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A Study on Relationship between Sperm Penetration and Egg Activation in the Medaka, Oryzias latipes

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Abstract

A physiological study has been made of the initial phase of fertilization in the medaka, *Oryzias latipes*. It was found that the effective attachment of a spermatozoon to the ovoplasm was established within 5 seconds after insemination. However, average reaction time for the breakdown of cortical alveoli evoked by stimulation of the spermatozoon or by pricking of the egg was 47±8 seconds and 20±3 seconds, respectively. Single stimulation by pricking is sufficient to evoke the excitation of ovoplasm. It was calculated, therefore, that the stimulus by the spermatozoon evoked the excitation of ovoplasm about 27 seconds after insemination.

From these results, it was concluded that the effective attachment of a spermatozoon to the ovoplasm was attained prior to the activation of ovoplasm.

Introduction

It has been shown in many kinds of animals, that the effective attachment of a spermatozoon to the ovoplasm is attained by the occurrence of acrosome reaction (*cf.* in marine invertebrates : DAN, 1956, 1960; COLWIN and COLWIN, 1961a, b, in lamprey and sturgeons : KILLE, 1960; GINSBURG, 1959, in rabbit and some rodents : AUSTIN, 1963). Prior to the sperm penetration, activation of egg is provoked by the spermatozoon. The first visible change in the egg is the breakdown of the cortical glanules (or cortical alveoli) followed by the elevation of fertilization membrane.

In teleosts, eggs have the micropyle(s) in the chorion. It is the only portal for sperm entry. The micropylar canal is so narrow that only a spermatozoon at a time is able to pass through. When the first has attained to the ovoplasm, a wave of alveolar breakdown passes through the ovoplasmic surface followed by the elevation of chorion (YAMAMOTO, 1939, 1944).

In salmonids, when ripe ova are inseminated in the isotonic salt solution, no cortical change takes place as long as they remain in the solution. On immersion in a hypotonic salt solution of fresh water, the activation takes place and subsequent development proceeds normally. KUSA (1950) performed an interesting experiment which suggests that the spermatozoon can enter the ovoplasm without activation of egg (*cf.* also GINSBURG, 1963). On account of the fact that salmonid eggs are activated by immertion in a hypotonic salt solution or in fresh water, it seems difficult to reveal whether the spermatozoon itself can stimulate the ovoplasm or not.

In the medaka, on the contrary, the ripe ova retain their fertilizability after they are immersed in the isotonic salt solution and normal fertilization takes place in it (YAMAMOTO, 1939).

In this paper, process of the initial event which takes place between spermatozoon and egg in the medaka is reported. Correlation between sperm entry and initiation of the breakdown of cortical alveoli is also considered.

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Material and Methods

Eggs and sperm of the orange-red type of the medaka, *Oryzias latipes*, were used. Egg-laden females were isolated from males the day before the experiments were made. The ripe ova were obtained from the ovaries isolated from the fish and placed in the isotonic salt solution for the medaka as formulated by YAMAMOTO (1939, 1944)*. This isotonic salt solution is referred to as YAMAMOTO's saline (Y-saline) in this paper.

Fresh sperm were obtained from the testes which were isolated from the fish and kept in Y-saline or Ca-free YAMAMOTO's saline (CF-saline) immediately before insemination. Though the sperm concentration was not estimated, the insemination was always carried out in a dense sperm suspensions.

The detailed procedure in the each experiments will be dealt with in the corresponding sections.

Results

1 *Effects of benzalkonium chloride on fertilization.*

In order to investigate the initiation of sperm entry, experimental procedure

* M/7.5 NaCl 100 parts+M/7.5 KCl 2.0 parts + M/11 $CaCl_2$ 2.1 parts, adjusted to pH 7.3 by $NaHCO_3$.

descrived by HAGSTRÖM and HAGSTRÖM (1954) was employed. The fertilization was inhibited after certain series of intervals by the addition of sperm inactivating agent.

For this purpose, benzalkonium chloride (BAC), a cationic surface active reagent, was tired and found to be suitable.

It is known that surface active reagents can activate the egg (*cf.* YAMAMOTO, 1961). Effect of BAC on ripe ova was first tested. The ripe ova were immersed in Y-saline or CF-saline containing 0.002–0.01% of BAC for 5 seconds, then washed twice with Y-saline and kept in it.

Both cases gave similar results. The data of the latter are shown in Table 1. In the concentration of solutions higher than 0.004% of BAC, some eggs were activated.

Table 1 Activation of Oryzias eggs with BAC CF-saline.
Rinsed in CF-saline for 30 min. → immersed in BAC CF-saline for 5 sec. → Y-saline

Temperature 21–23°C.

Concentration of BAC (%)	Total number of eggs	Number of activated eggs	Number of developed embryos
0	20	0	0
0.002	23	0	0
0.004	23	0	0
0.006	26	1	0
0.008	22	4	0
0.010	23	3	0
Normal control*	29	29	29

* Eggs were inseminated in Y-saline after rinsed in CF-saline.

In order to determine the minimal concentration of BAC which can immediately kill the spermatozoa around eggs and even in the micropyle, insemination was made in CF-saline. As is known, fertilization reaction does not take place in CF-saline (YAMAMOTO, 1944). The ripe ova were rinsed in CF-saline for 30 minutes and were inseminated in CF-saline. After 60 seconds, eggs were immersed in CF-saline containing 0.002–0.01% of BAC for 5 seconds, then washed twice with Y-saline and kept in it to develop.

The results are shown in Table 2. In the concentration of solutions higher than 0.006% of BAC, the spermatozoa were killed completely, but some eggs were activated (*cf.* also Table 1). In the concentration of solutions lower than 0.008% of BAC, elimination of the spermatozoa was not sufficient, *i.e.* some eggs were fertilized.

From these results, 0.006% of BAC solution was used for the following experiments to kill the supernumerary spermatozoa.

Table 2 Insemination of Oryzias eggs in CF-saline with a subsequent immertion in BAC CF-saline.
Rinsed in CF-saline for 30 min. → inseminated in CF-saline and after 60 sec. → immersed in BAC CF-saline for 5 sec. → Y-saline
Temperature 21-23°C.

Concentration of BAC (%)	Total number of eggs	Number of activated eggs	Number of developed embryos
0	29	29	29
0.002	35	32	29
0.004	35	11	11
0.006	35	3	2
0.008	34	8	0
0.010	35	9	0
Ca-free control*	20	0	0

* Eggs were inseminated in CF-saline and kept in it.

2 Inhibition of sperm entry by benzalkonium chloride.

a Insemination of eggs in Y-saline.

The time for effective attachment of a spermatozoon to the ovoplasm after insemination was measured. The ripe ova were inseminated in Y-saline and after certain series of intervals (5, 10, ……30 sec.) supernumerary spermatozoa were killed by immersion in 0.006% of BAC dissolved in Y-saline for 5 seconds. Then eggs were washed twice with Y-saline and kept in it to develop.

Tabre 3 Insemination of Oryzias eggs in Y-saline with a subsequent elimination of supernumerary spermatozoa.
Inseminated in Y-saline and kept in it for 0-30 sec → immersed in 0.006 % of BAC Y-saline for 5 sec. → Y-saline
Temperature 21-23°C.

Time after insemination(sec.)	Total number of eggs	Number of activated eggs	Number of developed embryos
0	30	4	0
5	40	28	27
10	31	25	24
15	29	24	23
20	28	27	27
30	30	29	27
Normal control*	26	26	26

* Eggs were inseminated in Y-saline.

The results in Table 3 indicate that, when eggs were inseminated in Y-saline, effective attachment of a spermatozoon to the ovoplasm was attained in 68% of

eggs 5 seconds after insemination, *i. e.* in these eggs, fertilization and cleavage occurred normally. Effective attachment between gamates was attained about 80% of the eggs, 10 seconds after insemination.

b Insemination of eggs in CF-saline.

More obvious results were obtained when the eggs were inseminated in CF-saline. The ripe ova were rinsed for 30 minutes in CF-saline, then inseminated in CF-saline. After 60 seconds, a drop of M/11 $CaCl_2$ solution was added and after certain series of intervals (0, 5, 10, ……30 sec.), supernumerary spermatozoa were killed by the same procedure mentioned previously. By this method, it is possible to measure the time for effective attachment of a spermatozoon to the ovoplasm after it was settled the bottom of the micropyle.

The results in Table 4 indicate that, 5 seconds after addition of calcium ions, the effective attachment between gamates was attained in almost all eggs.

Table 4 Insemination of Oryzias eggs in CF-saline with a subsequent elimination of supernumerary spermatozoa.
Rinsed in CF-saline for 30 min. → inseminated in CF-saline and kept in it for 60 sec. → calcium ions were added and after 0-30 sec. → immersed in 0.006 % of BAC Y-saline for 5 sec. → Y-saline
Temperature 21-23°C.

Time after addition of calcium ions(sec.)	Total number of eggs	Number of activated eggs	Number of developed embryos
0	32	4	0
5	31	31	30
10	30	30	30
15	30	30	27
20	31	31	31
30	30	30	30
Normal control*	30	30	30
Ca-free control**	30	0	0

* Eggs were inseminated in CF-saline and calcium ions were added subsequently.
** Eggs were inseminated in CF-saline and kept in it.

Throughout these experiments, nearly all of the eggs cleaved so long as they showed cortical changes. This fact indicates a possibility that the spermatozoon can enter the ovoplasm prior to the activation of egg similar to the salmonids.

3 Reaction time for the breakdown of cortical alveoli by insemination or by pricking.

In order to reveal the correlation between sperm entry and egg activation,

reaction time for the breakdown of cortical alveoli near the animal pole by insemination or by pricking was measured. In the latter case, the ripe ova were pricked near the site of micropyle with a fine glass needle whose tip was 20 μ in diamater (YAMAMOTO, 1939, 1944).

The results are shown in Fig. 1. Average reaction time for the breakdown of cortical alveoli near the animal pole by insemination or by pricking was 47±8 seconds and 20±3 seconds, respectively.

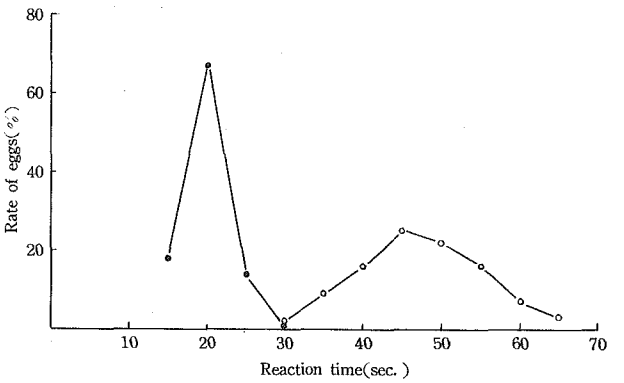

Fig. 1 Reaction time for the breakdown of cortical alveoli by insemination or by pricking.
Number of eggs used for insemination or for pricking was 100 and 128, respectively. Temperature 21–22°C. •——• : by pricking, ○——○ : by insemination.

A stimulation with a 15–20 μ needle point is sufficient to evoke the excitation of ovoplasm (YAMAMOTO, 1944). It was calculated, therefore, that the stimulus by the spermatozoon evoked the excitation of ovoplasm was about 27 seconds after insemination.

Discussion

The results of these experiments indicate that the effective attachment of a spermatozoon to the ovoplasm was attained within 5 seconds after insemination. On the other hand, the stimulus by the spermatozoon evoked the excitation of ovoplasm about 27 seconds after insemination. From these results, it was concluded that the effective attachment of a spermatozoon to the ovoplasm was attained prior to the activation of ovoplasm.

This conclusion is in agreement with the data obtained in salmonid eggs

(KUSA, 1950; GINSBURG, 1963). GINSBURG (1963) revealed cytologically that the spermatozoon reaches the egg surface and its head is half embedded in the cytoplasm, but subsequent sperm penetration proceeds only if they are activated by immersion in a hypotonic salt solution or in fresh water.

According to AKETA (1966), the spermatozoon of the medaka can enter the ovoplasm and monospermy is attained even if the breakdoun of the cortical alveoli is temporarily inhibited by treating the egg with an anesthetizing reagent, though the activation and subsequent development proceed after the recovery from anesthesia either autonomously or by stimulation of ovoplasm by pricking. This result seems to indicate that, like the salmonids, the spermatozoon is retained at the surface of ovoplasm when it was anesthetized.

Thus, it can be supposed in the medaka, that the effective attachment of a spermatozoon to ovoplasm is attained within a few seconds after insemination, but subsequent penetration proceeds after the ovoplasm is activated by the spermatozoon, *i. e.* abowt 47 seconds after insemination.

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