IV-3 Fertilization

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Meiotic maturation of gametes

1) Production of maturation inducing substance

Kanatani *et al.* (1969) isolated a meiosis- (or maturation-) inducing substance from the starfish and identified it as 1-methyladenine (1-MeAde), which is produced in follicle cells under the influence of a gonad-stimulating substance (Kanatani and Shirai, 1967). Ikegami, Kamiya and Shirai (1976) purified substances blocking "spontaneous" maturation of *Asterias* oocytes in seawater from the ovary and showed that they are steroid glycosides named asterosaponins A and B. Sano and Kanatani (1980a) revealed that when follicle cells in starfish are treated with trypsin for an appropriate period, the cells begin to produce 1-MeAde without a gonad-stimulating substance.

2) Early responses in the meiotic maturation of oocytes

Morisawa and Kanatani (1978) revealed that oocytes in starfish treated with seawater containing 0.01 % Triton X-100 lose their ability to respond to 1-MeAde. This ability was recovered by reconstitution of the eggs with the extracted factor. The treatment of the oocytes with Triton X-100 prolonged the hormone-dependent period (Ikadai and Kanatani, 1982).

Sulfhydryl content has been reported to increase after 1-MeAde treatment in oocyte-cortex proteins (Kishimoto, Cayer and Kanatani, 1976) and just before polar body formation in KCl-soluble proteins (Ikeda, Nemoto and Yoneda, 1976). Hamaguchi and Mabuchi (1978) reported that an intracellular Ca concentration in mature oocytes is lower than that in immature oocytes. By measuring the stiffness of starfish oocytes, Nemoto (1982) confirmed the presence of stable intermediate stages in oocytes treated insufficiently with 1-MeAde. He also showed that prior treatment of oocytes with isotonic CaCl₂, Ca ionophore, or methylxanthines shortens the hormone-dependent period. Nemoto and Ishida (1983) revealed that the intracellular level of cGMP transiently decreases after 1-MeAde treatment, but that of cAMP does not, suggesting that the control mechanisms in the initial phase of maturation differ between starfishes and amphibians.

Kishimoto and Kanatani (1976) revealed that 1-MeAde produces a maturation-promoting factor (MPF) in maturing starfish oocytes which induces germinal vesicle (nucleus of the oocyte) breakdown (GVBD) when injected into immature oocytes. Germinal vesicle materials were shown to be required for the amplification of MPF in the recipient oocyte of starfish (Kishimoto, Hirai and Kanatani, 1981). Kishimoto *et al.* (1982a) reported the generality of the action of MPF between meiosis and mitosis in starfish oocytes and eggs and among vertebrates and invertebrates.

Kishimoto *et al.* (1982b) showed that protease inhibitors, leupeptin and antipain, inhibit oocyte maturation in starfish by suppressing the production of MPF. In addition, Sano and Kanatani (1983) suggested that a chymotrypsin-like serine protease is involved in the formation of MPF later in the hormone-dependent period, from their inhibition experiments using chymostatin, N-benzoyl-L-tyrosine ethyl ester and other protease inhibitors. Nicotinamide inhibited both the GVBD induced by the injection of MPF and polar body formation (Sano *et al.*, 1979).

3) *Surface maturation and nuclear changes in oocytes*

Fujimori and Hirai (1979) suggested that a protection mechanism against polyspermy is gained after GVBD that is gradually lost after the first polar body formation in starfish. With electrophysiological studies, Miyazaki and Hirai (1979) showed that overripe eggs of starfish allow multiple sperm entries because of poor development of the activation potential. Using toad oocytes, Iwao, Ito and Katagiri (1981) showed that protein synthesis independent of the germinal vesicle is required for the occurrence of an activation potential and for cortical granule breakdown.

By injecting detergent-treated sperm of toad, Katagiri and Moriya showed that germinal vesicle materials are required for pronucleus formation and DNA synthesis in the injected spermatozoa (Moriya and Katagiri, 1976; Katagiri and Moriya, 1976). Katagiri (1980) further reported that the development of pronucleus-inducing activity requires both protein synthesis after progesterone treatment and an activation response in toad oocytes. On the other hand, using fish oocytes, Iwamatsu and Ohta (1980) reported that germinal vesicle materials are not required for male pronucleus formation, but they are required for chromosome formation from the male pronucleus. Using starfish, Hirai *et al.* (1981) reported that germinal vesicle materials are required for the formation of the amphiaster and sperm pronucleus.

4) *Changes in mechanical properties during oocyte maturation.*

Hiramoto (1976) showed by a compression method that the stiffness of starfish oocytes decreases during the early stage of meiotic division and transiently increases at the time of formation of the first and second polar bodies. With starfish oocytes, similar results were reported on surface tension (Ikeda, Nemoto and Yoneda, 1976), on surface stiffness using the suction method (Nakamura and Hiramoto, 1978), on the consistency of the endoplasm using the double-chamber method (Shoji, Hamaguchi and Hiramoto, 1978) and recently, Yamamoto and Yoneda (1983) suggested that germinal vesicle materials are involved in the formation of cyclic changes in tension during meiotic division. Nemoto, Yoneda and Uemura (1980) showed that cytochalasin B reduces the stiffness of immature starfish oocytes but does not interfere with the process of maturation. Shirai and Kanatani (1982a) revealed that 1-MeAde treatment of starfish oocytes brings about a shortening and an elongation of oocytes. They also showed that the prospective site of polar body formation is on the nearest surface to the position of the germinal vesicle (Shirai and Kanatani, 1980).

5) *Meiosis of spermatocytes*

In the newt, Abe (1981) developed an *in vitro* system in which primary spermatocytes undergo two successive meiotic divisions. The stages of prophase in living primary spermatocytes were determined by culture (Nishikawa and Abe, 1983). Cycloheximide was shown to inhibit the second meiotic division and to induce motile flagella in the secondary spermatocyte (Kiyotaka and Abe, 1983).

Fertilization

1) Control mechanism of sperm motility

Using sea urchin spermatozoa, Ohtake (1976a, b) showed that the diffusate from the egg jelly coat brings about a marked increase in the respiration and motility of spermatozoa at pH values lower than 7.8–7.9, and partially purified this material. Later, Suzuki *et al.* (1981) purified this substance and obtained two polypeptides with 9 and 10 amino acids. A polypeptide was synthesized and showed the same effect (Suzuki *et al.*, 1984). Morisawa and Suzuki (1980) used fresh and marine teleosts to show that a respective decrease or an increase in the osmolarity of the environment is the factor that initiates sperm motility in these species. Using rainbow trout, Morisawa and Okuno (1982) showed that the functionally immature (=immobile) axoneme of rainbow trout sperm is converted to the mature (=mobile) state by exposure to cAMP. Using starfish, Shirai *et al.* (1982) reported inhibitory factors of sperm motility in the seminal plasma. Using demembranated sperm models of mammals, Mohri and Yanagimachi (1980) showed that mature (cauda epididymal and ejaculated) spermatozoa are activated best by ATP followed by the caput epididymal spermatozoa, suggesting that the motor apparatus changes during epididymal transit. They also showed that movement of demembranated spermatozoa is very similar to the “hyperactivated” movement of capacitated spermatozoa.

2) Lysin of spermatozoa

Moriya and Hoshi (1979) revealed the presence of arylsulfatase in sperm and mature testes of sea urchins. They purified it partially (Moriya and Hoshi, 1980) and defined the arylsulfatase as a lysin (Hoshi and Moriya, 1980).

Using several protease inhibitors, Hoshi *et al.* (1979) showed that a chymotrypsin-like protease is presumed to be a lysin of the sea urchin, *Hemicentrotus pulcherrimus*. From spermatozoa of sea urchins, Yamada and Aketa (1981) extracted a factor which dissolves the vitelline layer of the egg with chymotrypsin-like activity, and they purified it (Yamada, Matsui and Aketa, 1982). The enzyme digested the jelly coat and vitelline layer and was released from the acrosome-reacted spermatozoa. They also purified a trypsin-like enzyme from sea urchin spermatozoa (Yamada and Aketa, 1982).

Using a solitary ascidian, Hoshi, Numakunai and Sawada (1981) showed that spermatozoa use trypsin-like and chymotrypsin-like proteases probably as lysin, by examining the effects of various protease inhibitors on the fertilization. Sawada *et al.* (1982) detected a mammalian acrosin-like protease in the extract of spermatozoa of a solitary ascidian and concluded it was lysin.

In mammals, Sakai, Yamamoto and Yasuda (1979) used peroxidase-labeled trypsin inhibitor to show that an acrosomal proteinase localizes in the acrosomal cap in sheep spermatozoa.

Using frog eggs, Yoshizaki and Katagiri (1981) showed that the susceptibility of the vitelline coat of the egg to spermatozoan lytic activity increases due to deposition of the substance secreted from the epithelial cells in the oviduct.

3) Sperm-egg binding

Tsuzuki *et al.* (1977) purified a substance from sea urchin eggs of *H. pulcherrimus* responsible for sperm-binding. This substance completely canceled the fertilizing capacity of homologous sperm only. From the spermatozoa of the sea urchin, Aketa *et al.* (1978) isolated a substance which interacts with the sperm-binding factor on unfertilized eggs. Using agarose beads coupled with the sperm-binding factor of the

eggs, Aketa *et al.* (1979) showed that on contact with these beads the spermatozoa exhibit the acrosome reaction and bind to them. They suggested that the sperm-binding factor functions as a species-specific inducer of the acrosome reaction when it is set up in the vitelline layer. Yoshida and Aketa (1979) prepared papain-digested, anti-sperm-binding factor serum and revealed that this serum deprives the egg of its fertilizability. They also partially purified the sperm-binding factor from eggs of *Anthocidaris crassispina* using an immunological method (Yoshida and Aketa, 1982).

Using sea urchins, Kato and Sugiyama (1978) showed that there is a direct relationship between the cross-fertilization of living gametes and the binding capacity of spermatozoa with fixed eggs.

4) *Acrosome reaction and agglutination of spermatozoa*

An acrosome reaction-inducing substance was shown to exist both on the surface of dejellied eggs and in the plasma membrane of fertilized eggs (Kimura-Furukawa, Suyemitsu and Ishihara, 1978; Suyemitsu, Suyemitsu and Ishihara, 1980). Using starfish, Ikadai and Hoshi (1981a) revealed that the acrosome reaction-inducing substance is a glycoprotein from the egg jelly that requires a diffusible cofactor(s) present in the egg jelly for full activity. The purified acrosome reaction-inducing substance is a sulfated glycoprotein, whose sugar moiety is important for the activity (Ikadai and Hoshi, 1981b). Kato and Sugiyama (1983) showed that 1 M urea can induce the acrosome reaction in sea urchins. In starfish, Shirai and Kanatani (1982b) reported that the responsiveness of spermatozoa to the induction of the acrosome reaction is increased by treatment with 1-MeAde. Using sea urchins, Sano (1983) showed that a calmodulin antagonist W-7 inhibits the induction of the acrosome reaction by egg jelly at 20–50 μ M, suggesting that calmodulin is involved in the triggering process of the acrosome reaction. Sugiyama and Kato (1977) revealed that trypsin deprives the acrosome-reacted spermatozoa of their fertilizing ability by removing the material covering the acrosomal tubule. With respect to cross-fertilizability, Aketa and Ohta (1979) suggested that the cross-fertilizability of the sea urchins *H. pulcherrimus* and *Pseudocentrotus depressus* depends highly on the inductivity of the acrosome reaction.

Uno and Hoshi (1978) isolated sperm agglutinin from the egg jelly of starfish and showed that it is similar to asterosaponin A. Isaka and Ikemori (1980) reported that sea urchin spermatozoa have several glycoside hydrolases and suggested their involvement in the reversible agglutination of sperm by egg water.

5) *Divalent cations, pH and activation of unfertilized eggs*

In sea urchins, Takahashi and Sugiyama (1973) revealed that eggs can be fertilized even in Ca-free seawater when spermatozoa first are treated with egg water to induce the acrosome reaction. Using the sea urchin, *H. pulcherrimus*, Sano and Kanatani (1980b) showed that a small amount of extracellular Ca ion, which can be introduced to the Ca-free seawater at the time of insemination (final conc. several tens μ M), are required for fertilization with the acrosome-reacted spermatozoa. Suppression of the fertilization rate due to the low concentration of extracellular Ca ions was clearly observed even in the absence of EGTA when there was an appropriate concentration of the reacted sperm (5×10^5 sperm per ml) (Sano, 1982) and the rate was not influenced by low temperature (Sano, unpublished data) in contrast to the results of Schmidt *et al.* in 1982 (Dev. Biol., 90, 284). With the isolated cortices of sea urchin eggs, Sasaki (1984) obtained a protein factor which sensitizes the cortical granules to Ca ions and partially purified this factor.

Sano and Mohri (1976, 1977) showed that fertilization of sea urchin eggs is inhibited in Mg-deficient seawater whether acrosome-reacted spermatozoa are employed or not. A recent study by Fujiwara, Yasumasu and Mohri (1983) showed that sea urchin spermatozoa inseminated in Mg-free seawater reduce their respiratory rate due to interaction with unfertilized eggs. These results support the view that under an Mg deficiency, sea urchin spermatozoa can bind to egg surfaces but cannot fuse or activate the eggs (Sano *et al.*, 1980) although the motility of sea urchin spermatozoa decreases in the absence of Mg (Iwasa, Shimizu and Mohri, 1981).

Sugiyama and Kato (1980) showed that fertilization failure at pH 6.0–6.5 is caused by the non-occurrence of the acrosome reaction.

Hamaguchi and Hiramoto (1981) examined the effects on unfertilized sea urchin eggs of changing the intracellular Ca concentration by microinjecting Ca buffers. They concluded that the rise in the Ca concentration in the cytoplasm to a definite value is the primary cause of the activation of the sea urchin egg at fertilization.

By microinjecting a pH indicator into its eggs, Hamaguchi (1982) examined the change in intracellular pH_i on fertilization of the sand dollar. The pH_i ranged from 6.5–6.75 and was raised 0.4–0.5 unit on fertilization. The arrest of the development of fertilized eggs due to Na-free seawater was released when the pH_i was elevated to the value of fertilized eggs. In contrast, Inoue and Yoshioka (1980) reported that the intracellular pH_i of unfertilized eggs is 6.2–6.3 and that the pH_i does not change after fertilization when pH_i is measured using ³¹P nuclear magnetic resonance.

Shimada *et al.* (1982) found that melittin, a stimulator of phospholipase A₂, causes the formation of fertilization membranes and an increase in the respiration of unfertilized eggs of sea urchins.

Using golden hamster eggs, Miyazaki and Igusa (1981) revealed that a sperm induces recurring hyperpolarizations, which constitute a fertilization potential. They showed that each hyperpolarizing response during fertilization is caused by an increase in the K conductance activated by an increase in the intracellular Ca ion concentration and that in the hamster egg a potential-dependent block of sperm entry to an egg does not occur (Miyazaki and Igusa, 1982).

6) *Metabolic changes during fertilization*

Tazawa and Yasumasu (1977) reported that the activation of phosphorylase, phosphofructokinase, and pyruvate kinase seems to be partly due to the increase in cAMP on fertilization of *Urechis* eggs. In addition, an initial decrease in the activity of fructose 1, 6-diphosphatase occurred upon fertilization in sea urchins (Fujimoto and Yasumasu, 1979). In the tricarboxylic acid cycle, the activity of the pyruvate dehydrogenase complex increased upon fertilization in sea urchin eggs (Yasumasu, 1976). In addition, the activity of arylesterase (Mita and Yasumasu, 1978) and thymidylate synthetase (Yasumasu *et al.*, 1979) initially increased upon fertilization.

Using fixed unfertilized eggs of sea urchins, Yasumasu and his co-workers studied changes in the respiration of spermatozoa upon fertilization. Sea urchin spermatozoa only adhered to fixed, unfertilized eggs of the same species and reduced their respiratory rate markedly (Hino, Fujiwara and Yasumasu, 1980; Yasumasu, Fujiwara and Hino, 1980). They obtained a heat stable and non-dialyzable substance, which inhibits sperm respiration, from eggs insufficiently fixed (Fujiwara, Hino and Yasumasu, 1980). The low respiratory rate of spermatozoa which had reacted with fixed eggs seemed to be due to a shortage of ADP and was regarded as stage 4 respiration in *H. pulcherrimus* (Hino *et al.*, 1980). The inhibition of respiration was due,

at least in part, to blockage of electron transport in a span between cytochrome b and c (Hiruma *et al.*, 1982). The mechanism of inhibition, however, seemed to differ among the species (Fujiwara, *et al.*, 1982). The arrest of movement in spermatozoa that reacted with fixed eggs seemed to be due to an increase in the amount of long chain fatty acyl-CoAs (Fujiwara *et al.*, 1983) which inhibit dynein ATPase.

7) *Morphological changes*

Using SEM and TEM, Usui, Sano and Mohri (1980) observed the ultrastructure of the expression of the cortical reaction and the penetration of a spermatozoon through the vitelline layer in the sea urchins eggs. Using medaka eggs, Iwamatsu and Ohta (1978) revealed morphological changes in the eggs from sperm penetration to pronucleus formation. With a differential interference microscope, Hamaguchi and Hiramoto (1980) observed the process of the penetration of spermatozoa and migration of the male and female pronuclei in the cytoplasm of sand dollar eggs and showed that the egg nucleus is carried to the center of the aster by the filamentous syrustructure formed between them.

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