilization membranes were removed in Ca-Mg-free sea water after the 1st fertilization. (Concentration of sperm in the 2nd insemination: 10^{-3})

Species	Temp.	Eggs 2–3 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
		Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
P. depressus	18° C.	0%	39%	61%	0%
H. crassispina	26° C.	2	11	87	0
T. toreumaticus	26° C.	0	25	75	1

from the membrane which covers the unfertilized eggs and the material which exists below the protoplasmic surface in the unfertilized egg. On the basis of this fact it is obvious that the vitelline membrane is at first permeable to the spermatozoon but becomes impermeable as it turns into the fertilization membrane, though the so-called "wave of negativity" may have taken place earlier. For this reason, in the preceding experiments the fertilization membrane was mechanically removed before re-fertilization. The question arises as to the possibility of re-fertili-

TABLE VI

Re-fertilization of once-fertilized eggs without removal of the fertilization membranes

Medium in which eggs were immersed	Temperature	Time from insemination to immersion in Ca-Mg-free medium (seconds)	Duration of immersion in Ca-Mg-free medium (minutes)	Eggs 2 hrs. after superimposed insemination			Polyspermic eggs in control (not inseminated again)
				Undivided eggs (not fertilized)	Normally divided eggs	Polyspermic eggs	
Ca-Mg-free sea water	10° C.	30	3	0%	6%	94%	1%
		50		0	15	85	
		90		0	99	1	
Urea solution (1 *M*)	11° C.	30	2	1	2	97	0
		50		1	4	95	
		80		0	4	96	
		120		0	93	7	

zation without removing the fertilization membrane when the hardening of the membrane has been inhibited. As stated before, it has been shown by several investigators that hardening of the fertilization membrane is dependent upon the presence of divalent ions such as Ca^{++} and Mg^{++} in the sea water, and lack of these ions causes a failure of hardening and thickening of the membrane (Hobson, 1932; Sugiyama, 1938a, 1938b). Accordingly an attempt was made to re-fertilize the once-fertilized eggs of *S. pulcherrimus* by the following procedure. The eggs are fertilized in the ordinary way and are put into a Ca-Mg-free medium just before the membranes begin to rise. In several minutes the membranes are elevated very high. When a molar urea solution is used the membranes become especially thin and delicate. Two to five minutes after, the eggs are put back into normal sea wa-

ter and sperm is gently added. The membranes sink to the surface of the eggs after being returned to sea water but they are not broken during this procedure. The count of polyspermic eggs is made after the first cleavage. Some of the results are shown in Table VI.

These results suffice to show that re-fertilization occurs without any mechanical removal of the membranes if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination. Too long a stay of the eggs in sea water before their removal into the Ca-Mg-free medium causes the failure of re-fertilization since the membranes become hard enough to prevent the penetration of the spermatozoa.

Discussion

It has long been accepted that fertilized eggs deprived of their fertilization membranes in normal sea water cannot be re-fertilized. This fact has been, as stated above, confirmed in the present work also. However, it has been found that fertilized eggs deprived of their membranes can, when washed in a Ca-Mg-free medium, be fertilized once again. It has also been demonstrated that when the hardening of the membrane has been inhibited, re-fertilization can take place without the removal of the membrane. In view of these findings it is justifiable to consider that in fertilized eggs there are two mechanisms of sperm exclusion. One of them is, of course, the fertilization membrane and the other is a certain substance covering the surface of the egg. The latter is so stable in sea water that it cannot be removed by any mechanical agitation. This is the reason why the removal of the fertilization membrane alone does not render the eggs re-fertilizable. However, it is noticed that this sperm-excluding mechanism becomes ineffective by washing with Ca-Mg-free sea water or non-electrolyte solutions. On the other hand, several kinds of reagents and solutions containing divalent cations were tried, but without success. These results seem to indicate that the sperm-excluding substance is soluble only in a medium which does not contain any divalent cation.

According to Just, the mechanism of monospermy in fertilization is attributed to the so-called "wave of negativity." The relationship between the wave of negativity and the sperm-excluding substance on the surface of fertilized eggs is an interesting problem. At present, however, no evidence has been furnished as to whether the sperm-excluding substance on the surface of the egg is the product of the wave of negativity or it is produced afterwards, independent of the wave of negativity.

In Urechis eggs, Tyler and Schultz (1932) have shown that the reversal of fertilization is possible by treatment with acid sea water. But with the eggs of the sea urchin no successful result has been achieved so far by means of acid sea water.

Bury (1913) reported that the fertilized eggs of the sea urchin could be fertilized again if they were exposed to a low temperature. A possible explanation of Bury's result is that the sperm-excluding substance on the surface of fertilized eggs may become ineffective at a low temperature, but a definite conclusion with respect to this point must await further investigation.

There have been several studies on the superposition of fertilization on parthenogenesis. Loeb (1913) found that the eggs of *Strongylocentrotus purpuratus*, in which membrane formation had been induced by butyric acid, could be fertilized by sperm if the membranes were torn by shaking. It was also found that the eggs

of the same form were fertilizable after activation by the treatment with hypertonic sea water. C. R. Moore (1916, 1917) showed that in the case of Arbacia there was no possibility of superimposing fertilization upon parthenogenesis after optimum exposure to the activating agent. Some other workers also came to a similar conclusion, using several kinds of marine eggs. Ishida and Nakano (1947, 1950) report that if the artificially activated eggs of *Strongylocentrotus pulcherrimus* are heavily inseminated after removal of the membranes, some of them can be fertilized, and that fertilization of the activated eggs is the more easily accomplished when the removal of the membrane is performed in Ca-Mg-free solutions. The results reported here on re-fertilization correlate closely with the findings of these two authors.

Attention may be called to the fact that re-fertilization can take place at any stage of the first cleavage or even at the 2-cell stage if the eggs have been allowed to develop in sea water after the removal of the fertilization membranes and their washing in a Ca-Mg-free medium. It would be of great interest to study the possibility of re-fertilization in the later developmental stages. Unfortunately, no experimental studies have yet been made on this problem.

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Summary

1. If the fertilized eggs of sea urchins (*Strongylocentrotus pulcherrimus, Pseudocentrotus depressus, Heliocidaris crassispina, Temnopleurus toreumaticus*) are inseminated again after removal of the fertilization membrane and washing with a Ca-Mg-free medium for a few minutes, re-fertilization can take place. Spermatozoa not only penetrate into the previously fertilized eggs but also take part in the formation of the mitotic figure, so that the cleavage of re-fertilized eggs is irregular and characteristic of polyspermy.

2. Re-fertilization can occur without removing the membrane mechanically if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination.

3. Re-fertilization can take place at any stage of the first cell division and even at the 2-cell stage.

4. It is justifiable to consider that there is a sperm-excluding substance on the surface of fertilized eggs. This substance is stable in sea water but is easily lost in Ca-Mg-free media.

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