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<Review>

Ultrastructures of Germ Cells Before and After Insemination in Rainbow Trout, *Oncorhynchus mykiss*

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수정전후 무지개 송어 (*Oncorhynchus mykiss*)의 생식세포의 미세구조

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ABSTRACT: Ultrastructure of the zona radiata, the micropyle and fertilization process in the rainbow trout (*Oncorhynchus mykiss*) were examined by light, scanning and transmission microscopes . The egg micropyle of rainbow trout consists of a funnel-shaped vestibule and a tapered canal transversing the zona radiata. The micropyle showed the type with a flat pit leading into a long canal and the micropylar wall showed the clockwise spiral structure. There were a great number of microvilli secreting adhesive materials having trapping function attracting the spermatozoa in the vicinity of micropyle. It was apparent that ridges extended between the projections. In the initial stages of penetration, the spermatozoon still within the micropylar canal attached perpendicularly at its apical tip to the egg surface, then the sperm head was rapidly engulfed by the folded egg surface with its many microvilli. The spermatozoon disappeared from the outer surface of the egg before the fertilization cone completely retracted 250 seconds after insemination. No interconnecting ridges was present in the egg surface. In short, the block to polyspermy to permit entry of a single sperm is considered to be mechanical by the morphological design of the micropyle and fertilization cone.

요 약: 광학, 주사 및 투과전자현미경을 이용하여 무지개송어 (*Oncorhynchus mykiss*) 성숙난자의 방사대와 난문 및 정자가 난문으로부터 난세포질까지 침투하는 과정의 미세구조를 조사하였다. 무지개송어의 난문은 깔때기 모양의 전정부와 방사대를 가로 지르는 나사선 모양의 도관으로 구성되어 있었다. 난문은 윗부분은 편평하면서 긴 도관 모양을 가지고 있었고, 난문벽은 시계방향 (우선형)의 구조를 나타내었다. 난문 주변부에 있는 난표면에는 정자를 접촉시키는 데 필요한 유인물질을 분비하는 무수히 많은 돌출물이 원을 이루면서 존재하였다. 침투 초기단계에 난문의 도관에 있는 한 마리의 정자는 난표면에 수직상태로 확인되었고, 곧이어 수정이 이루어진지 250초가 경과하자 정자두부는 사라지게 되었다. 정자두부가 난내부로 침투한 이후에 난표면에 있던 돌출물의 상호연결부위는 관찰되지 않았다. 다른 부위로 정자가 침투하는 지를 살펴보았지만 그러한 흔적은 확인되지 않았다. 난문과 수정추의 형태적인 구조를 관찰한 결과 이 미세구조물은 단 한 마리의 정자만을 허용함으로써 다정자침입을 방지하는 것으로 확인되었다.

INTRODUCTION

Ripe eggs of oviparous teleosts are covered with thick envelopes termed zona radiata or chorion. In most teleosts, the eggs usually have one micropyle and the canal of the micropyle is as narrow as the diameter of the sperm head. Only the first spermatozoon reaching the micropyle can come directly into contact with the zona radiata of the egg (Brummett & Dumont. 1979; Iwamatsu et al., 1993). However, average 7 micropyles are restricted to a 100~200 ㎛ region at the animal pole and penetrate the zona radiata of eggs in the white sturgeon, Acipenser transmontanus (Cherr et al., 1982). The micropyles and the surface pattern of fish eggs provide important characteristic criteria for identification of different teleosts (Dumont & Brumett, 1980; Ohta et al., 1983; Hart & Donovan, 1983; Kim

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et al., 1993; Guraya, 1986).

Studies have been done on fish spermatogenesis or spermiogenesis in *Clarias gariepinus* (Van Oordt et al., 1987), and *Lepidogalaxias salamandroides* (Leung, 1988). There have also been a few ultrastructural studies of teleost fertilization (Brummett & Dumont, 1979; Kim et al., 1993; Iwamatsu et al., 1993).

We have carried out transmission and scanning electron microscopic observations on micropyle, egg surface of unfertilized egg and initial stages of sperm penetration into the ripe egg of rainbow trout (*Oncorhynchus mykiss*) before and following artificial insemination from December to next January.

MATERIALS AND METHODS

Adult female and male rainbow trout, *Oncorhynchus mykiss*, were collected from the rearing tank (Dept. of Marine Biomedical Science, Kunsan University) under the normal environmental conditions from December to next January. A total of 50 fishes were sampled. Mature, unfertilized eggs were released from the ovarian lumen into freshwater. To add spermatozoa to unfertilized eggs, a sperm suspension was prepared by squeezing 2~3 testes in 50ml Tyrode's solution in a glass beaker. The values of GSI (gonadosomatic index) were employed to monitor gonadal maturation.

For transmission electron microscopy studies, the living specimens were fixed in 2.5% glutaraldehyde, buffered with 0.1 M PBS, pH 7.2, for 2 hrs at 4°C and postfixed in 2% osmium tetroxide in the same buffer for 2 hrs at room temperature, dehydrated by graded series of ethanol, and embedded in Epon 812. Semithin sections of unfertilized and fertilized egg, and testes stained with 1% toluidine blue dye were used to locate the cortical granules and sperm cells. Subsequently, ultrathin sections were obtained from the same block by ultramicrotome (No.2088, LKB, Bromma, Sweden) with a diamond knife, and The sections were picked up on copper grids and double-stained with aqueous 5% uranyl acetate and lead citrate solution, and examined in a transmission electron microscope (ISI-LEM 2000, Jeol, Japan) operated at 70 kV.

For SEM observations, 2.5% glutaraldehyde-fixed eggs and spermatozoa were attached to coverslips, washed in 0.1 M phosphate-sucrose buffer, postfixed in 2% osmium tetroxide, and dehydrated by graded series of ethanol and isoamyl acetate. The samples were then critical point dried with CO_2 in a Balzers CPD 030, and coated with 25 nm gold-palladium in a ion coater (Hitachi, Japan). The observations were made using by a scanning electron microscope (Hitachi, Japan) operated at 20 kV.

RESULTS AND DISCUSSION

1. Ultrastructures of Gametes

1) Sperm type

Spermatozoon, approximately 19 ㎛ in major axis, had their parachute-shaped and spheroidal head which exhibited a dense nucleus (Fig. 1A). These cells had an intensely and homogeneously-stained nucleus. The nucleus is moderately elongate, about 1.8 ㎛ long and 1.4 ㎛ wide (Tables 1 and 2). The chromatin consisted of an electron dense matrix containing scattered darker particles, and closely confirming to the double nuclear membrane. The relative ratio of the nucleus in this cell showed about 96.13 % (Table 1). However, the spermatozoa of carp and Korean loach have ovoid-shaped head. The rainbow trout sperm, as in the case of other teleosts has no acrosome (Leung, 1988; Guraya, 1996). An egg micropyle with a flat pit leading into a long canal was located in the egg surface of rainbow trout.

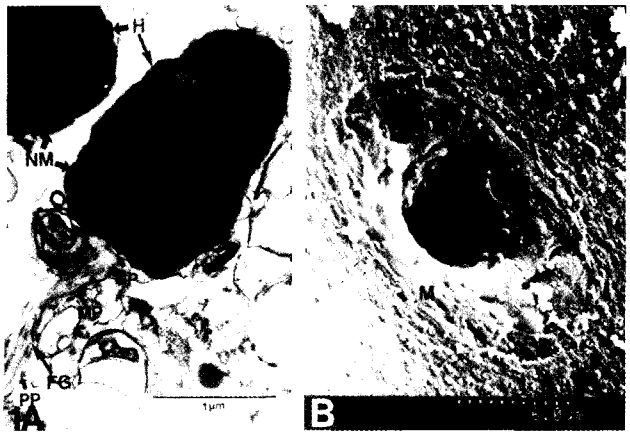

Fig. 1A and B. Longitudinal section through the sperm head, middle-piece and flagellum of a spematozoon. No acrosome can be seen around the nucleus of the cell (A). A micropyle of the shallow funnel-like structure in an unfertilized egg of rainbow trout (B). The wider distal opening leads into a canal which tapers to an small aperture. CP:central piece, FG:flagellum, H:sperm head, M:micropyle, MP:middle piece, NM:nuclear membrane, PP:principal piece

Table 1. Morphometric data for different stages of spermatogenesis in rainbow trout (*Oncorhynchus mykiss*) based on light microscopy, transmission, and scanning electron microscopy

Developmental stages	Head volume (μm^3)	Nucleus volume (μm^3)	Ratio (%)	Heterochromatin Type
Spermatogonia	5,739.6	437.58	7.62	Sparse
Primary Spermatocytes	1,217.5	278.52	22.88	Little sparse
Secondary Spermatocytes	417.25	160.89	38.56	Little sparse
Spermatids	73.38	58.65	79.93	Dense
Spermatozoa	40.35	38.79	96.13	Dense

Ratio (%): Nucleus volume/Head volume × 100
Each value is a result of four different experiments.

Table 2. Summary of properties of testis, semen, and spermatozoa of rainbow trout (*Oncorhynchus mykiss*) collecting in middle December

Testis			Semen		Spermatozoa	
GSI (%)	Appearance	Shape	Density (sperm ml^{-})	Osmolarity (mosmol kg^{-})	Length of head (μm)	Motility
4.95	Milky white	Long tubular	$10\sim15\times10^{8}$	257~325	1.8~2.2	75%

Each value is a result of four different experiment.

Approximately 140 nm long mitochondria of spermatozoa were arranged into a spheroid-shaped sheath that surrounded the axial elements of the midpiece (Fig. 1A). There appeared to be a short extension of the mitochondrion along one side of the basal region of the nucleus. Mitochondria surrounded a short, narrow cytoplasmic canal. The cytoplasmic canal, bounded by the usual fold of the plasma membrane, separated the midpiece from flagellum. The axoneme, inserted in the longitudinal axis of the head, has a 9 + 2 pattern of microtubules.

The gonadosomatic index (GSI) was approximately 4.95% during the breeding season, in December and January. The semen collected in the breeding season, in December, showed milky color and sperm concentration was $10\sim15\times10^{8}$ per ml of semen (Table 2). The motility ratio of spermatozoa showed 75%. The osmolarity of rainbow trout semen ranged from 257 to 325 mosmol kg^{-}.

2) Egg type

The micropyle consists of a funnel-shaped vestibule and a tapered canal transversing the zona radiata in rainbow trout (Fig. 1B). This micropyle could classified to the type of a flat pit and long canal which was distinguished by Riehl (1980) & Guraya (1986), and measured approximately 180 μm at the distal opening, 20 μm at the proximal opening and tapers from 8.2 μm to 1.3μm as it penetrates the zona radiata. Stehr & Hawkes (1979) reported that the 16 μm micropyle proximal opening in pink salmon (*Oncorhynchus gorbuscha*) egg is surrounded by an area of protrusion and the funnel-shaped canal tapers to 2 μm at its terminal structure. Since the diameter of the inner micropylar aperture in the egg this fish is slightly larger than the size of its sperm head, the block to polyspermy is considered to be mechanical and guaranteed by the morphological design of the micropyle (Iwamatsu & Ohta, 1981; Hart & Donovan, 1983).

The proximal opening of micropyles of eggs in the white sturgeon, *Acipenser transmontanus measures* 15 μm in diameter. The micropylar final canal contacting the oolemma tapers to 1.2 μm in the diameter (Cherr et al., 1982). The surface of the tapered micropylar canal is covered with numerous short microvilli (MV) and short projections, approximately 0.5~2.5 μm in height. Interconnecting ridges were present in the surface of unfertilized

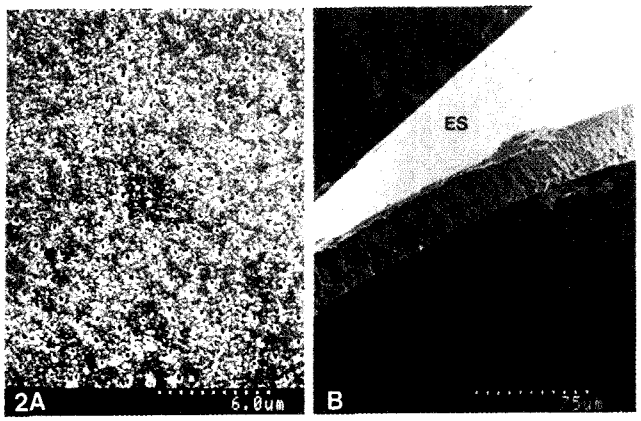

Fig. 2A and B. Note the interconnecting ridges in the egg surface of the unfertilized egg. No small beads can be seen on egg surface (A). The zona radiata has numerous pore canals (B). ES:egg surface.

egg (Fig. 2) (Ohta et al., 1983; Iwamatsu et al., 1993). Numerous short microvilli with irregular slug-like shapes covered the surface in *Fundulus heteroclitus* (Brummett & Dumont, 1979) and *Oryzias latipes* (Iwamatsu & Ohta, 1981). There were observed adhesive materials attracting the spermatozoa around micropyle of the egg surface (Dumont & Brumett, 1980; Iwamatsu & Ohta, 1981). The inner surface of the egg immediately underlying the zona radiata showed the bamboo basket-shaped structure in rainbow trout. The micropylar wall showed the clockwise spiral structure. The rotation direction of spermatozoa entering the micropyle in medaka (*Oryzias latipes*) were did not correspond to the counterclockwise spiral structure of the micropylar wall (Iwamatsu et al., 1993). Immediately upon fertilization by a sperm, the cortical granules begin to fuse with the vitelline membrane so as to release their contents into the zona radiata. The cortical granule material released by the egg interacted with the vitelline membrane material to produce the fertilization membrane (Tegner & Epel, 1973).

2. Time-course Ultrastructural Changes of the Fertilized Eggs Before and After Insemination

1) Ten Seconds After Insemination

The fertilized egg surface is surrounded by finger-like projections, called microvilli, extended from the plasma membrane through the vitelline membrane (Figs. 3A, and B) These are considered that many sperm adhere to the egg surface immediately after insemination. Immediately after rainbow trout are placed together, numerous spermatozoa standing in a row adhere to the surrounding micropyle. The sperm head, midpiece and flagellum are clearly defined. The materials attracting sperm are considered to be secreted from microvilli around the micropyle.

2) Twenty Seconds After Insemination

Five spermatozoa attached perpendicularly to the inner surface of the micropyle. The wrinkled appearance is illustrated in the inner surface in micropyle.

3) Thirty Seconds After Insemination

The head portion of the penetrating spermatozoon is completely wrapped by the egg surface with only the tail portion visible externally. The diameter of the inner opening of the micropyle indicates that only one sperm can gain access to the egg (Guraya, 1986). No sperm is attached to the egg surface in this region. Large cortical granules that have discharged their contents are observed around the animal pole area (Iwamatsu & Ohta, 1981). There are structural changes of cortical granules from spheroid type to irregular type. The fertilization membrane rises by the action of a cortical protease. The region of the zona radiata around the animal pole become thinner and elevated from the egg surface as soon as the cortical alveolar contents are secreted, as demonstrated by Guraya (1986). These changes are consistent with the expansion of the vitelline layer in the formation of the fertilization membrane. The cortical reaction producing block to polyspermy has covered the top half of this egg. The fertilization membrane is about half formed (Tegner & Epel, 1973; Iwamatsu & Ohta, 1981).

4) Sixty Seconds After Insemination

Small fertilization cones (about 4 ㎛ in average diameter) are identified in the central portion of the depressed inner micropyle. Most of the microvilli on the egg surface become slender. No supernumerary spermatozoa are found on the egg surface surrounding the sperm-penetrating region.

5) Ninety Seconds After Insemination

A large fertilization cone (about 6 ㎛ in average diameter) with relatively smooth surface protrudes from the central portion of the depressed inner micropyle (Fig. 4). The production of the

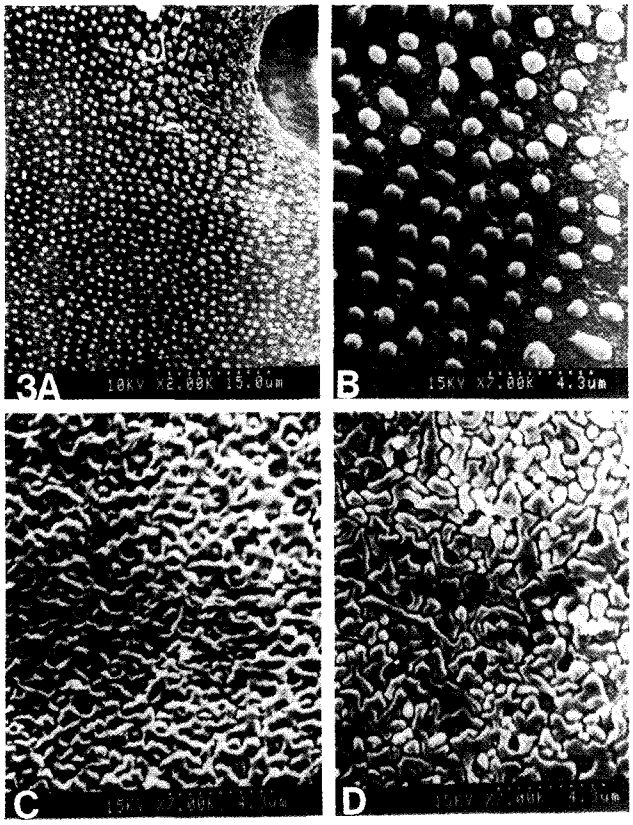

Fig. 3A, B, C and D. There were a great number of microvilli secreting adhesive materials having trapping function attracting the spermatozoa in the vicinity of micropyle of the egg surface. It was apparent that ridges extended between the projections (A). A high magnification of the microvilli in the egg surface (B). The microvilli appeared to decrease in height and then interconnecting ridges in egg surface. These structures gave the egg surface a reticulate aspect (C). No microvilli can be seen in the egg surface. No interconnecting ridges were present. All supernumerary sperm have been detached (D).

fertilization cone with the commencement of alveolar breakdown is completed in the period from 30 to 90 seconds after insemination. The fertilization cone is sometimes displayed the tail portion of the penetrating spermatozoon on the central portion of cone surface (Iwamatsu & Ohta, 1981). However, no sperm flagellum is identified in cone surface shortly before and after 90 seconds in rainbow trout. The microvilli of the fertilized egg appear to be shorter and farther apart than those of an unfertilized egg. These structural changes are considered to be consistent with the expansion of the vitelline layer in the formation of the fertilization membrane (Tegner & Epel, 1973).

6) One Hundred and Twenty Seconds After Insemination

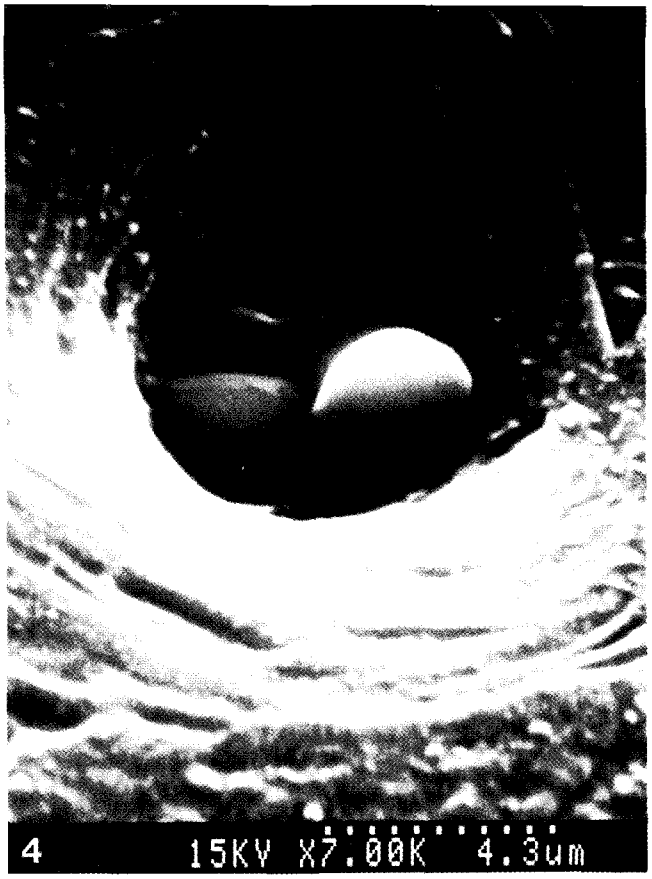

Fig. 4. The micropylar canal showing the smoothness of the surface 60 seconds after insemination. The smaller fertilization cones can be seen as a blister-like bulge.

The microvilli appeared to decrease in height and then interconnecting ridges in egg surface (Fig. 3C). These structures gave the egg surface a reticulate aspect. The cone can be shown as a spheroid ball without microvilli. The height of the micropylar vestibule with the shallow funnel-like structure is similar to that of egg surface.

7) One Hundred and Fifty Seconds After Insemination

The fertilization cones shown as a blister-like bulge have the rough and irregular outer surface. No microvilli can be seen in the egg surface (Fig. 3D). It was apparent that ridges extended between the projections. All supernumerary sperm have been detached.

8) Two Hundred and Fifty seconds After Insemination

The protruded fertilization cone is shown as an upended bowling pin. The egg surface in the vicinity of the protruded

cone is much smoother in appearance. The fertilization cone has diminished in size with the lapse of time. A retracting cone has a depressed central region. The disappearance of the cone is similarly synchronized with completion of the cortical reaction at the vegetal pole region of the egg.

The block to polyspermy in fish egg appears to be achieved by the morphological elimination of excess spermatozoa from the micropyle apparatus and the egg surface facing the inner opening of the micropyle, and the cortical reaction as described by many investigators (Brummett & Dumont, 1979; Iwamatsu & Ohta, 1981; Hart & Donovan, 1983).

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